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Original Article

An analytical description of distributions of HPV genotypes patients with cervical precancerous lesions and squamous cell carcinoma in Zhejiang and Jiangsu area

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Abstract: Objective: To investigate the distributions of human papillomavirus (HPV) infection type of women with cervical intraepithelial neoplasia (CIN) grade 2/3 and cervical squamous cell carcinoma (CSCC) in Zhejiang and Jiangsu Province, China. Methods: The research was based on the cross-sectional study. There were 219 cases of CIN2, 253 cases of CIN3 and 430 cases of CSCC from 14 local hospitals in Zhejiang and Jiangsu Province detected and genotyped by using HPV gene chip technology, and then various subtypes of infection were analyzed and compared. Results: The HPV positive rates of CIN2, CIN3 and CSCC were 58.90%, 88.93%, and 90.23%, respectively, which in CIN3 and CSCC were significantly higher than that in CIN2 (P<0.001). The infection rate of high-risk HPV in CSCC was significantly higher than that in CIN2 and CIN3 (P<0.001), but the concurrent infection rate of high & low-risk HPV was only 0.70%, which was significantly lower than that in CIN2 and CIN3 (P<0.001). The most common type of infection in CSCC was HPV16 (68.60%), HPV18 (11.16%), HPV33 (5.12%), HPV58 (4.41%), HPV59 (2.79%), HPV31 (2.56%) and HPV52 (2.56%), the sorts of HPV type in CIN3 and CIN2 was generally similar to that in CSCC, but the respective positive rates of the HPV type were not the same. Besides, the multiple infection rate in CSCC was significantly lower than that in CIN3 and CIN2 (P<0.001). Conclusion: HPV16, 18, 33, 58, 59, 31 and 52 are the most common types of CSCC in Zhejiang and Jiangsu Province. Further studies are needed to clarify the relationship between multiple infection and cervical cancer.

Keywords: Cervical squamous cell cancer, cervical intraepithelial neoplasia, human papillomavirus, genotype

Introduction

At present, cervical cancer is the fourth common cancer in women around the world. In 2012, the incidence and mortality of cervical cancer were about 527,600 and 265,700, respectively, of which nearly 90% occurred in the developing countries [1]. In China, with the rapid development of social economy, the change of life style, such as individuals' sex behavior, etc., as well as the increased risk factors of cervical cancer, the incidence and mortality of cervical cancer are increasing in general [2-4]. It is now clear that persistent HPV infection is a necessary cause of cervical cancer, and more than 99% of cervical cancer can be attributed to the infection of HPV [5, 6]. At present, more than 200 types of HPV have been found. According to the difference of the carcinogenicity of HPV, it can be divided into high-risk and low-risk types, and there are at least 15 high-risk types of HPV that associated with cervical cancer [6], in which HPV16 and HPV18 are the most common that cause 70%-80% cervical cancer in the world. Both in the world and China, there are obviously regional differences in HPV infection rates and distributions of types [7, 8]. In addition, the infection rates of HPV and the main types of infection are not the same in different grades of cervical intraepithelial lesions. Therefore, it is of great significance for the risk assessment of cervical

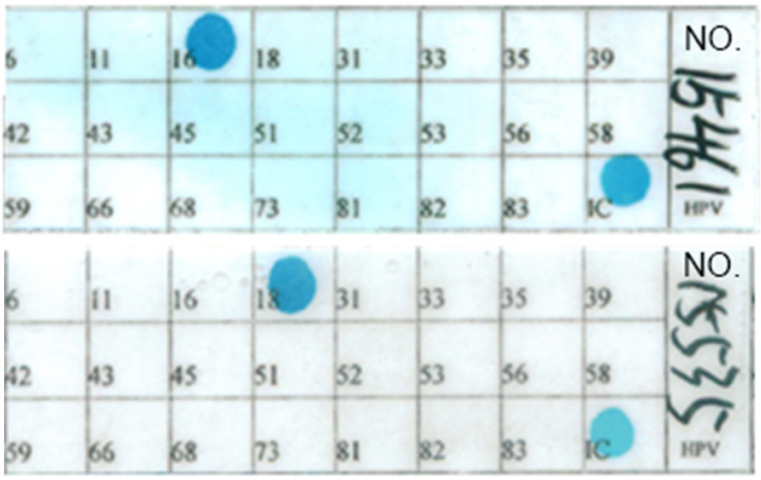

Figure 1. The positive membrane chips of HPV16 and HPV18 infections in the detection of cervical lesions.

cancer and the development of the vaccine to identify the main types of HPV infection for the women with varying degrees of cervical lesions in a region. The study aims to investigate the distributions of HPV infection types in women with different degrees of cervical lesions in Zhejiang Province, and to provide a theoretical basis for the control of cervical cancer and the development of HPV vaccine.

Materials and methods

Source of study population and specimens (the approval of Ethics Committee and informed consent were gained)

We collected the archived paraffin tissue samples of cervical intraepithelial neoplasia (CIN) and cervical squamous cell carcinoma (CSCC) which were diagnosed histologically by Departments of Pathology of Yinzhou People's Hospital, Jingjiang People's Hospital, Dantu People's Hospital, Nanjing Hospital of Traditional Chinese Medicine, Xuzhou Hospital of Traditional Chinese Medicine, Changshu Hospital of Traditional Chinese Medicine, Nanjing Liuhe People's Hospital, Changshu No. 1 People's Hospital, People's Hospital of Yangzhong City, Sheyang County People's Hospital, Xuzhou Maternity and Child Health Care Hospital, Shanghai Meishan Hospital, The First People's Hospital of Anqing, and Dangtu County People's Hospital from November 2006 to October 2015. Inclusion criteria: patients ≥18 years old, diagnosed with CIN2, CIN3 or CSCC, respectively, and had not undergone radiotherapy and chemotherapy; tissues with regular shapes, length <2 cm, width >2 mm; tissues were fixed by Faure Marin and embedded in paraffin. Exclusion criteria: too big, too thin or extremely irregular paraffin block; no satisfied section could be found for histological analysis; poor quality of paraffin block that couldn't be sliced; cervical specimens were from patients who had been treated by chemotherapy.

Specimen processing and detection

Specimen collection: The paraffin tissues removed excess paraffin wax, were cut into 4 μm-thick slices for 3 to 5 pieces. A special pair of forceps was used to gently clip the pieces into a small centrifuge tube. And the blade and forceps were wiped by sodium hypochlorite solution for 3 times respectively, before cutting the next paraffin tissue.

DNA extraction: The pieces of paraffin tissues were placed in a 1.5 ml centrifuge tube, where they were vortexed well with 150 μl lysis buffer. And the tube was incubated for 10 min at 100°C in metal bath, followed by centrifuging for 10 min at 13,000 rpm immediately, then the middle layer of DNA solution was collected and set aside for use when needed.

PCR amplification: The extracted DNA (5 μl) was added into the PCR reaction solution, and the total reaction volume was 25 μl. The PCR reaction conditions were 50°C for 15 min, 95°C for 10 min, 40 cycles (at 94°C for 30 s, 42°C for 90 s, and 72°C for 30 s), and at 72°C for 5 min.

Hybridization of PCR products and HPV genotyping chip: The system of HPV genotyping chip (Shenzhen Yaneng BIO Co. Ltd.), could detect 18 high-risk types of HPV DNA: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 83 and HPVMM4, and 5 low-risk types of HPV DNA: HPV6, 11, 42, 43 and 44. The specific methods were as follows: the chip signed with the sample number was put into a 15 ml plastic centrifuge tube, 5 ml solution A and the amplification product were added, and then the

Table 1. HPV infection rates in different cervical lesions

HPV	CIN2 (n, %)	CIN3 (n, %)	SCC (n, %)
Negative	90 (41.10)	28 (11.07)	42 (9.77)
Positive	129 (58.90)	225 (88.93)	388 (90.23)
Single high-risk infection	67 (30.59)	52 (20.55)	311 (72.32)
Multiple high-risk infection	48 (21.91)	118 (46.64)	63 (14.65)
High & low-risk concurrent infection	10 (4.57)	26 (10.27)	3 (0.70)
Single low-risk infection	4 (1.83)	29 (11.46)	11 (2.56)

system was denatured at 100°C for 10 min and hybridized at 51°C for 1.5 h. After washed with solution B at 51°C for 15 min, the chip was incubated at room temperature for 30 min. Then the chip was washed twice with solution A, 5 min for each, and placed in the coloration solution, avoiding the light for 15 min, and then the results could be observed. After the chip was cleaned up, whether there was infection of HPV or not could be known according to the presence of blue spots and the location of the spots. The existence of blue spots on the IC probe point of the chip could demonstrate the success of the experiment. For the single HPV infection there was one blue spot on the probe point of membrane chip of corresponding HPV genotypes; for the double HPV infection, there were two; for the multiple infection, there were several. The positive membrane chips of HPV16 and HPV18 infections in the detection of cervical lesions are shown in **Figure 1**.

Quality control: The slicing process and the whole detection process were carried out in a strictly clean laboratory. The blank wax block was used to detect whether there was cross contamination in the slicing process, and the reagent blank control, PCR negative control and PCR positive control were set to detect whether cross contamination in the detection process existed.

Statistical analysis

HPV infection rates of CIN2, CIN3 and CSCC tissues and the proportion of each type of HPV were analyzed by using HPV genotyping statistical software (Nanjing Beining Medical Equipment Co., Ltd.) The ages among patients in CIN2, CIN3 and CSCC were compared by using one-way ANOVA (α=0.05), and the categorical variables among these three groups were compared by using chi square test (α=0.05). The pairwise comparisons of the infection rates of the three groups were compared by using Bonferroni method.

Results

The specimens of 219 patients with CIN2, 253 patients with CIN3 and 430 patients with CSCC were obtained in our study. The mean ages of patients with CIN2, CIN3 and CSCC were 49.8±12.1, 51.1±10.6, and 51.7±7.6, respectively. There was no significant difference (F=2.77, P=0.06).

Distributions of HPV specific types in different degree of cervical lesions

Gene chip technology was adopted to detect the specimens of CIN2, CIN3 and CSCC. There were 129, 225 and 388 cases of positive HPV with the positive rates of 58.90%, 88.93%, and 90.23% respectively. The HPV infection rates in CIN3 and CSCC were significantly higher than those in CIN2, with significant differences among three groups (P<0.001). According to the high-risk condition of HPV type, the infection types were divided into four cases, including single high-risk infection, multiple high-risk infection (double infection included), high- & low-risk concurrent infection and single low-risk infection. And the distributions of HPV infection is presented in **Table 1**. The infection rate of single high-risk HPV in the CSCC was 72.32%, which was significantly higher than that in CIN3 (20.55%) and CIN2 group (30.59%) (all P<0.001), meanwhile, the infection in CIN2 was significantly higher than that in CIN3 (P=0.016). The multiple infection rate of high-risk HPV and the single infection rate of low-risk HPV in CIN3 were significantly higher than those in CIN2 and CSCC (P<0.001) but there was no significant difference between CIN2 and CSCC. The infection rate of high- & low-risk HPV in CSCC was only 0.70%, which was significantly lower than that in CIN2 and CIN3 (P<0.001). The infection rate of single low-risk in CIN3 (11.46%) was the highest, which was significantly higher than that in CSCC and CIN2 (P<0.001), and there was no significant difference between the CSCC and CIN2.

Table 2 shows the distributions of HPV genotypes in CIN2, CIN3 and CSCC (for patients with

Table 2. Distribution of HPV genotypes in different cervical lesions

HPV genotype	CIN2			CIN3			CSCC		
	Single infection	Concurrent infection	Total	Single infection	Concurrent infection	Total	Single infection	Concurrent infection	Total
HPV16	14 (6.39)	33 (15.07)	47 (21.46)	24 (9.49)	132 (52.17)	156 (61.66)	233 (54.19)	62 (14.42)	295 (68.60)
HPV18	18 (8.22)	27 (12.33)	45 (20.55)	13 (5.14)	50 (19.76)	63 (24.90)	25 (5.81)	23 (5.35)	48 (11.16)
HPV33	11 (5.02)	20 (9.13)	31 (14.16)	9 (3.56)	26 (10.28)	35 (13.83)	7 (1.63)	15 (3.49)	22 (5.12)
HPV58	11 (5.02)	13 (5.94)	24 (10.96)	11 (4.35)	37 (14.62)	48 (18.97)	5 (1.16)	14 (3.26)	19 (4.42)
HPV59	2 (0.91)	8 (3.65)	10 (4.57)	1 (0.40)	4 (1.58)	5 (1.98)	6 (1.40)	6 (1.40)	12 (2.79)
HPV31	7 (3.20)	5 (2.28)	12 (5.48)	4 (1.58)	19 (7.51)	23 (9.09)	3 (0.70)	8 (1.86)	11 (2.56)
HPV52	0 (0.00)	1 (0.46)	1 (0.46)	7 (2.77)	18 (7.11)	25 (9.88)	6 (1.40)	5 (1.16)	11 (2.56)
HPV45	0 (0.00)	1 (0.46)	1 (0.46)	0 (0.00)	1 (0.40)	1 (0.40)	4 (0.93)	4 (0.93)	8 (1.86)
HPV56	0 (0.00)	1 (0.46)	1 (0.46)	3 (1.19)	2 (0.79)	5 (1.98)	3 (0.70)	1 (0.23)	4 (0.93)
HPV53	1 (0.46)	0 (0.00)	1 (0.46)	1 (0.40)	4 (1.58)	5 (1.98)	4 (0.93)	0 (0.00)	4 (0.93)
HPV51	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	4 (0.93)	0 (0.00)	4 (0.93)
HPV73	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (1.19)	3 (1.19)	3 (0.70)	0 (0.00)	3 (0.70)
HPV66	0 (0.00)	1 (0.46)	1 (0.46)	1 (0.40)	2 (0.79)	3 (1.19)	3 (0.70)	0 (0.00)	3 (0.70)
HPV39	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (0.70)	0 (0.00)	3 (0.70)
HPV68	0 (0.00)	0 (0.00)	0 (0.00)	3 (1.19)	2 (0.79)	5 (1.98)	2 (0.47)	0 (0.00)	2 (0.47)
HPV45	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.40)	2 (0.79)	3 (1.19)	0 (0.00)	0 (0.00)	0 (0.00)
HPV43	4 (1.83)	7 (3.20)	11 (5.02)	0 (0.00)	3 (1.19)	3 (1.19)	4 (0.93)	0 (0.00)	4 (0.93)
HPV11	3 (1.37)	3 (1.37)	6 (2.74)	1 (0.40)	7 (2.77)	8 (3.16)	1 (0.23)	20 (0.47)	3 (0.70)
HPV42	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.40)	2 (0.79)	3 (1.19)	3 (0.70)	0 (0.00)	3 (0.70)
HPV6	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.40)	14 (5.53)	15 (5.93)	1 (0.23)	1 (0.23)	2 (0.47)
HPV81	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.47)	0 (0.00)	2 (0.47)
HPV (-)			42 (9.77)			28 (11.07)			90 (41.10)

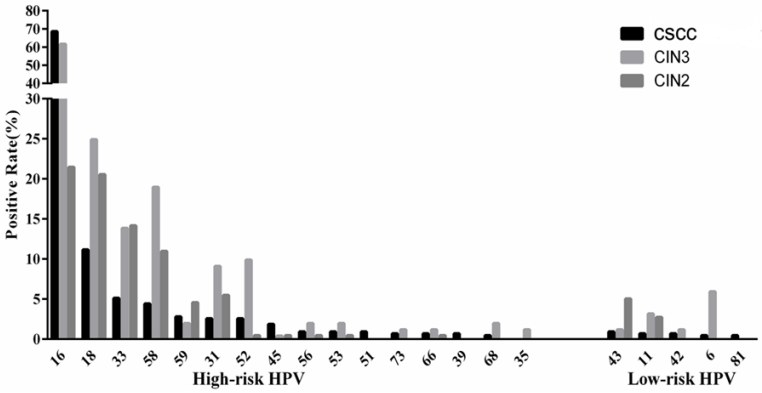

Figure 2. Distribution of HPV genotypes in CIN2, CIN3 and CSCC.

multiple infection, the positive rate of each type had been calculate repeated). In CSCC, the most common infection types were HPV16 (68.60%), HPV18 (11.16%), HPV33 (5.12%), HPV58 (4.41%), HPV59 (2.79%), HPV31 (2.56%) and HPV52 (2.56%). The sorts of CIN3 and CIN2 were similar with those of CSCC, but the positive rates of the HPV genotypes in the three groups were not the same (**Figure 2**).

Distributions of HPV multiple infection in different cervical lesions are showed in **Table 3**. In the 388 cases of positive HPV CSCC specimens, there were 322 cases (82.99%) of single infection, 66 cases (17.01%) of multiple infection. The multiple infection rates in CIN3 and CIN2 were 64% and 44.96% respectively, which were significantly higher than those in CSCC ($P<0.001$).

Discussion

The morbidity and mortality of cervical cancer in China have been maintained at a high level, and there is even a tendency to be younger for patients [9, 10]. HPV infection is closely related to the occurrence and development of CIN and cervical cancer. The genotypes of HPV infection in different cervical lesions were not identical, and the pathogenicity of different genotypes was also different. When using HPV examina-

Table 3. Distribution of HPV multiple infection in different cervical lesions

Cervical lesions	Positive (N)	Single infection (%)	Multiple infection				
			Double infection (%)	Triple infection (%)	Fourfold infection (%)	Fivefold infection (%)	Total (%)
CIN2	129	71 (55.04)	55 (42.64)	3 (2.33)	0 (0)	0 (0)	58 (44.96)
CIN3	225	81 (36)	105 (46.67)	35 (15.56)	4 (1.78)	0 (0)	144 (64)
CSCC	388	322 (82.99)	59 (15.21)	5 (1.29)	1 (0.26)	1 (0.26)	66 (17.01)

tion for cervical cancer screening, we must consider the differences in carcinogenicity of different HPV subtypes. Therefore, it is important to study the distributions of different types of HPV infection in cervical lesions and cervical cancer for the prevention and early diagnosis of cervical cancer.

The Meta-analysis shows that the types of HPV16 and HPV18 are the most widely distributed high-risk HPV types in the world, and there is no significant difference of distributions among all regions. In East Asia, there are higher prevalence of HPV58 and HPV52 [11]. In China, 84.5% of HPV related cervical cancer is caused by HPV16 and HPV18 subtypes, additionally, HPV58, 33, 52, 31, 45 and 59 are other 6 major high-risk HPV types of cervical cancer, but the distributions among regions also has significant difference [12-17]. Even in the Zhejiang area, the HPV infection rates and distributions of genotypes reported in various studies of CSCC are not exactly the same. In our study, the HPV infection rate of CSCC was 90.23%, and the most common type were HPV16, HPV18, HPV33, HPV58, HPV59, HPV31 and HPV52; besides, the HPV infection rates of CIN2 and CIN3 in Zhejiang were 95.0% and 81.9%, respectively, and the common types were HPV16, 18, 58, 31, 59, 52, 33 and HPV16, 58, 18, 52, 31, 33, respectively [18, 19]. The differences of age distributions among patients in different researches and detection methods may be the reason of slightly different order.

In our study, the HPV infection rates of CIN2, CIN3 and CSCC were 58.90%, 88.93% and 90.23%, respectively. The infection rate increased with the increasing severity of cervical lesions, which was similar to the results in other studies [16, 19]. However, the multiple infection rate of CSCC was significantly lower than that in CIN2 and CIN3. There is still controversy about whether HPV multiple infection increases and promotes the occurrence of cervical lesions. Some studies showed that compared with single HPV infection, the risk of high-grade cervical lesion and infiltrating cancer in women with multiple infection increased obviously [20, 21]. Lee studied the relationship between multiple HPV infection and cervical cancer, finding that the single high-risk infection increased the risk of cervical cancer by 19.9 times, and the multiple infection increased the risk to 31.8 times [21]. In a study in Inner Mongolia, China, the multiple infection rates of low- & high-grade squamous intraepithelial lesions and cervical cancer increased from 9.9% to 25% [16]. However, some scholars believed that, compared with single infection, HPV multiple infection did not increase the incidence of cervical cancer. In a previous study in Zhejiang, the multiple infection rate of CSCC was only 4.4%, which was significantly lower than that in CIN2 and CIN3 [19]. In view of the development from precancerous lesions to cervical cancer taking many years, most of the HPV infection can be cleared, and only a small part maintains sustainable, so some research showed that the multiple infection rate of intraepithelial neoplasia was higher than that in patients with cervical cancer [22, 23]. And Bosch believed that multiple infection would not increase the risk of cervical cancer and precancerous lesions, but only cause the risk of sustained disease [5]. In addition, in our study, the infection rate of high & low-risk concurrent type in CSCC was the lowest, while an American study showed that the co-infection of high & low-risk HPV type could reduce the risk of CSCC. The author thought that there was antagonism between high-risk type and low-risk type, and low-risk type could delay the progress from CIN to invasive cervical cancer [24]. Therefore, the relationship between multiple HPV infection and the risk of cervical cancer needs further study.

Prophylactic HPV vaccination is the most effective method for cervical cancer prevention in an

area where has not been carried out the systematic cervical cancer screening program [25]. At present, more than 100 countries around the world have allowed using prophylactic vaccines against cervical cancer, of which mainly are the bivalent vaccines (HPV16, 18) and quadrivalent vaccines (HPV6, 11, 16, 18), and China where has approved the bivalent vaccines listed included as well [26]. However, it should be noted that cervical cancer vaccine does not prevent all HPV infection. In this study, the bivalent vaccines could provide 79.8% of cervical cancer protection for the local women's HPV16/18, but could not cover HPV33, 58, 59, 52, 31, and other of the higher prevalence of subtypes in China. Therefore, it is one of the most important directions for the prevention and cure of cervical cancer in China to explore and design the related HPV vaccines that are suitable for China and its regions.

The paraffin waxes of cervical tissues in this study were obtained from 14 hospitals, which can well represent the distributions of HPV subtypes in women with different degrees of cervical lesions in Zhejiang area. However, there still exist some limitations, for example, the analysis of distributions of HPV subtypes didn't be made because of inadequate cases of cervical adenocarcinoma. Besides, although we have adopted stricter quality control measures, we might not be able to avoid the bias caused by the wrong diagnosis, due to the extensive sources.

In summary, we analyze the distributions characteristics of HPV primary infection type (HPV16, 18, 33, 58, 59, 31 and 52) of CIN and CSCC in women in Zhejiang area. Additionally, we find that the multiple infection rate of CSCC was significantly lower than that in CIN2 and CIN3, indicating that multiple infection might not be related to the occurrence of cervical cancer, but further research is needed. The results of our study provide data and reference for the prevention and treatment of cervical cancer in Zhejiang area.

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Disclosure of conflict of interest

None.

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