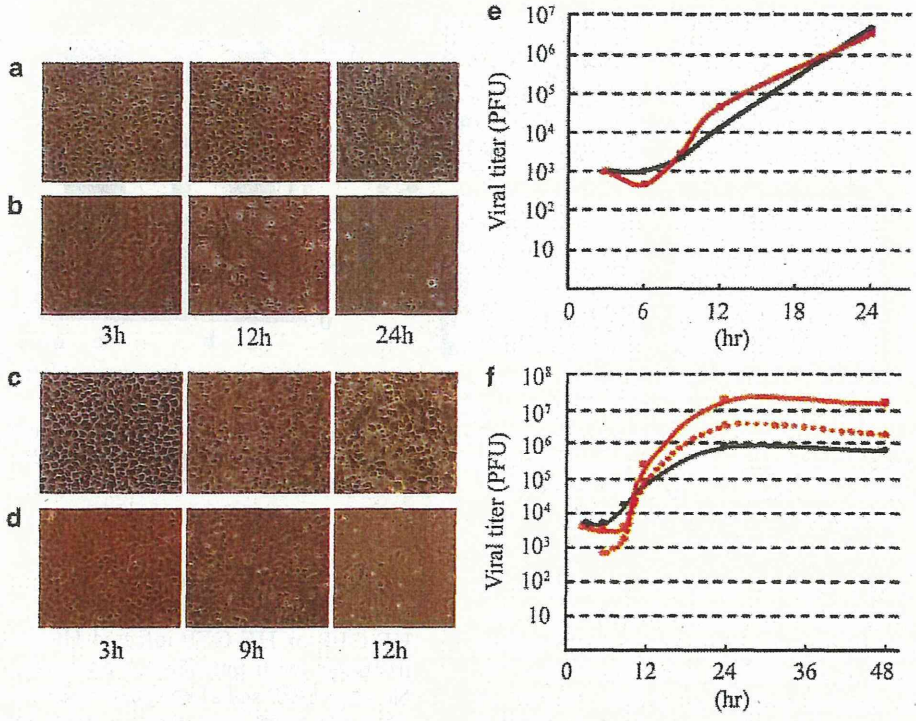

Figure 3 HF10 and Hh101 replication in mesothelial cells (MCs), human omentum mesothelial cells (HOmMCs) and SKOV3 cells. SKOV3 cells (**a**, black line) and MCs (**b**, red line) were infected with HF10 at multiplicities of infection (MOI) 3. SKOV3 cells (**c**, black line) and HOmMCs (**d**, red line) were infected with Hh101 at MOI 3. Representative cytopathogenic effects (CPE) are shown as time series. Cells were harvested and virus titer was determined by plaque assay. The red dotted line shows virus titers in MCs infected with Hh101 at MOI 0.03 (**e**, **f**). The values represent the mean of samples tested in triplicate. PFU, plaque-forming unit.

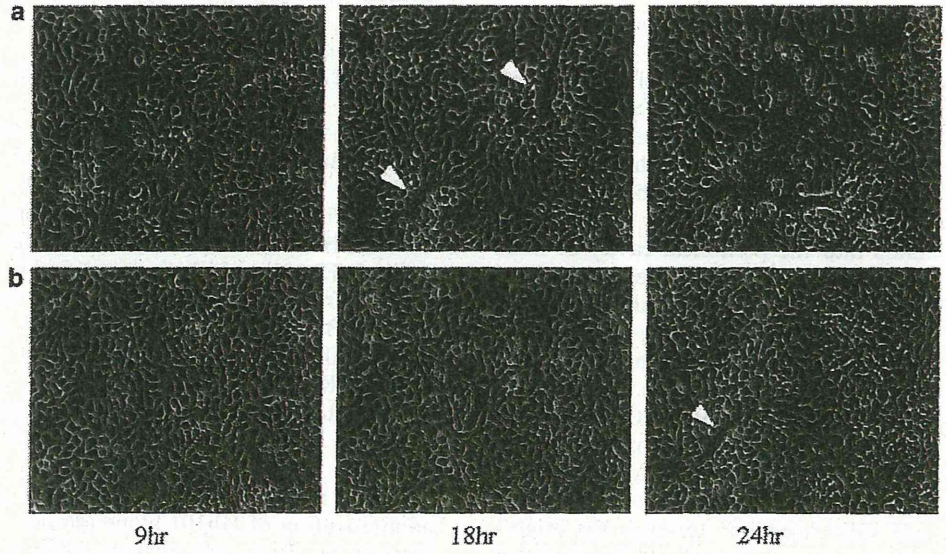

Figure 4 *In vitro* delivery of herpes simplex virus (HSV) using mesothelial cells as carriers. Hh101-infected carrier cells (**a**) or cell-free Hh101 (**b**) were added to the media of SKOV3 cells. Representative cytopathic effects (CPEs) are shown as time series. The white arrow heads show small CPEs.

against virus delivery by carrier cells, we performed *in vitro* experiments. We plated SKOV3 cells on 35-mm dishes, and after 24 h, HF-GFP (10^5 PFU per dish) or HF-GFP-infected carrier cells (10^4 cells per dish) were added to the culture media containing with anti-HSV-1 antiserum or control serum. As evidenced by virus-associated fluorescence, extensive replication was seen in SKOV3 cells 24 h following administration of HF-GFP-

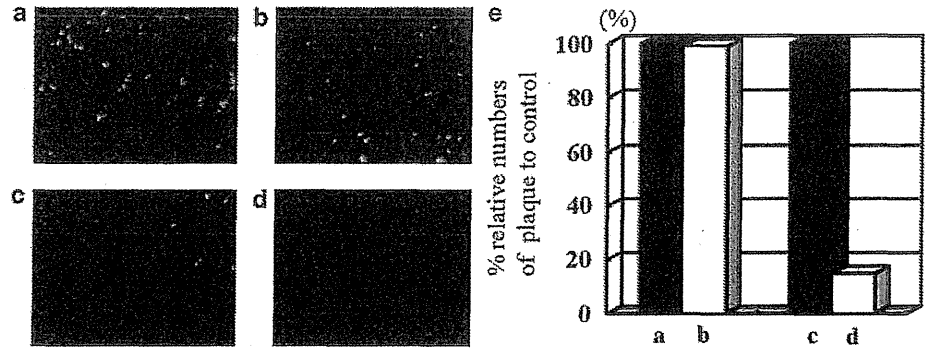

Figure 5 *In vitro* effects of anti-herpes simplex virus type 1 (HSV-1) antibody on HF-green fluorescent protein (GFP). SKOV3 cells were plated for 24 h, and HF-GFP-infected carrier cells (**a**, **b**) or HF-GFP (**c**, **d**) were added to the media containing control serum (**a**, **c**) or anti-HSV-1 antiserum (**b**, **d**). Anti-HSV-1 antiserum was added to the media to give a final dilution of 1:50. Representative cytopathogenic effect (CPE) images are taken at 24 h using the Leica M205FA fluorescence stereomicroscope with a standard GFP filter set. SKOV3 cells were then fixed, stained with 0.2% crystal violet solution and observed at 30 h. The number of plaques was counted and expressed as a percentage of number obtained in control cultures (**e**).

infected carrier cells in spite of anti-HSV-1 antiserum. In the absence of anti-HSV-1 antiserum (Figure 5a), however, virus-associated fluorescence was larger and brighter than in the presence of anti-HSV-1 antiserum (Figure 5b). In contrast, little virus-associated GFP was observed in SKOV3 cells 24 h following administration of HF-GFP in the presence of anti-HSV-1 antiserum (Figure 5d), although a number of GFP-expressing cells were detectable in the absence of anti-HSV-1 antiserum (Figure 5c). These results indicated that cellular carriers can efficiently shield oncolytic virus from neutralizing antibodies.

Localization of virally infected cell delivery in the presence of anti-HSV-1 antiserum

In order to visualize the distribution of the cellular vehicles in mice, we used HF-GFP, which allowed us to follow the biodistribution of virus-associated fluorescence using an *in vivo* imaging system. To assess the localization of intraperitoneally injected virus, we established a mouse model using ovarian cancer cells, in which 2×10^6 SKOV3 cells were inoculated into the peritoneal cavity of nude mice, leading to the formation of peritoneal disseminations. HF-GFP was injected into the peritoneal cavity 30 days after the initial inoculation of cancer cells. At 24 h after HF-GFP injection, nearly all visible tumor nodules in the peritoneal cavity were GFP positive. We detected GFP expression even in small tumors and the brightness of GFP varied in each tumor (Figure 6b). In contrast, no GFP expression was seen in the tumors in animals of the control group (Figure 6a). The GFP expression persisted for 7 days after HF-GFP injection; however, the brightness of GFP weakened as time passed. GFP expression persisted longer in mice injected with HF-GFP-infected carrier cells than in mice injected with HF-GFP alone. These findings suggested that viruses injected into the peritoneal cavity exhibited preferential and specific distribution in disseminated cancer foci in HSV-1 naïve animals.

Next, to examine the impact of a pre-existing immune response, we used the passive immunization method in which anti-HSV-1 antiserum was injected i.p. into mice. Intraperitoneal tumor-bearing mice were given cell-free HF-GFP or HF-GFP-infected HOmMCs i.p at 1 h after treatment with anit-HSV-1 antiserum or control serum. No clear GFP signal was observed in disseminated tumors when HF-GFP was given to mice pretreated with anti-HSV-1 antiserum (Figure 6c). In contrast, a significant GFP signal was detected in peritoneal tumors in spite of pretreatment with anti-HSV-1 antiserum, suggesting that infected carrier cells could bypass circulating antibodies and transfer virus to intraperitoneally disseminated ovarian tumors (Figure 6d).

Intraperitoneal administration of HSV-1 mutant-infected carrier cells improved survival of mice with ovarian cancer

To assess the suitability of carrier cells for the delivery of HSV-1 mutants *in vivo*, we conducted survival experiments to compare the effects of Hh101 administered directly with the effects of Hh101 delivered via HOmMCs. Because HF10 is lethal to immunodeficient animals, we utilized Hh101, which is a recombinant virus clone isolated from HF10 and hrR3 and is less virulent than HF10. PBS, Hh101 or Hh101-infected carrier cells were injected three times 3 days after the i.p. injection of 2×10^6 SKOV3 cells, a time at which tumors would be invisible to the naked eye. Three repeated therapeutic injections of Hh101-infected HOmMCs significantly improved the mean survival time of ovarian cancer-engrafted nude mice (55 days, $n = 10$) compared with the administration of Hh101 alone (46 days, $n = 9$; $P < 0.05$) (Figure 7a). Two-tenth of the animals in the carrier cell-treated group were completely protected from relapse of peritoneal tumor and ascites.

Next, PBS or Hh101-infected carrier cells were injected five times 6 days after the injection of tumor cells, a time at which numerous macroscopic white, 2 mm diameter tumors were seen at the diaphragm, at the mesentery and occasionally at the omentum. As shown in Figure 7b, all mice, irrespective of treatment, developed macroscopic

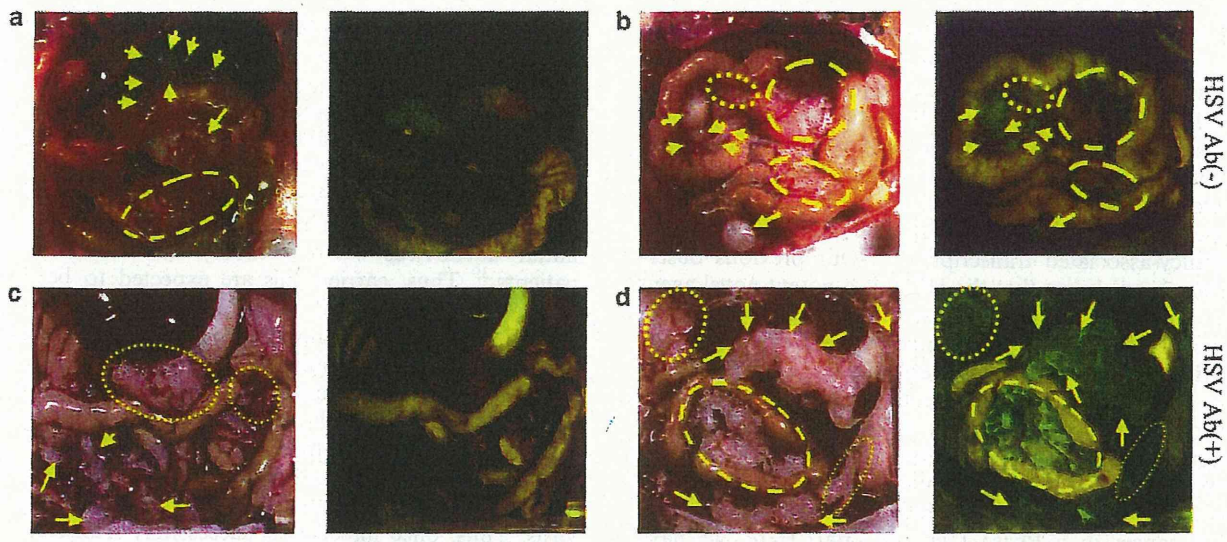

Figure 6 *In vivo* visualization of virally infected cell delivery in the presence of anti-herpes simplex virus type 1 (HSV-1) antiserum. Mice were bearing disseminated SKOV3 ovarian tumors. Mice were randomized into non-treatment group (**a**, **b**) and treatment group with anti-HSV-1 antiserum (**c**, **d**). Intraperitoneal tumor-bearing mice were given phosphate-buffered saline (PBS) (**a**), cell-free HF-green fluorescent protein (GFP) (**b**, **c**) or HF-GFP-infected human omentum mesothelial cells (HomMCs) (**d**) intraperitoneally 1 h after treatment. Representative photographs showing between tumor location and GFP signal. Each picture taken 24 h after viral injections is shown. The yellow arrows and dotted-line circles indicate disseminated ovarian tumor.

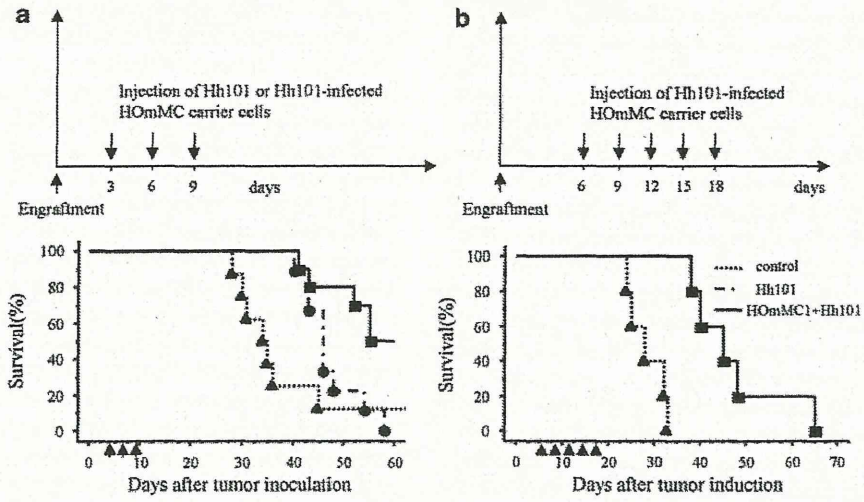

Figure 7 Therapeutic effects of Hh101-infected carrier cells in an ovarian cancer model. Nude mice were engrafted intraperitoneally with SKOV3 cells and (**a**) treatment was started 3 days later using three repeated i.p. injections. Three repeated therapeutic injections of Hh101-infected human omentum mesothelial cells (HOmMCs) improved the survival of ovarian cancer-engrafted nude mice. Injections of Hh101-infected carrier cells were more effective than Hh101 injections alone. Some of the mice treated with Hh101-infected carrier cells survived without symptoms or site injection tumors for >80 days. (**b**) Therapy was started on day 6 with five repeated i.p. injections. Median survival was significantly ($P=0.0018$) prolonged for the group of carrier-cell-treated animals compared with the control group (median survival, 45 days vs 28 days).

tumors in the peritoneal cavity and subsequently died. However, the survival time was extended by treatment with Hh101-infected carrier cells. This resulted in an approximate doubling of the median survival time (45 days; $n=5$) compared with that for control animals receiving PBS alone (28 days; $n=5$; $P<0.01$). Thus, in both experiments the prognosis was significantly improved by treatment with Hh101-infected carrier cells.

Discussion

Genetically engineered, conditionally replicating HSV-1 is a promising therapeutic agent for cancer therapy. The main antitumor mechanism of oncolytic viruses results from viral replication within infected tumor cells, resulting in cell destruction, and liberation of progeny virus particles that can directly infect adjacent tumor cells.[7]

Most clinical trials using oncolytic viral therapy have been performed using direct i.t. injection. However, almost all HSV-1 mutants were not so effective as expected when used clinically as antitumor cytolytic agents.[22–24] In an effort to develop more effective, well-tolerated, novel viral therapeutic agents, we have focused a highly attenuated oncolytic HSV-1 mutant, which lacks four accessory genes (*UL56*, *UL43*, *UL49.5* and *UL55*) and LAT (latency-associated transcript).[17–19] Our previous observation have shown that the HF10 is a potent novel agent for oncolytic therapy that is safe and effective for colon cancer, sarcoma and melanoma treatment in mouse models.[25–27] We have also performed a clinical trial of the treatment of recurrent breast cancer and head and neck cancer using i.t. injection of HF10.[28–30] These results revealed a potent oncolytic effect of HF10 without any side effects in human. Currently HF10 is being tested in the United States for the patients with advanced head and neck cancer in a Phase I clinical trial. Here, we have examined the ability of HF10 to control ovarian cancer in order to apply the HF10 therapy for peritoneally disseminated ovarian cancer. Firstly, we performed i.t. therapy of s.c. xenograft tumors using HF10, and tumor growth was significantly reduced. Moreover, in animals treated with six injections, a complete disappearance of the tumor was observed in some animals.

As a strategy to potentially enhance the delivery of HSV to disseminated tumors and to protect the virus from inhibitory factors (complement, anti-HSV antibodies) in the peritoneal cavity, we and others are exploring the use of carrier cells as Trojan Horses to deliver virus to tumors. The optimal carrier cell should be highly susceptible to HSV infection, not be rapidly killed by the virus, traffic to tumors and transfer the viral infection to the tumor cells via cell-to-cell heterofusion and/or by production of virus progeny.[11,31,32] An assortment of cells have been explored in this regard, including tumor cells,[24,33–35] outgrowth endothelial cells[36] and T cells.[31,37] In this work, we observed an ~10-fold higher amplification of the virus in HOmMCs than SKOV3 cells *in vitro*. Virus replication was the most efficient in HOmMCs, so we decided to use these cells as HSV carrier cells. Next, we estimated the efficacy of tumor killing caused by virus-loaded carrier cells *in vitro*. Our *in vitro* studies clearly demonstrated the efficacy of spread of infection between tumor cells and carrier cells. The transfer and spread of infectivity by MCs derived from the omentum was much higher than infectivity transfer by cell-free viruses. Taken together, these findings suggested that HOmMCs would be suitable for use as carrier cells to treat peritoneally disseminated ovarian cancer. However, the ultimate fear of carrier cells after intraperitoneal inoculation may pose some safety concerns. In this study, we immortalized normal human peritoneal MCs with non-viral human genes (mutant Cdk4, cyclin D1 and hTERT) and utilized as carrier cells. Such as the case, a carcinogenic potential of HOmMCs would not emerge thus far (data not shown). In the previous study,[21] we have developed immortalized ovarian surface epithelium with the same gene sets, and we did not observe any tumorigenesis up to doublings 60. The possibility of clinical application of carrier cells warrants that safety would be ensured.

Ascites frequently accumulate in patients who have tumor spread in the peritoneal cavity, and this fluid is expected to be rich in anti-HSV antibodies because the immunoglobulin G content of ascites fluid is known to reflect that of blood.[38] Also, it has been shown that pre-existing neutralizing antibodies in ascites may prevent initial adenovirus vector delivery in ovarian cancer patients.[39] Thus, carrier cells are expected to be useful not only for systemic virus delivery but also for intraperitoneal administration in patients with peritoneal metastases and pre-existing humoral immune response. We also examined the effect of antibodies against virus delivery by carrier cells. Our *in vitro* data demonstrated that direct cell-to-cell transfer of infectivity by HOmMCs was five to six times more resistant to neutralizing antibodies than infectivity transfer by naked virus. Thus, once infection is successfully transferred to the tumor, it is expected that antibodies will not stop i.t. virus spread. We also showed that HOmMCs infected with HF-GFP could target pre-established ovarian tumor nodules in mice (Figure 6), and this result is consistent with that of measles virus-infected cell carriers.[40] These data support the potential use of HSV oncolytic therapy using carrier cells in humans with pre-existing immunity to HSV.

This study supports the concept that the utilization of carrier cells may have a role in HSV-based oncolytic therapies. Inoculation of HOmMCs infected at MOI 3 or the equal titer of HSV particles should represent comparable viral loads initially administered to the animals. In the SKOV3 model, the carrier cell strategy led to a significant prolongation of animal survival compared with virus alone. Earlier treatment (3 days after engraftment) with infected carrier cells was even more effective. Hh101-infected carrier cells rescued few of the animals, because Hh101 is more attenuated than HF10, which is lethal for immunodeficient mice. If we use HF10 for carrier cell-based therapy in an immunocompetent model, we anticipate that the therapeutic effect would be better and be enhanced. Moreover, the immunogenicity of carrier cells may enhance therapy, as the activation of antitumor immunity during virotherapy appears to contribute to some degree to eliminating tumors and may help to protect from disease. To estimate the role of the immune response in oncolytic viral therapy, we would need to investigate this theory in a syngeneic immunocompetent mouse model of disseminated peritoneal ovarian carcinoma.

In conclusion, we establish that human peritoneal MCs are useful for carrier cells of oncolytic HSV in treating peritoneally disseminated ovarian cancer. Infected MCs and HOmMCs have the unique ability to produce a burst of virus upon delivery to the tumor site. In addition, this strategy allowed oncolytic HSV to escape neutralization by antibodies and complement, and subsequently to transfer the virus to tumor cells by *in situ* cell fusion. These findings may have significant implications for oncolytic virotherapy for ovarian cancer.

Conflict of interest

The authors declare no conflict of interest.

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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 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 established to be expressed in all layers of the epidermis except for horny layer in the lesions, quite different from the expression pattern restricted to basal and lower spinous layers seen 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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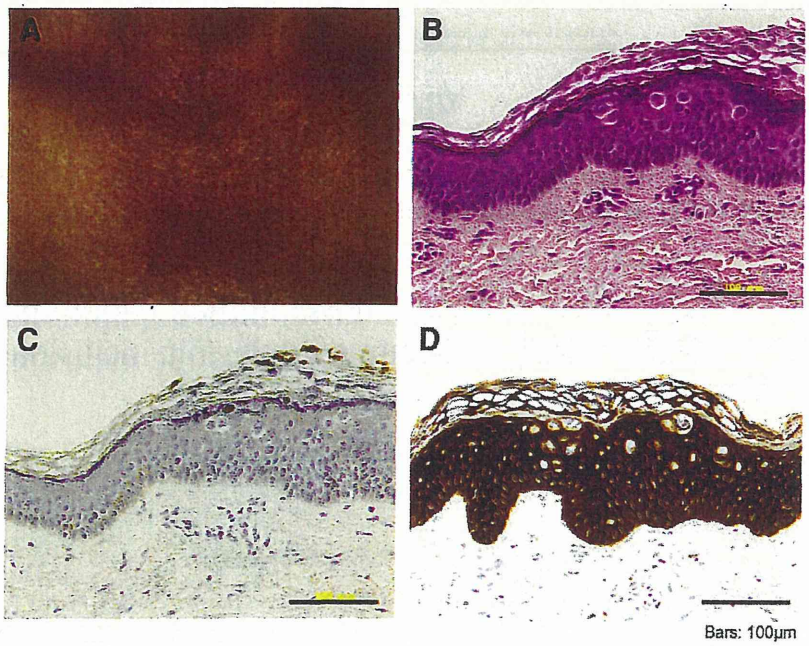

Fig. 1. Clinical (A), histopathological (B) and immunohistochemical (C and D) findings for the HPV 126-associated flat wart-like lesion. Macroscopic appearance of the flat wart-like lesions (A). In contrast to the normal skin adjacent to the flat wart-like lesion, Hg-ICBs are evident in balloon cells in the upper epidermal cell layers (B); strong positive signals for papillomavirus common antigen (L1 protein) in the nuclei of the cells in horny layer and cells with Hg-ICBs in granular layer (C); and positive signals for pan-cytokeratin are lacking in the cells with ICB (D). Scale bars; 100 μm.

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 (Fig. 1B) (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). 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 within a genus, we propose that HPV126, which has less than 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 the 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).

Table 1
Nucleotide sequence pairwise comparison of HPV 126 ORFs with those of representative genus gamma papillomaviruses.

ORF	E6	E7	E1	E2	E4	L1	L2
HPV type							
HPV 4	52.2	53.2	66.2	57.3	57.3	63.5	51.6
HPV 48	54.0	60.3	61.3	55.6	52.7	61.4	52.7
HPV 50	49.1	55.9	61.6	58.5	55.2	61.5	52.7
HPV 60	54.3	54.0	65.5	59.8	59.5	62.9	53.6
HPV 65	53.4	50.5	67.0	54.7	55.0	61.4	51.9
HPV 88	51.5	55.4	64.5	57.1	55.6	62.8	52.6
HPV 95	52.6	51.5	66.9	57.2	57.5	62.2	52.6
HPV 112	55.0	53.0	61.0	58.4	53.7	62.4	50.4
HPV 116	60.3	53.7	66.4	60.9	58.6	67.6	57.7
HPV 119	52.5	53.4	62.0	58.3	52.9	61.5	50.3
HPV 121	55.0	55.1	63.8	55.8	53.4	63.1	52.3
HPV 123	47.1	48.0	60.5	55.6	51.4	60.1	51.6
HPV 129	58.3	57.4	67.3	60.2	61.1	68.7	58.7

Similarities (%). Sequence for the genus gamma papillomaviruses were obtained from GenBank.

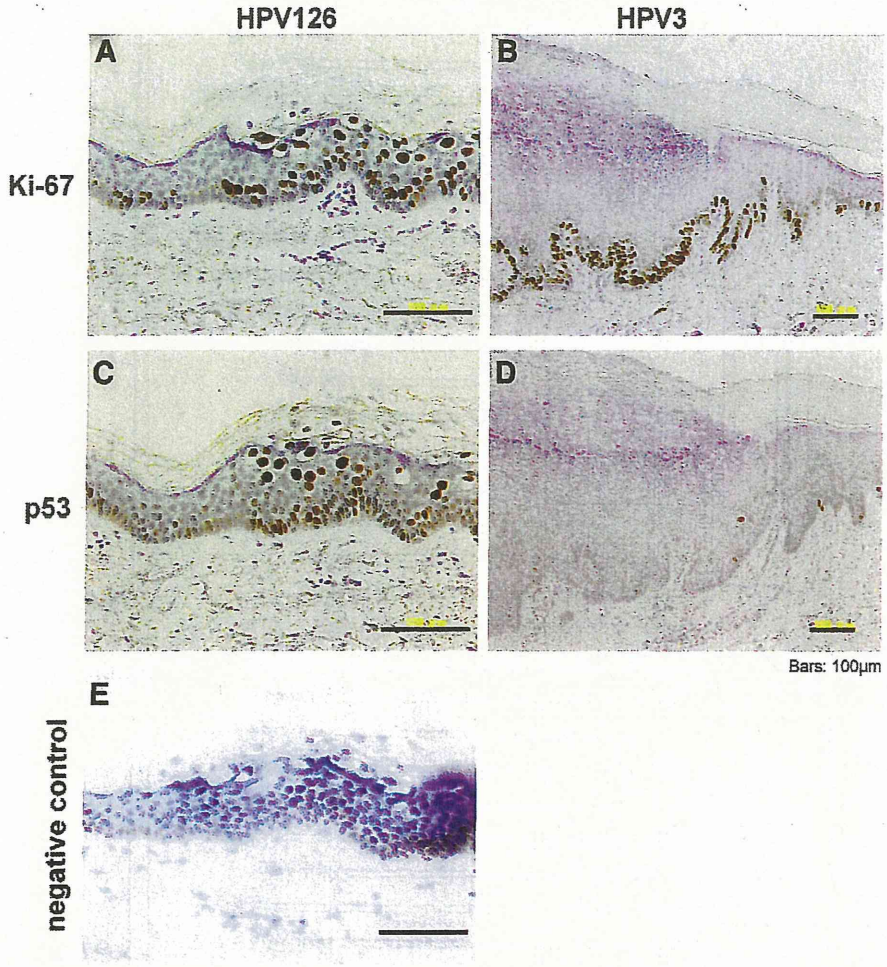

Fig. 2. Immunohistochemical features of the HPV 126-associated flat wart-like lesion. In contrast to the normal skin adjacent to the flat wart-like lesion (left side), positive signals for both Ki-67 (A) and p53 (C) are apparent in all compartments of the epithelium except for horny layer of the lesion (right side). However, a divergence is seen between Ki-67 and p53 staining. In contrast to that strong staining for Ki-67 is seen parabasal cells as well as cells of upper epidermal layers, the strong signal is seen predominantly in cells of upper epidermal cell layers. In contrast, weak (B) and faint (D) signals are restricted to the basal and parabasal (B) or lower spinous (D) layers of typical HPV 3-associated flat warts. Scale bars; 100 μm. As a negative control, no signals are observed in the staining of normal non-immune serum from the same source as the primary antibody (E).

Increased expression of Ki-67, an indicative marker of cycling cells, was observed in all compartments of the epithelium except for 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 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-asssociated inclusion warts studied thus far. These observations are reminiscence of high grade cervical intraepithelial neoplasia. However, neither the HPV 126-associated flat wart-like lesions nor ordinary flat warts showed positive staining for $p16^{INK4a}$, 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, HPV 126, 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,

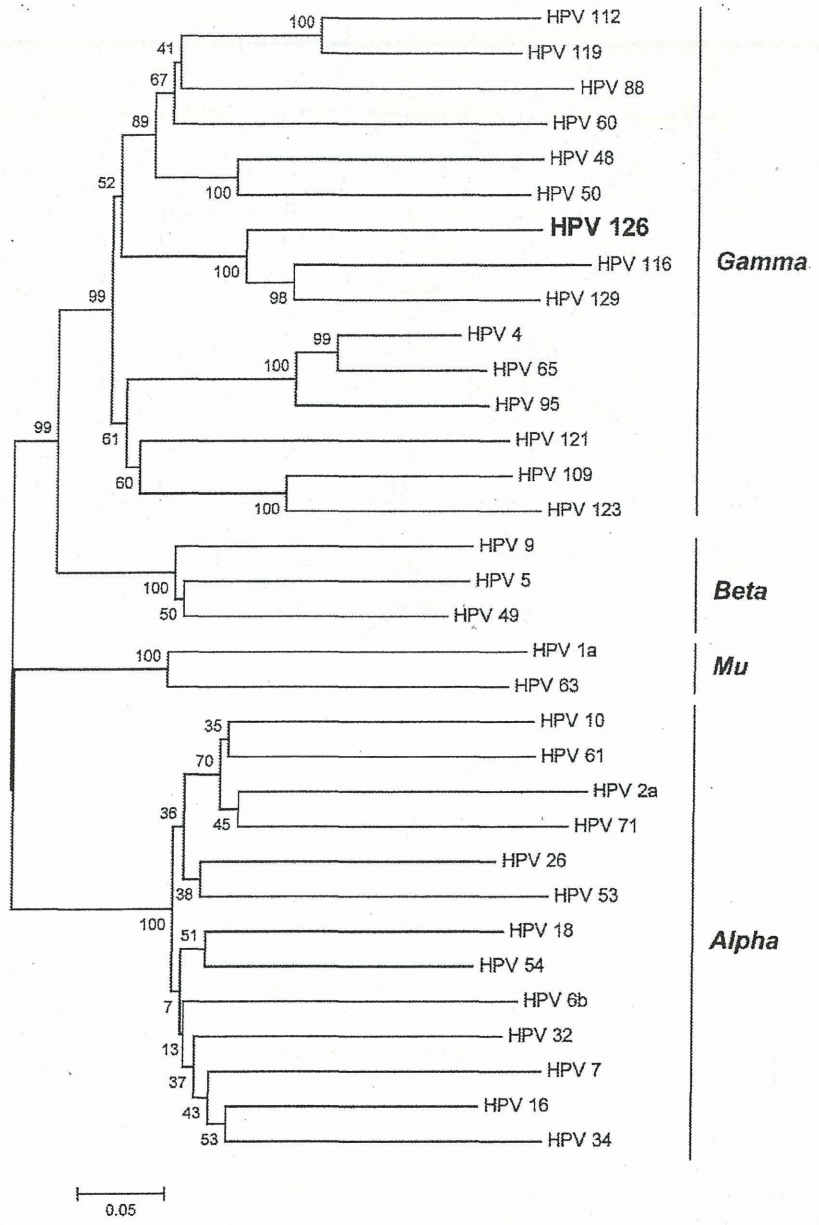

Fig. 3. Phylogenic relationships among HPV 126 and representative HPV types. A phylogenetic tree was constructed based on L1 ORF sequences using the neighbor-joining (NJ) method with 1000 bootstrap replicates. Numbers near branches indicate support index from NJ bootstrap percentage. Nucleotide sequences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

1994, 2005, 2007; Egawa et al., 1993). 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 $p16^{INK4a}$, cyclin D1, p53 and Ki-67. Abrupt inactivation of pRB can induce p53 accumulation though activation $p14^{ARF}$ (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 $p16^{INK4a}$