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Human Papillomavirus Type 16 L2 Gene Sequence Variation Analysis in Indonesian Cervical Cancer Specimens

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Research Article

Keywords: Human papillomavirus, HPV16, L2 gene, genetic variation, phylogeny

Posted Date: October 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-968609/v1

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Abstract

Background: Human papillomavirus type 16 (HPV16) is the most common etiological cause of cervical cancer in Indonesian women. HPV16 L2 genetic variation analysis is important for vaccine strategies of cervical cancer, yet the data in Indonesia is still limited. In this research, we determined the variation of the HPV16 L2 gene sequences in Indonesian cervical cancer specimens.

Method: We cross-sectionally observed a total of 23 DNA isolates of HPV16 positive specimens stored in the laboratory of the Center for Diagnostic and Research on Infectious Diseases (PDRPI Lab) Faculty of Medicine, Universitas Andalas, Padang, Indonesia. The HPV16 L2 genes were amplified, sequenced, and followed by DNA alignment, single nucleotide polymorphisms (SNPs) analysis, and phylogenetic tree reconstruction.

Results: As many as 35 SNPs were found, consist of 18 synonymous SNPs (sSNPs) and 17 non-synonymous SNPs (nsSNPs). Amino acid variations were mostly detected at S269P (100%) and L330F (43.48%) with no variation in the immuno-protective region near L2 N-terminus. A total of 5 HPV16 phylogenetic sub-lineages were found closely related to A1 (*n*=5), A2 (*n*=12), A3 (*n*=2), A4 (*n*=3), and C (*n*=1).

Conclusion: The variation of HPV16 L2 gene sequences was mostly located on the central region of the L2 sequences and the cross-protective region near the L2 N-terminus was particularly conserved. This study should enhance the information about HPV16 L2 gene variation in Indonesia.

Background

Cervical cancer is one of the main causes of morbidity and mortality in women (1). The incidence and the death rates of cervical cancer reached 15.1 and 8.2 respectively per 100,000 global population in 2018, which were the third-highest of all cancer cases (2). At the same time, Indonesia recorded 32,469 new cases and 18,279 deaths annually and had the highest incidence rates across South-East Asia countries (23.4 per 100,000 women) (3). The disease was caused by human papillomavirus (HPV) infection which is transmitted through sexual contact (4,5). This virus is found in 8.6% of women with cancers in the world (6). HPV infection has remained a global health problem that affects more than 24% population in many countries including Indonesia, especially in rural areas (2,7,8).

HPV belongs to the *Papillomaviridae* family that infects the skin and mucosal epithelial tissue. This virus is a circular double-stranded DNA virus with a genome around 8,000 bp and consists of early (E), late (L), and non-coding (NCR) regions (9). HPV carcinogenicity is mainly due to the activity of E5, E6, and E7 proteins in the tumor suppressor genes p53 and pRb of host cells (10). However, the L2 protein is crucial in viral entry into the host cell, avoidance to the immune system, and the integration of viral DNA into the host genome. L2 is the HPV minor capsid protein that has a molecular weight of around 67-78 kDa and is composed of approximately 500 amino acids. This protein is also necessary to release the virus from the endosomal vesicles after entering the host cell, transporting the viral genome into the nucleus, and

assembling viral components (11). Nevertheless, some studies have shown that the conserved region near the N-terminus of L2 protein may induce cross-neutralizing antibodies against highly divergent HPV types and can be single-expressed in bacteria. This finding proposed the HPV L2 to become a potential antigen candidate for the broad HPV vaccine (12–14).

Human papillomavirus type 16 (HPV16) is the most carcinogenic subtype of HPV (15). HPV16 infection is found in 47% of cervical cancer cases in Indonesia which is the most prevalent and may worsen the quality of life and decrease one's five-year life expectancy by 66.9% (3,16,17). This subtype belongs to the *Alphapapilloma* genus and alpha-9 species group. Based on phylogenetic analysis, HPV16 consists of four lineages (A, B, C, D lineage) and ten sub-lineages, namely: A1-A3 (European/ E); A4 (Asian/ As); B1 (African-1a/ Afr-1a) and B2 (African-1b/ Afr-1b); C (African-2a/ Afr-2b); D1 (North American-1 / NA), D2 (Asian-American-1/ AA1), and D3 (Asian-American-2/ AA2). The non-European (NE) lineages (B/C/D) are known to have a higher risk of infection persistence, progression to the pre-cancerous lesion, and development of cancer than the European (E) lineage (A) (18).

HPV16 L2 genetic variation and its phylogenetic analysis are vital to enhance the understanding of viral infectivity and pathogenicity. These analyses are also important for the clinical setting of cervical cancer management, especially in diagnostic and vaccine strategies (19). However, research on the HPV16 L2 gene in Indonesia is still limited. Here we determine the HPV16 L2 genetic variation and phylogenetic lineage from cervical cancer specimens in Indonesian women.

Materials And Method

Study design, population, and sample

This study was conducted with a cross-sectional approach using stored biological samples. The research population was the patients diagnosed with cervical cancer in Arifin Achmad Hospital, Pekanbaru, Riau Province, Indonesia. The specimens of the patients had already been obtained from cervical scrapings and reserved in the Center for Diagnostic and Research on Infectious Disease (PDRPI Lab), Faculty of Medicine, Universitas Andalas, Padang, West Sumatra Province, Indonesia. The viral DNA was isolated from these specimens and had been previously detected HPV positive using the PCR method with universal HPV primers GP5+/6+ (forward: 5’-TTT GTT ACT GTG GTA GAT ACT AC-3’; reverse: 5’- GAA AAA TAA ACT GTA AAT CAT ATT C -3’; product size: 150 bp). The HPV16 positive isolates were detected with PCR using specific genotyping primers (forward: 5’-GTC AAA AGC TGT GTC CT-3’; reverse: 5’-CCA TCC ATT ACA TCC CGT AC-3’; product size: 450 bp). A total of 23 HPV16 positive samples available were all examined in this research. This study has been ethically approved by the ethics committee at the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, Ref. No.: KE/FK/1273/EC/2019.

DNA Amplification

Amplification of the HPV16 L2 gene (NCBI reference sequence: NC_001526.4, nucleotide position: 3,373-4,794) was administered by conventional PCR method using three pairs of HPV16 L2 targeted primers: L2.1, L2.2, and L2.3 (Table 1) designed using AmplifX software and confirmed with NCBI-BLAST. The amplifications were performed separately for each primer set with a concentration of 10 µM (1 µL), DNA template (3 µL), and using TopTaq master mix kit Qiagen with a total PCR mix volume of 50 µL. The PCR protocols were 94°C of initial denaturation for 2 min, followed by 35 cycles of 94°C of denaturation for 45 s, different annealing temperature of each primer set for 45 s, and 72°C of extension for 1 min, then 72°C of final extension for 10 min. The DNA amplicons were electrophoresed at 7 µL each in 1% agarose well with 0.5 µL Gel Red staining. Electrophoresis was performed with a voltage of 100 V for 30 minutes. As an indicator, a DNA ladder of 1 kbp or 100 bp was used. PCR results were observed under UV transillumination or a Gel Doc machine and documented.

Table 1
Primer for amplification of HPV-16 L2 gene.

Primer*	Nucleotide sequences	Position	Product size (bp)	Tm (°C)	Ta (°C)
L2.1 F	5'-CAG CCT CTG CGT TTA GGT GTT T-3'	3143-3754	612	61.39	62.8
L2.1 R	5'-GGG GAA TGG AAG GTA CAG ATG TTG-3'			61.17	
L2.2 F	5'-TGT GGG CCC TTC TGA TCC TTC TAT-3'	3663-4281	619	63.00	59.6
L2.2 R	5'-GTA CCT AAT GCC AGT ACG CCT AGA-3'			61.52	
L2.3 F	5'-CTA GGC GTA CTG GCA TTA GGT ACA-3'	4259-4919	661	61.76	58.7
L2.3 R	5'-AGG GAT GTC CAA CTG CAA GTA G-3'			60.03	

*Primer: F, forward; R, reverse; bp, base pair; Tm, melting temperature; Ta, annealing temperature.

Gene sequencing and variation analysis

To acquire the gene sequences, the amplicons of the HPV16 L2 gene (40 µL of each sample) were sequenced in the 1st Base Singapore (41 Science Park Road #04-08, The Gemini, Singapore 117610) using the Bigdye® Terminator V3.1 Cycle Sequencing Kit and the three HPV16 L2 primer sets. The sequence products of each sample with different primer set were combined using MEGA X v10.0.5 software to obtain a maximum length of HPV16 L2 gene sequence (complete size 1,422 bp, nucleotide position 3,373-4,794). The combined HPV16 L2 sequences were aligned with the HPV16 genome (NCBI

reference sequence: NC_001526.4) to observe the single nucleotide polymorphisms (SNPs) and amino acid variations.

Phylogenetic analysis

To recognize the HPV16 L2 sub-lineages, the phylogenetic analysis was performed by aligning all the 23 HPV16 L2 sequences with the NCBI reference sample (NC_001526.4) and 10 HPV16 genomes representing different sub-lineages of variant prototypes (A1, K02718; A2, AF526179; A3, HQ644236; A4, AF534061; B1, AF536180; B2, HQ644298; C, AF472509; D1, HQ644257; D2, AY686579; D3, AF402678). The phylogenetic tree was reconstructed using MEGA X v10.0.5 software with the evolutionary analysis by maximum likelihood method and Tamura-Nei model with the number of bootstrap replications was 1,000.

Nucleotide sequence accession number

The HPV16 L2 gene sequences obtained were submitted to the GenBank with accession numbers from MW810431 to MW810453.

Results

HPV16 L2 sequence variations

From a total of 23 samples, the sequencing process generated as many as 9 complete and 14 partial HPV16 L2 gene sequences. All partial sequences were obtained at best from HPV16 L2 primer sets of L2.2 and L2.3 only, with a combined sequence size of 1,132 bp in a nucleotide position of 3,663-4,794. From these available sequences, a total of 35 SNPs was detected with 17 non-synonymous SNPs (nsSNPs) and 18 synonymous SNPs (sSNPs) (Table 2). The most variable sites of nucleotide were G4074A (100%), T4177C (100%), T4362G (52.2%) and T4362C (43.5%) compared to NCBI reference. According to these sequences, there were at least 10 unique genetic variations or variants were recognized. Mostly amino acid variation detected at the center region of HPV16 L2 protein, with predominantly were Serine to Proline (*n*=23/23; T4177C/ S269P) and Leucine to Phenylalanine (n=10/23; A4362C/ L330F) (Fig. 1). There were no SNPs and amino acid variations detected near the N-terminus region of HPV16 L2 in all complete sequences. There were also no deletions or insertions alongside the HPV16 L2 region.

HPV16 L2 phylogeny

The phylogeny of HPV16 L2 gene sequences between the samples, the GenBank reference, and 10 intra-typic variants representing the sub-lineage of HPV16 is depicted in the phylogenetic tree of the Tamura-

Nei model (Fig. 2). There were five phylogenetic sub-lineages of HPV16 L2 found closely related to sub-lineage A1/European (*n*=5), A2/European (*n*=12), A3/European (*n*=2), A4/Asian (*n*=3), and C/African-2a (*n*=1). There was no sample close to B and D sub-lineages. These results indicated that A lineage (European-Asian) was the most prevalent variant in this study with the highest dominance being A2 sub-lineage (European).

Discussion

HPV16 infection remained the leading etiological cause of cervical cancer worldwide, including in developing countries such as Indonesia (20). The L2 minor capsid protein is vital in HPV infection, especially for entering the host's cell, vesicular trafficking, nuclear entry, and supporting nuclear activities. L2 also proposes as a broadly neutralizing epitope, especially in the region near the N-terminus at amino acid residue 17-36, 58-64, 64-73, and some other segments beyond the first 120 residues (21–23). A study on HPV16 L2 gene variation should be essential for future research about their differences in pathogenicity in cervical cancer, as well as recognizing their phylogenetic sub-linages. It is also important in a clinical setting especially for vaccine strategies against HPV broad types and therapeutic modalities targeted on L2 (19,23–25). However, the data available for HPV16 L2 gene variation in Indonesia is still limited. Here we have shown that most SNPs in the 23 HPV16 L2 sequences from Indonesia were found at the middle to near C-terminus, yet the amino acid near N-terminus particularly at the first 120 residues was notably conserved.

Sequencing analysis revealed as many as 35 SNPs and resulted in ten unique variants that represent their specificity in the SNPs. For example, Variant 1 which was phylogenetically related to A1/European sub-lineage, had a distinctive SNP at T4548G, which is different from the NCBI reference sequence, another A1 sub-lineage member. The entire L2 sequence of Variant 1 is 100% similar to the Japan variant (NCBI accession number LC456632.1 (26). The most prevalent variant was Variant 4 from A2/European sub-lineage (39.13%), homolog with variants of Netherland (KY549169.1) and Japan (LC511106.1) (26,27). Yet, the most unique variant was Variant 10 from the C/African-2 sub-lineage, which is to the best of our knowledge, the 100% similarity is not available. Together, we assumed that there should be a historical relationship between Indonesian variants and these variants. However, further research needs to be done with larger sample size.

According to the type of point mutation, there were only transversion and translation detected in the HPV16 L2 sequence without any deletion or insertion. This is similar to the findings in most studies involving whole-genome sequencing of HPV16 in Asia, Europe, and America (19,26,28–30). However, deletions in L2 gene sequences have ever been reported in Central China as many as 3 sites per 51 samples (31). Although there was no deletion or insertion in our study, amino acid (AA) variations were generated from nsSNPs as many as 17/35 (48.57%). The most prevalent AA variation was S269P of L2 269 (100%) that still has an unknown effect on the L2 activities. However, AA variations in L2 428 (17.4%) and L2 424 (4.3%) may be related to the L1-L2 bond which is responsible for maintaining HPV virion (32). Other interesting sites of AA variation were detected in Variant 10, such as L2 419 and L2 420 which are

important locations for virus interactions with the ND10 subdomain in the host cell nucleus (33). Other AA variations, L2 309 and L2 311 were found in all variants of the A4 sub-lineage that regulates the viral genome complex to accumulate in the cell nucleus in vivo (34).

Based on the result of nine complete HPV16 L2 sequences obtained from the study, there was no AA variation in the region near N-terminus (AA 1-121). Despite a very low sample size due to the limitation of our research, this finding is likely supporting the evidence that the region near the N-terminus of HPV L2 is highly conserved (35–37). This region is known as essential for L2 infection mechanisms as cell surface exposure site, furin cleavage sites right before entering the host's cell, DNA binding domains, and localization of cell nucleus signals (11). Several studies have also shown that the L2 N-terminus region potentially induces cross-neutralizing antibodies and promises to be a broad HPV vaccine epitope candidate (14,22,37–39). The low-cost L2-based vaccine strategy may become a possible alternative compared to the high cost of the L1-based vaccine available (40). We hypothesized that L2-based vaccine strategies should be effective in lowering the morbidity and mortality of cervical cancer in developing countries like Indonesia, yet further research is necessary.

To address the variants and sub-lineages of Indonesian HPV16 L2 sequences, we conducted a phylogenetic analysis using the maximum likelihood method and Tamura-Nei model with the number of bootstrap replications of 1,000. As many as ten unique sequence variants were revealed, with a dominance of European sub-lineages (A), particularly A2. Our finding is following the result of a large phylogenetic study of HPV16 whole genome-sequencing which is also highlighted A sub-lineage as the world's most prevalent phylogeny (78.8%), with exception of A2 sub-lineage domination (41). However, a larger sample size should be included in a future study to confirm this finding.

Apart from the very limited samples observed and using long-stored HPV16 DNA isolates, the unavailability of adequate original identification data of the cervical cancer patients was the substantial limitation of our study. However, this research should be one of the preliminary studies that revealed the variation of HPV16 L2 gene sequence in Indonesia specifically and supporting evidence to further research on HPV-related disease.

Conclusion

In summary, our study determined the HPV16 L2 gene sequence variation and phylogeny of Indonesian isolates. We have shown that this variation was mostly located in the central region of the L2 sequences and the cross-protective region near the L2 N-terminus was notably conserved. This finding should support the opportunity of a cost-effective HPV L2-based vaccine strategy to deal with cervical cancer, especially in a developing country like Indonesia. Future research is necessary to explore HPV16 genetic variation and enhance our understanding of genetic-based strategies to reduce cervical cancer-related morbidity and mortality.

Abbreviations

AA: amino acid; HPV16: Human papillomavirus type 16; PDRPI: Center for Diagnostic and Research on Infectious Diseases; SNPs: single nucleotide polymorphisms; nsSNPs: non-synonymous SNPs; sSNPs: synonymous SNPs.

Declarations

Ethics approval and consent to participate:

The ethical approval was issued by the ethics committee at the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, Ref. No.: KE/FK/1273/EC/2019. Written informed consent had previously been derived from each patient to use their specimen data for laboratory studies. The research was conducted following the relevant ethical guidelines and regulations.

Consent for publication:

Not applicable

Availability of data and materials:

The data is available from the corresponding author and PDRPI but restrictions apply to the availability of these data. Data are available directly from the author (syandrez@med.unand.ac.id) and PDRPI (divisi_diagnostik_infeksi@med.unand.ac.id).

Competing interests:

The authors declare that there is no competing interest in this study.

Funding:

The funding was received from the Faculty of Medicine, Universitas Andalas (FK Unand) allocated to the project “Budget Implementation List (*DIPA*) of Fundamental Scheme”, with grant number 1097/UN16.02.D/PP/2020. The FK Unand had no role in the design of the study, collection, analysis, and interpretation of data, and in writing the manuscript.

Authors’ contributions:

SPP performed the research experiments, analyzed the data, and drafted the manuscript. AEP collected the specimens, prepared the HPV DNA isolates, designed the experiments, provided laboratory equipment, and drafted the manuscript. WA and IA contributed to reviewing the manuscript and guiding the research. All authors read and approved the final manuscript.

Acknowledgment:

We thank Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada for the ethical clearance approval. We thank all staff in Master Program of Tropical Medicine of the faculty who

contributed to the study. We also thank Faculty of Medicine, Universitas Andalas for the funding and all staff in Microbiology Department of the faculty for the support and facilitation of the experiments.

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Table

Due to technical limitations, table 2 docx is only available as a download in the Supplemental Files section.

Figures

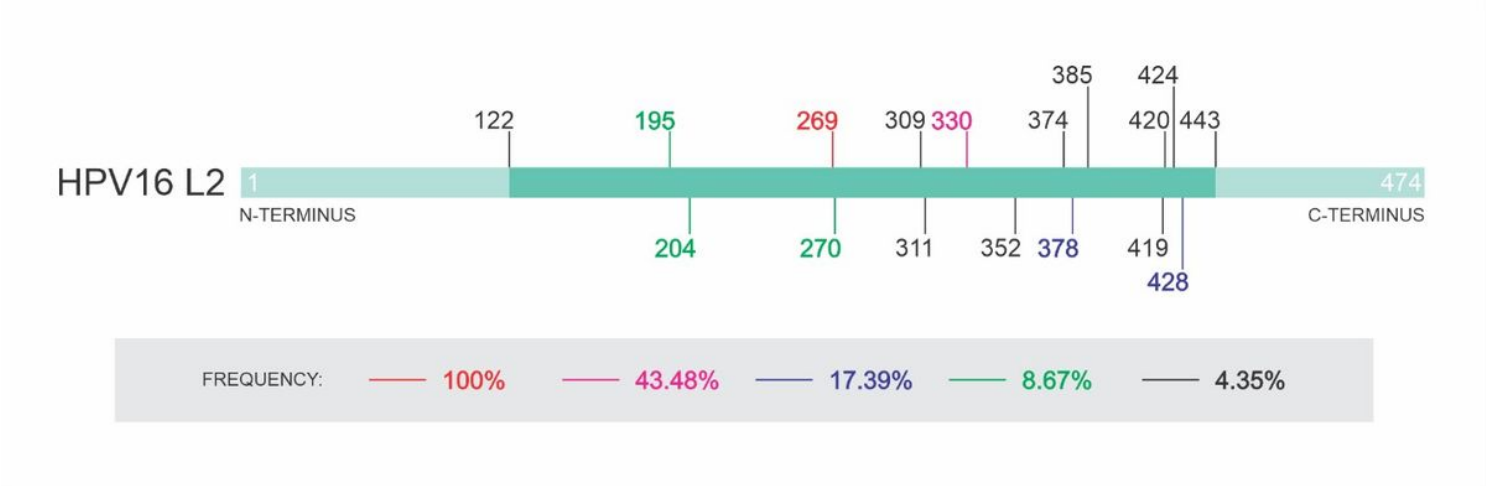

Figure 1

Position of amino acid variations in HPV16 L2 open reading frames. An illustrative green bar representing the L2 protein. The position site of amino acid variation is shown in line with number. The variation frequencies are described in percentage of representing color (red, blue, green, and black). Most of these variations were detected and figured in the central region (deep green). There is no variation near the N-terminus and C-terminus region of L2 (light green).

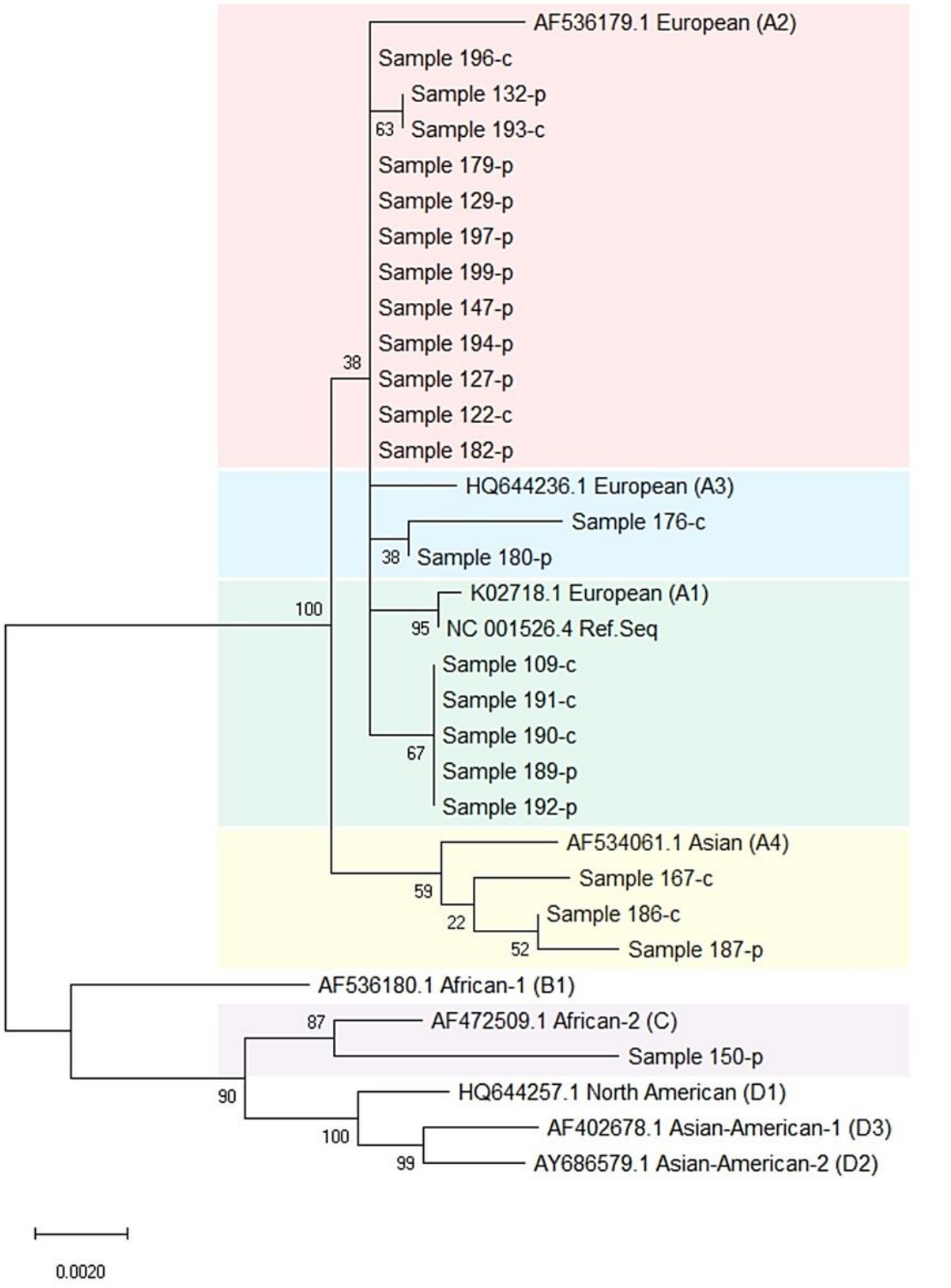

Figure 2

Phylogeny of HPV16 L2 sequences. The phylogenetic tree was constructed from 23 HPV16 L2 sequences compared with the whole-genome sequences of NCBI reference (NC_001526.4) and 10 intra-typic variants representing the HPV16 sub-lineages (A1, A2, A3, A4, B1, B2, C, D1, D2, D3). The analysis was performed with the maximum likelihood method and the Tamura-Nei model with 1,000 bootstrap replications. The

colored box represented the HPV16 sub-lineage: red (A2), blue (A3), green (A1), yellow (A4), purple (C). Scale bar was nucleotide substitution per site.

Supplementary Files

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- Table2.docx