Whole-Genome Sequence Analysis of Human Papillomavirus Type 18 from Infected Thai Women

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Key Words

Human papillomavirus type 18 • Whole genome, characterization

Abstract

Objective: The aim of this study was to attain molecular knowledge of human papillomavirus type 18 (HPV18) by sequencing the whole genome of HPV18 isolated from Thai women at various clinical stages of disease progression. **Method:** Our group analyzed 9 samples of whole-genome HPV18 in infected women ranging from normal to cervical cancer by PCR, a sequencing method and bioinformatics programs. **Results:** Phylogenetic analysis based on the whole genome showed that HPV18 samples were more closely related to the European and Asian-American type than the African type. The vaccine strain's L1 nucleotide (US patent 5820870) showed a close relationship to the African type. However, our data cannot indicate the correlation between cytological data and nucleotide or amino acid variation. **Conclusion:** Our group cannot draw any inference be-

tween the clinical stage of disease progression and amino acid alterations as there were only 1 or 2 samples available for each clinical trial. However, we hope that these new data on the HPV genome, which are representative of the entire genome of HPV in Southeast Asia, can serve as basis data for future research on the pathogenesis of cervical cancer. Additionally, the second-generation HPV18 vaccines should be tested on both HPV18-L1 and HPV18-L2 for increasing potential protection.

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Human papillomavirus type 18 (HPV18) is the second most prevalent genotype found in cervical cancer [1]. HPV18 has numerous variants which have been identified in different geographical locations [1–3]. Since the HPV18 genome displays numerous variations, HPV18 can be classified as a variant of three distinct groups: European, Asian-American and African [4]. HPV18 variants are associated with progression of cervical neoplasia, and the diversity of the HPV18 oncogenic potential de-

Table 1. Conserved primers for whole-genome amplification and sequencing of HPV18

Set	Primer name	Sequence (from 5' to 3')	Position	Product, bp
A	F15E6_HP18	CAATTgTAgTATATAAAAAAgggAgT	15	886
	R901E6_HP18	ggATgCACACCACggACACAC	901	
B	F828E7_HP18	CAGACgACCTTCgAgCATTCCA	828	803
	R1683E1_HP18	TTTACAgTCTAgACATTgAATATgg	1631	
C	F1612E1_HP18	AAAgTgATAAAAACCACgTgTACAg	1612	896
	R2508E1_HP18	gTTgCATCATCTAACATggCCAC	2508	
D	F2458E1_HP18	TTggTTggAACCGTTAACAgATAC	2458	935
	R3393E2_HP18	AATgTACTTCCCACgTACCTgTg	3393	
E	F3160E2_HP18	AAAggTggCCAAACAgTACAAGTA	3160	969
	R4129E4_HP18	TgCAATAgTAACATgggCAAT	4129	
F	F4016E4_HP18	TATgTgTgCgTATgCATgggTAT	4016	977
	R4993L2_HP18	TgTAATTAAAgAggATggACgTgT	4993	
G	F4871L2_HP18	CATTAgTAgTACCCCATtgcCTA	4870	954
	R5824L2_HP18	CTATATTggTATgCagAAACCTTAg	5824	
H	F5757L2_HP18	AgATTATTAACtGTTggTAATCCATA	5757	911
	R6668L1_HP18	TACAggAgACTgTgTAgAAgCAC	6668	
I	F6538L1_HP18	ggCTCTATTgTTACCTCTgACTC	6538	894
	R7432L1_HP18	ggATAAAATggATgCTgTAAggTg	7432	
J	F7296L1_HP18	gTTCTgTgTgTTATgTggTTgCg	7296	734
	R173E6_HP18	AgTgTTCAgTTCCgTgCACAgAT	173	

Position is based on the nucleotide sequence of HPV18 (X05015).

depends on geographical area and ethnicity of the population studied [5, 6]. HPV18 can be found in both squamous cell carcinoma and adenocarcinoma. However, HPV18 is more frequent in cervical adenocarcinoma than any other viral type, while HPV16 is more frequent in squamous cell carcinoma [7, 8]. HPV18 variants display different potentials of cancer progression. A previous study has revealed that infection by European variants tends to persist [9], whereas Asian-American variants induced tumors in vivo [5]. Moreover, HPV18 variants play different roles in the pathogenesis of cervical cancer [10]. Some previous projects have studied HPV16 at different stages of cervical cancer by examining the distribution of the long control region, E7, E6, L1, L2 and E2 polymorphisms. Thus, we have decided to examine the nucleotide and amino acid variations of HPV18 in critical regions as described in previous studies [1, 11]. The present research has been aimed at characterizing the whole genome (long control region, LCR; early gene and late gene) variability of HPV18 in Thai women at different clinical stages of cervical cancer based on cytological findings by focusing on the critical point. This project presents whole-genome results representative of Southeast Asia in comparison with HPV18 variants from other countries. Finally, it will provide useful information for identifying the nucleo-

tides associated with viral function, viral persistence and pathogenicity.

The study protocols were approved by the Ethics Committee of the hospital and Faculty of Medicine, Chulalongkorn University. All specimens were collected for cytology by liquid-based cytology (Thinprep®; Hologic, UK) and tested for HPV DNA by using Hybrid capture II (Digene). Subsequent to BLAST analysis of the whole genome amplified by polymerase chain reaction (PCR), the respective genotypes were determined by direct sequencing. All HPV samples were stored at -70° until used. From 515 samples, 16 were classified as CA group, with HPV18 present in 3 samples (3/16, 18.75%), 98 samples were classified as HSIL group, with HPV18 found in 3 samples (3/98, 3.06%), 136 samples were classified as LSIL group, with HPV18 detected in 2 samples (2/136, 1.47%), 186 samples were classified as normal group, with HPV18 present in 3 samples (3/186, 1.61%). Thus, we randomly selected HPV18-positive Thai women representing patients with different cytological data. DNA was obtained by organic extraction (phenol-chloroform) of the samples as described previously [12]. PCR was performed to amplify the HPV genome. The reaction mixture comprised 2 µl DNA, 0.5 µM of each primer shown in table 1, 10 µl of 2.5× Eppendorf master mix (Eppendorf, Hamburg,

Germany) and nuclease-free water to a final volume of 25 μ l. The amplification reaction was performed in a thermal cycler (Eppendorf) under the following conditions: initial denaturation at 94° for 3 min, followed by 40 amplification cycles consisting of denaturation at 94° for 30 s, primer annealing at 55° for 45 s and extension at 72° for 1.30 min, and concluded by a final extension at 72° for 7 min. HPV primer positions and PCR products are depicted in table 1. The housekeeping gene β -globin was selected to serve as an internal control for DNA extraction, using conventional PCR as a detection method. Primer sequences for the β -globin gene have been previously described [13].

The PCR products were obtained as described above and used for directed sequencing. Determination of the nucleotide sequences was performed in duplicate and analyzed in both directions using forward and reverse primers to ensure that variations of nucleotide sequences were not due to sequencing errors. When a difference was observed, triplicate sequences were determined in order to confirm the consistency of the sequencing result. Nucleotide sequences were analyzed and assembled using the Lasergene 6 Package® (DNASTar Inc., Madison, Wisc., USA) and BLAST analysis tool (<http://www.ncbi.nlm.gov/BLAST>). Complete genome sequences were prepared and aligned using Clustal W applied by the Bioedit program (version 7.0.4.1). Phylogenetic trees were constructed by neighbor-joining analysis with the Tamura-Nei model executed by the Mega3® program [14]. The rate between nonsynonymous (dN) and synonymous (dS) substitutions was measured by the single likelihood ancestor counting method in Datamonkey elsewhere [15]. Rates of dN/dS refer to selective pressure acting on a protein-coding gene. A dN/dS ratio >1 indicates that this gene is evolving under positive selection. All nucleotide sequences of HPV18 obtained in the course of this project were submitted to the Genbank database under designated accession No. GQ180784–92.

Nine HPV18-positive samples (CU8–CU16) displayed different cytology results such as normal (CU8–CU9), LSIL (CU10–CU11), HSIL (CU12–CU13), squamous cell carcinoma (CU14–CU15) and adenocarcinoma (CU16). HSIL and cervical cancer (CU15–CU16) samples have a histological outcome to confirm the cytological result whereas normal and LSIL samples lacked histological outcome. This study was performed on women between 19 and 70 years of age. All 9 samples had shown positive results on application of a commercially available test kit (Hybrid capture II) except for CU14 and CU16 which had yielded an abnormal Pap smear detected by a cytopathol-

ogist. Whole-genome sequences of HPV18 found in Thai samples (accession No. GQ180784–92) were aligned with X05015 [16], NC001357, Y262282, EF202152–EF202155 (African type), EF202147–EF202151 (European type) and EF202143–EF202146 (Asian-American type). Analysis of HPV nucleotide sequences showed 97.9–99.9% similarity among the Thai and all reference sequences (data not shown). Phylogenetic analysis revealed that HPV18 in Thailand was closely related with European and Asian-American types (fig. 1).

The complete genome of CU11 comprised 7,844 bp, whereas other samples consisted of 7,857 bp indicating variable genome lengths of HPV18 isolated from Thai women. We observed deletion in the LCR (7245–7256) and E4 gene (position 3630–3635) of CU11 samples (data not shown). These deletions occurred in the African type, and phylogenetic tree analysis showed that CU11 was closely related with the African type whereas other samples were related with the European and Asian-American types (fig. 1). In contrast to existing data on HPV16 variants, HPV16 in Thailand was more closely related to the European than the Asian-American and the African types [17]. Thus, our study showed that HPV18 variants in Thailand related two distinct groups of HPV18 variants (European and Asian-American types). Nevertheless, due to the small sample size, this finding should be verified by larger studies.

Based on nucleotide variation, we focused on the LCR segment at positions 41, 104 and 7,726. A previous study had reported that nucleotide variations in this segment should affect transcription factor binding sites such as Sp1 (selective promoter factor 1), YY1 (yin-yang factor 1) and Oct1 (octamer binding factor), respectively [1]. Based on our results, nucleotide variations at these critical positions occurred with 2 samples (CU10 and CU11). Nucleotide variation of CU10 changed both positions 41 (A to G) and 104 (T to C), whereas the nucleotide sequence of CU11 changed at positions 104 (T to C) and 7726 (C to T). Variation of nucleotides 41 and 104 was associated with a higher activity of the E6/E7 promoter by modulating Sp1 and YY1 activities [11]. A previous report indicated that nucleotide alterations at position 104 were less likely to promote tumor recurrence than virus with the reference sequence [11] and variation was usually found in the European type. Based on the pattern of nucleotide variation, all samples displayed more similarity to the European (especially CU10) and Asian-American type than the African type. We could detect these changes in CU10 and CU11 that indicated LSIL cytological data.

Fig. 1. Phylogenetic tree based on whole-genome analyses of 9 samples and reference sequences from the Genbank database. Phylogenetic tree construction by neighbor-joining analysis with the Kimura model implemented in the Mega® program (version 3.1).

Based on amino acid analysis, the E6 region, position N129K, had not undergone amino acid variation in our samples whereas in CU10 and CU11, the L1 region, position T149N, was identical to the African and European types. The amino acid at position 129 is highly conserved within HPV18, and in vitro analysis has shown that this change of amino acid does not affect the ability to promote p53 degradation [9]. Amino acid alteration at position 149 of L1 is commonly found in many HPV genotypes and is located near the surface of the capsid protein [18]. Hence, it does not affect the function of the L1 gene. In addition, the proteins displaying most nonsynonymous amino acids were detected in E2 and L2 as reported for HPV16 in a previous study [17]. López-Saavedra et al. [19] indicated that the E2 viral protein is essential for regulating LCR activity and transcription of viral genes. Some researchers reported that E2 variations were insignificant between controls with no past and current history of cervical abnormalities and tumor cases [20]. Multiple nucleotide substitutions found in HPV18-E2 variants were not affected by activities over homologous LCR among HPV18 variants [19]. Moreover, based on variation, the rate of dN/dS alterations was not found significant

for all coding regions. Thus, our data did not perform and analyze amino acid variation on the E2 gene. However, E2 variation could affect other biological functions such as viral replication or interaction with other cellular proteins [19].

Alignment of the vaccine strain's (US patent 5820870) polypeptide with all our samples has shown that the L1 vaccine strain is closely related to the African type, whereas most of our samples' sequences are related to the Asian-American and European types. Some previous studies revealed that oncogenic potential and HPV18 variants demonstrated that the African type is usually found in patients with less abnormal pathology [21]. Although bivalent and quadrivalent vaccine cross-reactivities have been reported, long-term stimulation of immune response remains to be established in direct comparison [22]. A report revealed that protective and broadly cross-neutralization should be emphasized on L2 [23]. Thus, a second-generation HPV18 vaccine should be tested on both HPV18-L1 and HPV18-L2 for increasing potential protection. Due to our small sample size, we could not observe any significant correlation between HPV18 variants detected in this study and the results

from cervical cytology. In addition, we did not see any difference in the distribution of HPV18 variants when comparing squamous cell carcinoma with adenocarcinomas. Our data support the proposed association of non-European variants (especially the Asian-American variant) with adenocarcinoma as suggested elsewhere [24]. However, our project was limited by the small sample size. It had been intended as a preliminary study aimed at HPV18 genome characterization in Thailand which would represent HPV18 in Southeast Asia.

In conclusion, we found numerous nucleotide and amino acid variations in the genome of HPV18 isolated from infected Thai women. Whole-genome analysis of these samples showed HPV18 from Thailand to be more closely related to the European and the Asian-American than African type. This study revealed that our group cannot draw any inference between the clinical stage of

disease progression and amino acid alterations as there were only 1 or 2 samples available for each clinical trial. However, we hope that these new data on the HPV18 genome which are representative of the entire genome of HPV18 in Southeast Asia can serve as basic data for scientific research on the pathogenesis of cervical cancer.

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