Molecular cloning and characterization of a novel human papillomavirus, HPV 126, isolated from a flat wart-like lesion with intracytoplasmic inclusion bodies and a peculiar distribution of Ki-67 and p53

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Abstract

Infection with certain human papillomavirus types induces warts with specific macroscopic and microscopic features. We observed multiple flat wart-like lesions on the chest, neck and extremities of an adult T-cell leukemia patient. Histologically, atypical intracytoplasmic inclusion bodies currently known to be pathognomonic for genus gamma or mu papillomaviruses were disclosed in some cells of the epidermis showing histological features compatible with flat warts. In the present study, a novel human papillomavirus was identified and its whole genome, 7326 bp in length, was cloned and characterized. Phylogenetic analysis showed the virus designated as HPV126 to be a novel type of genus gamma papillomavirus. Strikingly, Ki-67 and p53 expression was found to be increased in all layers of the epidermis except for the horny layer, contrasting to expression restricted to the basal and lower spinous layers in ordinary flat warts.

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Introduction

So far, more than one hundred twenty human papillomaviruses (HPVs) have been characterized based on nucleotide sequence diversity (Bernard et al., 2010). Infections of distinct types of HPVs are characterized by type-specific cytopathic/cytopathogenic effects (CPEs), i.e., macro- and microscopic features, pathological properties, and tissue tropisms. Hence, unusual CPEs which had not previously been described may suggest that lesions could be induced by a novel type of HPV (Egawa, 2005). We recently observed intracytoplasmic inclusion bodies (ICBs) resembling the HPV 4/60/65-associated homogenous ICB (Hg-ICB) (Egawa, 1994, 2005; Egawa et al., 1993) in flat wart-like lesions of a patient with adult T-cell leukemia (ATL). However, the clinical features of the lesions proved quite different from those of HPV 4/60/65-associated skin lesions, i.e., pigmented warts (Egawa, 1988; Egawa et al., 1993) or ridged warts (Honda et al., 1994), suggesting the presence of a previously unidentified papillomavirus. While the HPV type-specific CPEs are important in understanding the biological nature of the viruses, many of the novel HPV genotypes recently isolated lacked specific cell biological aspects.

The present report describes not only isolation and molecular biological characterization of a novel HPV genotype, HPV126, but also a clinical, histopathological and immunohistochemical characterization of HPV 126-associated skin lesions, revealing this novel human genus gamma papillomavirus to induce flat wart-like lesions with Hg-ICBs. Strikingly, Ki-67 and p53, well-known cell cycle proteins, were expressed in all layers of the epidermis except the horny layer, contrasting to expression restricted to the basal and lower spinous layers in ordinary flat warts.

Results

Histopathological features of wart lesions

Disseminated hypopigmented macules clinically resembling flat warts or epidermodysplasia verruciformis-related tinea versicolor-like lesions (Jablonska and Orth, 1985) were seen on the chest, neck, and extremities of a 56-year-old Japanese patient (Fig. 1A) (Kawai et al., 2009). A biopsy was taken from the disseminated fused lesion and adjacent normal-looking skin. Microscopically, at least two independent wart-

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like lesions separated by normal epidermis were included in the specimen. The epidermis showed mild acanthosis with basket-weave-like hyperkeratosis, partial hypergranulosis and mild papillomatosis, basic histological features compatible with those of flat warts (Jablonska et al., 1985). However, additional unique histopathological features were also seen, i.e., keratinocytes with an enlarged nucleus, abundant blue-gray cytoplasm, occasional perinuclear haloes, and prominent keratohyalin granules observed in the granular and spinous layers, which are histopathological features consistent with EV (Jablonska and Orth, 1985). In addition, large clear cells contained homogeneous eosinophilic ICBs (Fig. 1B) resembling the homogeneous ICBs (Hg-ICBs) previously described in HPV 4/60/65-associated cutaneous warts (Egawa, 1994, 2005; Egawa et al., 1993).

Cloning and characterization of the HPV 126 genome

Although highly sensitive PCR failed to detect the DNA of either genus beta or mu papillomaviruses from the frozen biopsy specimen, a segment of a putative novel type genus gamma papillomavirus was amplified with a gamma papillomavirus-specific degenerate primers (Kawai et al., 2009) (Supplementary Fig. 1). Based on the nucleotide sequence, the full genome was cloned as described in Materials and methods. Sequencing of two clones from independent PCR reactions revealed the full genome consists of 7326 bp in length with a GC content of 50.5%. With a cutaneotropic papillomavirus primer set FAP59/FAP64 (Forslund et al., 1999), only the corresponding region of the cloned genome was amplified, further indicating the HPV is a single type in the lesions of this patient. The cloned HPV was found to be closely related to genus gamma papillomavirus types with an L1 ORF nucleotide similarity ranging from 60.1% to 68.7% (Table 1).

Table 1

<table>
<thead>
<tr>
<th>ORF</th>
<th>HPV type</th>
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<tbody>
<tr>
<td>E6</td>
<td>52.2</td>
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<tr>
<td>E7</td>
<td>53.2</td>
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<tr>
<td>E1</td>
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<tr>
<td>L1</td>
<td>63.5</td>
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<tr>
<td>L2</td>
<td>51.6</td>
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According to the established criteria for a new type of papillomavirus that a new type should have 10% divergence of the L1 ORF nucleotide sequence from that of any other papillomavirus type (de Villiers, 2004 #16), the cloned HPV qualified as a new type of papillomavirus designated as HPV126. According to the proposed criteria for species that should share between 60% and 70% nucleotide identity with any other papillomaviruses, constitutes a new species of genus gamma papillomavirus. Generation of a phylogenetic tree based on complete L1 nucleotide sequences of representative HPV types indicated that HPV 126 is most closely related to HPV 129 (Fig. 3), with similarity of 68.7% (Table 1). HPV 126 has a typical genomic organization for a genus gamma papillomavirus, and it has seven ORFs, E6, E7, E1, E2, E4, L2 and L1, but no E5 (Supplementary Fig. 2).

Immunohistochemical features of the wart lesions

Strong signals of L1 capsid proteins were seen in the nuclei of the cells in horny layer and cells with ICBs in granular layer (Fig. 1C), suggesting active production of virions. In the cells with ICBs, little cytokeratin staining was observed while strong staining was observed in all epidermal cell layers of the lesions as well as its adjacent normal skin (Fig. 1D).
Increased expression of Ki-67, an indicative marker of cycling cells, was observed in all compartments of the epithelium except for the horny layer of the lesions, whereas its expression was restricted to the basal proliferative compartment of the adjacent normal epidermis (Fig. 2A) and to basal to parabasal cells in the typical HPV 3-positive flat warts (Fig. 2B).

Increased p53 staining was also observed in all compartments of the epithelium except for the horny layer of the lesions in the HPV 126-associated lesions. However, unlike Ki-67, strong signals were not seen in parabasal cells for p53. In the adjacent normal epidermis, weak staining for p53 was restricted to the basal proliferative compartment (Fig. 2C), and faint staining was in the basal and lower spinous layers in HPV 3-positive typical flat warts (Fig. 2D). Five cases of typical HPV 3-associated flat warts were examined for comparison to confirm the unusual distribution of Ki-67 and p53 expression in the present flat wart-like lesion though the present case is the only patient with HPV 126-associated inclusion warts studied thus far. These observations are reminiscent of high grade cervical intraepithelial neoplasia. However, neither the HPV 126-associated flat wart-like lesions nor ordinary flat warts showed positive staining for p16INK4a; while cervical cancer biopsy examined as a positive control exhibited strong positive signals (data not shown).

Discussion

In the present study, the full-length genome of a novel papillomavirus, HP126, was cloned from flat wart-like lesions arising in a Japanese ATL patient and characterized. The DNA genome of HPV 126 consists of 7326 base pairs and shows the gene arrangement characteristic for a cutaneous HPV. The nucleotide sequence of the L1 ORF of HPV 126 shares the highest homology of 68.7% to that of HPV 129, a genus gamma papillomavirus, thereby defining HPV 126 a novel type possibly constituting a novel species of the genus gamma papillomavirus (de Villiers et al., 2004). The HPV 126-associated cutaneous lesions on the chest, neck and extremities of our Japanese ATL patient were disseminated hypopigmented macules clinically resembling flat warts or tinea versicolor-like lesions seen in epidermodysplasia verruciformis (EV) and acquired EV patients (Jablonska and Orth, 1985; Lutzner et al., 1983).

On microscopy balloon cells with pale blue cytoplasm like those seen in flat wart-like lesions of EV or acquired EV patients (Jablonska and Orth, 1985; Lutzner et al., 1983). Additional features characteristic for the present case were ICBs most resemble those associated with HPV 4/60/65, which are members of species 1 (HPV 4/65) and species 4 (HPV 60) of genus gamma papillomaviruses. (Egawa,
It is well known that distinct ICBs are pathognomonic for genus gamma and mu papillomaviruses (Egawa, 2007), although the histological features of recently isolated genus gamma papillomaviruses, including HPV 129 and HPV 116, have yet to be described (Bernard et al., 2010; Li et al., 2009). Cytokeratins were absent from cells containing Hg-ICBs (Fig. 1D), in which E4 proteins are thought to be a major component, though E4 protein expression was not examined in the present case. Thus like HPV16 E4 (Doorbar et al., 1989), HPV 126 E4 might be involved in interference with keratin filament assembly.

Another striking feature of the present case was its peculiar immunohistochemical localization of Ki-67 and p53, namely, they were expressed strongly and distributed in all compartments of the epithelium except for horny layer of the HPV 126-associated lesions (Fig. 2). Antigen Ki-67 is expressed during all phases of the cellular cycle, G1, S, G2, and M, of proliferating cells, but is absent in quiescent cells (G0). It is, therefore, a marker of cellular proliferation, which can be detected with monoclonal antibodies. Interaction of human papillomavirus oncoproteins E6 and E7 with cell cycle proteins leads to disturbance of the cell cycle and subsequent alteration in expression of some cell cycle proteins, such as p16INK4a, cyclin D1, p53 and Ki-67. Abrupt inactivation of pRB can induce p53 accumulation though activation p14ARF (Bates et al., 1998). Like other HPVs, E7 protein of HPV 126 conserves the pRB binding motif and potentially inactivates pRB. Indeed some of the E7 proteins of cutaneous HPVs, such as HPV1 E7, can strongly bind and inactivate pRB (Hiraiwa et al., 1996; Schmitt et al., 1994). Thus it will be interesting to examine the activity of HPV 126 E7 protein and relationship among expression levels of HPV 126 E7, Ki-67 and the p53 accumulation. Unlike high grade CIN lesions where a positive correlation between the expression of the p16INK4a

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**Fig. 3.** Phylogenetic relationships among HPV 126 and representative HPV types. A phylogenetic tree was constructed based on L1 ORF sequences using the neighbor-joining (NJ) method with 1000 bootstrap replicates. Numbers near branches indicate support index from NJ bootstrap percentage. Nucleotide sequences of representative HPVs were obtained from GenBank.

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and Ki-67 has been reported (Nam et al., 2008; Queiroz et al., 2006), accumulation of p16\(^\text{INK4A}\) was not detected in the Ki-67 positive cells (data not shown) in the present case whose clinical behavior and histopathological findings were benign (Kawai et al., 2009).

In conclusion, HPV 126, isolated and characterized in the present study, is a novel type of genus gamma papillomavirus and associated with flat wart- or EV-related tinea versicolor-like clinical features: histological ICBs as with other genus gamma papillomaviruses; and immunohistochemical expression of Ki-67 and p53 in characteristic manner not typical for benign cutaneous warts. It is probable that conditions accompanying immunosuppression in this ATL patient may have contributed to stimulate viral production of HPV 126, thus leading to wart formation, as known for acquired EV (Lutzner et al., 1983). To ascertain the true nature of HPV 126 and its associated warts, we need to perform epidemiological as well as further clinicopathological and virological studies on a larger number of lesions and patients, including immunocompromised individuals.

**Materials and methods**

**Patient**

A 56-year-old Japanese man was referred to us in August 2008 for evaluation of a 5-year history of disseminated hypopigmented macules clinically resembling flat warts or epidermodysplasia verruciformis-related tinea versicolor-like lesions (Jablonska and Orth, 1985) on the chest, neck, and extremities (Fig. 1A) (Kawai et al., 2009). The patient might have been suffering from immunodeficiency, because he was diagnosed as having chronic type of adult T-cell leukemia at the age of 52 years and manifested recurrent fungal pneumonia, rapidly progressing oral squamous cell carcinoma and multiple brain abscesses. A biopsy specimen was taken from the flat wart-like lesions with adjacent normal skin under suspicion of acquired EV (Lutzner et al., 1983). The biopsy specimen was cut into two pieces, one of which was fixed in 20% buffered formalin and embedded in paraffin for conventional histopathological and immunohistochemical analyses, and the other was frozen and stored in −70 °C for further analyses including DNA extraction.

**Microscopical examination**

Four-micrometer thick sections were obtained from the formalin-fixed and paraffin-embedded biopsy specimen, stained with hematoxylin and eosin (H&E), and examined microscopically.

**Cloning and characterization of HPV DNA**

Degenerate primers to detect genus gamma papillomaviruses were as described previously (Kawai et al., 2009). The amplified sequence turned out to correspond to nt 4641 to 5632 of the cloned HPV126 (Supplementary Fig. 1). Then abutting primers were designed juxtaposing the Hpai site present in the L1 region (forward primer: 5′-GTATAACAGTGCCATCCCTATTTTGATATTGTTG-3′; reverse primer: 5′-GTATAACAGTCTTCCAGTATTTGCGCATGAAATATTCTG-3′). The genome was amplified by 30 cycles of PCR using KOD plus DNA polymerase (Toyobo, Japan) according to the supplier’s instruction; annealing at 60 °C, elongation at 68 °C for 8 min. About 8 kbp PCR products were purified and then cloned into pBluescriptSK(−) (Stratagene, La Jolla, CA), in which 15-bp overlapping sequence of HPV 126 was added to the NotI site by PCR (forward primer: 5′-TGAAGACTGTGAACGGCG-CGCCCTTACAGAATGGTGAC-3′, reverse primer: 5′-TGCCCTACTGTAACGGCGCCGCCGGTGTGC-3′), by In-Fusion reaction (Clontech, Mountain View, CA). The complete genomic sequence of a clone was initially determined using primer walking by Nihon Gene Research Laboratories Inc. With the same set of primers (Supplementary Table 1), we confirmed the sequence of another clone from an independent PCR reaction to be identical (Supplementary Fig. 1). The DNA clone was submitted to the Human Papillomavirus Reference Laboratory (Heidelberg, Germany) for official designation, HPV 126, and the sequence was reconfirmed. HPV 126 sequence was submitted to DNA Data Bank of Japan (DDBJ) under accession number AB646346. Nucleotide sequence pairwise comparison of HPV 126 ORFs with types representing genus gamma papillomaviruses and L1 nucleotide global multiple sequence alignments were analyzed using ClustalW program (Thompson et al., 1994). Each gap was included and counted as one position. Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007).

**Immunohistochemical examination**

Formalin-fixed and paraffin-embedded tissue sections (4 μm-thick) were deparaffinized in xylene and rehydrated through a series of graded ethanol (100–70%). For antigen retrieval, slides were immersed in citrate buffer (pH6.0) and were heated for 20 min in a microwave. The slides were then incubated in methanol containing 0.3% H2O2 to inhibit endogenous peroxidase activity. After washing, primary antibodies (Anti-papillomavirus common antigen, DAKO, clone K1H8, 1:200; Ki-67, DAKO, Clone MIB-1, 1:50; p53 protein, DAKO, Clone D07, 1:50; p16\(^{\text{INK4A}}\), Santa Cruz, Clone JC8, 1:200; cytoketarin, Nichirei, polyclonal,1:2) were applied for 1 h and binding was detected using an Envision Kit (Dako Cytomation; K4006). Color development was achieved with 3, 3-diaminobenzene (DAB) as the chromogen and hematoxylin counterstaining was performed to aid in orientation. As a negative control, normal non-immune serum from the same source as the primary antibody was applied. Formalin-fixed, paraffin-embedded sections from an invasive uterine cervix squamous cell carcinoma biopsy served as a positive control for p16\(^{\text{INK4A}}\).

Supplementary materials related to this article can be found online at doi: 10.1016/j.virol.2011.10.011.

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**References**


Hiraiwa, A., Kiyono, T., Suzuki, S., Ohashi, M., Ishibashi, M., 1996. E7 proteins of four human papillomaviruses, irrespective of their tissue tropism or cancer...
association, possess the ability to transactivate transcriptional promoters E2F site dependently. Virus Genes 12, 27–35.


