An epidermotropic canine papillomavirus with malignant potential contains an E5 gene and establishes a unique genus

Hang Yuan a, Shinje Ghim b, Joe Newsome c, Tania Apolinario a, Vanessa Olcese a, Mary Martin a, Hajo Delius d, Peter Felsburg c, Bennett Jenson b, Richard Schlegel a,*

a Department of Pathology, Georgetown University Medical School, 3900 Reservoir Road, NW, Washington, DC 20007, USA
b Brown Cancer Center, University of Louisville, Louisville, KY, USA
c Division of Laboratory Animal Resources, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
d Division for the Characterization of Tumorviruses, Deutsches Krebsforschungszentrum, Heidelberg, Germany
e Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

Received 25 April 2006; returned to author for revision 1 June 2006; accepted 11 August 2006
Available online 10 October 2006

Abstract

A novel canine papillomavirus, CfPV-2, was cloned from a footpad lesion of a golden retriever. Unlike the known canine oral papillomavirus (COPV), which has a double-stranded DNA genome size of 8607 bps, the genome of CfPV-2 is 8101 bps. Some of this size difference is due to an abbreviated early-late region (ELR), which is 1200 bps shorter than that of COPV. However, CfPV-2 has other differences from COPV, including the presence of an E5 ORF between the E2 gene and the ELR and an enlarged E4 ORF (one of the largest PV E4 open reading frames). The genome of CfPV-2 shares low homology with all the other papillomaviruses and, even in the most highly conserved ORF of L1, the nucleotide sequence shares only 57% homology with COPV. Due to this highly divergent DNA sequence, CfPV-2 establishes a new PV genus, with its closest phylogenetic relatives being amongst the Xi and Gamma genuses. CfPV-2 also has unique biological features; it induces papillomas on footpads and interdigital regions which, if infection is persistent, can progress to highly metastatic squamous cell carcinoma. CfPV-2 does not induce oral papillomas in immunocompetent animals and antibodies generated against COPV and CfPV-2 are type-specific. The availability of a new canine papillomavirus with differing genetic and biological properties now makes it possible to study type-specific host immune responses, tissue tropism and the comparative analysis of viral gene functions in the dog.

Keywords: Papillomavirus; COPV; CfPV-2

Introduction

Papillomaviruses are small, non-enveloped, double-stranded DNA viruses that induce hyperproliferative lesions of cutaneous and mucosal epithelia. In humans, more than 100 different human papillomaviruses (HPVs) have been isolated and cloned (zur Hausen, 2002). A subset of HPVs, the high-risk types, is associated with the development of malignancy, most frequently with cervical cancer. Approximately 99% of human cervical cancers contain HPV DNA of the high-risk types (Walboomers et al., 1999). Papillomaviruses are also found in many other vertebrate species and have been used as experimental models for analyzing virus–host interactions, including humoral and cellular immune responses to virus infection. Indeed, the canine oral papillomavirus (COPV) has been used as a prototype mucosal papillomavirus for developing a vaccine based upon the viral L1 capsid protein. Studies with COPV (Suzich et al., 1995) provided the pre-clinical foundation for the current development of a human papillomavirus vaccine (Harper et al., 2004).

Canine oral papillomavirus (COPV) was first isolated and sequenced from oral papillomas in dogs in 1994 (Delius et al., 1994). Canine oral papillomatosis is a contagious disease of young dogs and papillomas usually appear 3–4 weeks after infection and last for about 4 weeks before spontaneous regression. COPV infection can be prevented by immunization.
with either COPV virus like particles (VLPs) (Suzich et al., 1995), GST-L1 protein (Yuan et al., 2001) or L1 DNA vaccines (Stanley et al., 2001).

Recently, we became aware of several dogs with chronic papillomas of the footpad that exhibited histologic features characteristic of papillomavirus infection. In some cases, the dogs had been vaccinated against COPV, suggesting that another type of canine papillomavirus might be the etiologic agent. These footpad lesions were also much more persistent than COPV lesions, in some cases lasting more than 6 months. Usually the footpad lesions were associated with a clinical history of immunosuppression or with a stressful environmental setting. To determine whether there was a new canine papillomavirus associated with this disease, we cloned, sequenced and characterized the viral DNA from a footpad papilloma of a Golden retriever that had been in intensive training as a guide dog for the blind. Our results verify the existence of a new and divergent canine papillomavirus. We have designated this new virus as Canis familiaris papillomavirus type 2 (CfPV-2).

Results

Histology and immunohistochemistry of a canine footpad lesion

The food pad lesion of a golden retriever was biopsied, fixed in formalin and embedded in paraffin using standard histological techniques. Slides of these paraffin-embedded biopsies were then stained and examined microscopically (Fig. 1A, 10× and 40×). The tumor was determined to be an inverted or endophytic papilloma, differing from the normal exophytic papillomas induced by COPV. To determine if this papilloma were induced by papillomavirus, we stained additional sections of the tumor with DAKO papillomavirus L1 antibody (Fig. 1B, 10× and 40×), which was generated against denatured papillomavirus L1 protein and reacts with most papillomavirus isolates (regardless of species origin). The immunohistochemistry slides of the tumor showed positive staining for papillomavirus L1 protein within the nuclei of the differentiated keratinocytes. The strong staining with this antibody (specifically within the differentiated layers of the epidermis where the L1 protein is synthesized) confirmed that the lesion contained and expressed a papillomavirus.

To further substantiate the presence of papillomavirus, we extracted the footpad tumors and attempted to isolate viral particles as described in Materials and methods. When preparations were examined by electron microscopy, we observed abundant non-enveloped, 55 nm diameter virions which were very similar to COPV and characteristic of papillomaviruses in general (Fig. 1C). Finally, to determine if this viral isolate might be different than COPV, we examined the reactivity of isolated virions of COPV and the new isolate with a monoclonal anti-AU1 antibody. This monoclonal antibody recognizes the COPV L1 amino acid sequence, DTYRYI. As shown in the Western blot experiments in Fig. 1D, the reactivity of the new viral isolate with anti-AU1 antibody was significantly weaker than with COPV virions, suggesting that a different type of canine papillomavirus might be present.

Complete DNA sequence of the CfPV-2 genome

Viral genomic DNA was purified from either food pads warts or purified virions propagated in a xenograft system and sequenced as described in Materials and methods. The complete nucleotide sequence (GenBank accession numberAY722648 of this virus consists of 8101 base pairs (bps) and has a GC-content of 45%. The size is substantially smaller than that of COPV (8607 bps) which has the largest genome among papillomaviruses (Delius et al., 1994). We named this new virus, CfPV-2 (Canis familiaris papillomavirus type 2).

Phylogenetic analysis

The L1 ORF is the most conserved gene with the papillomavirus genome and has been used for the classification of papillomaviruses (de Villiers et al., 2004). In order to make an optimal phylogenetic analysis, L1 proteins from 29 HPVs and 17 animal papillomaviruses were selected according to their genus (de Villiers et al., 2004), and aligned using Clustal W (Thompson et al., 1994) with MEGA version 3.0 (Kumar and Nei, 2004). Based on the alignment, a phylogenetic tree (Fig. 2) was assembled by using the neighbor joining method with MEGA version 3.0 (Kumar and Nei, 2004). CfPV-2 was most closely related to the genus xi-papillomaviruses (BPV3, 4 and 6) and genus gamma-papillomaviruses (HPV4, 48, 50 and 60) and only distantly related to the genus lambda-papillomaviruses (COPV and Felis domesticus papillomavirus).

Open reading frames of viral genes

Similar to COPV and all other papillomaviruses, CfPV-2 has all of its open reading frames (ORFs) on the same coding strand of its circular double-stranded DNA genome. A diagram comparing the ORFs of CfPV-2 and COPV is shown in Fig. 3. CfPV-2 has eight ORFs that encode six early (E) proteins: E1, E2, E4, E5, E6 and E7. In addition there are two late (L) proteins: L1 and L2. The location of the ORFs and the predicted size of the encoded viral proteins are compared with the corresponding ones from COPV in Table 1.

Comparison of CfPV-2 ORFs to those of related papillomaviruses

Based upon the preceding phylogenetic analysis and ORF identification of CfPV-2, we compared its ORFs to those of related papillomaviruses. Comparisons were made with COPV (which infects the same host), BPV-1 (which is another animal papillomavirus encoding a small E5 protein), HPV-16 (which is an E5-containing human papillomavirus), BPV-6 (a xi-papillomavirus, a closely related animal papillomavirus) and HPV-4 (a gamma-papillomavirus, a closely related human papillomavirus). Comparisons were investigated by the pairwise alignment of corresponding ORFs nucleotides and proteins, as
calculated by using the Needle program (Needleman and Wunsch, 1970) of Emboss Pairwise Alignment Algorithms (http://www.ebi.ac.uk/emboss/align/). Low identity of nucleotide sequences (<60%) and in amino acid sequences (<72%) were observed (Table 2) with all the papillomaviruses analyzed.

The difference between the CfPV-2 and COPV genome size and organization was striking. The size of CfPV-2 genome (8101 bps) was much smaller than that of COPV (8607) and is more similar in size to most papillomaviruses. CfPV-2 has an early-late-region (ELR) of 368 bps, which is approximately 1200 bps shorter than that of COPV (which has the longest ELR sequence of any papillomavirus). In contrast to COPV, CfPV-2 contains an E5 ORF located between E2 and ELR. CfPV-2 also has an enlarged E4 ORF, which is one of the longest E4

Fig. 1. Canine footpad tumors contain and express a papillomavirus. (A, 10× and 40×) Histology of footpad tumors. The foot lesions, which were biopsied and examined histologically, showed the microscopic appearance of benign squamous cell papillomas. The tumors were inward-growing (endophytic) and the squamous epithelial cells showed evidence of cytoplasmic and nuclear vacuolation in the more differentiated layers of the epidermis, consistent with a papillomavirus infection. (B) The differentiated squamous cells of the tumor express intranuclear papillomavirus L1 protein. Immunohistochemistry demonstrated that the nuclei of the differentiated cells reacted specifically with DAKO anti-papillomavirus capsid antibody (brown staining of nuclei), indicating the expression of papillomavirus L1 capsid protein and suggesting viral replication. (C). Footpad tumors contain papillomavirus virions. The footpad lesions were extracted as described and virus was purified, negatively stained, and examined by electron microscopy. Virions were observed that exhibited the size (55 nm diameter) and structure of papillomavirus. (D) COPV and the new viral isolate (CfPV-2) show different reactivity with an L1 monoclonal antibody. The indicated amounts of viral protein from purified COPV and CfPV-2 were separated by SDS-PAGE and immunoblotted with the AU1 anti-L1 monoclonal antibody. The new viral isolate showed reduced reactivity compared to COPV, suggesting the presence of a unique papillomavirus type.
Fig. 2. Phylogenetic analysis of the L1 sequences of CfPV-2 with those from 29 human papillomaviruses and 18 animal papillomaviruses. The L1 gene of CfPV-2 was compared with a variety of divergent papillomaviruses in order to establish its closest relatives. The analyzed papillomavirus genomes included were FcPV (K02019), bovine BPV-1 (NC_001556), BPV-2 (NC_001551), BPV-3 (NC_004197), BPV-4 (X05817), canine oral COPV (NC_001619), Canine papillomavirus type 2 CfPV-2 (AY722648), cotton rabbit CRPV (NC_001541), deer DPV (NC_001522), Equus caballus EEPV-1 (AF498323), European elk EEPV (NC_001524), Felis domesticus FdPV-1 (AF480454), Hamster oral HaOPV (E15110), HPV-1 (NC_001556), HPV-3 (NC_001558), HPV-4 (NC_001457), HPV-5 (NC_001531), HPV-6b (NC_000904), HPV-9 (NC_001596), HPV-10 (X74465), HPV-16 (NC_001526), HPV-18 (NC_001557), HPV-26 (X74472), HPV-29 (NC_001685), HPV-32 (X74475), HPV-34 (X74476), HPV-41 (X56147), HPV-42 (M73236), HPV-48 (NC_001690), HPV-49 (NC_001591), HPV-50 (NC_001691), HPV-53 (NC_001593), HPV-54 (U37488), HPV-60 (NC_001693), HPV-61 (U31793), HPV-63 (X70828), HPV-90 (AY057438), HPV-92 (AF531420), HPV-94 (AJ620211), HPV-96 (AY382779), Mastomys natalensis MnPV (NC_001605), Ovine OvPV-1 (NC_001789), OvPV-2 (NC_001790), Psittacus erithacus PePV (AF502599), Phocoena spinipinnis PsPV-1 (NC_003348), Rhesus RhPV-1 (NC_001678) and rabbit oral ROPV (NC_002232).

Fig. 3. Linear representation of the ORFs of the COPV and CfPV-2 genome. The open reading frames of the COPV and CfPV-2 virus genomes are represented diagrammatically.
sequences found among the papillomaviruses. Even in the highly conserved L1 ORF, the CfPV-2 nucleotide sequence only shares 57% homology with that of COPV. According to the papillomavirus classification system (de Villiers et al., 2004), this defines CfPV-2 as a distinct papillomavirus genus (where less than 60% homology defines a new genus).

The E6 proteins of both CfPV-2 and COPV contain four C-X-X-C motifs. However, unlike COPV, the predicted CfPV-2 E6 protein also contains a carboxyl-terminal PDZ domain protein binding motif (D-S-C-V-COOH). This suggests that CfPV-2 E6 may behave like high-risk HPV-16 which can bind to PDZ proteins and, in some cases, lead to their E6AP-mediated ubiquitination and proteolysis (Lee et al., 2000; Nakagawa and Huibregtse, 2000; Thomas et al., 2005).

The predicted E7 proteins of CfPV-2 and COPV have two C-X-X-C motifs within their carboxyl terminal half. However, the CfPV-2 E7 protein lacks an Rb binding site (L-X-C-X-E) which is present in COPV E7. This implies that CfPV-2 E7 may not be able to bind and degrade the retinoblastoma tumor suppressor protein. Interestingly, Narechania et al. (2004) have observed that papillomaviruses that induce fibropapillomas often have characteristic E5 and E7 proteins. Specifically, the E7 proteins of PVs that induce fibropapillomas lack an Rb binding domain. However, despite lacking this Rb-binding domain in E7, CfPV-2 does not induce fibropapillomas, suggesting that there may be complex interactions between the E5 and E7 proteins for inducing the fibromatous component of tumors.

The CfPV-2 E1 protein shares the highest similarity with the other papillomavirus early proteins. Like COPV E1, it contains a cyclin interaction motif (the RXL motif) which mediates E1 interaction with and phosphorylation by cyclin/Cdk complexes and is required for efficient HPV replication in vivo (Ma et al., 1999). The ATP-binding site (GGPDGTGKS) of an ATP-dependent helicase is also conserved.

CfPV-2 E2 has a predicted transactivating domain within its amino-terminal half and a DNA-binding and dimerization domain located in the carboxyl-terminal region. The known E2 proteins bind the consensus sequence, ACCN6GGT (Androphy et al., 1987), and regulate transcription from promoters containing E2-binding sequence (Haugen et al., 1988). CfPV-2 has three E2-binding sites within the upstream regulatory region (URR) sequence. There are also three polyadenylation sites (AATAAA) in the upstream URR and a TATA box in the downstream URR.

The CfPV-2 E4 ORF is 933 bps and overlaps the E2 region, but in a different reading frame. The E4 gene exhibits low sequence homology to the E4 genes of other papillomaviruses, although it does have a characteristic high proline content (more than 15%).

CfPV-2 encodes a 41 amino acid E5 protein which is missing in COPV. This protein is a highly hydrophobic protein with an average hydrophaticity (GRAVY) index of 1.985 and Ile+Leu+Val content of 48.8%. It has a putative transmembrane domain between amino acids 10–32. The CfPV-2 E5 protein does not share a high level of amino acid homology with other E5 proteins, including those from BPV-1 or HPV-16 (Table 2). Interestingly, the CfPV-2 E5 protein is not closely related to any of the four proposed E5 groups (Bravo and Alonso, 2004), although it does resemble the BPV-1 E5 protein in size, the presence of C-terminal cysteine residues and the existence of polar residues in the transmembrane domain.

The DNA sequence and organization of the L1 and L2 proteins of CfPV-2 are similar to other papillomavirus capsid proteins. Translocation of the papillomavirus L1 and L2 proteins to the nucleus is mediated by nuclear localization signals (NLS) containing a high proportion of positively charged residues (K and R). CfPV-2 L1 contains a bipartite NLS, KRPATAKRAPTSKKRRK, at its carboxyl-terminus. The highest L1 sequence homology was observed with BPV-6 (59%) and HPV-4 (58%). The L2 protein contains two NLS domains, one at the N-terminus and one at the C-terminus. Otherwise, the L2 protein sequence homology between CfPV-2 and other papillomaviruses was low.

**CfPV-2 does not induce oral warts**

It is clear from clinical cases that CfPV-2 induces papillomas on the footpad whereas COPV seems to be selective for oral and genital mucosa. However, to further verify these apparent differences in tropism, we challenged the oral mucosa of dogs with both viral types. We could not challenge dogs at the

### Table 1

<table>
<thead>
<tr>
<th>ORF</th>
<th>Virus</th>
<th>Start ORF</th>
<th>Stop codon</th>
<th>Bases</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>COPV</td>
<td>102</td>
<td>536</td>
<td>435</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>105</td>
<td>512</td>
<td>408</td>
<td>135</td>
</tr>
<tr>
<td>E7</td>
<td>COPV</td>
<td>533</td>
<td>826</td>
<td>294</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>515</td>
<td>811</td>
<td>297</td>
<td>98</td>
</tr>
<tr>
<td>E1</td>
<td>COPV</td>
<td>816</td>
<td>2609</td>
<td>1794</td>
<td>597</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>795</td>
<td>2618</td>
<td>1824</td>
<td>607</td>
</tr>
<tr>
<td>E2</td>
<td>COPV</td>
<td>2551</td>
<td>3708</td>
<td>1158</td>
<td>383</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>2557</td>
<td>4152</td>
<td>1596</td>
<td>531</td>
</tr>
<tr>
<td>E4</td>
<td>COPV</td>
<td>3116</td>
<td>3461</td>
<td>345</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>2912</td>
<td>3907</td>
<td>996</td>
<td>331</td>
</tr>
<tr>
<td>E5</td>
<td>COPV</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>4169</td>
<td>4294</td>
<td>126</td>
<td>41</td>
</tr>
<tr>
<td>L2</td>
<td>COPV</td>
<td>5288</td>
<td>6829</td>
<td>1542</td>
<td>513</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>4662</td>
<td>6188</td>
<td>1527</td>
<td>508</td>
</tr>
<tr>
<td>L1</td>
<td>COPV</td>
<td>6837</td>
<td>8348</td>
<td>1521</td>
<td>503</td>
</tr>
<tr>
<td></td>
<td>CfPV-2</td>
<td>6202</td>
<td>7713</td>
<td>1512</td>
<td>503</td>
</tr>
</tbody>
</table>
footpad site with both viruses since CfPV-2 induces tumors that are painful and debilitating. Four dogs were divided into 2 groups, and each group was challenged on the oral mucosa with either purified COPV or purified CfPV-2. Dogs were evaluated weekly for 14 weeks after challenge for the appearance and size of oral papillomas. Four weeks after viral challenge, the COPV group developed typical oral papillomas (Fig. 4A). The group challenged with CfPV-2 remained free of papillomas for at least 14 weeks. It appears, therefore, that CfPV-2 cannot induce tumors in the oral mucosa of immunocompetent dogs.

CfPV-2 induces type-specific antibodies

Our sequencing data suggest that the capsid proteins of CfPV-2 and COPV are only distantly related. To verify this at the immunological level, we used ELISA to screen for possible cross-reactivity between antibodies against intact CfPV-2 and COPV virus. COPV antiserum was already available from our previous studies (Chen et al., 1998). CfPV-2-specific antibodies were generated by injecting 100 μg of purified CfPV-2 virions into a rabbit intradermally 3 times at 2 weeks intervals. The rabbit was phlebotomized 2 weeks after the third immunization, and the serum fraction collected. When the CfPV-2 antiserum was tested in ELISA, it demonstrated strong reactivity against CfPV-2 virus but only background reactivity against COPV (Fig. 4B). Similarly, antiserum against COPV virus reacted strongly with COPV but not significantly with CfPV-2. It appears, therefore, that these antisera are type-specific and that CfPV-2 and COPV induce distinct, non-cross-reactive antibodies. It is presumed, therefore, that previous infection with COPV will not protect dogs against CfPV-2 infection, which is consistent with our clinical data.

Discussion

Papillomas have been observed clinically at multiple anatomic sites on the dog, including the mucosa of the oral cavity and conjunctiva as well as the epithelium of the penis, vulva, and epidermis (Nicholls and Stanley, 1999). Studies suggest an etiologic role for a canine papillomavirus in the induction of cutaneous squamous papillomas (Campbell et al., 1988), cutaneous inverted papillomas (Shimada et al., 1993) and pigmented epidermal nevus (Tanabe et al., 2000). It has been postulated that there is more than one type of canine papillomavirus responsible for these neoplastic lesions. To date, however, only COPV has been completely cloned, sequenced and characterized (Sundberg et al., 1994). The L1 region of a canine papillomavirus causing pigmented epidermal

Fig. 4. COPV and CfPV-2: tissue tropism and type-specific antigenicity. (A) COPV and CfPV-2 differ in their ability to induce oral papillomas. Dog oral mucosa was abraded and infected with either COPV or CfPV-2 as described. At 4 weeks, tumors were observed in the COPV-infected animals (arrow) but not in the CfPV-2-infected animals. Animals were observed for an additional 2 months until COPV tumors had completely regressed, at which time the CfPV-2 challenged animals were still tumor-free. (B) COPV and CfPV-2 virions induce type-specific antibodies. Rabbit antisera were generated against purified COPV or CfPV-2 virions as described in Materials and methods and the reactivity of the antisera with purified virions was tested by ELISA. COPV-induced antibodies reacted preferentially with COPV and vice versa.
nevus has been partially sequenced (Tanabe et al., 2000), and it is more related to HPV than COPV or CfPV-2.

COPV induces benign mucosal papillomas in the oral cavity of young dogs. The lesions have a typical exophytic phenotype, and usually spontaneously regress within 4 to 8 weeks. In contrast, cutaneous papillomas caused by CfPV-2 occur uncommonly in the dog, have an endophytic phenotype and take a longer time to regress. Cutaneous papillomas have been seen primarily in dogs during immununosuppression. Recently, CfPV-2 has also been identified in cutaneous papillomas from X-linked severe combined immunodeficiency (XSCID) dogs (Goldschmidt et al., 2006). Although these hematopoietic stem cell transplanted dogs reconstitute normal T cell function as well as normal humoral immune function, they are susceptible to infection by CfPV-2. Most significant, however, is the observation that persistent CfPV-2 infection in these dogs leads to the progression of papillomas into invasive, metastatic squamous cell carcinoma. The distant metastasis of epidermal squamous cell carcinomas in dogs is extremely unusual.

Papillomaviruses are known to have a high degree of host and tissue specificity and our studies indicate that this applies to COPV and CfPV-2 as well. CfPV-2 cannot induce tumors on the oral mucosa of immunocompetent animals. In addition, these two viruses induce type-specific antibodies that do not cross-react.

Unlike COPV, CfPV-2 has an E5 ORF. The E5 protein of BPV-1 is a strong oncoprotein (reviewed in DiMaio and Mattoon, 2001; Suprynowicz et al., 2006) and may have a role in the initiation and maintenance of benign tumors in vivo. In HPV, the in vivo role of the E5 protein is less well defined, but recent studies have shown that the high-risk HPV-16 E5 protein induces rapid tumor formation and progression to cancer in transgenic mice (Genther Williams et al., 2005). Using the CfPV-2 model, it should now be possible to directly examine the role of E5 in vivo and in the context of the intact virus.

The oncogenic activities of high-risk HPV E6 and E7 have been documented extensively (reviewed in Mantovani and Banks, 2001; Munger et al., 2001). Like high-risk HPVs, the CfPV-2 E6 protein contains a carboxyl-terminal PDZ ligand motif. PDZ domain proteins play a major role in many cellular signal transduction pathways (van Ham and Hendriks, 2003) and studies with a transgenic mouse model demonstrate that the PDZ ligand domain on E6 is required for transformation (Nguyen et al., 2003). In contrast to high-risk HPV E7, CfPV-2 E7 does not contain an Rb-binding motif. Since the ability of the HPV E7 proteins to bind and degrade RB is closely coupled with malignant potential and cell transformation (Munger et al., 2001), it is unclear at this time how CfPV-2 induces tumors that progress to cancer. It will be important in the future to analyze the ability of the CfPV-2 protein to functionally interact with the Rb pathway.

Our phylogenetic analysis indicates that CfPV-2 is distantly related to COPV as well as all other papillomaviruses (Fig. 4). The highest L1 sequence similarity was found with BPV-6 and HPV-4 (59% and 58%, respectively). This dissimilar sequence homology, therefore, places CfPV-2 in a novel papillomavirus genus. From a biological standpoint, the striking difference in genetic and biological properties between COPV and CfPV-2 makes possible new inquiries into type-specific host immune responses, tissue tropism and the comparative analysis of viral gene functions.

**Materials and methods**

**DNA isolation and sequencing**

CfPV-2 genomic DNA was isolated from a footpad papilloma by alkaline lysis and purified by cesium gradient centrifugation (Delius et al., 1994). The genome was digested with digested with BglIII and subcloned into a modified pBR322 vector. In addition, viral DNA was also extracted from purified virions, digested with SacI and subsequently cloned into a modified pBR322 vector. Sequencing was performed by fluorescent sequencing in an ABI model 377 sequencer. The sequences from both sources are identical. The nucleotide and cDNA sequence data reported in this paper were submitted in GenBank using the BankIt submission tool (NCBI, Bethesda, MD) with accession number AY722648.

**Viral propagation**

CfPV-2 virus was propagated using a mouse xenograft system (Bonnez et al., 1993). In brief, fresh dog buccal mucosa was incubated with wart homogenate for 2 h, and then implanted under the renal capsule of three SCID mice. Ten weeks later, the mice were sacrificed and the grafts were collected. The tissue was cut into small pieces and homogenized in extraction buffer (0.02 M Tris, pH 7.5, 1.0 M NaCl, 30 μM PMSF) using a Dounce homogenizer. The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C using an HB-4 rotor (Sorvall centrifuge). The supernatant (about 3 ml) was transferred to a Ti centrifugation tube and ultracentrifuged at 4 °C, 35,000 rpm for 101 min (70 Ti rotor). The pellet was resuspended in a total of 20 ml of CsCl (D = 1.333) using a Dounce homogenizer. The CsCl solution (5 ml each tube) was then centrifuged at 30,000 rpm for 20 h (55 Ti rotor) at 4 °C. The band containing virions was aspirated from the side of the centrifuge tube with a 3 ml syringe (23 gauge needle) and dialyzed against phosphate-buffered saline containing phenylmethylsulfonylfluoride (overnight at 4°C).

**Electron microscopy of viral particles**

The purified COPV and CfPV-2 viruses were adsorbed to glow-discharged, carbon-coated grids (EM Sciences, Fort Washington, PA) and stained with 2% uranyl acetate. A JEOL 100-CX electron microscope was used for visualization.

**Immunohistochemical staining**

Formalin-fixed, paraffin-embedded tissue was used for histology (hematoxylin and eosin) and immunohistochemical
staining. The sections were deparaffinized with xylene and hydrated with ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The slides were first incubated with rabbit anti-bovine papillomavirus antibodies (dilution 1:1000, B508, Dako, Carpinteria, CA) at 4 °C for 18 h. The slides were then incubated with biotinylated secondary antibody (1:1000, DAKO) in a humid chamber at 37 °C for 30 min, followed by incubation with streptavidin–biotin–peroxidase complex (StreptABC kit, DAKO). The chromogenic substrate was 60% dianobenzidine in phosphate-buffered saline, and hematoxylin was used for counterstaining.

**Immunoblot analysis of viral capsid protein expression**

Proteins were separated in 4–20% gradient SDS-PAGE gels and then electrophoretically transferred to a polyvinylidenedifluoride membrane. The primary antibody, a mouse anti-UC1 monoclonal antibody, was used at 1:1000 dilution. The secondary antibodies, alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit antibodies (Tropix, Bedford, MA), were also used at 1:1000 dilution. Immunoblots were developed using the CDP-Star chemiluminescent substrate (Tropix).

**DNA and protein sequence analysis**

The putative open reading frames of CfPV-2 were identified with ORF Finder (http://www.ncbi.nlm.nih.gov/orf/orf.html). Pairwise sequence alignments were calculated using the Needle program of EMBOS Paivwise Alignment Algorithms (http://www.ebi.ac.uk/emboss/align/) at European Bioinformatics Institute (EBI) of European Molecular Biology Laboratory (EMBL). Multiple sequence alignments of L1 were prepared using Clustal W. Phylogenetic analysis was conducted using the Mega version3.0 (Kumar and Nei, 2004).

**Viral challenge of dogs**

Four beagles (Marshall Farm, North Rose, N.Y.) were randomly distributed into two groups. Groups A and B were challenged with COPV and CfPV-2 respectively as described in earlier studies (Yuan et al., 2001). Briefly, the maxillary buccal mucosa of the dogs was abraded with a sterile wire brush and wart homogenate applied to the excoriated mucosa with a cotton swab. All animal experiments were approved by the Georgetown University Animal Care and Use Committee, and the procedures are consistent with Public Health Service guidelines (20). This research was supported in part by the National Cancer Institute, R01CA53371.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.08.029.

**References**


