Conservation of Major L1 and Variability of Minor L2 Capsid Late Protein Genes in Human Papillomavirus of Indonesia Variants

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Abstract

Human Papilloma Virus (HPV) has an outstanding feature for its vast intraspecies variability. Of all known 100 types or more, 15 types of them are classified as high risk because of their occurrence in more than 95% of cervical cancer cases. Among all genes in their genome, E6 and E7 genes are considered oncogenes and have close relevance with their pathogenicity, whilst L1 and L2 genes produce capsid proteins that directly interact with their host receptors. Considering the importance of L1 and L2 in host-receptor relationship, we tried to investigate their molecular variability thereby uncover their specificity as Indonesian variants. Here we reported about the conservation of L1 minor capsid protein and variability of L2 capsid protein among high-risk types Human Papilloma Virus (HPV). The results indicated that L1 DNA was relatively more conserved than its L2 counterpart. Also it was indicated that the middle part of either L1 or L2 CDS’ showed more DNA variability than those at their upstream sequences. It is concluded that L2 middle sequences are important factors for intraspecific variations found in HPV of Indonesian variants.

Keywords: Human Papilloma Virus (HPV), L1 gene, L2 gene, cervical cancer, high risk HPV

Introduction

Cervical cancer is an “infectious” cancer caused by persistent infection of Human Papilloma Virus (HPV) of oncogenic types which could potentially lead to cancer. More than 95% of cervical cancer cases are caused by several types of high-risk HPV (Bekkers et al., 2004; Motoyama et al., 2004). In Indonesia, cervical cancer is the leading cause of death in women followed by breast cancer (Novel et al., 2010).

Since the advent of molecular methods, over 100 HPV genotypes have been isolated and characterized. Among these, more than 40 have been shown to infect the genital tract, and 15 of them (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) have been found to be associated with cervical cancer or High-Grade Cervical Intraepithelial Neoplasia (CIN) and therefore classified as high-risk types (Munoz, 2000; Munoz et al., 2003).

For decades, early detection method of cervical cancer has been relied on cytological technique known as Papsmear developed by Dr. Papanicolau (Novel et al., 2010). Recently HPV DNA tests have been developed that make use of molecular biology techniques. The FDA approved HPV detection kit of Digene/Hybrid Capture 2 (HC2) manufactured by Digene (MD, USA) was presently the best example of HPV-DNA test available in the market. The HC2 procedure includes several steps using genotype-specific probes that are mixed in a high-risk or a low-risk RNA cocktail, and RNA-DNA hybrids are recognized by an antibody, used both for the capture step and for a signal amplification detection method. This technique is used to screen high-risk types from low-risk and other types; however it cannot discriminate among different genotypes (Burd, 2003).

HPV belongs to the Papovaviridae family, has a diameter of 55 μm with isohedral protein structure that are arranged from 72
capsomeres. It has circular double-stranded DNA with a length of approximately 8000 base pairs (de Villiers et al, 2004). The linearized representation of HPV 16 genome is shown in Figure 1.

Studies of HPV genome variation are very important to provide biological component as diagnostic or immunogenic materials originated from diverse sources of local variants. This study investigated molecular variation of L1 and L2 DNA sequence of HPV type 16 of Indonesia origin. At the end, this study may provide genetic information about genomic variations of L1 and L2 genes of HPV type 16 of Indonesian variants so that it opens up opportunities in the design and manufacture of recombinant vaccine as well as diagnostic kits based on the local database.

Figure 1. Linearized representation of HPV genome consists of Early (E1-E7), and Late (L) genes (L1 = 1421 bp & L2 = 1595 bp) genes and long controlling region (LCR).

Table 1. Oligonucleotide primers for PCR and DNA sequencing for the amplification of partial L1 and L2 regions.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer name</th>
<th>5'-3' sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HPV 16 F</td>
<td>GGT CGG TGG ACC GGT CGA TG</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>HPV 16 R</td>
<td>GCA ATG TAG GTG TAT CTC CA</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>L1 LEFT F</td>
<td>TGC AGC AGG TGA CTT TTA TTT ACA TCC</td>
<td>27</td>
</tr>
<tr>
<td>4.</td>
<td>L1 LEFT R</td>
<td>ACC AGC ACC TAC CTC ACC TAC</td>
<td>21</td>
</tr>
<tr>
<td>5.</td>
<td>L1 MIDF</td>
<td>GCT GGT TTG GGC CTG TGT AG</td>
<td>20</td>
</tr>
<tr>
<td>6.</td>
<td>L1 MIDR</td>
<td>TTG GCC TTT AAT CCT GCT TGT AG</td>
<td>23</td>
</tr>
<tr>
<td>7.</td>
<td>L2 LEFT F</td>
<td>TTT TTG GTG GGT TAG GA</td>
<td>17</td>
</tr>
<tr>
<td>8.</td>
<td>L2 LEFT R</td>
<td>AAA GTG GGA TTA TTA TGT GTA GTA</td>
<td>24</td>
</tr>
<tr>
<td>9.</td>
<td>L2 MIDF</td>
<td>AGA CCC CCT TTA ACA GTA GAT</td>
<td>21</td>
</tr>
<tr>
<td>10.</td>
<td>L2 MIDR</td>
<td>TAG GCA GCC AAA GAG ACA</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 2. Temperature program for real time PCR followed by HRM analysis of L1 and L2 PCR fragments. LP= Left primer; M= Middle primer. *=denotes sec/increment

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Temperature (°C)</th>
<th>Time (min:sec)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>L2</td>
<td>L1</td>
<td>L2</td>
</tr>
<tr>
<td>Enzyme activation</td>
<td>98</td>
<td>98</td>
<td>03:00</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>98</td>
<td>00:05</td>
</tr>
<tr>
<td>Annealing/extension</td>
<td>61.4 (LP)</td>
<td>64.9 (M)</td>
<td>55-60</td>
</tr>
<tr>
<td>HRM</td>
<td>75-95</td>
<td>75-95</td>
<td>00:05*</td>
</tr>
</tbody>
</table>

Materials & Methods

Sampling & HPV DNA preparation. Cervical swab sample suspensions in the Specimen Transport Medium (Digene™, USA) were spotted on filter paper (Whatman FTA™ Cards) to immobilize their DNA and then air-dried for long term storage afterwards. DNA template was prepared from at least 3 punched paper discs by elution with FTA elution buffer (Whatman™, USA). DNA
concentration was measured using a spectrophotometer.

**Hybrid Capture Technology.** Second generation Hybrid capture technology (HC-II; The Digene Corporation, USA) was used to screen high risk HPV types to detect nucleic acid targets directly. This screening discriminates the following high-risk types (as initially defined by Digene and supported by epidemiological studies): HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56. The HC-II procedure includes: DNA denaturation (1 hr), Mixing with RNA probe cocktail followed by hybridization at 65°C (1.5 hr), Hybrid Capture (1.5 hr), Detection for labeling (1 hr) and finally Data collection, validation and interpretation (1 hr).

**PCR primers.** PCR and sequencing primers were designed using a standalone primer design software (PrimerSelect™, DNA Star™, Hitachi, Japan) generated from L1 (bases 5560-7155) and L2 (bases # 4236-5657) CDS’ of Human Papillomavirus of East Asia Type as reference (GenBank Accession number AF534061.1). Several alternative primers covering at least the first 300 bp upstream regions of both L1 and L2 genes are shown in (Table 1).

**Standard PCR, Real Time PCR and High Resolution Melting (HRM) Analysis.** Standard PCR was done using Master Gradient Thermal Cycler (Eppendorf™, Germany). Real Time PCR was performed on CFX96 machine attached to its C1000 base unit (BioRad, USA). PCR mix (GoTaq Green, BioRad USA) was used throughout the experiment to prepare DNA for genotyping of HPV 16 and DNA sequencing. The typical PCR cycle was as follows: Initial denaturation 95°C, 5 min; Denaturation 95°C, 30 sec; Annealing 54°C, 30 sec; Extension 72°C, 30 sec. The denaturation, annealing and extension steps were repeated to 39 cycles followed by final extension at 72°C for 10 min. High Resolution Melting (HRM) Analysis was done in a Real Time PCR setting using dsDNA specific dye (Sso Fast™ EvaGreen® Supermix, BioRad USA) which was chosen as a preferred alternative to SYBR Green dye (Mao et al, 2007). HRM temperature scheme was immediately attached following the PCR temperature protocol as shown in Table 2.

**DNA Sequencing and analysis.** Analysis of the L1 and L2 were done using modified dideoxy Sanger Method in the PCR cycle sequencing to determine the polymorphism in detail, with Automated DNA Sequencer ABI 3730 XLs (Applied Biosystem, USA). Sequence analysis such as multiple alignments and phylogenetic clustering were performed using Bio Edit or MegAlign™ DNA Star (Hitachi, Japan). The Clustal V method groups sequences into clusters by examining sequence distances between all pairs. Clusters are aligned as pairs then collectively as sequence groups to produce the overall alignment. After finishing the multiple alignment, a neighbor-joining method was employed to reconstruct phylogeny for the putative alignment.

**Results**

**L1 HPV DNA Analysis.**

High resolution melting (HRM) analyses were performed following Realtime PCR amplification of L1 HPV. The results indicated that there were 12 different types of polymorphisms grouped into 12 clusters. (Figure 2A) on the first 300 bp upstream regions. The same analysis was performed on L1 middle regions and finally grouped them into 8 polymorphic clusters (Figure 2B). All clusters were used as basis for choosing sequencing samples thereby avoiding too many samples to sequence.

**Phylogenetic analysis of L1 HPV DNA sequences**

Based on the multiple alignment analyses, DNA distances were grouped to construct a phylogenetic tree of upstream region of L1 HPV DNA sequence (Figure 3). As seen from the tree construct, upstream region of L1 DNAs were relatively homogenous with tight grouping. These results coincide with DNA sequence analyses shown on Figure 4 where Indonesian variants had relatively conserved L1 sequences at their upstream region. However Indonesian variants grouped together with the majority of them were separated from L1 HPV from other countries.
Figure 2. L1 HPV-DNA clusters generated by High Resolution Melting (HRM) analysis resulting in 12 clusters of L1 upstream regions (A) and 8 clusters of L1 middle regions (B) respectively.

Figure 3. Phylogenetic analyses of L1 HPV-DNA of upstream sequence region.

In contrast, the L1 middle regions were more variable than the upstream regions as seen from the phylogenetic construct on Figure 5. Again, Indonesian variants grouped together to form a distinctive major clusters with only HPV from China was the closest relative. Surprisingly, the Indonesian variants had farthest genetic distance with those of East Asia type.

L2 HPV DNA analysis

As with L1 DNA, the L2 DNA was first analyzed by High Resolution Melting following Realtime PCR and the resulting melting curves were converted into temperature shifted difference curves to see clearer polymorphic characteristics. The L2 DNA upstream regions were grouped into 3 clusters on the basis of melting temperature differences (Figure 6A). The middle sequences, however, showed more variable sequences with 14 clusters (Figure 6B).

Figure 4. Multiple alignment of L1 HPV DNA

Figure 5. Phylogenetic of HPV DNA of middle L1 regions
Multiple alignments of the first 300 bp L2 upstream sequences is shown in Figure 7. Here, several sequence variations were seen as single bases differences. Overall, L2 upstream regions showed more variable than L1 upstream region (Figure 8).

The middle part of L2-HPV DNAs were also positioned in separate clusters from other HPV from East Asia, Africa, Brazil, Thailand, Germany as well as from China and USA (Figure 9).

Figure 7. Multiple alignment analysis of L2 upstream sequence

**Discussion**

HPV genome consists of early proteins genes (E1, E2, E4, E5, E6, E7), in which the E6 and E7 plays an important role in cancer formation, hence oncogene term applied. The other 2 genes are late proteins (L1, L2) which are immunogenic and have been recently much explored in the design of HPV bivalent and quadrivalent vaccines (Harper et al., 2004; Koutsky et al, 2002; Schiller et al, 2004; Villa et al, 2005; Zheng et al, 2006).

It was reported that nucleotide variation rate of 2% was found in HPV type 16 (Bernard et al, 2006). The fact that mutations occurred in L1 and L2 genes lead to the change of amino acid sequence of the capsid protein. This also supports the possibility of an immunogenic and oncogenic diversity between the variants of all types of HPV (Prado et al, 2005). L2 minor capsid protein
has a more reactive antibody than the L1 major capsid protein, making it a suitable candidate for the development of modern vaccines against HPV infection. Virus-like particle (VLP) is a DNA-less capsid protein used in the construction of prophylactic bivalent and quadrivalent vaccines against both high risk and low risk HPV marketed as Gardasil (Merck & Co) and Cervarix (Glaxo Smith Kline) which finally gained FDA approval (Lowy & Schiller, 2006; FDA, 2008).

L2 HPV plays an important role in capsid assembly by introducing the HPV DNA to the virus particles. It is also a binding protein that can interact with any DNA without any requirement of specific DNA sequences which helps in the formation of virions with viral DNA and kaposmer linking. HPV L2 also has an important role in viral entry into cells, localization of viral components into the core, capsid formation, and stability of DNA binding (Ishii et al, 2005; Zhou et al, 1994).

Compared with L1, L2 is much smaller but the DNA sequence is more variable. L2 has been used as sources of antigen for type-specific HPV antibody (Motoyama et al, 2004). L2 HPV types 11 and 16 require L1 in the process of virus penetration into the nucleus (Bordeaux et al, 2006; Kieback et al, 2006). L2 can function in the uncoating of virions and virus delivery to the core genome (Richard et al, 2001).

Previously, it was reported that there were three mutations found in L2 sequence of HPV types 6 (Wang et al, 2001). Mutation from A to G at position 7081 in the genome of HPV types 6 and from G to A in 7099 represent the regional characteristics of L2.

The result of this study indicated that L2 sequences are more varied than the L1, either the upstream or the middle regions. This means that larger possibility of variations could be expected from this region including those found in the Indonesian variants. In general, Indonesian variants formed separate clusters on the basis of single base differences found in both L1 and L2 genes. Characterization of the L1 & L2 sequence variation from the high-risk HPV group of the Indonesian variants can provide further information of their immunogenic properties, biomarkers potential and future development of novel therapeutic agents against cervical cancer.

**Figure 8.** Phylogenetic analysis of upstream L2 HPV-DNA sequence

**Figure 9.** Phylogenetic analysis based on SNP of the middle L2 sequences

**Conclusion**

It was understood that L1 gene represented the best conserved part of HPV genome because of its major contribution in the physical structure of the capsid protein so that the main integrity of the virus was maintained. L2 gene on the other hands, showed intraspesific DNA variability which was found in most HPV type 16 of Indonesian variants. These facts opens up possibilities of diagnostic methods as well as novel vaccine containing immunogenic determinants which are developed based on L1 and L2 of HPV of Indonesian variants. The new generation of HPV vaccine has also been prepared from modified L1 & L2 regions generating pseudovirions or virus-like particle (VLP).
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