

RESEARCH ARTICLE

Low Prevalence of HPV in Male Sexual Partners of HR-HPV Infected Females and Low Concordance of Viral Types in Couples in Eastern Guangdong

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Abstract

Objective: To investigate the prevalence of genital high-risk human papillomavirus (HR-HPV) in male sexual partners of HR-HPV infected women and the concordance of viral types in couples in China, and comprehend the role of men play in HPV transmission to women. **Methods/Materials:** 94 asymptomatic women and their husbands from rural Chaozhou participated in epidemiologic screening for HPV infection. Cervical cells from females were collected for high risk HPV screening by real time-PCR, and they were positive for at least 1 of 13 HR-HPV subtypes, then these samples were genotyped. Approximately one month later, penile epithelial cells from 94 asymptomatic husbands were collected for HPV genotyping. At the same time, a cross-sectional study was conducted in 366 male patients from sexually transmitted disease (STD) outpatient clinic in China for the prevalence of genital HR-HPV infection in men having frequent sex behavior. Penial epithelial cells were collected for HPV 6/11 and HPV 16/18 detection by fluorescent real-time quantified PCR. **Results:** Among 94 couples, the prevalence of genital HR-HPV infection in men whose wife was positive for cervical HR-HPV was 5.32% (5/94). Only 2.63% (2/76) had the same high risk viral type presented by their wife. HPV 16 proved to be the most prevalent viral type in men and in couples. Of 366 male patients from STD outpatient clinic, the prevalence of HPV 16/18 infection in men with or without HPV 6/11 was 6.85% and 8.16%, respectively. The incidence of HPV 16/18 was higher in men aged more than 35 years than the young men (18-35 years). **Conclusion:** The prevalence of genital HR-HPV infection in male sexual partners of HPV-positive women in China was lower than that expected, and the concordance of high risk viral type between couples was extremely low. These data suggested that infected men constitute an important viral reservoir, contributing to transmission of HR-HPV to women and maintenance of infection, but HR-HPV infection may be less likely to persist in men than in women.

Keywords: Genital HR-HPV - prevalence - men - China - type-specific concordance - couples

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Introduction

Human papillomavirus (HPVs) are members of the papillomaviridae family that was capable of infecting humans. Currently, over 120 subtypes have been identified. About 30 to 40 subtypes can transmit through sexual contact and infect the anogenital region (Dunne et al., 2006). Anogenital HPV types have been classified into low-risk types, which were associated with anogenital warts and mild dysplasia, and high-risk types, which compared with low-risk types, may lead to the development of cervical intraepithelial neoplasia (CIN), vulvar intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN) and anal intraepithelial neoplasia (AIN) (Foresta et al., 2011). HPV infection is very common among men and women across all geographical, racial and socio-economic subgroups worldwide.

Most previous studies have focused on HPV infection

in women because of the connection between HPV infection and cervical cancer. The cause-effect relationship between HPV and cancers of the uterine cervix, vagina, and vulva has been deeply studied, indicating very clear mechanisms of interaction (Cheung et al., 2009). At present, the interest in understanding the burden of HPV infection and disease among men is increasing. In earlier work, much of this interest focused on the role of the male sexual partners of women with genital HPV infection in the progression of cervical cancer. Over the past several years, many studies found that HPV causes substantial disease in men, such as PIN and AIN (Giuliano et al., 2010).

The prevalence of genital HPV varies depending on world region and population source, ranging from 14% to more than 90% in women and from 1.3% to 72.9% in men (Flores et al., 2008). In China, the HPV prevalence in women ranges from 8.8% to 19.6%, and the prevalence of HR-HPV infection in women ranges from 7.2% to

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15.2 %, but there aren't any study for HR-HPV infection in asymptomatic men. At present, no data is available concerning natural history of genital HR-HPV infection and progression to disease in men. Most importantly, there is a knowledge gap with regard to the concordance of HR-HPV status between the sexual partners. According to our knowledge, information on HPV infection and serologic responses to infection in men is informative for evaluations of the potential impact of prophylactic HPV vaccines. Here, we reported a study to investigate the prevalence of genital high-risk HPV in men whose wife presented cervical HPV infection, and to evaluate the type-specific concordance in a predominantly monogamous population in China.

Materials and Methods

Study Population

An epidemiologic screening for cervical HR-HPV infection was organized by Chaozhou municipal government (Chen et al., 2012). Eligible women underwent a gynecological examination, exfoliated cervical cells were collected for HPV screening by real time-PCR (real time-PCR could detect 13 subtypes of HR-HPV, but could not genotype). And then the HR-HPV positive samples received further examination for HPV genotype by HPV GenoArray test (Chen et al., 2012). A total of 48559 eligible rural Chaozhou women participated in the HR-HPV screening, real time-PCR revealed that 3830 cases were positive for HR-HPV DNA, and the crude

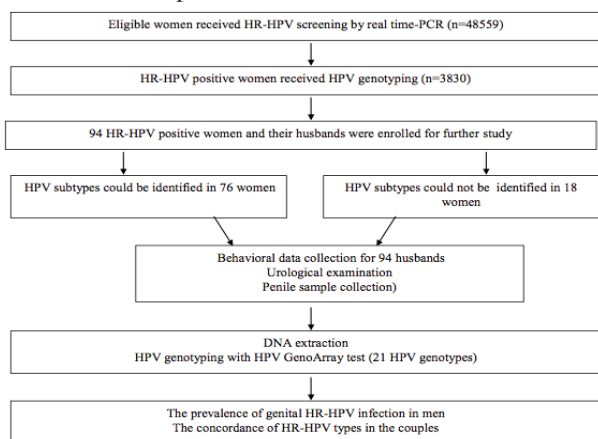


Figure 1. The Designed Details of HPV Type-specific Concordance in the Couples

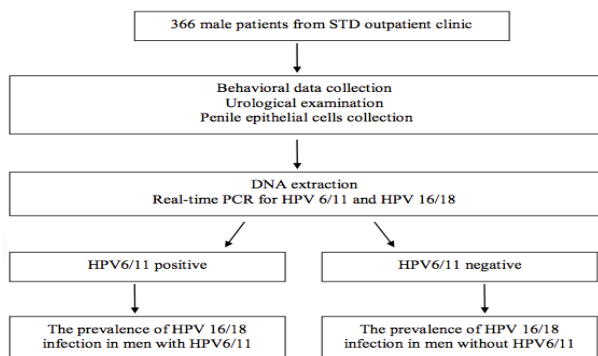


Figure 2. The Designed Details of Genital HR-HPV Prevalence in Male Patients from STD Outpatient Clinic

infection rate was 7.89% (3830/48559) (Chen et al., 2012). 94 HR-HPV positive women and their husbands (aged more than 35 years) were enrolled for the prevalence of genital HR-HPV infection and the concordance of high risk viral types in couples. It was to emphasize that the 94 women were permanent residents of Chaozhou rural area, and had given birth at least once and 80 of them received the tubal ligation. All of the 94 couples were in a monogamous relationship, which was defined as a stable sexual partner for more than one year. The designed details of HPV type-specific concordance in couples were shown in Figure 1.

Furthermore, to understand the incidence of genital HR-HPV infection in men having frequent sexual behaviors in eastern Guangdong Province, 366 male patients (aged from 18 to 65 years) from STD outpatient clinic in Chaozhou Central Hospital were willing to participate in the study. The participants underwent the urological examination, and penile epithelial cells were collected for HPV 6/11 and HPV 16/18 detection by real time PCR. The designed details of genital HR-HPV prevalence in male patients from STD outpatient clinic were shown in Figure 2.

All studies were approved by Chaozhou Central Hospital Ethics Committee. Information concerning the research project was provided to all participants, and all signed a free and informed consent form approved by the institutional ethics committee of Chaozhou Central Hospital.

Penile Epithelial Cell Collection

Male participants, including husbands of HR-HPV positive women and STD outpatients, were instructed not to urinate 2 hours before the urological examination. Their external genitalia, including the urethral meatus, glans, corona and prepuce, were submitted to visual inspection. Urethral epithelial cells were collected by inserting a sterile and small brush (DaAn Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China) into the urethral meatus and 2 to 4 cm of the urethra and rotating it (Della et al., 1992). Then more sufficient epithelial cells were brushed from the glans and prepuce internal surfaces, including the sulcus and corona (De et al., 2008). All the material was placed in transport tubes. Cells were suspended in 2 ml phosphate buffered saline (PBS) solution and stored immediately at 4 °C. All the specimens were finally sent to our clinical laboratory for HPV DNA analysis.

Flow-through Hybridisation and HPV GenoArray test

The samples from 94 couples received HPV genotyping. HPV genotypes were detected by a commercial HPV GenoArray test Kits (Chaozhou Hybribio Biotechnology Limited Corporation, China), which could detect 21 HPV genotypes, including 15 high-risk HPV (HR-HPV) (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66 and 68) and 6 low-risk HPV (LR-HPV) (HPV 6, 11, 42, 43, 44 and CP 8304).

The DNA Extraction from samples was described previously (Lin et al., 2008). HPV DNA was amplified with the L1 consensus HPV PGMY09/PGMY11 primer

Table 1. The HPV Genotypes of the 94 HR-HPV Positive Women

| HR-HPV genotype | n | Age (years) |
|--------------------------|----|-------------|
| HPV 52 | 15 | 36-53 |
| HPV 16 | 9 | 38-53 |
| HPV 58 | 8 | 40-55 |
| HPV 18 | 4 | 40-56 |
| HPV 31 | 3 | 42-57 |
| HPV 33 | 2 | 53,56 |
| HPV 51 | 2 | 40,45 |
| HPV 56 | 2 | 41,55 |
| HPV 68 | 2 | 46,51 |
| HPV 35 | 1 | 51 |
| HPV 39 | 1 | 58 |
| HPV 53 | 1 | 44 |
| HPV 59 | 1 | 37 |
| Multiple HPV (2-5 kinds) | 25 | 37-57 |
| Non-determined genotype | 18 | 36-59 |

set, as previously described (Lin et al., 2008). 1 μ L HPV DNA was mixed with 24 μ L reaction system (HybriBio Biotechnology PCR Kit). Reaction system was prepared with 4 mmol/L of MgCl₂, 50 mmol/L of KCl, 7.5 U of AmpliTaq Gold DNA polymerase, 200 μ mol/L of each dNTP, 600 μ mol/L of dUTP, 1 U of uracil-N-glycosylase, 100 pmol of each biotinylated PGMY primer, and 2.5 pmol of each 5'-biotinylated β -globin primer GH20 and CP04. The amplification process was performed in Thermal Cycler MJ Mini (BIO-RAD Company), and consisted of 40 cycles of denaturation at 93 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 40 s.

Genotyping for HPV was accomplished by flow-through hybridisation and gene chip using HybriMax (Lin et al., 2008; Chen et al., 2012). The gene chip contained type-specific oligonucleotides immobilised on a nylon membrane. The final results were detected by colourimetric change on the gene chip under direct visualization.

Fluorescent Real-time Quantified PCR for HPV 6/11 and HPV 16/18

366 male patients from STD outpatient clinic received real time PCR detection for HPV 6/11 and HPV 16/18. The brush in PBS solution was vigorously vortexed. Then the brush and the supernatant were removed after the cells were centrifugated with relative centrifugal force of 9660 g for 5 min. The sediments obtained were extracted by Alkali lysis using DNA extraction Kits (DaAn Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China).

HPV 6/11 (HPV 6 and/or HPV 11) and HPV 16/18 (HPV 16 and/or HPV 18) were detected by TaqMan MGB fluorescent real-time quantitative PCR in LightCycler® 2.0 Real-Time PCR System (Roche Applied Science, Germany), using HPV 6/11 and HPV 16/18 PCR fluorescent diagnostic kits (DaAn Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China), respectively. Each reaction was comprised of 1 \times Roche Lightcycler TaqMan master mix, HPV specific primer pairs and fluorescently-tagged probes for HPV 6/11 or 16/18 in a total reaction volume of 10 μ l (Seaman et al., 2010). Thermal cycle conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of alternating 95 °C for 15

Table 2. The Concordance of HR-HPV Types in the 5 Couples

| Men | | Women | |
|--------------|-------------|---------------------|-------------|
| HPV genotype | Age (years) | HPV genotype | Age (years) |
| HPV 16 | 54 | HPV 16 | 53 |
| HPV 16 | 40 | HPV 16 | 38 |
| HPV 18 | 42 | HPV 33 | 42 |
| HPV 33 | 45 | HPV 58 | 41 |
| HPV 58 | 59 | Non-determined type | 59 |

s and 60 °C for 30 s. For standard curves, real-time PCR was performed on a 10-fold dilution series of each purified plasmid containing a type-specific L1 amplicon ranging from 2 \times 10¹ to 1 \times 10⁶ copies. A melting curve, starting at 40 °C and increasing by 0.5 °C every 10 s until 120 °C, was reached to verify the specificity of the obtained amplicons.

Statistical Analysis

Statistical significance for the prevalence of different HPV types between age groups was tested by using Pearson χ^2 test and considered significant when $p < 0.05$.

Results

HPV GenoArray test

94 HR-HPV positive women and their husbands (aged more than 35 years) were enrolled for the prevalence of genital HR-HPV infection and the concordance of high risk viral types in couples. All of the 94 women were positive in HPV screening by real time-PCR. However, when HPV GenoArray test was used for HPV genotyping, only 76 cases could be genotyped, and the others (n=18) could not be genotyped, possibly due to lower viral loads. The detailed data of the 94 HR-HPV-positive women was shown in Table 1.

Among 94 husbands whose wife presented cervical HR-HPV infection, 5.32% (5/94) were identified with penile HR-HPV infection. The remaining 89 samples were negative for HR-HPV. Each positive male sample presented only one type of HR-HPV infection. HPV 16 proved to be the most prevalent viral type in men (n=2).

Among 76 females in which HPV genotypes could be identified, their husbands were tested for HPV infection by HPV GenoArray test, 4 cases were identified with genital HR-HPV infection, including 2 cases of HPV 16, a case of HPV 18 and a case of HPV 33. The two men with HPV 16 had the same HPV type presented by their wife, while the man with HPV 18 (his wife with HPV 33) and the man with HPV 33 (his wife with HPV 58) did not share the concordance of high risk viral type with their wife. Therefore, 4 couples were concurrently infected with HR-HPV, and 2 out of 4 couples showed the concordance of high risk viral type. The prevalence of genital HR-HPV was 5.26% (4/76), and the concordance of at least one high risk viral type was observed in 2 of 76 couples (2.63%). HPV 16 was the most prevalent in couples where both were HR-HPV positive. The concordance of HPV genotype between the 94 couples was shown in Table 2. In 18 husbands of HR-HPV positive wives whose HPV genotypes could not be determined, only one man was

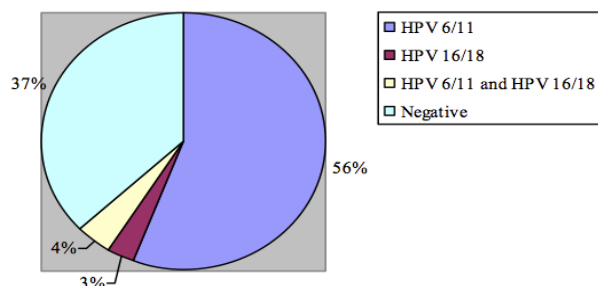


Figure 3. The Prevalence of the Different HPV Types among the 366 Male Patients from STD Clinic

Table 3. The Prevalence of the Different HPV Types among the 366 Male Patients from STD Clinic According to Age

| Age (years) | HPV type n (%) | | |
|-------------|----------------|--------------|------------------------|
| | HPV 6/11 | HPV 16/18 | HPV 6/11 and HPV 16/18 |
| Total | 204/366 (56%) | 12/366 (3%) | 15/366 (4%) |
| 18-35 | 133/241 (55%) | 3/241 (1.2%) | 7/241 (2.9%) |
| >35 | 71/125 (57%) | 9/125 (7.2%) | 8/125 (6.4%) |

detected with genital HR-HPV infection, the genotype was HPV 58. The prevalence was 5.56% (1/18).

Fluorescent Real-Time Quantitative PCR for HPV 6/11 and HPV 16/18

Among 366 male patients from STD outpatient clinic, the prevalence of HPV 6/11, HPV 16/18, and the mixed infection (HPV 6/11 and HPV 16/18) were 55.73% (204/366), 3.28 (12/366), and 4.1% (15/366), respectively (Figure 3).

The prevalence of HPV 6/11 infection did not change greatly by comparing men aged 18-35 years and those aged more than 35 years ($p=0.825$, $p>0.05$) (Table 3). However, it was observed that the prevalence of HPV 16/18 or the mixed infection (HPV 6/11 and HPV 16/18) was higher among men more than 35 years than the younger men (18-35 years) ($p=0.004$, $p<0.05$).

The incidence of HPV 16/18 in men with or without HPV 6/11 infection was 6.85% (15/219) and 8.16% (12/147), respectively. No significant difference was observed for the prevalence of high risk HPV between men with and without low risk HPV infection ($p=0.686$, $p>0.05$).

Discussion

The prevalence of HPV infection in men varies depending on world region and population source, ranging from 1.3% to 72.9% (Simon et al., 2010). A high prevalence of genital HR-HPV among male sexual partners of women with and without cervical HPV infection in America (such as Mexico and Brazil) and Europe (such as the Netherlands and Denmark), as compared to those detected in Asian countries (Japan and South Korea), has been also reported in the previous literature (Simon et al., 2010). In Brazil and the Netherlands, the prevalence of HPV in men was 70% and 72.9%, respectively (Bleeker et al., 2005; Nicolau et al., 2005). In Japan, the incidence of HR-HPV in men absolutely decreased, ranging from

1.3% to 5.9% (Takahashi et al., 2003; Takahashi et al., 2005). In South Korea, 4.2% of the male students analyzed presented penile HR-HPV infection (Shin et al., 2004). The most possible explanation for this discrepancy may be due to the difference in sexual behavior between different countries. The risk factors for HPV infection in men are similar to their female counterparts: age at the initial sexual intercourse, number of sexual partners, high frequency of sexual intercourse and immuno-deficiency syndrome (Svare et al., 2002). Life-time number of sexual partners is the most significant risk factor for the acquisition of HPV infection (Silins et al., 2000). According to the previous report, men in American and European countries mostly had a history of 10 or more sexual partners over their lifetime, and they even had sexual relations with prostitutes (Parada et al., 2010). This sexual behavior is more popular than that in Asian countries. However, in China, no data is available concerning the incidence of genital HR-HPV infection in men. The actual incidence and natural history of HR-HPV infection in men are less understood than in women obviously.

In this study, 94 rural women presented cervical HR-HPV infection, and most of them have received the tubal ligation, and did not use condom during sexual intercourse. However, the prevalence of genital HR-HPV in male sexual partners of HR-HPV infected women was lower than that expected, and the concordance of high risk viral types between couples was extremely low. Based on our current knowledge, high risk sexual behaviors were strong determinants of HR-HPV infection in men, and the male patients with HPV 6/11 infection from STD outpatient clinic mostly had high frequency of sexual intercourse with multiple sexual partners, even prostitutes over lifetime (Lu et al., 2011). But there was no statistical significance in the incidence of genital HR-HPV infection between men with (6.85%) and without (8.16%) HPV 6/11 in our study. All of our results were in agreement with the previous literatures about the incidence of genital HR-HPV infection in men in Asian countries such as Japan and South Korea, but lower than those detected in American and European countries (Simon et al., 2010). In addition, studies evaluating HPV infection in men who were sexual partners of women with intraepithelial squamous lesions associated with HPV showed that 13% to 63.2% of partners were infected by the same viral type, suggesting that the concordance was more frequent than that expected by chance (Burchell et al., 2010; Reiter et al., 2010). But different from their studies, only 2.63% (2/76) of the male subjects analyzed in our study shared at least one HR-HPV subtype with their wife.

In our study, the HR-HPV infected women and their husbands (aged between 35 and 60 years) were permanent residents of Chaozhou rural area, and they were in a monogamous relationship, suggesting that men mostly had a stable sexual partner. We could not get the numbers of sex partner of the couples, because they refused to answer these questions. Chaozhou was a heavily influenced area by Confucius moral, and peoples here was very conservative in sex (Lin et al., 2008; Chen et al., 2012). Having only one stable sexual partner may be the main reason for the lower prevalence of penile HR-HPV in

men in China than that observed in America and Europe.

There is an increasing body of evidence to suggest that men with HPV infection may play an important role in the transmission of HPV to women and the development of cervical cancer in women as well as cancer at several sites in men (Giuliano et al., 2007; Giraldo et al., 2008). In general, the prevalence of genital HR-HPV infection in men ought to be higher than that in women. However, HR-HPV prevalence and the frequency of HPV-related lesions of the external genitalia in male sexual partners of infected women are reported to be lower. There are many reasons to explain this phenomenon:

Firstly, the detection of HPV in female samples, as compared to male samples, was higher, suggesting that a higher cellular representation may be present in cervical samples, in turn improving the chances of detecting HPV. In men, the presence of DNA-HPV in samples may escape clinical examination. The best anatomic sites for sampling in men - taking into consideration convenience of sampling, adequacy of samples, and detection of DNA-HPV - appear to be the glans, corona, prepuce, and shaft of the penis (Weaver et al., 2004). 5.5% of the circumcised men presented HPV infection, lower than 19.6% in non-circumcised men, suggesting that prepuce was the suitable site for sampling (Castellsague et al., 2002). Urethra, urine and semen often have adequate DNA-HPV, but are difficult and sometimes uncomfortable to collect, and the rate of HPV detection is lower than those collected from glans, coronas, prepuces, and penile bodies (Forslund et al., 1993; Dunne et al., 2006). Moreover, sampling at multiple penile sites, when lesions are not visible, apparently increases the sensitivity of HPV detection. In our laboratory, we collected samples from the glans, coronas, prepuces, penile bodies and urethra, it may be optimal and the minor reason for the low prevalence of HPV in men.

More importantly, the lack of concordance in a majority of couples may be explained by difference in the time required for clearance of HPV infection in men and women (Partridge et al., 2006). Just like infected women, HPV infection usually clears spontaneously over time and only persists in a small proportion of men. However, HPV infection may be less likely to persist in men than in women. In a Dutch study, the persistent infection which was defined as detection of DNA-HPV of a specific type at two consecutive visits over a period of 1 year, was observed in 20% of infected women and 6% of infected men (Van et al., 1994). Short-lasting infection with HPV was demonstrated in 49% of infected men and 31% of infected women (Van et al., 1994). In a Finnish study, a majority of women cleared HPV infection during the prospective follow-up of 6 years (median, 62.4 months; range, 1.6 to 94.5 months) (Louvanto et al., 2010). However, 75% of HPV infection in men disappeared within a 12-month period (median, 5.9 months), without any difference between HPV 6/11 and HPV 16/18 (Giuliano et al., 2008). On the other hand, difference between viral types regarding clearance time may also affect the concordance of HPV between couples. HPV 16 and 18 infection seem to be more persistent than other HR-HPV types (the median duration was 12.19

months for HPV 16), consistent to recent study (Giuliano et al., 2011). Consequently, HR-HPV infection in men are usually transient and cleared to undetectable levels in short duration, this may be the main reason for the low prevalence in men of our study.

In addition, the lack of concordance in a majority of couples may be explained by the fact that the transmission of HPV infection occurs mainly at the beginning of sexual life and is associated with immunity (Giraldo et al., 2006). Immune responses may influence the viral load, the alternation of viral types, the individual propagation of HPV types, and thus the concordance of HPV types between couples with long-term relationships (Maria et al., 2012). Moreover, the gradual development of an effective immune response is thought to be the likely mechanism for HPV DNA clearance (Molano et al., 2003).

The genital HR-HPV requires specific conditions provided by certain sites for infection to occur and develop. HPVs are a large family of small double-stranded DNA viruses that infect the squamous epithelia (Kreider et al., 1985). In particular, the squamocolumnar junction, where basal or reserve cells are undergoing expansion in the form of squamous or columnar cell differentiation (transformation zones), is most vulnerable to HR-HPV. The columnar epithelia in male genitourinary tract are not the suitable site for HR-HPV infection to develop. As a consequence, HPV infection duration is long-term in the squamous epithelia in cervix, while short-term in the columnar epithelia in male genitourinary tract.

As previously described, HPV infection among men followed a bimodal pattern with respect to age, with the peak prevalence among individuals aged 30 to 34 years and 60 to 64 years (Gillison et al., 2012). On the other hand, some literatures revealed that HPV prevalence did not change greatly among men from 20 years old to at least 50 years old (Anna et al., 2008). Here, we found that the prevalence of HPV 6/11 infection did not change greatly between men aged 18-35 years and men older than 35 years, while the prevalence of HPV 16/18 or the mixed infection (HPV 6/11 and HPV 16/18) was higher among men \geq 35 years than the younger men (18-35 years).

In summary, the HR-HPV prevalence of the external genitalia in male sexual partners of HR-HPV infected women was lower than that expected, and the concordance of HR-HPV types between couples was extremely low. The main reason is that genital HR-HPV infection may be less likely to persist in men than in women, in which the squamous epithelia are most susceptible to HR-HPV infection. Men have a significant role in the transmission of HR-HPV to women. The male partners of HR-HPV positive women should undergo a penile HPV test, and HR-HPV positive males should undergo a clinical follow-up. This is important to limit the spread of HR-HPV in couples.

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