Short Communication

Isolation and characterization of the first American bottlenose dolphin papillomavirus: *Tursiops truncatus* papillomavirus type 2

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Received 13 July 2006
Accepted 18 August 2006

A novel papillomavirus (PV) was isolated from a genital condyloma of a free-ranging bottlenose dolphin inhabiting the coastal waters of Charleston Harbor, SC, USA: *Tursiops truncatus* papillomavirus type 2 (TtPV2). This novel virus represents the first isolated North American cetacean PV and the first American bottlenose dolphin PV. After the viral genome was cloned, sequenced and characterized genetically, phylogenetic analyses revealed that TtPV2 is most similar to the only published cetacean PV isolated and characterized thus far, *Phocoena spinipinnis* PV type 1 (PsPV1). A striking feature of the genome of TtPV2, as well as that of PsPV1, is the lack of an E7 open reading frame, which typically encodes one of the oncogenic proteins believed to be responsible for malignant transformation in the high-risk mucosotropic human papillomaviruses (HPVs). TtPV2 E6 contains a PDZ-binding motif that has been shown to be involved in transformation in the case of high-risk genital HPVs.

Papillomaviruses (PVs) have been classified as a separate virus family, *Papillomaviridae* (Howley & Lowy, 2001), and a new complex system of genera based on their phylogenetic relationships has recently been developed (de Villiers *et al.*, 2004). PVs are small DNA viruses that infect the basal cells of the skin and start to induce an increased epithelial proliferation (zur Hausen & de Villiers, 1994). The activities of the viral proteins E6 and E7 conduce to the induction of a hyperproliferation of the suprabasal cells by interfering with the pathways of cellular tumour-suppressor proteins (Chow & Broker, 1994; Howley & Lowy, 2001). So-called intermediate- and high-risk PVs contribute to malignant progression of epithelial tumours.

Mainly during the last decade, an intensive search for novel animal PVs has been conducted. We recently isolated the first sirenian PV, *Trichechus manatus latirostris* PV1 (TmPV1; GenBank accession no. NC_006563), from cutaneous lesions of a captive Florida manatee (Bossart *et al.*, 2002; Rector *et al.*, 2004). Based on clinical, histopathological, transmission electron microscopic and immunohistochemical findings, PVs have also been suspected to be the primary agent of genital and/or lingual tumours in several cetacean species, which comprise the families of dolphins and whales (Bossart *et al.*, 1996; Flom *et al.*, 1980; Lambertsen *et al.*, 1987; Van Bressem *et al.*, 1999). However, the only cetacean PV genome sequence published hitherto is *Phocoena spinipinnis* PV1 (PsPV1; GenBank accession no. NC_003348), a strain derived from a genital condyloma of a Burmeister's porpoise from the coast of Peru. We recently reported lingual papillomas, squamous-cell carcinomas and genital papillomas in Atlantic bottlenose dolphins (*Tursiops truncatus*) (Bossart *et al.*, 2005) and also isolated *Tursiops truncatus* PV1 (TtPV1) from a genital lesion of a captive European male bottlenose dolphin (Rector *et al.*, 2006). The TtPV1 sequence has not been published to date.

Here, we describe the isolation, cloning and genomic characterization of TtPV2. A detailed method description is

The GenBank/EMBL/DDBJ accession number for the genome sequence reported in this paper is NC_008184.

Detailed methods and a table containing GenBank accession numbers of sequences used for phylogenetic analysis are available as supplementary material in JGV Online.

available as supplementary material in JGV Online. During a bottlenose dolphin health-assessment study in 2004, conducted in estuarine waters near Charleston, SC, USA, specimens of genital condylomas were collected. Isothermal, multiply primed rolling-circle amplification (RCA) was carried out with extracted whole DNA from these lesions by using a TempliPhi 100 amplification kit (Amersham Biosciences). A DNA fragment of about 8 kb was identified and cloned for sequence analyses. Investigating transposon integrations with an EZ-Tn5 <KAN-2> insertion kit (Epicentre), 32 colonies were sequenced forward and backward and aligned by using DNASTAR Lasergene SeqMan software. The resultant complete TtPV2 genome nucleotide sequence was submitted to GenBank under accession no. NC_008184.

With respect to the chronological appearance of the PV proteins during the viral life cycle, corresponding genes and proteins are classified as early (E) and late (L). Products of L genes represent the structural components L1 (major capsid protein) and L2 (minor capsid protein), whereas products of E genes fulfil regulatory tasks during cell transformation, replication and transcription, as well as during capsid packing, virus release and more (E1–E9 depending on the type). Located between the open reading frames (ORFs) of L1 and E6 is a non-coding region (NCR) containing the most important *cis*-control elements for the regulation of viral transcription and replication. A schematic presentation of the TtPV2 genome organization is shown in Fig. 1. The complete nucleotide sequence comprises 7866 bp with a GC content of 45·98 mol% and contains at least five ORFs (E6, E1, E2, L2 and L1) and two putative genes (E4 and E5).

The TtPV2 E6 protein bears two zinc-finger motifs of the type CX_2C–X_{29}–CX_2C, separated by 36 aa. These motifs have been shown to be important for the stability and activity of the protein (Smola-Hess & Pfister, 2002). The C termini of E6 proteins of mucosal high-risk PVs contain the PDZ-binding motif XS/TXV/L, in which X can represent any amino acid [e.g. human PV (HPV) 16, ETQL; HPV18, ETQV]. This domain enables the E6 proteins of mucosotropic HPVs to bind to PDZ motif-containing proteins and direct them to proteolysis (Gardiol *et al.*, 1999; Lee *et al.*, 2000; Thomas *et al.*, 2002). The ability of E6 to bind to PDZ domain-containing proteins correlates with oncogenic potential and is essential for the induction of hyperplasia in *in vivo* models (Nguyen *et al.*, 2003). In the C terminus of TtPV2 E6, such a motif (ETEL) can also be found, whereas both PsPV1 and TmPV1 lack a PDZ-binding motif. This domain suggests the possibility for TtPV2 E6 to bind to corresponding PDZ motif-containing cellular proteins, as was shown for other PV E6 proteins, and to direct them to proteolysis, which might indicate that TtPV2 represents a high-risk type.

The E6 and E7 proteins of human mucosal high-risk PV types, such as HPV16 and 18, are able to transform immortalized cells independently (Halbert *et al.*, 1991; Phelps *et al.*, 1988; Sedman *et al.*, 1991) by binding to cellular proteins such as p53, pRb, $p21^{CIP1}$ and $p27^{KIP1}$ (Gonzalez *et al.*, 2001; Jones & Münger, 1997; Münger *et al.*, 2001; Scheffner *et al.*, 1990). Altering signal-transductional pathways and inhibiting cell cycle-controlling capabilities of these cellular proteins are believed to be the main causes of the transforming potential of the viral oncogenes E6 and E7. It was suggested that the continuous expression of both proteins is necessary for optimal proliferation of cervical carcinoma cells and that the two viral proteins exert distinct effects on cell survival and proliferation (DeFilippis *et al.*, 2003). However, only the E6 proteins of epidermodysplasia verruciformis-associated, high-risk PVs exhibit transforming capabilities, whilst corresponding E7 proteins show weak transforming potentials only (Iftner *et al.*, 1988; Schmitt *et al.*, 1994). One striking feature of the cetacean PVs isolated so far, including TtPV2, is the lack of an E7 ORF. This represents an uncommon feature among PVs

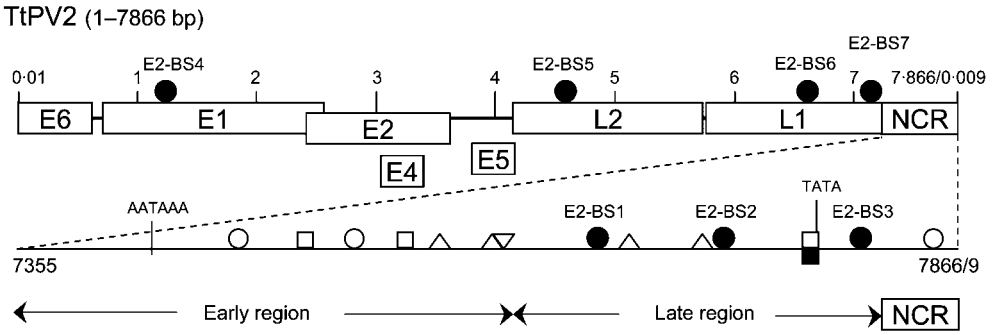

Fig. 1. Schematic representation of TtPV2 genome organization, including all ORFs. An enlarged section of the NCR shows the distribution of BSs for viral proteins (filled symbols) and putative recognition sites for cellular transcriptional activators (empty symbols). Shown are the consensus E1-BS (■) and seven consensus E2-BSs (●), as well as predicted recognition sites for SP1 (○), NF1 (△, ▽) and AP1 (□). Positions of a perfect TATA box and a polyadenylation signal site are indicated. Abbreviations: BS, binding site; NCR, non-coding region; E, early; L, late; SP1, GC box-binding protein; AP1, activator protein 1; NF1, nuclear factor 1.

only observed in the parrot PV *Psittacus erithacus* PV and the chaffinch PV *Fringilla coelebs* PV, which both lack the classical E6 and E7 ORFs (Terai *et al.*, 2002). A few PVs have also been described to lack an E6 ORF (bovine PVs 3, 4 and 6) (Jackson *et al.*, 1991; Patel *et al.*, 1987). To our knowledge, TtPV2 represents the only isolated PV worldwide missing an E7 ORF and encoding an E6 ORF containing a PDZ-binding domain. Depending on the corresponding protein potentials, it may be sufficient for some PVs only to express one rather than both oncogenes to display transforming capabilities. Alternatively, the length of the dolphin PV E6 might also play a role. When compared with the E6 proteins of some human high-risk PVs (types 16, 18, 6, 11, 5, 8) and TmPV1, which all contain an E6 ORF of 414–477 nt, the corresponding TtPV2 and PsPV1 ORFs consist of 621 and 636 nt, respectively. As the E7 ORF usually starts directly within the area downstream of the E6 ORF or within the C-terminal region of E6, sequence similarities of TtPV2 E6 with several E7 proteins of other PVs were investigated. No significant similarities could be found. Additionally, the PDZ-binding motif of TtPV2 E6 is located at the end of the ORF, as it is with other PV E6 proteins such as HPV16 and 18, making the evolutionary loss of a stop codon at the end of the E6 ORF unlikely to be the cause for the longer E6. There also are PVs [e.g. Rhesus monkey PV1 (RhPV1)] that encode both proteins and have a larger E6 ORF (576 nt). Furthermore, the so-called pRb pocket (LXCXE motif) was shown to be present in proteins binding pRb, such as HPV16 E7 and adenovirus E1A (Dick *et al.*, 2000; Phelps *et al.*, 1988). TtPV2 sequence investigations revealed that no similar motif is present in the corresponding E6 protein. Thus, another explanation for tumour pathogenesis might be the possible development of new pathways for interfering with cellular-control mechanisms by cetacean PV E6 proteins, which could also be true for E6 and/or E7 proteins of other PVs containing only one of the corresponding ORFs. To investigate whether cetacean PV E6 proteins are sufficient to lead to genital tumours without a corresponding E7 will be of great interest in the future. Appropriate investigations could lead to a better understanding of the importance of the cooperation of E6 and E7 with their corresponding cellular interaction partners.

As a dimer, the viral E2 protein binds to its palindromic consensus sequence 5′-ACC(N)$_6$GGT-3′ (Androphy *et al.*, 1987; Dostatni *et al.*, 1988) within PV genomes and thus regulates viral transcription. E2 also activates virus replication by interacting with E1 and stabilizing the sequence-specific, but weak, binding of the latter to the origin of replication (Frattini & Laimins, 1994; Sedman & Stenlund, 1995). The NCR of TtPV2 contains three consensus E2-binding sites (E2-BSs), whereas four additional consensus E2-BSs are located within the E1, L2 and L1 ORFs (Fig. 1, ●). Positions of a perfect TATA box (TATATAAA, nt 7787–7795) and a polyadenylation signal (AATAAA, nt 7422–7427) were also identified. A palindromic consensus E1-BS (Stenlund, 2003), 5′-ATTGTTACTAACAAT-3′ in between two E2-BSs at position 7772 directly upstream of the TATA box, is indicated (Fig. 1, ■). The viral helicase, the E1 protein, usually contains an ATP-binding domain in which three conserved motifs (A, B and C) are located. The formation of a phosphate-binding loop (P-loop) of motif A is implicated in binding the triphosphate moiety of ATP and was shown to be essential for the binding of E2 (Titolo *et al.*, 1999). All three conserved motifs are present in TtPV2 E1 (A, GPSNTGKS, nt 451–458; B, IGLLDD, nt 492–497; C, VTSNY, nt 538–542).

A number of cellular proteins involved in the activation of transcription have been shown to play an essential role in the regulation of PV oncogene expression (Apt *et al.*, 1993; Bednarek *et al.*, 1998; Butz & Hoppe-Seyler, 1993; Chong *et al.*, 1991; Demeret *et al.*, 1994; O'Connor *et al.*, 1996; Tan *et al.*, 1994; Thierry *et al.*, 1992). By using the DNASTAR Lasergene software package, a search for DNA-binding sites recognized by known transcription factors revealed possible recognition sites for numerous cellular proteins. BSs for selected activators of gene expression (SP1, GC box-binding protein; AP1, activator protein 1; NF1, nuclear factor 1) only

Table 1. Amino acid sequence similarities of TtPV2 proteins to corresponding proteins of other PVs

Listed are percentages of amino acid sequence similarity of TtPV2 ORFs with the respective ORFs of another dolphin genital PV (PsPV1), a marine mammal cutaneous PV (TmPV1), one high-risk type associated with mucosal carcinomas in monkeys (RhPV1), the four most prevalent high-risk (HPV 16 and 18) and low-risk (HPV6 and 11) human genital PVs and two human high-risk types associated with the skin-cancer disease epidermodysplasia verruciformis (HPV 5 and 8). NA, Not alignable because of insufficient similarity or not applicable due to a lack of the respective ORF.

TtPV2 ORF	PsPV1	TmPV1	RhPV1	HPV16	HPV18	HPV6b	HPV11	HPV8	HPV5
E6	29	17	22	22	24	22	22	19	20
E1	44	37	40	35	32	39	39	37	36
E2	29	24	30	28	27	31	29	21	19
Putative E4	16	5	NA	20	17	17	21	NA	5
Putative E5	NA	NA	NA	19	8	17 (E5a)	20 (E5a)	NA	NA
L2	28	26	21	23	26	22	25	28	28
L1	48	44	44	41	39	42	42	44	47

within the NCR are indicated in Fig. 1. Possible SP1-BSs (5′-CCCGCC-3′, nt 7467, 7530 and 7855) and AP1-BSs (5′-TGANTMA/TTANTAA-3′, nt 7507 and 7551/7776) are depicted as ○ and □, respectively. Whilst SP1 and AP1 were shown to play an essential role in the regulation of PV oncogene expression, the numerous members of the NF1 family might regulate viral transcription differently (Rehtanz, 1999). NF1 is able to bind to its palindromic recognition sites as homo- or heterodimers and to half of its BS as a monomer. Recognition sites important for activation of transcription were shown to be NF1 monomer-BSs (Chong *et al.*, 1991; O'Connor & Bernard, 1995; Rehtanz, 1999; Taniguchi *et al.*, 1993). Three putative NF1 monomer-BSs (5′-TTGGC-3′, nt 7582, 7687 and 7723), shown to be recognized by NF1 in other PV genomes, are located within the NCR, in addition to one NF1 dimer consensus-BS (nt 7610) (Fig. 1, △, ▽).

Sequence similarities were investigated by pairwise alignments comparing TtPV2 proteins with a number of corresponding PV amino acid sequences of other types (Table 1). TtPV2 amino acid sequence similarities to corresponding sequences of all other characterized PVs are generally low. As expected, several TtPV2 protein

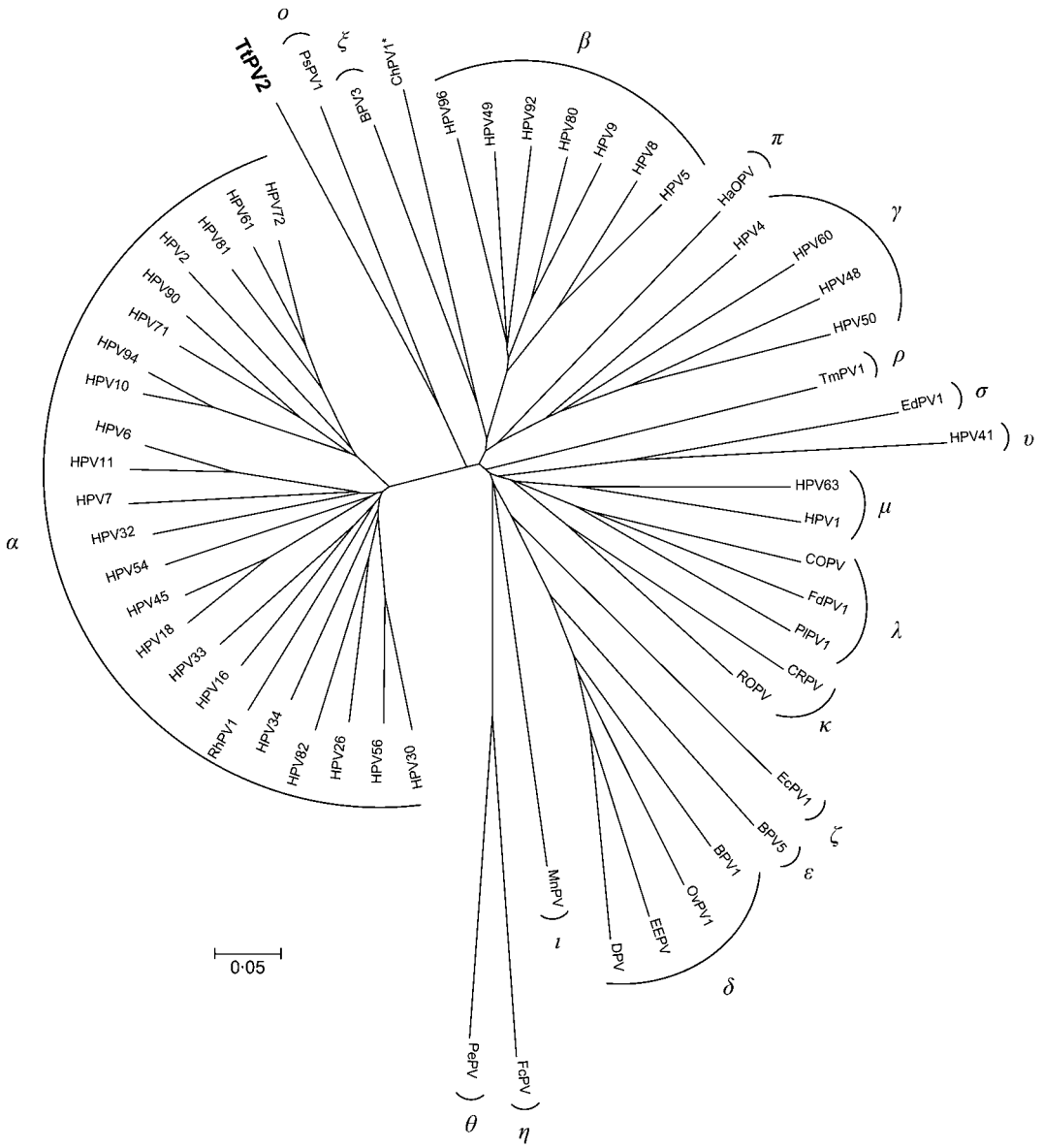

Fig. 2. Neighbour-joining phylogenetic tree of 58 PVs based on concatenated E1–E2–L2–L1 nucleotide sequences. Alignments of TtPV2 with 57 known PVs were investigated, with 2898 nt evaluated. Corresponding PV genera are indicated by their Greek symbols. GenBank accession numbers of included animal ($n=22$) and human ($n=36$) PVs are available in Supplementary Table S1 in JGV Online.

sequences show the highest similarities to corresponding PsPV1 proteins, with 48 % between L1 and 44 % between E1. All TtPV2 proteins, being less conserved than L1, E1 and L2, show higher similarities to the investigated genital types (PsPV1, RhPV1, HPV6, 11, 16, 18) than to cutaneous PVs (HPV5, 8, TmPV1). A neighbour-joining phylogenetic tree was prepared by using corresponding ORF-based E1–E2–L2–L1 concatenated nucleotide sequence alignments of 58 PVs, with 2898 nt evaluated in total. In accordance with the sequence-similarity investigations, the phylogenetic tree revealed that TtPV2 is related most closely to PsPV1, whereas it does not show a close relationship to TmPV1 (Fig. 2). Based on the recently introduced classification system, according to which different PV genera share $<60\,\%$ nucleotide sequence similarity, TtPV2 represents a novel genus that has not yet been assigned a name. The relationship of TtPV2 to the other isolated dolphin PV, PsPV1, was expected to be due to co-evolution of PVs with their hosts (Chan *et al.*, 1995). Marine mammals are believed to have evolved at various times from their dry-land ancestors. Cetaceans were already well diversified by about 50 million years ago. Although there is no conclusive fossil evidence to ascertain the origin and divergence of the genera *Tursiops* and *Phocoena*, they are estimated to have diverged about 10–13 million years ago (Perrin *et al.*, 2002), making the early time of separate evolvement of TtPV2 and PsPV1 plausible. The lack of a relationship of TtPV2 to TmPV1 can be explained by the manatee branching off the phylogenetic tree of marine mammal evolution early on. About 45–50 million years ago, several genera of the order Sirenia already existed and the first truly manatee-like sirenian fossils were dated to be about 15 million years old (Perrin *et al.*, 2002). The tropism of the respective PV within a family of more closely related hosts also plays a role in the corresponding clustering. Thus, the generally low sequence similarity of the genital mucosa-infecting TtPV2 to mucosotropic representatives of the family *Papillomaviridae*, but higher when compared with cutaneous types, was expected. A genetic and phylogenetic characterization of TtPV1 in relation to TtPV2 will contribute to determine cetacean PV relationships further.

To analyse whether the identified TtPV2 should be classified as a high-risk virus based on the potential of corresponding lesions to become malignant will help to identify the relevance and significance of PV infections for dolphins. Aside from PVs, it is believed that many cofactors contribute to the development of cervical cancer in humans, including smoking, human immunodeficiency virus infection, diet, hormonal factors and the presence of other sexually transmitted infections (Anttila *et al.*, 2001; Castellsagué *et al.*, 2002; Gerberding, 2004). With such cofactors in mind, concurrent infections with other unidentified pathogens could also contribute to condylomas in dolphins.

Acknowledgements

This work was supported by a postdoctoral fellowship of the Harbor Branch Oceanographic Institution to M. R. Funding for this study was provided in part by the State of Florida Protect Wild Dolphins License Plate, a Florida Ocean Initiatives grant, and the NOAA Fisheries Marine Mammal Health and Stranding Response Program and NOAA/NOS/CCEHBR. Dolphin lesions were collected under National Marine Fisheries Service scientific research permit no. 998-1678, issued to G. D. B. as part of the Health and Risk Assessment of Bottlenose Dolphin Project conducted in the Indian River Lagoon, FL, USA, and estuarine waters of Charleston, SC, USA. Sincere appreciation also is extended to Dr G. Steger and Sherry S. Willer for critically discussing the manuscript and to Andrew C. Marsh for editing.

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