Prevalence of Single and Multiple Human Papillomavirus Types in Cervical Cancer and Precursor Lesions in Hubei, China

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**Introduction**

Cervical cancer is the second most common type of cancer in females worldwide. In 2000, approximately 468,000 new cases were diagnosed, of which 80% were from developing countries [1]. About 132,300 new cases of cervical carcinoma were reported from China, constituting an overall incidence rate of over 27 per 100,000 women [1]. The incidence, however, varied between 2.54 per 100,000 in Beijing and 1,073.34 per 100,000 in Wufeng in Hubei, a region of central China. The rate in Wufeng is 422 times that of Beijing [2]. This high incidence of cervical carcinoma may reflect a poor screening program [3] and differences in the type of human papillomavirus (HPV) infecting the Chinese population.

Molecular, clinical, and epidemiological studies have implicated HPV infection in the pathogenesis of cervical carcinoma. Approximately 90% of cervical carcinoma contains DNA sequences of specific HPV types, especially HPV 16 and HPV 18 [4]. The prevalence of HPV association with cervical carcinoma can vary significantly in distinct geographical areas and histologic type [5, 6]. Globally, it has been shown that HPV 16, 18, 45 and 31 are the most prevalent HPV types associated with cervical cancer [7, 8]. However, few studies have assessed the prevalence and diversity of HPV in cervical carcinoma and its precursor in Chinese women. Published reports...
were limited to samples from Hong Kong, Taiwan and Southern China [9–11]. Little is known about the prevalence of specific HPV types in cervical carcinoma in Hu- 
bei, central China.

With HPV vaccination studies currently under way, it is important to map the epidemiology of HPV infection. To design vaccination strategies suitable for the Chinese population, an inventory of the prevalence of HPV is essential. The WHO has recommended that further studies be performed to investigate and broaden our knowledge base concerning the prevalence of the different types of HPV, especially in populations at high risk of cervical carcinoma, in order to define a future vaccine formula [12]. Moreover, the distribution and frequency of different HPV types have implications in the choice of diagnostic methods and epidemiological studies involving disease control.

Due to the limited published data on HPV prevalence rates, it is not yet known which HPV types are more prevalent in cervical carcinoma and its precursor in Hubei. The aim of this study, therefore, was to establish which HPV types are more prevalent in cervical carcinoma and cervical intraepithelial neoplasia (CIN) in central China.

Materials and Methods

Study Subjects

From June 2004 to December 2006, 126 patients with cervical squamous-cell carcinoma (SCC) and 65 patients with CIN II–III were referred to Zhongnan Hospital. Among these, 112 inpatients with SCC who received radical hysterectomy and bilateral pelvic lymphadenectomy and 60 inpatients with CIN II–III (CIN II, n = 40; CIN III, n = 20) who received hysterectomy as a primary treatment were tested for HPV. The mean age of the patients with cervical cancer was 44 years (range 22–65 years), while the mean age of the patients with CIN II–III was 37 years (range 21–52 years) (table 1). The clinical stages of cervical cancers were IB–IIA (stage IB, n = 68; stage II A, n = 44). All patients underwent physical and gynecological examinations and were asked privately if they had any questions regarding participation in the study.

These cervical samples were obtained from women undergoing biopsy or surgery. Following cervical punch biopsy, a small piece of tissue was sent for histopathologic examination by two independent pathologists. The rest of the specimens were stored at 4°C for no more than 24 h before being cut into small fragments and stored as multiple aliquots at ~80°C. The study protocol was approved by the ethical committee of Wuhan University. Written informed consent was obtained from the study subjects.

Detection and Typing of HPV

DNA was extracted from the samples by a standard SDS-proteinase K-phenol chloroform-ribonuclease-ethanol precipitate method [13]. The DNA samples were quantitated spectrophotometrically at 260 nm and examined by 0.8% agarose gel electrophoresis.

All the specimens were first subjected to PCR amplification with a pair of primers MY11/MY09 (5'-GCA CAG GGT CAG AAC AAT GG-3' and 5'-GCA CAG GGT CAG GGAGGTAAT TGA TC-3'). DNA quality and integrity were monitored through amplification of part of the β-globin gene in replicate tubes. Amplification without a DNA template was used to monitor contamination in both HPV and β-globin reactions. PCRs were performed using sterile 0.5-ml RNase-/DNase-free tubes and each PCR was made up to a final volume of 50 µl, which contained 100 mM KCl, 20 mM Tris-Cl pH 8.0, 2.0 mM MgCl₂, 2.5 mM of dNTP, 1.5 units of Taq polymerase (Promega Corp., Madison, Wisc., USA), 25 pmol of each primer, and 2 µg (1 µg/µl) of DNA. After thermal cycling (initially for 90 s at 94°C for 1 cycle; then 40 cycles at 55°C for 1 min, 72°C for 1 min, and at 94°C for 1 min, and finally 1 cycle at 72°C for 10 min); the amplified DNA fragments were identified by electrophoresis in 1.5% agarose gel with ethidium bromide.

The HPV-positive specimens were subjected to cycle-sequencing PCR with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. Sequencing reactions were then run on the ABI Prism 310 Genetic Analyzer. Viral sequencing was analyzed by sequence analysis software and a sequencing navigator.

The specimens negative for HPV in the first-round PCR or those with ambiguous sequences were further tested by a second PCR with type-specific primers OXL007/OXL008 (primer 1: 5'-ATGTTCAGGGCACCAGAGA-3'; primer 2: 5'-CAGCTGG-GTTTCTCACTGTGT-3') derived from the E6 open reading frames of HPV DNA. This method avoided false-negative results due to the disruption or deletion of the L1 region.

Statistical Analysis

Categorical data were analyzed for statistical significance by the χ² or Fisher exact test, as appropriate. A p value <0.05 was considered significant. Statistical analysis was performed using SPSS software (version 11.0).

Results

The rates of HPV infection and the distribution of the different HPV types are shown in tables 1 and 2. HPV genotypes including HPV 16, 58, 31, 18, 52, 33, 59, 35, 11 and 6 were detected in patients with cervical carcinoma and CIN II–III. Out of the 112 patients with SCC, HPV DNA was identified in 105 cases, HPV 16 being detected in 91 cases, HPV 58 in 7 cases, HPV 31 in 5 cases, HPV 18 in 4 cases; the remaining 7 patients were HPV negative. Among the 60 patients with CIN II–III, HPV DNA was detected in 50 cases, HPV 16 being detected in 37 cases, HPV 58 in 5 cases, HPV 31 in 3 cases, HPV 18 in 2 cases; the remaining 10 patients were HPV negative. When the prevalence rates of the HPV types detected in the cervical cancer specimens were compared with those
Prevalence of Single and Multiple Human Papillomavirus Types

The development of HPV vaccines holds tremendous promise for developing countries like China, where cervical cancer is the most common malignancy in middle-aged women [1]. The availability of an HPV vaccine will not only help in curbing the incidence and mortality of cervical cancer, but it will also bring down the cost burden for cervical cancer screening programs [14]. To maximize the cost effectiveness of HPV vaccination programs in China, it is important to understand the distribution of the major HPV types in various geographical regions.

We therefore evaluated the prevalence and genotypes of HPV in cervical cancer and precursor lesions in Hubei, central China, where there is a high incidence of cervical cancer. HPV DNA was identified in 93.8% (105/112) of cervical cancers, which is close to the reported prevalence rates of HPV DNA in women from southern China [10, 11, 15], Taiwan [16] and Korea [17, 18], and lower than the 99% reported previously in southern India [19]. The HPV prevalence rate in CIN II–III was 83.3%, which falls within the reported prevalence rates of HPV DNA (84.3–90.2%) in women from Asia [12, 20]. These results may reflect differences in HPV detection sensitivity. The method used in India amplified an HPV target of –150 bp [19], whereas our amplification product using the MY09/11 primer is much larger (450 bp). Therefore, DNA degradation in some of the samples could have led to a false-negative result and an underestimation of the prevalence of HPV. Moreover, the inability to detect HPV DNA in patients could be due to sample collection and storage or to yet unknown HPV types, not amplified by the existing PCR primers or the presence of only few copies of HPV DNA.

detected in the CIN specimens, only women with HPV 16 infection showed a statistically significant higher rate of cervical cancer (χ² = 7.87, p = 0.005). HPV 18 was not common in this study (table 2). The negative samples using the primer MY09/11 remained negative when primer OXL007/OXL008 was used.

Multiple HPV types were identified in 17 of 172 cases (table 2). Five cases of CIN II–III and 12 cases of cervical cancer exhibited multiple infections. There was no statistically significant increase in multiple infections in the cervical cancer specimens. HPV 16 was not significantly associated with multiple infections (p = 0.528) although HPV 16 was the most common genotype detected in multiple infections, with 15/17 multiple infections containing HPV 16; the most common multiple infection was HPV 16 with HPV 31 (5/17 multiple infections). HPV 31 was significantly associated with multiple infections (p = 0.001) and was detected in 8 patients (5 with cervical cancer and 3 with CIN II–III), of whom 7 had multiple infections (5 patients with cervical cancer and 2 patients with CIN II–III). HPV 58 was always found as single infection.

Discussion

The development of HPV vaccines holds tremendous promise for developing countries like China, where cervical cancer is the most common malignancy in middle-aged women [1]. The availability of an HPV vaccine will not only help in curbing the incidence and mortality of cervical cancer, but it will also bring down the cost burden for cervical cancer screening programs [14]. To maximize the cost effectiveness of HPV vaccination programs in China, it is important to understand the distribution of the major HPV types in various geographical regions.

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The application of a PCR strategy designed to amplify a larger HPV target sequence here could have decreased our HPV detection rate. Therefore, the HPV-negative specimens or specimens with undetermined sequences were further tested by a second PCR with type-specific primers OXL007/OXL008 derived from the E6 open reading frame of HPV DNA, which did not yield any additional positive cases.

In this study, the most prevalent HPV type in Hubei, China, was HPV 16. The HPV types identified were, in order of decreasing prevalence, HPV 58, 31, 18, 52, 33, and 59. Other HPV types were detected in no more than 1% cervical cancers. The most striking feature of the genotype distribution in the biopsies was the high prevalence rate of HPV 16 infection (81.3% in cervical cancer and 61.7% in CIN II–III), a proportion that is similar to that found in surveys by Wu et al. (79.6%) [11], and somewhat higher than the prevalence rates found by others, which range from 56.2 to 64.1% in parts of Europe and the USA [5].

Geographical differences in HPV types have been reported to exist among countries [21]. According to a recently published meta-analysis by the International Agency for Research on Cancer [4], HPV types 18, 52 and 58 are more prevalent in Asia (Japan, Taiwan, Hong Kong and some southern provinces of China); however, HPV types 18, 31, 33 and 45 are more frequently seen in the other continents. HPV 58 has been found in only 2% of cervical cancers in the western world. Our results show that HPV 58 was the second most common type, accounting for 6.3% of cervical cancers and 8.3% of CIN II–III. The prevalence of HPV 18 (3.6% in cervical cancer, 3.3% in CIN) was relatively low in Hubei, China. The distribution of HPV types found in our study is different from previous reports in Asia [4, 11, 15, 20]. Such HPV typing information is very important for further HPV vaccine design and applications. It would have been important to evaluate a control population (a screening population) as well to evaluate the prevalence and type distribution of HPV at a more general level in women in Hubei.

The clinical role of multiple types of HPV infection is still unclear. Ho et al. [22], investigating the natural history of cervicovaginal HPV infections in young women, defined an odds ratio of 4.1 (95% CI = 2.7–6.3) associated with the presence of multiple types over a 6-month period for persistent HPV infections that is considered as a major factor of progression to cervical preinvasive lesions. Two other studies [23, 24], on the contrary, showed that persistence of HPV infection was independent of coinfection with other HPV types. The explanation is that not all PCR-based methods are equally sensitive in detecting multiple infections because of limitations in the number of HPV types detectable and assay performance [20]. Another possibility is the difference in sensitivity for distinctive HPV types between different test systems used and the reproducibility of different HPV tests for determining the exact HPV type in the sample. The rate of multiple infections in this study was not significantly higher than that of single infections (p = 0.79), and the percentage of mixed-type HPV infections was relatively low as compared to other published data. Whether the rate of mixed-type HPV infection in Hubei is really low or whether the PCR assay to detect multiple-type HPV infections was not sensitive enough would need further evaluation.

**Conclusion**

The high prevalence of HPV 16 and HPV 58 in Hubei, China, deserves special attention in future vaccination programs to effectively lessen the burden of cervical cancer in China. The findings also point to the necessity of organizing screenings for HPV detection, in particular HPV types 16 and 58.

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