Epidemiological study of high-risk human papillomavirus infection in subjects with abnormal cytological findings in cervical cancer screening

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Abstract. The present study aimed to determine the genotype and age distribution of high-risk human papillomavirus (HR-HPV) and evaluate HPV-DNA in subjects with abnormal cervical cytology results by using crowd-based cervical cancer screening cytology data. The Thinprep liquid-based cytologic test (TCT) was performed from January 2013 to January 2014 in the permanent residents of Liaocheng (China) aged 21-65 years who were married or had sexual intercourse. The number of screened women totaled 20,017, among whom 937 had abnormal results, 785 of which were recalled. For subjects in the age range of 21-65 years, an HR-HPV typing test using the fluorescence hybridization method. Among the 785 cases with abnormal TCT findings, repeated testing identified atypical squamous cells of unknown significance/atypical glandular cells in 478, low-grade squamous intraepithelial lesions in 175, high squamous intraepithelial lesions in 127 and squamous cell carcinoma/adenocarcinoma in 5 cases. Among these types, infection rates of HR-HPV were 50.2, 77.1, 89.0 and 100%, respectively. Of the 785 cases with abnormal TCT results, 493 (62.8%) were HR-HPV-positive. A total of 16 types of HR-HPV were detected: HPV-16, -18, -31, -33, -35, -39, -45, -51-53, -56, -58, -59, -66, 68 and 73. Subjects infected with ≥2 types were defined as having a multi-type infection. The infection rate was high in the age groups of 26-30 and 51-55 years, accounting for 87.7% (71/81) and 79.7% (51/64), respectively, while it was lower in the >55 years group at 28.6% (14/54). The top five types of HR-HPV (stated in a decreasing order regarding positivity rate) were HPV16 (21.5%, 169/785), HPV52 (12.2%, 96/785), HPV58 (9.8%, 77/785), HPV33 (9.7%, 76/785) and HPV18 (7.5%, 59/785). Single-type infection was encountered in 45.0% (353/785) and multi-type infection in 17.8% (140/785), among which 98 cases had a two-type infection, 37 had a three-type infection, 2 had a four-type infection, 2 had a five-type infection and 1 case had a six-type infection. In the present study, differences in multi-type HR-HPV infection between groups with different TCT results were statistically significant. In conclusion, compared with CTC screening on its own, complementary HR-HPV testing is an effective method for screening for cervical cancer. The infection rate of HPV16, -52, -58, -33 and -18 was high among patients with cervical cytological abnormalities. Multi-type infection adds to the risk of malignancies. In Liaocheng, high-risk groups were aged 26-30 and 51-55. Attention should be paid during the screening and follow-up visits of these groups.

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Introduction

Cervical cancer, the third most common malignant tumor type endangering women's health, has a high incidence rate in developing countries due to limitation of medical treatment for health conditions and lack of cervical cancer screening (1,2). An annual estimate of 10,000 cases are newly diagnosed and 30,000 succumb to cervical cancer in China (3). With the development of detection methods, early-stage diagnosis, which has an important role in the prevention and treatment of cancer, has been successfully performed and been of benefit to the patients.

The implementation of the Pap smear test, a single method to screen for cervical cancer, has decreased the incidence rate of this malignancy. However, due to the low coverage, low dependence, high specificity subjective judgment and the discomfort of the patients, the method is not widely used in the clinic (4-7). The Thinprep liquid-based cytological test (TCT) provides the screening efficiency of cervical cytology with a better medium for cervical cells and avoidance of the disadvantages of the Pap smear test. However, cytological screening cannot be widely used for the prevention and treatment of cervical cancer, due to a series of weaknesses, such as limitation of morphological examination, tiredness of vision as well as low sensitivity and specificity (8,9).

In the past decade, cervical cancer was evidenced to be closely correlated with HR-HPV infection (10). HPV

testing has been approved to be a reasonable choice and to be widely used in the screening of cervical pre-cancerous diseases (11,12). The risk of disease also depends on HPV types, along with the patient's genotype and environmental factors such as smoking (10). To date, >200 types of HPV have been recognized and it has been suggested that HR-HPV including types 16, 18, are able to induce cervical cancer, particularly in patients with chronic HR-HPV infection (13).

More than 50 years of experience in the US and European countries have revealed that cytological screening on its own or combined with HPV testing for feedback obviously decreased the incidence rate of cervical cancer (14). However, HPV testing during the first line of cervical cancer screening has remained controversial. HPV testing was reported to be more effective and sensitive than cytological methods and may replace cytology as the first-line screening of cervical cancer (15). However, HPV testing may also have high probability of ignoring cervical diseases that may be diagnosed by other methods of detection. The American Cancer Society, the American Society for Colposcopy and Cervical Pathology and the American Society of Clinical Pathology have proposed that the combination of cytology and HPV testing significantly increase the sensitivity in cervical cancer screens (16). In addition HR-HPV testing in subjects with abnormal TCT results may increase the sensitivity of cervical cancer detection and decrease the missed diagnosis rate (10).

Further studies suggested that the controversial role of HPV testing in the screening of cervical cancer may be due to differences in study populations. A meta-analysis revealed that subjects display significant inter-regional differences (17).

As a screening guide must take several factors and the real conditions of the district into consideration, the present study was performed. In 2008, Beijing commenced the screening of women for cervical and breast cancer free of charge and implemented this screening in the entire city in 2009 with good social feedback. Through this cervical cancer screening, the detection rate of cervical pre-cancerous lesions and early cervical cancer was significantly increased (18). Liaocheng People's Hospital (Lioacheng, China) in Shandong province began to screen women for cervical and breast cancer in January 2013; this service was provided free of charge in the first instance and once every two years thereafter. Until January 2014, nearly 20,000 married women residing in Liaocheng underwent cervical cancer screening. The present study focused on subjects with abnormal cervical cytology findings. HR-HPV testing was performed and an epidemiological analysis was conducted in order to effectively identify individuals with a high risk of cervical cancer. The present study may increase the screening efficacy, save resources and provide data for screening individuals with abnormal cytology results.

Materials and methods

Ethics statement. All of the testing procedures were in accordance with the ethical committee of Liaocheng People's Hospital and the international ethical guidelines for biomedical research involving humans (19). Patients provided informed written consent and agreed with their data being included in the present study.

Study design. According to the plan of Liaocheng (China) to screen women who were permanent residents for cervical and breast cancer, Liaocheng People's Hospital (Liaocheng, China) subjected 20,017 women to cervical cytology testing from January 2013 to January 2014, among which 937 cases had abnormal results. Abnormalities on liquid-based cytology are defined according to the Bethesda 2001 directive and include atypical squamous cell of unknown significance (ASCUS), atypical glandular cells of unknown significance (AGCUS), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesion (HSIL), squamous cell carcinoma (SCC) and adenocarcinoma (ACA). In the present study, liquid-based cytology results of ASCUS or worse (ASCUS+) were considered abnormal (20). Patients with abnormal results were informed by trained staff via telephone communication, and were advised to undergo further treatment at the hospital. A file was generated for each of these patients and an information management platform was built in order to screen high-risk patients. A total of 785 patients were recalled for repeated CTC and HR-HPV-DNA testing. Their age ranged from 21 to 65 years with an average age of 36±9 years and none of them were pregnant.

Cytology testing. The sampling of the secretion specimens was strictly performed during the non-menstrual period. No operation was performed within 3 days prior to sampling. For sampling, a cotton swab was used to obtain a secretion specimen from the cervix by wiping, and a cervical brush (ThinPrep 2000; Hologic, Inc., Beijing, China) was then applied to the cervical mouth and rotated clockwise for 4-5 circles. The cervical brush head was then put into a tube containing cell preservation liquid (PreservCyt® Solution; Hologic, Inc.). Tubes were kept in an upright position. Cervical liquid-based cytology tests were then performed by experienced cytology experts at the Department of Pathology of Liaocheng People's Hospital.

HPV DNA testing. Methods clinically used for HPV testing include cytology, immunohistochemistry, *in situ* hybridization, dot blot hybridization, nucleic acid blotting and polymerase chain reaction (PCR). In 2014, the US Food and Drug Administration (FDA) approved Cobas® HPV testing applied on its own in the first-line screening of women aged ≥25 years (20). This method will decrease the status of cervical cytology in screening and become a method for diagnostically stratifying patients. At present, two major HR-HPV testing methods are used in China: The first one is the Hybrid Capture 2 HPV test for quantitative testing of HR-HPV DNA, which is an established standard worldwide, and served as a comparator in the guidelines for accurate HPV testing methods (21-23). The second is a gene chip technology-based test, which combines PCR and reverse dot blot hybridization (24). The two methods are used to collect information about the probands' HPV infection status of the genital tract at different time-points, and they are widely used in initial screens for cervical diseases and follow-up visits.

In the present study, cervical cell samples from patients were kept for 2 weeks, within which one equal part of the samples of patients with abnormal cytology findings was transported to the central laboratory and stored at a temperature of 4˚C,

Table I. Comparison between TCT results and positivity rate of high-risk human papillomavirus infection.

TCT typing	Total cases (n)	Positive (n)	Negative (n)	Positive rate (%)	χ^2	P-value
ASCUS/AGC	478	240	238	50.2	88.1	0.001
LSIL	175	135	40	77.1		
HSIL	127	113	14	89.0		
SCC/ACA	5	5	0	100.0		
Total	785	493	292	62.8		

TCT, Thinprep liquid-based cytological test; ASCUS, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ACA, adenocarcinoma.

Table II. Comparison of infection rate [n, (%)] of single- and multi-type high-risk human papillomavirus in subjects with different TCT results in Liaocheng.

TCT type	Cases tested (n)	Single-type	Multi-type	χ^2	P-value
ASCUS/AGC	478	207 (43.3)	33 (6.9)	94.7	<0.01
LSIL	175	101 (57.7)	34 (19.4)[a]		
HSIL	127	45 (35.4)	68 (53.5)[a,b]		
SCC/ACA	5	0 (0.0)	5 (100.0)[a,b]		

[a]P<0.05 compared with ASCUS/AGC group; [b]P<0.05 compared with LSIL. TCT, Thinprep liquid-based cytological test; ASCUS, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ACA, adenocarcinoma.

where HPV-DNA testing was performed. Regular HPV testing included HPV-DNA sampling, DNA amplification, *in situ* hybridization detection and data processing with Luminex Data Collector version 1.7 (Luminex Corp., Austin, TX, USA). The procedure was performed according to the protocol of the Nucleic Acid Genotyping kit for Human Papillomavirus (cat. no. HPV27) provided by Shanghai Tellgen Life Science Co., Ltd. (Shanghai, China).

HPV genotype testing. Cervical cell samples were subjected to PCR for *in vitro* amplification of HPV DNA using the Nucleic Acid Genotyping kit for Human Papillomavirus. The Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was used as a platform, and according to the hybridization principle, DNA probes were hybridized on a low-density gene chip with attached nucleic acid probes. This experiment can test 27 HPV sub-types, which are divided into 17 high-risk and 10 low-risk HPV types (23). Through analysis of high-risk sub-type cases, HR-HPV includes HPV16, -18, -31, -33, -35, -39, -45, -51-53, -56, -58, -59, -66, -26, -68 and -73. Positivity is defined as ≥103 copies/ml HR-HPV DNA. Infection with ≥2 of the 17 types was defined as a multi-type infection. In the current study, differences in multi-type HR-HPV infection between groups with different TCT results were statistically significant.

Statistical analysis. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used for comparative and descriptive analysis. χ^2 testing was used when comparing more than two groups, and data were compared in a line x column Table. P<0.05 was considered to indicate a statistically significant difference.

Results

Cases with abnormal TCT results. Among the 785 cases with abnormal findings on TCT, repeated examination identified ASCUS/AGC in 478, LSIL in 175, HSIL in 127, and SCC/ACA in 5. In these groups, the infective rate of HR-HPV was 50.2, 77.1, 89.0 and 100%, respectively. The HPV positivity rate was significantly associated with the degree of cervical cell abnormality according to the TCT (χ^2=88.1; P<0.05; Table I).

Epidemiological distribution of HR-HPV. Of the 17 genotypes of HR-HPV covered by the testing kit, all were found in the screened subjects except HPV26. The infection rate of HR-HPV was 62.8% (493/785). Fig. 1 presents the 5 most frequently identified HR-HPV infection types, including HPV16 (21.5%, 169/785), HPV52 (12.2%, 96/785), HPV58 (9.8%, 77/785), HPV33 (9.7%, 76/785) and HPV18 (7.5%, 59/785). The HPV infection rate was high among cases of HSIL and cases of SCC and ACA. Single-type HR-HPV infection was encountered in 45.0% (353/785), while multi-type HR-HPV infection (including ≥2 types) was found in 17.8% (140/785). There were 98 cases of two-type infection, 37 cases of three-type infection, 2 of four-type infection, 2 of five-type infection and 1 case of six-type infection. As presented in Fig. 1 and Table II, there were 478 cases of ASCUS and AGC, 207 of which possessed single-type infection (43.3%)

and 33 cases possessed two-type infection (6.9%). A total of 175 cases of LSIL were identified, among which 101 cases had single-type infection (57.7%) and 34 had multi-type infection (19.4%; two- and three-type). There were 127 cases of HSIL, 45 of which had single-type HR-HPV infection and 68 had multi-type infection (53.5%; two- and four-type infection). A total of 5 cases of SCC and ACA were identified, all of which had multi-type infection; among them, 2 cases were of three-type infection, 1 of four-type, 1 of five-type and 1 of six-type infection (Tables II and III, and Fig. 2).

Age distribution of HR-HPV-infected patients. As presented in Fig. 2, different age groups had a different infection rate of HPV. χ^2 (line x column) testing revealed χ^2=377 and the difference was statistically significant (P<0.05). Further comparison at α=0.0014 demonstrated that the age groups with the highest infection rates were those of 26-30 and 51-55 years, while the infection rate was lowest in patients aged 56-60 and 61-65 years.

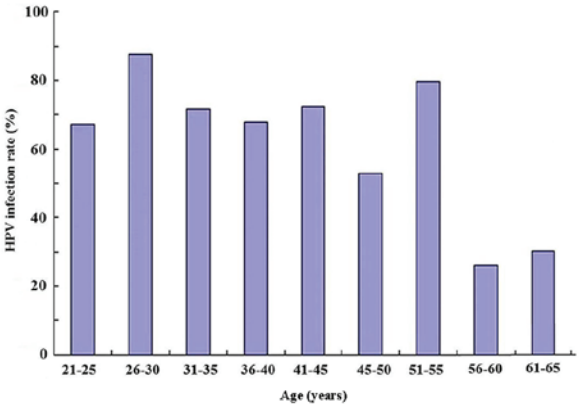

Figure 1. Rate of HPV infection in different age groups recalled for Thinprep liquid-based cytological testing. HPV, human papillomavirus.

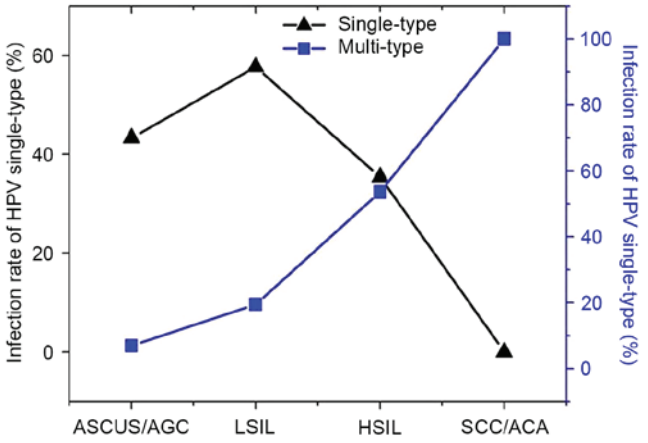

Figure 2. Comparison of infection rate of single- and multi-type high-risk-HPV in subjects with different Thinprep liquid-based cytological test results. HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ACA, adenocarcinoma.

Discussion

Epidemiological and biological data have confirmed that continuous HR-HPV infection is the main cause of cervical cancer and its pre-cancerous lesions. There is a dispute with regard to whether HPV testing should be used as a primary screening method for cervical cancer. It is worth mentioning that cytology is not effective in the screening of cervical intraepithelial neoplasia grade 2+ (CIN2+) lesions. Analysis of European and North American cases indicated that the sensitivity rate is only 53%, giving rise to a new era of molecular testing for HPV DNA (24). In May 2003, the US FDA approved the joint application of TCT and HPV-DNA HC2 testing for the primary screening of women aged ≥30 years (17). In 2005, the World Health Organization's International Agency for Research on Cancer (IARC) recommended HR-HPV testing for use in the primary screening of cervical cancer (22). Stratified management of HR-HPV-positive patients is performed according to cytological testing results. Once women were tested as double negative, they are not tested for ≥5 years, but a test is mandatory after 10 years (25); these guidelines are applied in Liaocheng People's Hospital. Cervical cancer screening guidelines by the American College of Obstetrics and Gynecology was published in 2014 proposed that HR-HPV testing is not recommended for women under the age of 30 years. The reason is that transient infections are common and even if they test positive, the infection will be cleared over a period of time. HPV infection may be transient and only a small number of patients infected will develop cancer after a long incubation period. As there are >20 sub-types (13), testing for single HPV types is not sufficient and a typing test is necessary. In recent years, the Chinese government has listed cervical cancer screening for women at appropriate ages as a major public health service, which is funded by the government. Therefore, the screening effect was evaluated in different regions, institutions and job positions, which may promote continuous improvement of cervical cancer screening work and ensure that the government's policy is implemented. The present study screened patients with abnormal TCT findings and subjected them to HR-HPV testing. It provided an evaluation of the epidemiological distribution of HPV in the local area and allows for comparison with previous studies. The age for testing was 21-65 years, with the lower limit of the age range being earlier than that reported in recent studies (10,26). The purpose of the present study was to identify HR-HPV infection rates in patients with different abnormal TCT results as well as age distribution of infected patients and common genotypes of HPV in the local area through joint testing. The results may lead to the improvement of the effective screening rate of cervical lesions and ensure the future prevention and control of cervical cancer. In the follow-up study, patients whose cytology result was ASCUS were not further assessed in order to avoid unnecessary and intrusive inspection and decrease vaginal examination for HPV-negative patients. However, this did not reduce the discovery of CIN2+ and CIN3+ in patients with ASCUS (27). The diagnostic reliability is improved by eliminating suspicious ASCUS or low-grade lesions. Patients who had an increased risk of developing cervical cancer were differentiated from those with a low risk, which promoted the appropriate use of vaginal examination and pathological

Table III. Epidemiological distribution of HR-HPV [positive rate in n (%)] for subjects with different TCT results in Liaocheng.

HR-HPV type	Screened subjects (n=785)	ASCUS/AGC (n=478)	LSIL (n=175)	HSIL (n=127)	SCC/ACA (n=5)
HPV16	169 (21.5)	76 (15.9)	35 (20)	54 (42.5)	4 (80)
HPV18	59 (7.5)	21 (4.4)	19 (10.9)	15 (11.8)	4 (80)
HPV31	36 (4.6)	15 (3.1)	6 (3.4)	14 (11)	1 (20)
HPV33	76 (9.7)	31 (6.5)	20 (11.4)	23 (18.1)	2 (40)
HPV35	2 (0.3)	2 (0.4)	0 (0)	0 (0)	0 (0)
HPV39	9 (1.1)	9 (1.9)	0 (0)	0 (0)	0 (0)
HPV45	2 (0.3)	1 (0.2)	1 (0.6)	0 (0)	0 (0)
HPV51	24 (3.1)	9 (1.9)	3 (1.7)	12 (9.4)	0 (0)
HPV52	96 (12.2)	28 (5.9)	25 (14.3)	40 (31.5)	3 (60)
HPV53	23 (2.9)	14 (2.9)	6 (3.4)	2 (1.6)	1 (20)
HPV56	51 (6.5)	20 (4.2)	15 (8.6)	15 (11.8)	1 (20)
HPV58	77 (9.8)	24 (5)	22 (12.6)	29 (22.8)	2 (40)
HPV59	9 (1.1)	5 (1)	4 (2.3)	0 (0)	0 (0)
HPV66	18 (2.3)	6 (1.3)	9 (5.1)	2 (1.6)	1 (20)
HPV68	24 (3.1)	10 (2.1)	11 (6.3)	2 (1.6)	1 (20)
HPV73	2 (0.3)	2 (0.4)	0 (0)	0 (0)	0 (0)

Patients with abnormal TCT results included those with combined multiple HR-HPV infection. HR-HPV, high-risk human papillomavirus; TCT, Thinprep liquid-based cytological test; ASCUS, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ACA, adenocarcinoma.

biopsy. In the follow-up study, screening time was relaxed for women negative on HPV testing.

Recent random comparisons among follow-up visits for HPV screening indicated that HPV DNA testing detects more high-level lesions than cytology (22). HPV-negative women have a low risk of developing CIN, and screening for them is performed at intervals of ≥5 years (27). This largely reduces the screening cost as well as the psychological and economic burden of women screened. The present study found that HPV genotypes in different stages of cervical pre-cancerous lesions were significantly different. The infection rate of HR-HPV increased with the degree of cytological abnormality. This verified that HR-HPV infection is associated with the development of cervical lesions and it is the cause of cervical cancer. This result indicated that HR-HPV infection may predict high-level cervical lesions and high-degree abnormalities on cytology may suggest a high probability of HPV infection. Therefore, it is important for patients with abnormal cytological findings to be subjected to a HPV typing test. Individual evaluation may be performed in order to predict the risk of cervical lesions and determine a personal treatment plan. This is in consistent with the results of previous studies (28,29).

In the present study, multi-type infection mostly comprised two-type infection, but hybrid infection of three, five or six types also occurred. Multi-type HR-HPV infection is more dangerous than single-type infection with regard to the generation of cervical cancer and HSIL, which has been demonstrated by previous studies (30,31). In the present study, differences in multi-type HR-HPV infection between groups with different TCT results were statistically significant. Although the impact of multi-type infection on the molecular mechanism and epidemiology of cervical cancer remains to be fully elucidated, follow-up visits for multi-type infection patients should be increased and further examination should be performed when necessary. The HPV genotype distribution has inter-regional differences and the 15 common sub-types reported by the IARC are HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68 and -73 (32).

The present study found that HPV16 was common in Liaocheng (China), followed by HPV52 and -58, while HPV18 was uncommon. However, HPV52 and -58 were associated with a lower incidence rate of cervical cancer than HPV18, indicating that HPV18 may have a greater carcinogenic effect or accelerate the development of cancer to a greater extent than the former types.

A recent study demonstrated that in a susceptible population, the 9vHPV vaccine prevented diseases associated with HPV31, -33, -45, -52 and -58, led to the production of antibodies against HPV6, -11, -16 and -18 and did not perform worse than the qHPV vaccine (33). HPV typing revealed the genotype distribution in the region, which may provide scientific evidence for future screening and vaccine development.

A large-scale epidemiological survey identified age differences among HPV-infected individuals in different regions and populations (34). In line with this, the present study also found that the HR-HPV infection rate differs among different age groups. Among the 785 patients with abnormal TCT findings, the age groups of 26-30 and 51-55 years had the highest infection rates of 87.7 and 79.7%, respectively. The infection rate was low women aged >55 at 28.6%. The

rate of HPV infection is high in women aged >30 years due to frequent sexual intercourse. However, their immune function is relatively strong, and most of the HPV infections are only transient and may be eliminated by the immune system. With increasing age, the function of the immune system decreases and the HPV infection rate increases. Changes of estrogen and progesterone levels in the peri-menopausal period are relatively large and is affect the metaplasia of the cervical epithelium. HPV replication may be supported by squamous epithelial metaplasia through its natural proliferation and differentiation process, thus increasing the HPV infection rate. Therefore, follow-up visits are important for women in the peri-menopausal period. A previous study demonstrated that with the polarization of cervical pre-cancerous lesions regarding the incidence age of cervical cancer, attention should be paid to women around the age of 30 years and those after the age of 50 years (35). Follow-up visits should be increased for HPV-positive patients. This has an important role in the prevention and treatment of cervical cancer.

In conclusion, the present study focused on the epidemiology of HPV infection in a population of patients with abnormal findings on cervical cytology in Liaocheng (China). According to the screening results, as compared with cytological screening on its own, HR-HPV typing is an effective screening method for cervical cancer. Identification of high-risk patients may strengthen the management of cervical cancer screening in the region in order to perform individualized treatment. Patients with abnormalities on cervical cytology in the region had a high infection rate of HPV16, -52, -58, -33 and -18. Multi-type infection was found to increase the risk of disease. In the region, high-risk individuals are mostly aged 26-30 and 51-55 years, and should therefore be subjected to stringent screening and follow-up visits. The genotype distribution in the region may provide scientific evidence for future screenings and vaccine development.

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