

Genetic variability in E5, E6, E7 and L1 genes of human papillomavirus type 31

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Abstract. Human papillomavirus (HPV) type 31 is an important pathogenic subtype associated with cervical cancer. The aims of the present study were to analyze E5, E6, E7 and L1 gene mutations of HPV-31 among females, and to elucidate the evolutionary associations between them. In total, 87 positive samples were collected. The E5, E6, E7 and L1 genes were amplified by polymerase chain reaction and sequenced. Subsequently, two phylogenetic trees were constructed from the nucleotide sequences of the E5, E6 and E7 and the L1 variants of HPV-31. In total, 31 mutation sites of E5, E6 and E7 genes were identified, of which 16 were non-synonymous. T4053A (F80I), C285T (H60Y), C520T (A138V) and A743G (K62E) were the most common non-synonymous mutations. A total of 30 mutation sites of L1 genes were identified, of which four were non-synonymous. The most common non-synonymous mutations of L1 genes were A6350G (T29A) and C6372A (T36N). By phylogenetic analysis, A and C variants were most frequently detected, while B variants were less frequently detected in this population. The sequence variation data obtained in the present study provides a foundation for future research regarding HPV-induced oncogenesis, and may prove valuable for developing diagnostic probes and in the design of HPV vaccines for targeted populations.

Introduction

Cervical cancer is a common form of tumorigenesis among females globally. The most important risk factor for cervical cancer is persistent infection with human papillomavirus (HPV) (1). HPVs with <90% nucleotide sequence homology in the L1 gene are considered different species (traditionally referred to as 'types'), HPVs with 90-98% L1 sequence homology are different subtypes and HPVs with >98% L1 sequence homology are considered mutants of the same subtype (2,3). Over 150 HPV types have been identified, of which 60 types are predominantly detected in the genital tract (2,3). HPV types are divided into high risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 83) and low risk (HPV-6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81) according to their oncogenic potential. Low risk HPV causes mild genital warts and no oncogenic risk. The majority of high risk (HR) types, including 16, 18, 31, 33, 52 and 58, have the potential to lead to invasive cervical cancer (4-6).

When the HPV DNA integrates it inactivates tumor-suppressor genes, stimulating oncogene expression (7,8). The E7 protein of HR HPV binds to and inactivates retinoblastoma protein (pRB), a tumor suppressor protein (9,10). E6 protein has been demonstrated to mediate the degradation of p53 through the E6-associated protein (9,11). High protein expression levels of epidermal growth factor receptor and erb-b2 receptor tyrosine kinase 4 are promoted by the E5 protein, which has also been demonstrated to promote cell proliferation and signal transmission (12,13).

The study of HPV variants is on the increase, and multiple reports have confirmed that HPV variants differ in biology and etiology (14). At present, multiple studies have investigated HPV-16, 18, 52 and 58 variants, however the HPV-31 variant, which is the one of the HR oncogenic types, has been rarely studied, particularly in China. Due to the differences in biological characteristics, it is necessary to identify the HPV-31 variants (15). The results of the present study may provide an effective reference for further clinical application, and may aid assessment of the prognosis of patients with HPV.

The distribution of HPV subtypes differs geographically and across populations, and the primary aim of the present study was to assess the single nucleotide polymorphisms

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and/or amino acid polymorphisms of the E5, E6 E7 and L1 proteins of HPV-31 in Sichuan (China). To the best of our knowledge, this is the first analysis of HPV-31 oncogene variants in patients in Sichuan.

Materials and methods

Ethical statement. The present study was approved by the Ethics Committee of Sichuan University (Chengdu, China). All participants provided informed consent prior to the collection of experimental specimens.

Study subjects and specimen collection. Between January 1, 2009 and September 14, 2015, a total of 13,283 patients aged between 18 and 65 years old provided specimens. They were obtained from maternity hospitals in Sichuan, China (The Affiliate Reproduction Hospital of Sichuan Genitalia Hygiene Research Center, Chengdu; Chengdu Medical College Attached Infertility Hospital, Chengdu; Jinjiang Maternity and Child Health Hospital, Quanzhou; Angel Women and Children's Hospital, Chengdu; Chengdu Zongnan Gynecology Hospital, Chengdu; and Chengdu Songziniiao Sterility Hospital, Chengdu) and the patients were neither in the menstrual period nor had undergone cervical conization prior to the present study. A brush and a colposcope were used to collect cervical scrapings, which were placed in Cell Preservation Liquid (Yaneng Bioscience Co., Ltd., Shenzhen, China), and stored at -20°C until DNA extraction, HPV detection and typing were performed. Further experiments primarily took place in the Institute of Medical Genetics, College of Life Science, Sichuan University (Chengdu, China).

DNA extraction. DNA was extracted using the Human Papillomavirus Genotyping kit for 23 Types (PCR-RDB; Yaneng Bio-Technology (Shenzhen) Co., Ltd., Shenzhen, China) according to the manufacturer's protocols. The cervical specimens, which were stored at -20°C, were thawed to room temperature. Cells were suspended in Cell Preservation Liquid and 1 ml suspension transferred to a 1.5 ml Eppendorf tube (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China) and subjected to centrifugation for 10 min at 16,155 x g at 4-6°C in a Hema 14D high speed centrifuge. The supernatant was removed and 100 µl cell pyrolysis liquid (KCl, Tris-HCl, Triton X-100) was added to the sediment, which was incubated in a boiling water bath for 10 min. Centrifugation was conducted again, at 16,155 x g for 10 min at 4-6°C, and the supernatant was removed. The supernatant was stored at 4°C and used for polymerase chain reaction (PCR) analysis. All of the extracted DNA samples were stored at -20°C until examination. None of the samples remained at room temperature for more than 2 h, or at 4°C for more than 24 h, to avoid DNA degradation.

HPV genotyping. HPV genotyping was accomplished using the Human Papillomavirus Genotyping kit for 23 Types (Yaneng Bioscience Co., Ltd.), which exploits chip technology and employs reverse membrane hybridization technology, where the probe is fixed on a membrane strip, to identify 23 different HPV genotypes in one reaction (18 HR and five low risk subtypes). Viral DNA was extracted, amplified and

genotyped according to the manufacturers' protocol, and negative and positive specimens were displayed in each reaction. All HPV-31 positive specimens were subsequently subjected to variant analysis.

PCR amplification and sequencing. The specific primers used to amplify E5, E6, E7 and L1 genes in HPV-31 positive DNA are listed in Table I and were designed using Primer 5.0 bioinformatics software (Premier Biosoft, Palo Alto, CA, USA) according to the published GenBank reference sequence (accession no. J04353. <https://www.ncbi.nlm.nih.gov/nucleotide/333048>). The length of the L1 sequence was 1,550 base pairs, so it was divided into two sections (L1Q and L1H). PCR amplification for each gene was set up in a 25 µl reaction volume containing 4.0 µl DNA (10-100 ng) 10X PCR buffer, 2.5 mM/l deoxynucleotide triphosphates, 25 mM/l MgCl₂ (TransBionovo Co., Ltd., Beijing, China; <https://www.transbionovo.com>), 50 µM/l of each primer (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.) and 2.5 U *Taq* polymerase (TransBionovo Co., Ltd.). PCR amplification was conducted at 95°C for 5 min, 38 cycles of denaturation at 94°C for 45 sec, annealing at various temperatures for 50 sec (Table I), extension at 72°C for 1 min and a final extension step at 72°C for 10 min. PCR products were checked on 2% agarose gels (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.), visualized using UV fluorescence (GeneGreen; Tiangen Biotech Co., Ltd., Beijing, China) using a WFH-202 fluorometer (Wenzhou Fuhua Instruments, Inc. wenzhou068795.11467.com) and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Data analysis. Following direct sequencing, all sequences were aligned with an HPV-31 prototype sequence (GenBank accession no. J04353) available from the National Center for Biotechnology Information (NCBI). The samples with mutational sites were amplified and sequenced again to rule out the possibility of error due to mismatched bases in the PCR process. All E5, E6, E7 and L1 sequences were separately aligned by Clustal X 2.1 (<ftp://ftp.ebi.ac.uk/pub/software/clustalw2/>) (16). Maximum-likelihood phylogenetic trees of respective HPV-31 E5, E6, E7 and L1 variation patterns were subsequently constructed by Molecular Evolutionary Genetics Analysis 6 software (17), using Kimura's two-parameter model (18). To estimate the selection pressure acting on the HPV-31 E5, E6, E7 and L1 gene sequences, non-synonymous and synonymous nucleotide divergence for coding regions was inferred by the Nei and Gojobori method with Phylogenetic Analyses by Maximum Likelihood (PAML; <http://abacus.gene.ucl.ac.uk/software/paml.html>) software version 4.8 (19-22).

Nucleotide sequence accession numbers. All sequences of the E5, E6, E7 and L1 genes were submitted to the NCBI GenBank database and assigned accession numbers. The HPV-31 E5, E6 and E7 sequences, 31EPL01-31EPL16, were published with the GenBank accession codes KU163553-KU163568. The HPV-31 L1 sequences, 31LPL01-31LPL16 are published with the GenBank accession codes KU163569-KU163575, KU163584 and KU163576-KU163583.

Table I. Primer used for the molecular characterization of HPV-31 E5, E6, E7 and L1.

Primer name	Sequence primers	Sequenced region (bp)	ORF size (bp)	Annealing temperature (°C)
HPV-31 E5 F	5'-gcacaaaccaacaaggct-3'	3,531-4,200 (670)	255	59.5
HPV-31 E5 R	5'-agtgcgtttttagcgtt-3'			
HPV-31 E6 F	5'-gaaagtggatgaaccgaaac-3'	41-740 (700)	450	58
HPV-31 E6 R	5'-actgacaacaaaaggtaa-3'			
HPV-31 E7 F	5'-gaccgttggtccagaagaa-3'	430-963 (534)	297	59
HPV-31 E7 R	5'-ctctgaaatgtgccctg-3'			
HPV-31 L1-Q F	5'-cccctacaacgccacaagt-3'	5,441-6,373 (933)	750	58
HPV-31 L1-Q R	5'-agtagggaccgattcacc-3'			
HPV-31 L1-H F	5'-aatgctattaccctgg-3'	6,062-7,098 (1,037)	800	54.5
HPV-31 L1-H R	5'-atacaatacagcacaagcac-3'			

Due to the length of the L1 sequence, it was divided into two sections for amplification (L1Q and L1H). HPV, human papillomavirus; ORF, open reading frame; bp, base pairs; F, forward; R, reverse.

Table II. Nucleotide sequence mutations of HPV-31 E5.

Category	Variation of E5 at nucleotide position											n
	3827	3828	3956	3957	3980	3981	4005	4052	4053	4059	4064	
Reference nt	A	A	G	A	T	C	G	T	T	A	A	
31EPL01	-	-	A	-	-	-	-	-	A	-	-	13
31EPL02	G	-	-	G	A	-	-	C	A	-	-	1
31EPL03	-	-	-	-	-	-	-	-	A	-	-	1
31EPL04	-	-	-	-	-	-	-	-	A	-	-	12
31EPL05	G	-	-	G	A	-	-	C	A	-	-	2
31EPL06	G	-	-	G	A	-	-	C	-	-	-	4
31EPL07	-	G	-	-	-	G	A	C	A	-	G	1
31EPL08	G	-	-	G	A	-	-	C	A	-	-	5
31EPL09	G	-	-	G	A	-	-	C	-	-	-	1
31EPL10	-	-	-	-	-	-	-	-	A	-	-	1
31EPL11	G	-	-	G	A	-	-	C	-	-	-	1
31EPL12	-	-	-	-	-	-	-	-	A	-	-	1
31EPL13	-	-	A	-	-	-	-	-	A	G	-	2
31EPL14	-	-	A	-	-	-	-	-	A	-	-	1
31EPL15	-	-	A	-	-	-	-	-	A	G	-	1
31EPL16	G	-	-	G	A	-	-	C	A	-	-	1
AA mutations		N		I		P	V		F	S		
		5		48		56	64		80	82		
	-	D	-	V	-	A	I	-	I	G	-	

Nucleotide positions are shown where sequence alterations were detected in comparison with the sequence of the reference HPV-31 isolate (GenBank accession no. J04353). Nucleotide changes are presented by the corresponding letters. Dashes indicate no nucleotide exchange from the HPV-31 reference sequence. The number of amino acid mutation is the location in the corresponding genes. HPV, human papillomavirus; nt, nucleotide; AA, amino acid.

Results

Distribution of HPV-31. A total of 13,283 specimens from Sichuan (China), were collected for the present study, and 4,130 (31.1%) were HPV positive. Of these 4,130 samples,

141/4,130 (3.4%) were positive for HPV-31. There were 70/141 (49.6%) samples with single HPV-31 infection, 35/141 (24.8%) samples with double infection and 36/141 (25.5%) samples with multiple infection. The ages of the patients infected by HPV-31 ranged between 18-70, with a median age of 32 years.

Table III. Nucleotide sequence mutations of HPV-31 E6.

Category	Variation of E6 at nucleotide position														n
	134	176	248	285	297	301	312	321	326	335	404	428	475	520	
Reference nt	T	C	T	C	A	A	T	A	A	T	G	A	A	C	
31EPL01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
31EPL02	-	-	-	T	-	G	-	T	-	-	A	-	-	T	1
31EPL03	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
31EPL04	-	T	-	-	-	-	-	-	-	-	-	-	-	-	12
31EPL05	-	-	-	T	-	-	-	T	G	-	A	G	-	T	2
31EPL06	-	-	-	T	-	-	-	T	G	-	A	G	-	T	4
31EPL07	-	-	C	-	G	-	-	T	-	-	-	-	G	T	1
31EPL08	-	-	-	T	-	-	-	T	-	-	A	G	-	T	5
31EPL09	-	-	-	T	-	-	-	T	G	C	A	G	-	T	1
31EPL10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
31EPL11	-	-	-	T	-	-	-	T	G	-	A	-	-	T	1
31EPL12	-	-	C	-	G	-	-	T	-	-	-	-	G	T	1
31EPL13	A	-	-	-	-	-	-	-	-	-	-	-	-	-	2
31EPL14	A	-	-	-	-	-	C	-	-	-	-	-	-	-	1
31EPL15	A	-	-	-	-	-	C	-	-	-	-	-	-	-	1
31EPL16	-	-	-	T	-	-	-	T	-	-	A	G	-	T	1
AA mutations				H	T	K	F						K	A	
				60	64	65	69						123	138	
				Y	A	R	L						R	V	

Nucleotide positions are shown where sequence alterations were detected in comparison with the sequence of the reference HPV-31 isolate (GenBank accession number J04353). Nucleotide changes are presented by the corresponding letters. Dashes indicate no nucleotide exchange from the HPV-31 reference sequence. The number of amino acid mutation is the location in the corresponding genes. HPV, human papillomavirus; nt, nucleotide; AA, amino acid.

E5 sequence variations. In total, 141 HPV-31 positive samples were detected, of which 87 samples were used for further exploration; 54 samples were excluded due to incomplete data. In the present study, sequences of HPV-31 E5, E6, E7 genes and the L1 gene were obtained from 48/87 and 37/87 patients, respectively. The failure of some genes to be amplified or sequenced was due to either a short copy of HPV or instability of the amplicon.

Compared with the HPV-31 reference sequence (J04353), 11 nucleotide variations of the E5 gene were observed in the 48 HPV-31 E5 sequences studied (Table II). In total, six missense mutations of A3828G (1/48), A3957G (15/48), C3981G (1/48), G4005A (1/48), T4053A (42/48) and A4059G (3/48) were revealed, which resulted in amino acid changes of N5D, I48V, P56A, V64I, F80I and S82G, respectively (Table II). The remaining five variations, A3827G (15/48), G3956A (17/48), T3980A (11/48), T4052C (16/48) and A4064G (1/48), were synonymous variations. T4053A was the most common non-synonymous variation, followed by A3957G. Excluding A4064G, which had only one specimen, the synonymous mutations were of similar quantity.

E6 sequence variations. The present study detected 14 mutation sites in the E6 gene in 34/48 (70.8%) samples through comparative analysis with the HPV-31 reference sequence

(J04353). No variations were observed in 14/48 (29.2%) samples, which were referred to as ‘E6 prototype-like’ sequences. There were six non-synonymous variations: C285T (15/48), A297G (2/48), A301G (1/48), T312C (2/48), A475G (2/48) and C520T (17/48), which led to amino acids changes of H60Y, T64A, K65R, T69L, K123R and A138V (Table III). The frequencies of C285T (15/48) and C520T (17/48) mutations were 15/48 (31.3%) and 17/48 (35.4%), respectively. These resulted in changes at the same point in the genetic code, however expressed different amino acids. The remaining eight mutations were synonymous (Table III).

E7 sequence variations. Compared with the HPV-31 reference sequence (J04353), six mutation sites were presented in the 48 samples (Table IV). There were four non-synonymous variations: C626T (33/48), G695A (15/48), A743G (47/48) and C737G (1/48) which resulted in amino acid changes of H23Y, E46K, K62E and Q60E (Table IV). A743G was the most common non-synonymous mutation across all specimens. The remaining two mutations were G580A (15/48) and C670T (15/48), and were synonymous.

The specimens were divided into 16 species, termed 31EPL01-31EPL16 (Tables II, III and IV). According to the frequency of E5, E6 and E7 mutations, based on the statistics from the present study, 31 mutation sites were detected,

Table IV. Nucleotide sequence mutations of HPV-31 E7.

Category	Variation of E7 at nucleotide position						n
	580	626	670	695	743	737	
Reference nt	G	C	C	G	A	C	
31EPL01	-	T	-	-	G	-	13
131EPL02	A	-	T	A	G	-	1
31EPL03	A	T	-	-	G	-	1
31EPL04	-	T	-	-	G	-	12
31EPL05	A	-	T	A	G	-	2
31EPL06	A	-	T	A	G	-	4
31EPL07	-	T	T	A	G	-	1
31EPL08	A	-	T	A	G	-	5
31EPL09	A	-	T	A	G	-	1
31EPL10	-	T	-	-	G	-	1
31EPL11	A	-	T	A	G	-	1
31EPL12	-	T	-	-	G	-	1
31EPL13	-	T	-	-	G	-	2
31EPL14	-	T	-	-	G	-	1
31EPL15	-	T	-	-	G	-	1
31EPL16	A	-	T	A	G	G	1
AA mutations		H		E	K	Q	
	-	23	-	46	62	60	
		Y	-	K	E	E	

HPV, human papillomavirus; nt, nucleotide; AA, amino acid.

of which 16 sites were non-synonymous mutations and the remaining 15 were synonymous mutations.

Phylogenetic trees of respective HPV-31 E5, E6 and E7 variation patterns were subsequently constructed by Molecular Evolutionary Genetics Analysis 6 software (17), using Kimura's two-parameter model (18). Phylogenetic analysis of HPV-31 variant lineage distribution (n=48) in Sichuan, China (23), demonstrated that A variants were most commonly detected (66.7%; Fig. 1), followed by C variants (31.3%; Fig. 1) and B variants (2.0%; Fig. 1).

L1 sequence variations. Compared with the HPV-31 reference sequence (J04353), the specimens were divided into 16 species, named 31LPL01-31LPL16 (Tables V and VI). In total, 30 nucleotide variations of the L1 gene were observed in strains from 37 patients, of which four were missense mutations: T6131A (1/37), A6350G (14/37), C6372A (17/37) and A6840G (1/37), which resulted in amino acid changes of S206T, T29A, T36N and Q192R, respectively (Table III). The remaining 26 variations were synonymous, of which C6367T (37/37) and C6817A (37/37) were detected in samples. The non-synonymous mutations A6350G (14/37) and C6372A (17/37) were more common than the others. No nucleotide substitutions resulting in premature termination codon or frameshift mutations were detected.

Phylogenetic trees of respective HPV-31 L1 variation patterns were subsequently constructed by Molecular Evolutionary Genetics Analysis 6 software (17), using Kimura's two-parameter model (18). Phylogenetic analysis of HPV-31

variant lineage distribution (n=37) in Sichuan, China, revealed that A variants were most commonly detected (54.0%; Fig. 2), followed by C variants (37.8%; Fig. 2), and B variant (8.2%; Fig. 2).

Selective pressure analysis of all sequences. PAML 4.8 software was used to test for variable dN/dS rate ratios among the lineages. There was no evidence of negative selection in the sequence alignment of HPV-31 E5, E6 and E7 genes or L1 genes (P>0.05 and P>0.05, respectively; Tables VII and VIII, respectively).

Discussion

The present study demonstrated that 31.1% of the females with cervical cancer studied were infected with at least one subtype of HPV. Globally, ~2-20% of healthy females have detectable levels of HPV DNA in their cervical tissue, as detected by epidemiological studies (24). The higher rate recorded in the present study may reflect the design of the study, which selected only females with active cervical cancer. Previous studies have demonstrated that smoking habits, the number of sexual partners, history of sexually transmitted diseases and abnormal cervical cytology collectively increase the risk of HPV infection (25).

Knowledge of HPV genetic variants may aid in the understanding of the pathogenic mechanisms and progression of cervical cancer. It has previously been suggested that variants

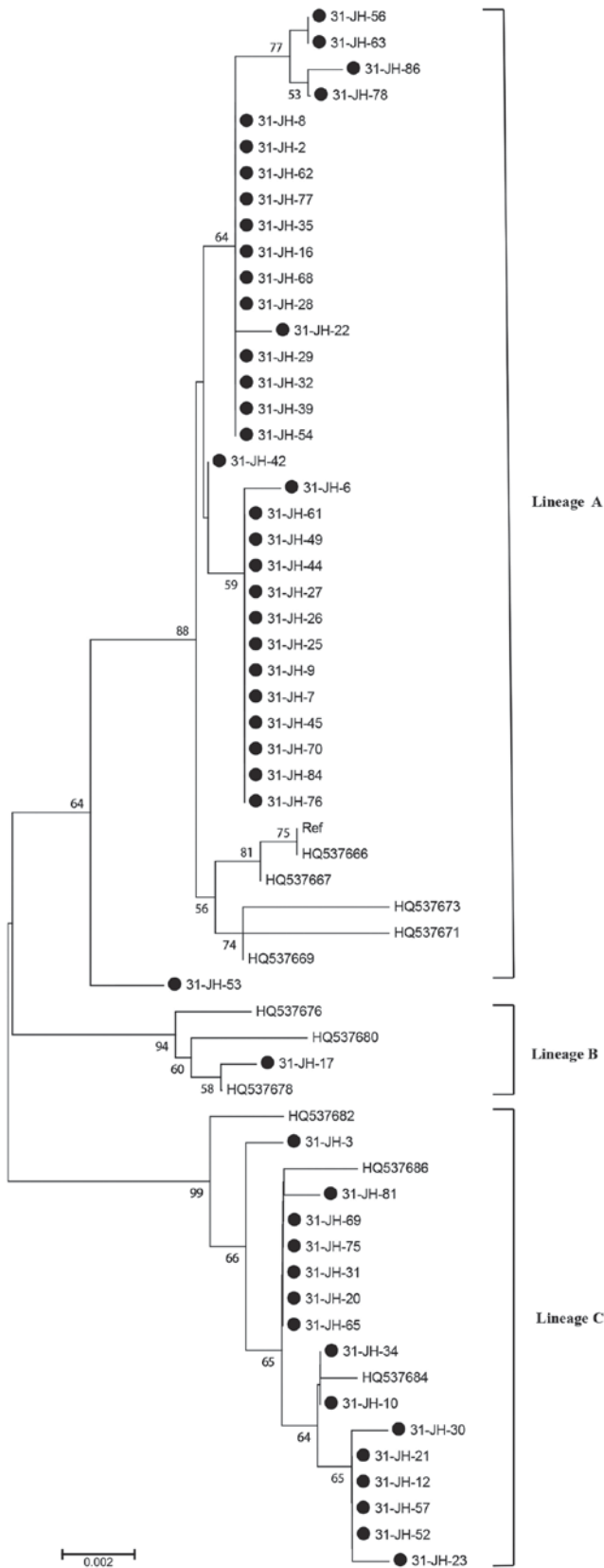


Figure 1. Sequences from the present study are labeled with black circles (n=48). Other sequences (n=11) were the variants previously reported in the whole genome study (23) and were used as the standard sequences. Viral lineages analyzed in the present study are clustered into A, B and C branches.

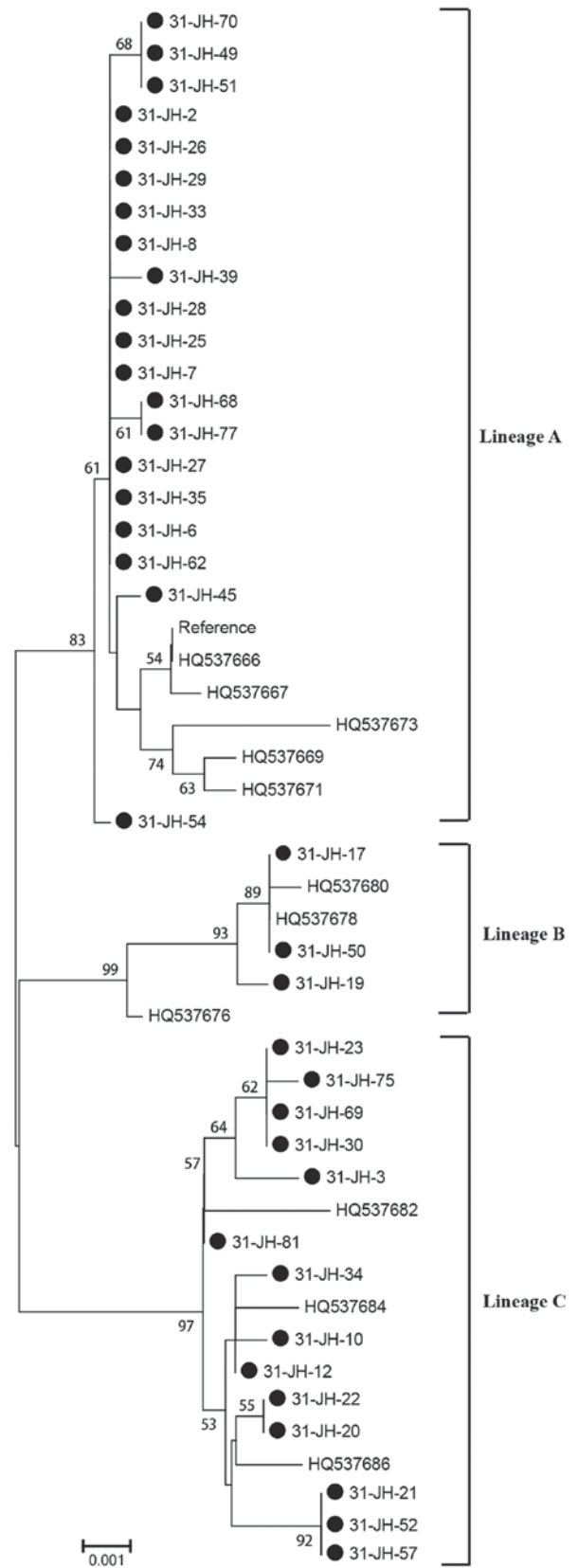


Figure 2. Sequences from the present study are labeled with black circles (n=37). Other sequences (n=11) were the variants previously reported in the whole genome study (23) and were used as the standard sequences. Viral lineages analyzed in the present study are clustered into A, B and C branches.

of the same HPV type are biologically distinct and may have different pathogenic risks (26). HPV-16 is the most frequent

HPV type globally, followed by HPV-18 (8). In Sichuan, China, HPV-16 is also the most frequent, however the second most

Table V. Nucleotide sequence mutations of HPV-31 L1.

Category	Variation of L1 at nucleotide position															n	
	5581	5752	5797	5839	5848	5866	5920	5921	5998	6019	6067	6085	6127	6131	6199		
Reference nt	T	A	C	T	A	T	A	T	A	A	A	C	A	T	C		
31LPL01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
31LPL02	A	G	-	-	-	-	-	C	-	G	G	T	-	-	-	-	1
31LPL03	A	-	T	-	-	-	-	C	-	G	-	T	-	-	-	-	1
31LPL04	A	-	-	-	-	-	-	C	-	G	-	T	-	-	-	-	2
31LPL05	-	-	-	-	G	-	-	C	-	-	-	-	G	-	-	-	2
31LPL06	-	-	-	-	-	-	-	C	-	-	-	-	G	A	-	-	1
31LPL07	A	-	-	-	-	-	G	C	-	G	-	T	-	-	-	-	2
31LPL08	A	-	-	-	-	C	-	C	-	G	-	T	-	-	-	-	3
31LPL09	A	-	-	-	-	-	-	C	-	G	-	T	-	-	-	-	3
31LPL10	A	-	-	C	-	-	-	C	-	G	-	T	-	-	-	-	1
31LPL11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
31LPL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
31LPL13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	3
31LPL14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
31LPL15	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	2
31LPL16	A	-	-	-	-	-	-	C	-	G	-	T	-	-	-	-	1
AA mutations														S	206		

Nucleotide positions are shown where sequence alterations were detected in comparison with the sequence of the reference HPV-31 isolate (GenBank accession number J04353). Nucleotide changes are presented by the corresponding letters. Dashes indicate no nucleotide exchange from the HPV-31 reference sequence. The amino acid mutation number is the location in the corresponding genes. HPV, human papillomavirus; nt, nucleotide; AA, amino acid.

frequent is HPV-58 (27). The incidence of HR HPV types in Sichuan were demonstrated to be as follows: HPV-16 (28.1%), HPV-58 (16.0%), HPV-33 (9.2%), HPV-52 (8.4), HPV-18 (7.3%) and HPV-31 (3.4%), with HPV-18 only being the fifth most common (27). In the present study, the observed HPV-31 prevalence was 1.1%. This was similar to the prevalence reported by Xi *et al* (15), which enrolled 5,060 females from the ASC-US and LSIL Triage study in the US and observed a prevalence of 1.1%. However, this differs from the rate (0.4%) reported by a meta-analysis of females with normal cytology from Asia (28), which was in accordance with an international study that reported that the prevalence of HPV-31 is lower in Asia (0.3%) compared with the global average (0.8%), particularly in European (2.3%) and Latin American (1.2%) females with normal cytology (29). However, the rate of HPV-31 prevalence in Sichuan, China, differed from the 0.52% previously reported in Northern Chinese females (30). Future studies regarding geographic variation and ethnic differences may be worthwhile.

HPV E5, E6 and E7 proteins are important for replication and transcription of viral DNA, and are involved in interacting with the cytoskeleton network, cell immortalization and transformation (31). E6 and E7 are known for their ability to bind to and inactivate p53 and pRB, respectively, however they also interact with a wide range of cellular proteins (11,32,33).

To the best of our knowledge, the present study was the first to examine gene mutations of HPV-31 E5. All the mutation sites detected by the present study were novel.

Nucleotide changes in the HPV-31 E6 oncogene at positive 134, 301, 312 and 335 were discovered, to the best of our knowledge, for the first time in the present study. When considering the HPV-31 E7 oncogene, a novel nucleotide change at positive 737 was reported by the present study. According to the frequency of E5, E6, E7 gene mutations, the samples were divided into six, nine and five species, respectively. However, when the E5, E6 and E7 sequences of the samples were integrated, 16 species were detected. E5, E6 and E7 oncogenes displayed characteristics of the alternative model, Kimura's two parameter model, suggesting that these mutations may be cyclic in frequency in the Sichuan population. In these genes, the non-synonymous mutations C285T, A297G, A475G, C520T, C626T, G695A, A743G, C737G, A3975G, C3981G, G4005A, T4053A and A4059G can lead to changes to polarity, hydrophobic potential and the amino acid side chain, which potentially altered the folding of the oncoprotein (34).

In the L1 region, one or more amino acid changes may lead to a conformational change of the capsid protein, and interfere with the conformation of epitopes relevant to viral neutralization. A previous study in Central Brazil reported six mutations (34), fewer than the 30 mutations reported by the present study, of which four were non-synonymous mutations. This previous study reported the presence of the mutation C6862T in Brazil, however the present study did not observe this mutation in Sichuan, China.

Table VI. Additional nucleotide sequence mutations of HPV-31 L1.

Category	Variation of L1 at nucleotide position															n
	6238	6328	6350	6367	6372	6379	6568	6574	6586	6647	6664	6772	6796	6817	6840	
Reference nt	T	G	A	C	C	A	T	C	T	A	T	G	G	C	A	
31LPL01	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	T
31LPL02	-	A	G	T	A	-	-	T	G	-	-	-	A	A	-	-
31LPL03	-	A	G	T	A	-	-	-	G	-	C	-	A	A	-	-
31LPL04	-	A	G	T	A	-	-	-	G	-	C	-	A	A	-	-
31LPL05	A	-	-	T	A	G	C	-	-	-	-	A	A	A	-	-
31LPL06	A	-	-	T	A	G	C	-	-	-	-	A	A	A	-	A
31LPL07	-	A	G	T	A	-	-	-	G	-	C	-	A	A	-	A
31LPL08	-	A	G	T	A	-	-	-	G	C	C	A	A	A	-	-
31LPL09	-	A	G	T	A	-	-	T	G	-	-	-	A	A	-	-
31LPL10	-	A	G	T	A	-	-	-	G	-	C	-	A	A	-	-
31LPL11	-	-	-	T	-	-	-	-	-	-	-	-	-	A	G	-
31LPL12	-	-	-	T	-	-	-	-	-	C	-	-	-	A	-	-
31LPL13	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-
31LPL14	-	-	-	T	-	-	-	-	G	-	-	-	-	A	-	-
31LPL15	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-
31LPL16	-	A	G	T	A	-	-	-	G	-	-	-	A	A	-	-
AA mutations			T		T										Q	
			29		36										192	
			A		N										R	

Nucleotide positions are shown where sequence alterations were detected in comparison with the sequence of the reference HPV-31 isolate (GenBank accession number J04353). Nucleotide changes are presented by the corresponding letters. Dashes indicate no nucleotide exchange from the HPV-31 reference sequence. The amino acid mutation number is the location in the corresponding genes. HPV, human papillomavirus; nt, nucleotide; AA, amino acid.

Table VII. Site-specific tests for positive selection on HPV-31 E5-E6-E7.

Model	InL	Estimates of parameters	2Δl	Positively selected sites
M1	-1731.91	P=0.774, 0.226; w=0.000, 1.000		NA
M2	-1724.28	P=0.919, 0.000, 0.0815; w=0.000, 1.000, 5.227	15.26 P<0.05	138 A ^a , 195 E ^a

InL, the log-likelihood difference between the two models; 2Δl, twice the log-likelihood difference between the two models; the positively selected sites were identified with posterior probability ≥0.9 using the Bayes empirical Bayes approach. NA, not available. NS, sites under positive selection not reaching the significance level of 0.9. HPV, human papillomavirus.

Table VIII. Site-specific tests for positive selection on HPV-31 L1.

Model	InL	Estimates of parameters	2Δl	Positively selected sites
M7	-2556.01	P=0.009; q=0.149		NA
M8	-2544.39	P0=0.995; P=1.600; q=99.000 P1=0.005; w=11.648	23.24 P<0.05	267 T ^a , 274 T ^a

InL, the log-likelihood difference between the two models; 2Δl, twice the log-likelihood difference between the two models; the positively selected sites were identified with posterior probability ≥0.9 using the Bayes empirical Bayes approach. NA, not available; NS, sites under positive selection not reaching the significance level of 0.9; HPV, human papillomavirus.

Xi *et al* (15) reported the proportion of A, B and C variants were 41.7, 21.1 and 37.2% in the USA female population (28). Chagas *et al* (35) examined five HPV-31 positive specimens from Brazil and reported that the proportions of A, B and C variants were 57.2, 5.7 and 37.1%, respectively (35). However, previous investigations have only observed variant lineages A (64.3%) and C (35.7%) in Southern China (30). As above, in the present study the variant lineage A of HPV-31 was the most commonly detected variant, followed by C. However, the present study also observed the presence of variant lineage B, which has not been previously reported from Southern China. The results of one previous study by Ferenczi *et al* (36) differ substantially from the above; when 41 HPV-31 positive specimens were collected from Italian females, the proportions of A, B and C variants were 4.9, 29.3 and 65.8%, respectively (36). Therefore, geographic variation and ethnic differences cannot be ignored.

In conclusion, the present study reported sequence variations in the E5, E6, E7 and L1 genes of HPV-31 isolates from Sichuan, China. The sequence variations in the genes examined may contribute to HPV oncogenesis. These data will provide a solid foundation for further biological and clinical studies, and may also be employed in epidemiological studies where sequence variations are used as markers for monitoring HPV infections in target populations.

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