

RESEARCH COMMUNICATION

Whole Genome Analysis of Human Papillomavirus Genotype 11 from Cervix, Larynx and Lung

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Abstract

The prevalence of human papillomavirus genotypes differs in various target organs. HPV16 is the most prevalent genotype in the cervix while genotypes 6 and 11 are highly prevalent in skin and aero-digestive tract infections. In this study HPV11 positive specimens were selected from cervix, larynx and lung biopsy tissue to analyze the whole genome by PCR and direct sequencing. Five HPV11 whole genomes were characterized, consisting of two cervical specimens, two laryngeal specimens and one lung specimen. The results showed high homology of HPV11 in these organs. Phylogenetic analysis showed that all HPV11 derived from various organs belonged to the same lineage. Molecular characterization and functional studies can further our understanding of virulence, expression or transmission. Additional studies on functional protein expression at different organ sites will also contribute to our knowledge of HPV infection in various organs.

Keywords: Human Papillomavirus - whole genome

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Introduction

Papillomaviruses belong to the family *papillomaviridae* and are found in various mammals without inter-species transmission. Proliferation of these viruses can cause benign tumors or invasive cancer of the genital tract, oral cavity, skin and upper aero-digestive tract (Burd, 2003). More than 100 types of human papillomavirus (HPV) have been identified and characterized at the molecular level with approximately 40 infecting the epithelium of the genito-anal tract and other mucosal areas (Tiersma et al., 2005). Based on their associations with the potential risk for cancer and precancerous lesion progression, these HPV can be divided into low-risk types such as HPV6, 11, 40, 43 and 44, and high-risk types such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (Burd, 2003). Since 1983, many studies have shown that oral cavity and larynx cancer may be caused by HPV (Syrjanen K et al., 1983). Moreover, some non-malignant diseases are mostly related with HPV6 and HPV11 especially recurrent respiratory papillomatosis (RRP) and oral papillomas (Donne et al., 2010). A meta-analysis of head and neck squamous cell carcinoma (HNSCC) has shown a 24% prevalence of HPV DNA in laryngeal squamous cell carcinoma (Kreimer et al., 2005). HPV DNA isolated from upper aero-digestive tract tumors varies in prevalence compared with each area. Malignancy of lower respiratory tract epithelial cells developing into lung cancer has been associated with HPV infection. Many studies have shown that the association of HPV infection to lung cancer is still controversial. In European and Asian

countries, most squamous cell carcinomas of the aero-digestive tract are caused by exposure to carcinogens such as poor oral hygiene, genetic predisposition, tobacco and alcohol consumption (Kreimer et al., 2005). RRP, known as laryngeal papillomatosis, is a common benign tumor of the airways (Derkey, 2001) characterized by exophytic proliferative lesions of connective tissue. In most cases, this condition is spontaneously resolved with less than 1% progressing to malignancy (Goon et al., 2008). The papillomas can grow anywhere in the respiratory tract such as trachea, bronchi and lung but most commonly in the larynx (Lacey et al., 2006). The disease primarily affects the larynx but also various extralaryngeal sites, and the role of HPV in progression to laryngeal cancer has so far remained unclear. These groups of patients require extensive healthcare and present a challenge to the treating physician (Lindman et al., 2005). Based on molecular analysis, the cause of RRP has been established as multiple benign growths (papillomas) in the middle and lower respiratory tract. The risk factors for malignant progression are smoking, radiotherapy, alcohol consumption and specific HPV genotypes. The low-risk HPV genotypes, HPV6 and HPV11 are the major causes of RRP (Dickens et al., 1991) while HPV16 and 18 are less common. HPV6 and 11 are the causative agents of almost 100% of juvenile and adult onset RRP. Juvenile onset RRP affects young children (usually under the age of five) and initial research has suggested these infections may be due to vertical HPV transmission from mother to child (Silverberg et al., 2003). Adult onset is usually observed in 20-40-year olds and sexual transmission has

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been speculated on (D'Souza et al., 2011). Supported by clinical evidence, HPV11 is associated with a more severe form and earlier presentation (Wiatrak et al., 2004). HPV6 isolates can be characterized into three variants of HPV6a, HPV6b and HPV6c with differences in degrees of virulence while HPV11 isolates can be divided into two variants of HPV11a and HPV11b (Heinzel et al., 1995). The HPV genome has three domains: upstream regulatory regions (URR), early region (E) and late region (L). HPV has eight viral genes which encode the expression of all early (E) and late (L) proteins. The six E proteins affect cell growth and function while the two L proteins contribute to the viral capsid structure (Hebner et al., 2006). All E and L proteins of HPV6 and 11 display profound sequence similarity. The less conserved regions such as the long control region (LCR), are useful for determining genetic variants. To this day, the association between genetic variation and clinical disease has remained unclear. There is evidence to suggest that rearrangements in the URR of HPV6 and HPV11 contain binding sites for transcriptional activators associated with malignancies without displaying any major differences in the early promoter activities (Chin et al., 1989; Rubben et al., 1992). The E6 protein has been described as an intrinsically disordered protein (IDP) which implies that any small amino acid change can have an effect on tertiary structure (Uversky et al., 2006). The E6 amino acid sequences of HPV6 and 11 are conserved while showing significant differences to HPV16. The high-risk HPV E6 proteins induce degradation of p53 through the ubiquitin-proteasome degradation pathway while the low-risk E6 proteins abolish the p53 function in in-vivo experiments (Band et al., 1993). This suggests that the E6 protein may affect its function. The high-risk HPV genomes usually integrate into the host genome which is associated with the risk of malignant transformation while low-risk HPV genomes form circular episomal DNA (Schneider-Maunoury et al., 1987). The virulence of HPV depends on E6, E7 and L1 genes to effect clinical severity. Moreover, the molecular characterization and functional assays of HPV could assist in understanding the significance of mutations, diagnosis and treatment.

The aim of this study has been molecular characterization of HPV11 in cervical, larynx and lung tissue by whole genome analysis and to elucidate the differentiation of HPV11 in these infected tissues.

Materials and Methods

The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB 49/55). Samples were obtained during the patients' examination and treatment between March 2011 and January 2012 from Samitivej Srinakharin and King Chulalongkorn memorial hospital. The anonymous samples consisted of the known HPV11 positive tissues from papilloma tumors from lung (1 sample), larynx (2 specimens) and cervix (2 HPV11 positive specimens from 1097 asymptomatic routine checkup samples). The patient (No.3) with pulmonary papilloma was the same person as the patient (No.2) with recurrent laryngeal papilloma which the lesions extended from the larynx to

the lung. (Table 1) Permission was also granted by the director of the King Chulalongkorn Memorial Hospital. The specimens were stored at -20°C until tested.

DNA extraction

Two cervical swab, two larynx and one lung tissue samples were subjected to DNA extraction and subsequent HPV detection. The standard organic method (phenol-chloroform) and alcohol precipitation of the specimens as described by Broccolo F et al. (Broccolo et al., 2005) was applied for DNA extraction. The purified substance was re-suspended in a final volume of 30 µl of deionized water.

HPV detection and typing

HPV DNA was detected by using consensus polymerase chain reaction of the E1 and L1 regions as described by Chansaenroj et al. (Chansaenroj et al., 2010; Chinchai et al., 2011). HPV DNA positive samples were identified by electrophoresis in 2% agarose gel (FMC Bioproducts Rockland, ME) and purified using the agarose gel extract mini kit (5PRIME, Hamburg, Germany) according to the manufacturer's specifications. The purified DNA was sequenced by FirstBASE Laboratories SDNBHD (Selangor Darul Ehsan, Malaysia). The

Table 1. Demographic Characteristics

No.	Sex/ Age	Method of sample collection	Organ	Diagnostic	Accession No.
1	M/30	Biopsy	Larynx	Laryngeal papilloma	JQ773408
2	F/14	Biopsy	Larynx	Laryngeal papilloma	JQ773411
3	F/15	Biopsy	Lung	Pulmonary papilloma	JQ773412
4	F/26	Scraping	Cervix	HSIL, CIN III	JQ773410
5	F/37	Scraping	Cervix	Atypical Squamous Cells of Undetermined Significance (ASCUS)	JQ773409

Table 2. Conserved Primers for Whole Genome Amplification and Sequencing of HPV11

Primer name	Sequence (5'-3')	Position ¹	Product (bp)
*A_F104	GGAAAGTAAAGATGCCTCCACG	104-126	769
*A_R851	TTTCTGTACCTGAATCGTCCGC	851-873	
*B_F714	ACAGCAACGTCCGACTGGTTGT	714-736	878
*B_R1569	ATGATGTATACCAAATCTGCAA	1569-1592	
*C_F1378	GACGACAGCACCCGAGAGCAT	1378-1399	789
*C_R2144	CCTACACTGTCAACTTTAGTACC	2144-2167	
*D_F2104	CAATGGATTAAGTATAGGGGTA	2104-2126	923
*D_R3005	GTAATGTCCAAGGTTCCACACC	3005-3027	
*E_F2902	ACCATTAAGTGTGTCAGAGACT	2902-2924	1045
*E_R3922	ATATCAATGTTGTAGTTGTTGCTGC	3922-3947	
*F_F3784	CATTAGGCATAAGGTGGGGTT	3784-3805	1182
*F_R4945	TGTGACCACTGGCCTCCACAG	4945-4966	
*G_F4840	GATGTGTCTGTTACCAATCAC	4840-4861	1025
*G_R5844	GCATCCGTGGCAACAACCTTG	5844-5865	
*H_F5769	AGATGTGGCGGCTAGCGACA	5769-5790	966
*H_R6712	CTGAGCCTTTTGAAGCCAATATG	6712-6735	
*I_F6637	ACATACACCTAGTGGCTCATTGG	6637-6660	1136
*I_R7751	TGTACATTAGTCATGCAATACC	7751-7773	
*J_F7646	TTGTACTAATCCCATATGTTGTG	7646-7669	579
*J_R272	ACACGCTGCAAAGGGAAAGTTG	272-294	

*HPV11, ¹Nucleotide position based on nucleotide sequence of HPV11 (Accession No. M14119)

nucleotide sequences were analyzed by Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

HPV11 Whole genome sequencing and phylogenetic tree construction

The polymerase chain reaction was performed to amplify the whole HPV11 genome. Primer sequences are shown in Table 2. The reaction mixture contained 2 μ l DNA, 0.5 μ l of each primer, 10 μ l 2.5X PerfectTaq Plus MasterMix (5PRIME, Hamburg, Germany), and nuclease-free water to a final volume of 25 μ l. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94°C for 5 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 45 s, and extension at 72°C for 1.30 min, and concluded by a final extension at 72°C for 7 min. HPV11 primer positions and PCR products are depicted in Table 2. The nucleotide sequences were edited using Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and assembled by The Lasergene 6 Package (DNASTAR, Inc., Madison, WI). The complete genome sequences were compared with the reference sequences available at GenBank. The alignments were performed by using Clustal W applying the BioEdit program (version 7.0.4.1) (Hall TA, 1999). The phylogenetic trees were constructed for whole genome sequences of HPV11 and for each gene by neighbor-joining (NJ) from MEGA 4.0 program (Tamura et al., 2007) and support tree topologies by boots-trapping applied with 1,000 replicates.

Results

Of the 1097 cervical specimens, two samples were found HPV11 positive upon amplification by consensus polymerase chain reaction in the E1 and L1 regions. The HPV11 whole genome sequence was also detected in 2 larynx papilloma samples and one papilloma from lung tissue. The demographic data, method of sampling, diagnosis of samples are shown in table 1. All sequences were submitted to the Genbank database under accession numbers JQ773408-JQ773412. The complete genomes of HPV11 were subjected to phylogenetic tree analysis as shown in Figure 1. The nucleotide sequences of all samples were edited and aligned with the reference sequences of HPV6, HPV11 and their variants (accession numbers: FR872717, HE574704, FN907964, HE611274, M14119, EU918768, NC001355, FR751323, L41216,

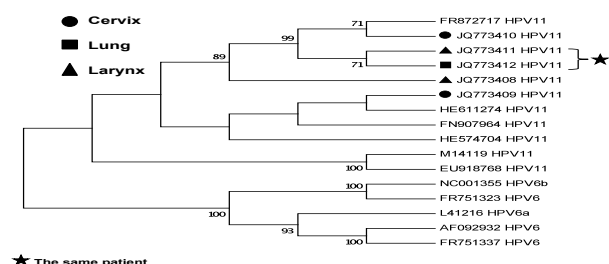


Figure 1. Phylogenetic Analysis of HPV11 whole Genome

Table 3. Nucleotide and Amino Acid Sequences Change of HPV11 in E7, E5 and E1 Compared to the Reference Sequences.

Gene	Nucleotide sequence		Amino acid residue
	Position ¹	Change	Change
E7	662	G --> T	A --> S
E1	1783	C --> G	R --> A
	1784	G --> C	
E5	3952	A --> T	I --> F
	3991	G --> C	V --> L

¹The Nucleotide positions and numbering are based on the HPV11 reference sequence (Accession No. M14119).

AF092932, FR751337). The HPV11 whole genomes derived from cervix, larynx and lung clustered in the same lineage. All HPV11 isolates in this study showed more than 99% similarity when compared with the reference sequences. Moreover, 24 nucleotide polymorphisms have been detected. The 4 mutations in non-synonymous nucleotide sequences were found at E7, E5 and E1 genes while the remaining mutations were silent (Table 3).

Discussion

RRP is a squamous lesion within the respiratory tract which in the majority of cases is caused by HPV6 and 11 at a prevalence rate varying between 50 and 90% (Gissmann et al., 1983; Major et al., 2005). The reported variation in prevalence rate of HPV can be attributed to differences between detection methods, sensitivity and types of samples. The sensitivity of the technique applied for HPV detection is relevant for analysis of sub-types or variants. PCR is the most sensitive method for HPV detection in larynx samples. The MY09/MY11 and GP5+/GP6+ primer pairs are generally used for epidemiological studies (Zehbe et al., 1996). In children, the incidence of RRP is approximately 4 per 100,000 compared to 2 per 100,000 in adults (Reeves et al., 2003). RRP usually affects children below the age of 5 years with 25% comprising infants (Stamataki et al., 2007). A meta-analysis of head and neck cancers by Kreimer et al. (Kreimer et al., 2005) detected HPV DNA in 24% of laryngeal tumors. Hobbs et al. studied the association between HPV and cancer in cases of head and neck squamous cell carcinoma. The strongest association was detected for tonsil (OR=15.1, 95% CI, 6.8-33.7), intermediate for oropharynx (OR=4.3, 95% CI, 2.1-8.9) and weakest for oral and larynx primary tumors (OR=2.0, 95% CI, 1.2-3.4 and 1.0-4.2 respectively) (Hobbs CGL et al., 2006). HPV11 is the most common cause of RRP and associated with a greater risk for progression to lung cancer than HPV6 (Dilorenzo et al., 1992; Rimell et al., 1997). The levels of HPV6 and 11 viral loads tend to stabilize over time in most children with RRP (Maloney et al., 2006). Comparison of DNA sequences between HPV6b and HPV11 showed strong conservation. A nucleotide sequence analysis may provide molecular determinants of virulence. In the non-coding regions of HPV6 and HPV11, polymorphisms may affect expression of the E6 and E7 genes and transformation of host cells and deletions and insertions may be caused by

slippage or DNA synthesis error to produce variations in two HPV types (Chan et al., 1995). The nucleotide changes in the URR may be responsible for viral replication by influencing the E2 and E1 proteins (Hubert, 2005). In this study, HPV11 from three organ lesions were selected to analyze their whole genome. Sequences from samples were analyzed and compared to reference sequences of HPV6 and HPV11. The similarities between the individual HPV11 isolates were in excess of 99% even in the non-coding region. The four mutation points were identical to those reported by Gall T et al (Gall et al., 2011) and may contribute to therapy resistance and virulence of HPV11. These results imply that viral factors and environment factors have an effect on tumor development, HPV infection and prevalence. The management of RRP treatment depends on the degree of airway involvement and surgical removal. Surgery is the primary treatment of RRP while effective treatments for patients with an aggressive disease comprise drug therapy with cidofovir, interferon, acyclovir and ribivirin (Gallagher et al., 2009). The most suitable treatment may consist of surgery, radiotherapy, chemotherapy and the quadrivalent HPV vaccine developed to protect against HPV6 and 11 (Barr et al., 2007). The management in children is more difficult than in adults as the tumors are more aggressive and hence, preservation of laryngeal function and voice is more problematic. In children, vertical transmission of HPV is considered to be a major route of contracting the disease especially if the mother has anogenital warts or underlying HPV infection (Silverberg et al., 2003). Testing for HPV can be helpful in prognosis and management of RRP during childhood. Finally, the prevalence of the HPV types is important for prognosis. Data on molecular variability and in vitro functional studies may help clarify virulence, expression and transformation of host cells.

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