Molecular Epidemiological Study on Prevalence of Human Papillomaviruses in Patients with Common Warts in Beijing Area

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Objective To study the circulation, distribution, and genomic diversity of HPVs in common warts in Beijing area of China. **Methods** Forty eight patients with pathologically diagnosed common warts were screened for the presence of HPV with HPV type-specific PCR and direct sequencing analysis. The genomic diversity of HPVs prevalent in Chinese patients was analyzed based on LCR. **Results** Forty one (85.5%) samples were positive for HPV DNA, 13(31.7%)-HPV-57, 12(29.3%)-HPV-1a, 7(17%)-HPV-27 and 5(12.2%)-HPV-2a. Four cases were infected with two different HPV types, two (4.9%) with HPV-1a and HPV-27, one (2.4%) with HPV-1 and HPV-57 and one (2.4%) with HPV-27 and HPV-57. In contrast to the prevalence of single strain of novel HPV-57 variant and HPV-1 prototype, two HPV-2 and three HPV-27 novel variants were found to circulate in Beijing. **Conclusion** HPV-1, -2, -27 and -57 are predominantly prevalent in patients with common warts in Beijing.

Key words: Human papillomavirus; Genotype; Common warts; Variants

INTRODUCTION

Common warts are the most frequently encountered benign cutaneous lesions which are closely associated with infections of human papillomavirus (HPV) worldwide. More than 100 different types of HPV have been isolated, according to at least 10% nucleotide sequence diversities in late gene L1[1]. HPVs have been classified into cutaneous, mucosal, and epidermodysplasia verruciformis (EV) types according to their location and clinical context from which they are initially isolated[2], of which HPV-1, -2, -4, -7, -27, -57, and -65 are frequently associated with common warts[3-7].

Despite the fact that the clinical morphology of infecting viruses is associated with their histological patterns, this issue remains controversial[8-12]. HPV-1 usually induces warts on the hands, especially in the periungual and palmar locations. HPV-2 induces so-called intermediate warts displaying clinical features of verrucae vulgares with pronounced papillomatosis. The warts induced by HPV-3, -10, and -28 behave like plane warts[13]. HPV-60, -63, and -65 are found in cystic or punctate warts[14]. It was reported that HPV-2, -27, and -57 are more commonly found in patients with warts from Europe[4, 7, 12], while HPV-1, -4, and -65 are more frequently seen in patients with warts from Japan[5]. Compared with studies on mucosal HPVs, only a few molecular epidemiological studies on cutaneous HPVs have been conducted.

Although HPV genomes are rather stable, nucleotide exchanges are often seen in many clinical isolates. HPV variant is defined as one that differs from other viruses of the same type having only about 2% in conserved regions of the genome, like E1 or L1, and having up to 5% in the LCR[15]. To identify a HPV variant more conveniently, one procedure is to sequence a small part of the HPV genome, for example, a 400-bp of long control region (LCR) or a 450-bp of E6 open reading frame (ORF). If an isolate differs from the prototype in at least one nucleotide, it can be

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considered a variant[1]. In fact, the distribution of variants of high-risk HPVs, HPV-16, and -18, has been reported and some mutations in the genomes confer a differential risk for tumorigenesis[15-16].

In order to observe the circulation and distribution of HPVs in common warts in Beijing area, 48 skin biopsies from patients with common warts were screened for the presence of HPV with the HPV type-specific polymerase chain reaction (PCR) and sequence analysis. Four types of HPV DNA, including HPV-1, -2, -27, and -57, were observed in patients with common warts. In contrast to the unique strain of HPV-1 or HPV-57 in the patients, more diversiform HPV-2 and HPV-27 variants circulate in Beijing area.

MATERIALS AND METHODS

Patients and Samples

Common wart biopsies from 48 patients attending the Department of Dermatology, China-Japan Friendship Hospital, Beijing, from April 2006 to December 2006, were enrolled in this study. All patients were diagnosed by dermatological and/or histopathologic examinations with the presence of obvious hyperkeratosis, parakeratosis, and acanthosis in epidermis. All biopsies were collected at surgical removal of the skin lesions. Informed consent was obtained from each of the 48 patients (38 males and 10 females), with their age ranging from 18 to 72 years (41.5 years on average). None of the patients complained of any other severe disorders or immunosuppressive status.

DNA Extraction

Liquid nitrogen-frozen specimens were minced and total DNA was extracted using a QIAamp DNA mini kit (Qiagen). The fixed tissue samples were sectioned and total DNA was purified using the MagneSil genomic, fixed tissue system kit (Promega) according to the manufacturer's protocol. To avoid DNA contamination, the DNA extraction was separately performed and filter pipette tips were used in all manipulations.

Design of Primers

Consensus HPV primer pair MY09/11 was synthesized according to the published sequences[17]. To amplify the specific DNAs of HPVs related to common warts, HPV-1, -2, -4, -7, -27, -57, and -65 were included. The sequences of each HPV were referred from Genbank (Genbank accession no. NC001356, NC001352, NC001457, NC001595, NC001584, NC001353, and NC001459) and aligned with computer software DNAStar. The type-specific primers targeted to each HPV were designed and synthesized based on the more diversified parts of their genomes (Table 1).

TABLE 1

Sequences and Location of HPV Type-Specific Primers

HPV Type	Sequence	Position	Location
HPV-1a	P1: 5'-GTCTGGTTACCAGCGCAGAAT-3'	nt 5437-5357	L1
	P2: 5'-CTACCTATCTCTATCCCTCTT-3'	nt 5730-5750	
	P3: 5'-GCATGACACAACGTACTGCTA-3'	nt 6851~6871	LCR
	P4: 5'-TGGGATACAGAGGCTTTCGGA-3'	nt157~137	
HPV-2a	P3: 5'-TATGTCTTGTGGCCTAAACGACG-3'	nt5741～5763	L1
	P4: 5'-TGAGAGCTAACGCCTTACCCG -3'	nt7260～7280	
	P5: 5'-GGCCTATATACATAATATGGATCC 3'	nt6934-6958	LCR
	P6: 5'-TAGGGCATGGATTCTCCTCAGACA 3'	nt108-134	
HPV-4	P1: 5'-GTATGAGATTACAGAGGAGGA-3'	nt628-648	E7
	P2: 5'-CAATTATTCCCTTCTAAGTCA-3'	nt839-859	
HPV-7	P1: 5'-GTGTGATACTCCCTTGCACTC-3'	nt7332-7353	LCR
	P2: 5'-GGAACTTTGACCTAGCAATTTG-3'	nt7749-7770	
HPV-27	P1: 5'-TGTGCATTCCTAGGCTGA-3'	nt7651-7668	LCR
	P2: 5'-CCACACTACAGACAGTTC-3'	nt258-275	
	P3: 5'-GTTATATCCTGTGGCCTA 3'	nt 5713~5730	L1, LCR/E6E7
	P4: 5'-CGTGCACCACAAAGAACAG 3'	nt 7430~7411	
	P5: 5'-TTACGTGTGAGTGTCTCG 3'	nt 7243~7260	
	P6: 5'-TAATGGCCTCCACATGGAAC 3'	nt 900~883	
HPV-57	P1: 5'AACTGCAGCGGCGCCCACTGCTAA-3'	nt 7190-7213	LCR
	P2: 5'-GTAGGAATGTGCACGCCCACA-3'	nt 7630-7650	
	P3: 5'-CTGCCAGCACACTCATATCCT-3'	nt7529~7549	
	P4: 5'-ACCATACTCTCTGCACAGCAG-3'	nt161~141	
HPV-65	P1: 5'-ATGGCGAGTTGGTTATCTGCA-3'	nt5326-5346	L1
	P2: 5'-CATCCTCAATATATGTGTTCA-3'	nt5900-5920	

Polymerase Chain Reaction (PCR)

DNA extraction, preparation of reagents, PCR set-up and analysis of PCR products were performed in four separate rooms with appropriative equipments. PCR amplifications with HPV consensus primer MY09/11 were conducted for 40 cycles as described elsewhere[18]. Thirty-five cycles of HPV type-specific PCR was performed in 25 μL of reaction containing 2.5 U of Ex Taq DNA polymerase (TaKaRa, DaLian, China), 100 μmol/L concentration of each dNTP, 200 ng of each HPV type-specific primers and 2 μL extracted DNA, in the condition of denaturing at 94 ℃ for 40 s, annealing at 58 ℃ for 40 s, extension at 72 ℃ for 1 min.

Direct Sequencing

PCR products were analyzed in 2% agarose gel and recovered from gel with a QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the same PCR primers and the base sequences were read on the ABI PRISMTM 3730XL DNA analyzer.

Phylogenetic Analysis

Phylogenetic analyses were based on multiple alignments that were done using CLUSTALX software. Phylogenetic tree was constructed with Maximum Parsimony methods using PHYLIP software.

Statistic Analysis

Statistic analysis was performed using the Fisher's exact test or Kruskal-Wallis test where appropriate. $P<0.05$ was considered statistically significant.

RESULTS

A total of 48 clinical biopsies of common warts were firstly screened by PCR with the consensus HPV primer pair MY09/11, among which 21 were frozen tissue samples and 27 were fixed tissue samples. In parallel, 20 biopsy samples from normal skin tissue (5 cases), pimented naevus (8 cases) and acrochordon (7 cases) were employed in PCR screening assays. Only 12 samples of common warts showed amplified products (25%, 12/48), while none of the samples of normal skin or other dermatological diseases was positive.

Prevalence of HPV DNAs in Tissues of Common Warts

All extracted DNA preparations were separately surveyed by PCR with six common wart-related HPV specific primers, followed by sequence analysis. To avoid artificial positive reactions, DNA was extracted from each sample and each sample was tested at least twice with independently extracted DNA preparations and the sequence data of each target fragment were read at least from two independent PCR amplifications. The results showed that 41 of 48 (85.5%) samples were positive for HPV DNAs, including 13 for HPV-57 (31.7%), 12 for HPV-1a (29.3%), 7 for HPV-27 (17%), and 5 for HPV-2a (12.2%). Besides, four cases (9.8%) were found to be infected with two different HPV types, two cases (4.9%) with HPV-1a and HPV-27, one case (2.4%) with HPV-1 and HPV-57, one case (2.4%) with HPV-27 and HPV-57 (Table 2). Neither the HPV-4 nor the HPV-65 specific fragment was found. Meanwhile, 20 non-common-wart samples were all negative.

TABLE 2

Distribution of HPV DNAs in Patients with Common Warts from Beijing Area

	No	Percent (%)
Positive	41	85.5
HPV-1	12	25.0
HPV-2	5	10.4
HPV-27	7	14.6
HPV-57	13	27.1
HPV-1/HPV-27	2	4.2
HPV-27/HPV-57	1	2.1
HPV-1/HPV-57	1	2.1
Negative	7	14.6

Relationship between Main Clinical Features and Infection with HPV Types

Out of the 41 HPV positive patients, 31 (75.6%) were male and 10 (24.4%) were female. All HPV-2 (5/5, 100%) and most of HPV-27 (6/7, 85.7%) and HPV-57 (11/13, 84.6%) infections were identified in male patients. However, HPV-1 infections seemed to involve both genders (7 males, 5 females) (Table 3). No statistical difference ($P=0.279$) was detected at the onset age of patients infected with each of the four HPV genotypes (Table 3). In line with other reports, the lesions were predominantly observed in hands and feet, without marked difference in the four HPV types. To observe the relation between infection with HPV types and the sizes of common warts, the patients were divided into three groups based on the size of common warts (smaller than 0.5 cm, 0.5-1.0 cm and larger than 1.0 cm). Meanwhile, the time from onset of the warts to medical treatment was

taken as another index. Kruskal-Wallis test showed that the sizes of warts were correlated with the clinical duration of the disease (P=0.016), but not with the infection with HPV types (Table 3).

TABLE 3

Clinical Data Obtained from Patients with Common Warts

		HPV-1	HPV-2	HPV-27	HPV-57	HPV-1/ HPV-27	HPV-27/ HPV-57	HPV-1/ HPV-57	Total
Gender	Male	7	5	6	11	1	1	0	31
	Female	5	0	1	2	1	0	1	10
Average (Years)		34.3	46.3	43.3	42.5	50	31	58	-
Location	Hands and Feet	10	4	6	8	1	1	1	31
	Others	Upper Arm 1 Abdomen 1	Leg 1	Upper Arm 1	Face 3 Back 1 Limb 1	Face 1	0	0	10
Sizes of Wards	<0.5 cm	8	1	6	8	2	0	1	26
	0.5-1 cm	4	0	1	3	0	0	0	8
	>1 cm	0	4	0	2	0	1	0	7

Genetic Diversities of Various HPV Isolates

To address the possible genetic diversity of HPV in wart tissues, the nucleotide sequences of PCR products were aligned with each other, as well as with the relevant HPV LCR sequences from Genbank. All HPV-1 LCR sequences amplified from the patients in this study possessed exactly the same sequence as HPV-1a, indicating the unique prevalence of prototype HPV-1a in patients with common wart in Beijing.

An identical HPV-57-related LCR sequence with 19 nucleotide exchanges was found in the 15 samples containing HPV57 (Table 4). This HPV57 variant, nominated as HPV-57CV1, possessed 97.6% homology in the region of LCR with the HPV-57 prototype and differed from all variants in Genbank. Three different HPV-27-related LCR sequences were observed in 10 skin lesions, none of them was HPV-27 prototype. The new HPV-27 variants, termed as HPV-27CV1, -27CV2, and -27CV3 respectively, contained distinguishable nucleotide exchanges but shared five identical point mutations at positions of nt 7246, 7247, 7259, 7260, and 7263 (Table 5). Seven samples contained HPV-27CV2, two contained HPV-27CV1 and one had HPV-27CV3. In the five HPV-2 positive samples, two contained HPV-2a LCR sequences, two had HPV-2-related LCR sequences with two point mutations and one deletion (HPV-2CV2), one had a more divergent HPV-2 related segment with six point mutations, one deletion and one insertion (HPV-2CV1, Table 6). Additionally, the HPV sequences in the four double-infection cases were HPV-1-prototype/HPV-27CV1, HPV-1-prototype/ HPV-27CV2, HPV-1-prototype/HPV-57CV, and HPV-27CV3/HPV-57CV, respectively.

TABLE 4

Nucleotide Mutations of HPV-57 Variant Isolated from Patients with Common Warts within LCR

Position(nt)	HPV-57 Prototype	HPV-57 CV1	Position(nt)	HPV-57 Prototype	HPV-57 CV1
7237	G	A	7599	A	G
7238	A	G	7666	C	A
7241	C	A	7714	G	A
7261	T	G	7704	C	C deletion
7411	T	C	7722	T	C
7458	C	A	7798	G	C
7471	A	G	15	A	T
7488	A	C	22	C	T
7581	T	G	38	C	T
7589	G	A			

TABLE 5

Nucleotide Mutations of HPV-27 Variants Isolated from Patients with Common Warts within LCR

Position(nt)	HPV-27 Prototype	HPV-27 CV1	HPV-27 CV2	HPV-27 CV3
7246	C	G	G	G
7247	G	C	C	C
7259	C	G	G	G
7260	G	C	C	C
7263	A	C	C	C
7428	A	-	-	C
7480	G	-	-	A
7485	C	-	-	A
7774	G	T	-	T

TABLE 6

Nucleotide Mutations of HPV-2 Variant Isolated from Patients with Common Warts within LCR

Position (nt)	HPV-2 Prototype	HPV-2 CV1	HPV-2 CV2
7298	C	T	-
7320	T	C	-
7350	-	T insertion	-
7564	G	A	A
7708	T	T deletion	T deletion
7720	G	C	C
7768	G	A	-
63	T	A	-

Using the PHYLIP software, a phylogenetic tree was established based on the LCR nucleotide sequences of the isolates in this study, as well as HPV-1, -2, -27, and -57 prototypes and other relative variants in Genbank. All HPV variants from the tested wart tissues were distinct from their corresponding prototypes and other reported variants (Fig. 1), indicating that HPV-2, HPV-27, and HPV-57 strains circulating in Beijing are novel variants. Two HPV-2 variants spanned a relatively large distance in their LCR sequences, while three HPV-27 variants were located much closer.

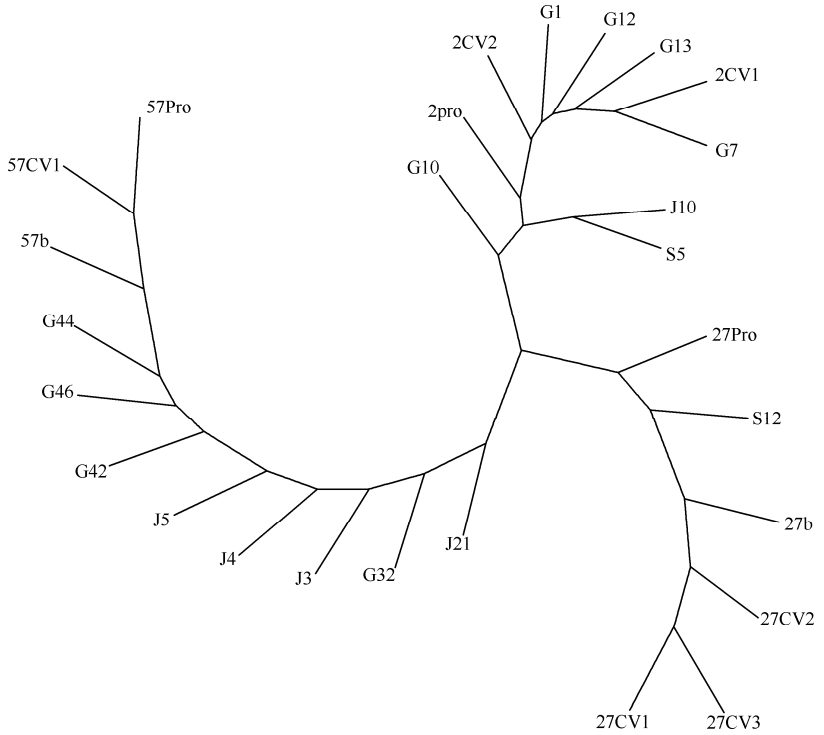

FIG. 1. Phylogenetic Tree of HPV-2, HPV-27, and HPV-57 Prototype and Variants Constructed Based on the LCR Segment Using Maximum Parsimony Methods.

DISCUSSION

In this study, four HPV types, HPV-57, -1, -27, and -2, were confirmed to be the principally prevalent HPV genotypes in patients with common warts from Beijing, Northern China. An earlier survey in Taiwan, China, showed that the prevalence of HPV-1, -2, -4, and -5 was 13%, 7%, 16%, and 2% respectively, in 61 skin warts by DNA hybridization[19]. It was reported that the most prevalent HPV types in

patients with common warts are HPV-1 (44.1%), HPV-4 (16.4%), and HPV-65 (14.1%)[5]. A study in Germany showed that 91% of the studied individuals are positive for HPV DNA, and their HPV-1, -27, and -2 are 27.3%, 12.9%, and 9.6%, respectively[6]. Porro *et al.*[7] reported that HPV DNA could be detected in 64% of HIV-infected patients with cutaneous warts (*n*=24) and in 79% of samples from non-HIV-infected patients (*n*=13) in Brazil. HPV 2／27／57 were found in 81% of HIV-infected patients with common warts and in 82% of the controls.

Our study revealed that the prevalence of HPVs in patients with common warts in Beijing had two forms: circulation of a single HPV-1 and -57 or circulation of HPV-2 and HPV-27. All HPV-1 positive lesions contained LCR sequences of HPV-1a prototype. HPV-1 is the first identified HPV from a single wart in 1980[20]. Since then, only a few HPV-1 variants have been described, except for a HPV-1 variant genome isolated from a plantar wart in 1994[21]. Our data indicate that HPV-1 genome is quite stable during the long-term circulation. All HPV-57 positive specimens contained the same LCR sequences, suggesting that circulation of one unique HPV-57 strain is distinct from either HPV-57 prototype or from other known variants in Beijing. HPV 57 was originally isolated from an inverted papilloma of the maxillary sinus in 1989[22] and several variants have been identified subsequently[4,23-24]. The HPV-57 phylogenetic tree established based on LCR sequences presents dichotomic clusters, in which the variants from Germany and Japan seem to be located separately. The Chinese HPV-57 variant in this report is closer to the German isolates. Whether there is geography-related distribution of HPV-57 needs a whole genome sequence comparison of more isolates nationwide and worldwide. HPV 27 was first found in a common hand wart from a renal transplant patient in 1989[25]. Since then, HPV-27 subtype and variants have been reported in Japan and Singapore[4, 26]. Analysis of HPV-27 LCR sequences of the three novel variants revealed that a large portion of the same mutation was similar to variant HPV-27b identified in Japan, possibly indicating the same nascence. Three different variants circulating in Beijing also imply a relatively fast evolution of HPV-27. A similar situation was observed in the circulation of HPV-2 in Beijing. HPV 2, originally detected in a common wart[27], is also occasionally found in benign and malignant genital and mucosal lesions[28-30]. Comparison of the LCR sequences of HPV-2 strains in Genbank confirmed that these two variants are novel.

The data obtained from this study do not show any correlation between infection with HPV genotype and other factors, including patients' gender, age and lesion's anatomic location. In addition, no notable difference was observed in clinical morphology or size of common warts among infections with HPV-1 prototype, HPV-57 variant, -27 variants and HPV-2 prototype, except for the infection with HPV-2 variants. Compared with other cutaneous HPVs-related benign proliferative lesions, HPV-2 is often prone to induce multiple verrucae vulgaris and responds only modestly to treatment[12]. Actually, two HPV-2 positive cases in this study had a clinical manifestation of unusual large verrucae vulgaris with huge extensive clustered cutaneous horn[31]. Our previous study has identified that nucleotide changes within LCR and E2 ORF make HPV-2 variants possess relatively stronger promoter activities than prototype HPV-2a, that may be linked with special clinical phenotypes[32]. The association between infection with cutaneous HPVs and clinical and epidemiological characteristics of common warts needs further molecular epidemiological studies.

The different positive rate of HPV infections in common warts may be partially due to the processing procedure of lesion tissue. It was reported that a significantly higher HPV DNA prevalence and a broader spectrum of HPV types with the use of frozen vs paraffin-embedded materials, are most likely because DNA is insufficiently purified, either quantitatively or qualitatively, for PCR amplification[5,33]. However, with the protocol described in this study, a satisfied result was obtained from the fixed tissues, although its positive rate (77.8%, 21/27) was still lower than that of the frozen fresh tissue (95.2%, 20/21). Certainly, fresh specimens may reflect the actual situation of HPV prevalence in common warts other than the skin lesion specimens. Nevertheless, appropriately processing of fixed tissues is definitely helpful for extending the sample ranges for evaluation of HPV infection, especially the origin and fore-passed circulation of HPV with the samples stored for decades.

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