

CANINE ORAL PAPILLOMAVIRUS INFECTION: CLINICAL COURSE, PATHOLOGY, L1 GENE AND NCR2 GENE SEQUENCING

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ABSTRACT

Canine oral papillomatosis is a self-limiting and spontaneous-regressing neoplastic disease caused by canine oral papillomavirus (COPV). In this report, five warts from the oral mucosa of five dogs suffering from canine oral papillomatosis were removed surgically for histopathologic examination, immunohistochemical analysis, L1 gene and NCR2 gene sequencing. Histopathology revealed various degrees of epithelial hyperplasia, hyperkeratosis and basophilic keratohyalin granules in the stratum lucidum and granular layer. COPV-L1 gene was highly conserved in all the five samples and several mutations of non-coding region (NCR2) gene were detected in three out of five samples. Immunohistochemistry revealed that cell division took place from stratum basale to stratum spinosum, even reaching the granular layer with COPV-L1 protein mainly distributed in stratum corneum and adjacent granular layer. The tendency of canine oral papillomatosis to transform into a squamous cell carcinoma is also discussed.

Keywords: Canine oral papillomatosis, histopathology, immunohistochemistry, sequencing.

INTRODUCTION

Canine oral papillomatosis is a self-limiting neoplastic disease caused by canine oral papillomavirus (COPV) (1). Major lesions are observed in the canine oral cavity characterized by the formation of papillary or cauliflower-like tumors. The most significant cytopathic feature caused by COPV is the vacuolization of cells in the granular layer and prickle cell layer (2).

COPV is a double-stranded DNA virus of 8,607 base-pairs (bp), and its genome contains several major early (E6, E7, E1, E2 and E4) and late (L2 and L1) open reading frames, and two non-coding regions (NCR1 and NCR2). The early viral proteins E1 and E2 play a role in replication of the viral genome. E4 may play some part in viral DNA replication or release of viral particles from infected cells. E6 and E7 control cell growth and the cell cycle which maximizes viral DNA replication, and the late proteins L1 and L2 form the viral capsid and package viral DNA. The function of two non-coding regions is not known (3,4,5,6,7,8,9).

In the study, five cases from canine oral papillomatosis were chosen for histopathologic examination, immunohistochemical analysis, L1 gene and NCR2 gene sequencing. Our aim was to provide evidence for the clinical diagnosis from a pathological view point with reference to the relationship between the proliferation of papillomavirus, the structure of the histiocyte, the development and regression of the tumor, and its tendency of inducing carcinoma. To the best of the authors knowledge this is the first time that research on the pathology with

reference to the viral gene of canine oral papillomatosis has been undertaken in China.

MATERIALS AND METHODS

Patient selection

The 5 samples in this study were obtained from 5 domestic dogs of different ages and breeds. They were all diagnosed with canine oral papillomatosis at the Animal Hospital of the China Agricultural University (Table 1).

Histopathology

The papilloma tissue were collected by biopsy, fixed in 10% neutral buffered formalin solution and processed routinely in paraffin wax. Sections (5 µm) were cut and stained with haematoxylin and eosin (H&E).

Electron microscopy

The papilloma tissue were collected by biopsy, immersed in 2.5% glutaraldehyde solution, washed with 0.1M PBS buffer (pH7.2), fixed by 1% osmic acid solution, dehydrated and embedded. Ultrathin slides were prepared and double-stained with uranyl acetate and lead citrate, and examined under a JEM-1230 electron microscope (Jeol, Tokyo, Japan).

Immunohistochemistry

Papilloma and normal canine oral mucosa epithelial tissue were treated following routine process, fixed in 4% paraformaldehyde, embedded and cut at 3µm. The samples were dewaxed, the antigens repaired and the endogenous biotin removed by 3% H₂O₂. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed

using a mouse anti-PCNA antibody (WuHan Boster Bioengineering co. Ltd.) as the first antibody and a horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG as the second antibody. The staining intensities were observed and photographed microscopically. The positive signal was dark brown and the negative signal colorless to very light yellow. The same method was used to perform COPV L1 gene immunohistochemical staining (rabbit anti-human HPV L1 antibody as the first antibody and HRP-conjugated sheep anti-rabbit IgG as the second antibody).

For the control groups, slides were taken from the papillomatous lesions and PBS was used to replace the first antibody. Tissue from normal oral epithelium was used as a negative control.

Polymerase chain reaction and sequencing

Viral DNA was extracted with DNAzol kit (Vigorous Biotechnology Beijing Co. Ltd., China) according to the manufacturer's instructions. The primers for L1 gene and NCR2 sequence were designed based on sequenced COPV genome (GenBank Accession No.D55633) as shown in Table 2. Specific polymerase chain reaction (PCR) was performed using primers described in Table 2. Briefly, a 20 μ l reaction system was composed which included the template DNA 3 μ l, 0.5 μ l of upstream and downstream primer, 10 μ l of PCRmix (Mylab Corporation, Beijing, China) and 6 μ l tri-distilled water. This was uniformly mixed and then started with an initial heating step of 5 minutes at 94 $^{\circ}$ C. Further cycles consisted of a denaturation step of 45 seconds at 94 $^{\circ}$ C, followed by 45 second primer annealing at 59.9 $^{\circ}$ C, and 90 second primer extension at 72 $^{\circ}$ C. After 35 cycles, the final extension step was set at 10min/72 $^{\circ}$ C. The amplified products were examined and target DNA were recovered using the Axygen kit, then directly sequenced at a commercial sequencing facility (Beijing Sunbiotech Co. Ltd., Beijing, China).

Phylogenetic analysis

The available sequences of COPVs and other major PVs were downloaded from NCBI to compare their relationship with the determined sequences in the study. Multiple sequence alignments were performed with DNAMAN computer software and phylogenetic trees were constructed with MEGA 4.0 program.

RESULTS

Clinical aspects

On visual inspection single or multiple pink filiform papillae-like growths were seen in or around the lip, tongue, buccal mucosa and hard palate. The diameter of the growths was often less than 1 cm (Fig.1A). Highly mature tumors could be removed easily. All five dogs had undergone surgery to remove their warts; no recurrences were observed.

Histological examination

Under light microscopy, hyperplasia of the epithelium, hyperkeratosis, basophilic keratohyalin granules in stratum lucidum and granular layer were observed. Large koilocytes were found in the granular layer and prickle cell layer. Compared with the three dogs which had tumors for only a short time, koilocytes in the two dogs with tumors of 5 and 6 weeks were larger in both number and size. Among tumor cells, exposed intercellular bridges were significantly elongated. Mitotic figures and intranuclear inclusion bodies formed by virus could also be seen in cells from dogs which had had

tumors of 3 or 4 weeks (Fig.1B).

Electron microscopy (EM)

Under electron microscope, small polygonal particles aggregated in the nucleus were observed in tumor cells of all cases. Other changes noted were swelling of organelles, vacuolar degeneration, distortion of nuclear appearance, shrunken, margination and dissolution of chromatin, microfilament aggregated in the cytoplasm or connections with abnormal hyperplastic desmosomes around the cell membranes (Fig.2).

PCNA immunohistochemical staining

After PCNA staining, positive cells were detected only in stratum basale of normal canine oral epithelial tissue (Fig.3C), while in all of the oral papillomatosis sections of dogs, positive cells were distributed from stratum basale to stratum spinosum, and even to granular layer (Fig.3A). This demonstrated that the prickle cell layer and granular layer both were undergoing cell division in addition to the stratum basale in the case of oral papillomatosis. This phenomenon might have resulted from virus proliferating in tumor cells.

COPV L1 protein immunohistochemical staining

No positive signal was found in control group, while in each section of the experimental group positive signals were present mainly distributed in the stratum corneum and adjacent granular layer. In the koilocytes of the granular layer and prickle cell layer, only in those cells which were close to the stratum corneum could a positive signal be detected. Furthermore, the closer the cells were to the surface layer, the easier it was to detect a positive signal. Under higher magnification, a greater number of deeply stained irregular particles could be seen. Thin strong-positive deposit areas could often be seen in parakeratotic area of some dogs. Positive cells of all cases in the non-stratum corneum mostly showed a weak positive diffusively stained reaction with the cytoplasm, however some cell presented with a strong positive nuclear reaction (Fig.3B,D).

PCR Amplification and Sequencing

COPV L1 gene

In all five cases, the same size L1 DNA sequence could be amplified by PCR, as shown in Fig.4A confirming the presence of COPV. All the L1 genes were sequenced and submitted to the GenBank. GenBank accession numbers were HM054511, HM054512, HM054513, HM054514 and HM054515. Compared with all other COPV sequence loaded in GenBank by DNAMAN computer software there was no variation among L1 gene sequences.

Specific NCR2 sequence

All the NCR2 sequences were obtained and submitted to the GenBank. GenBank accession numbers were HM054516, HM054517, HM054518, HM054519 and HM054520. The amplified NCR2 sequence had a length of 849bp (Fig.4B) and was rich in base A and base T (about 65%, which was about 56% in the entire genome of COPV). Compared with the isolate Y62 (GenBank Accession No.D55633) (10), point mutations existed in sample 2, sample 3 and sample 4 in the NCR2 gene (Table 3).

Phylogenetic analysis

The L1 gene sequences determined in the study were compared and phylogenetically analyzed with all other PVs' L1 gene sequence obtained from GenBank (Fig.5). Compared with other viruses from papillomavirus family, COPV

had the most closely evolutionary relationship with feline papillomavirus (FPV). COPV was also evolutionary related to the *Canis familiaris* papillomavirus type 2 (CfPV-2) which is also a variety of canine papillomavirus (11).

DISCUSSION

Generally, clinical signs of canine oral papillomatosis appear 1 to 4 weeks after infection and disappear 6 to 12 weeks later (12). All five dogs used in the study had tumors for more than 3 weeks of tumor growth indicating that the tumors were in the maturation period.

Observation with light microscopy revealed that koilocytes were larger than normal cells. In affected cells the cytoplasm was found to be hollow and transparent, the nucleus pyknotic and the nucleolus heavily stained. Under electron microscopy, koilocytes were pyknotic and the nuclear membrane severely wrinkles with halos appearing around the nucleus. At different growth stages the appearance of the tumor changed. In the cytoplasm, vacuoles of different sizes replaced swelling denatured organelles occupying a major portion of the cell. Compared with the disappearance of many inherent structures in the cytoplasm, microfilament increased and aggregated. In more mature papillomas, koilocytes were filled with homogeneous rarefied substances which obliterated the nucleus. A few cells even appeared to undergo apoptosis, which may represent the spontaneous regression of the tumor.

The formation of koilocytes may have resulted from the joint change of nucleus and cytoplasm infected by COPV. Reproduction of virus possibly compromised normal cell activities resulting in changes in material exchange and energy consumption appearing as an enlarged cell with pathological changes in mitochondria and endoplasmic reticulum (13). Changes in the nucleus presented as nuclear membrane wrinkling and perinuclear space broadening. With growth of the papilloma, cells infected by virus gradually underwent necrosis resulting in the disappearance of all kinds of structures and finally presenting as a completely homogeneous cavitation-like framework.

Sundberg et al. (8) reported that squamous cell carcinoma might occur at injection site in dogs which had been vaccinated for COPV, and that in some dogs with some degree of immunodeficiency, benign canine oral papillomatosis could transform into squamous cell carcinoma (14). This suggests that canine oral papillomatosis and squamous cell carcinoma might have a closer relationship than previously thought (15, 16). An increase in microfilaments and apparent intercellular bridge and multi-layer keratinized keratin pearls has been reported in carcinogenic tissues of squamous cell carcinoma type-1 (17). In this study we found similar characteristics as mentioned above. Electron microscopic observation showed an abundance of stacking desmosomes, step-type intercellular bridges and related microfilament aggregates which stratified inside the cell membrane. Large blocks or concentric circular multi-layer structures composed of keratin microfilament there were also seen (Fig. 6). The findings mentioned above illustrated that the development of canine oral papillomatosis has caused damage to microfilaments and intercellular bridges, which resulted in abnormal hyperplasia of these structures. Under the situation of immunosuppression or immunodeficiency, where the papilloma could not regress normally these hyperkeratotic structures could possibly become induced to develop into

squamous cell carcinomas.

Results of COPV L1 protein staining showed that a strong positive signal was distributed in the stratum corneum. L1 protein is one of PVs' major proteins and its appearance suggests that the virus is reproducing. Former research has shown that only the stratum corneum and granular layer have the essential materials for virus assembly, so that the stratum corneum and granular layers are the major sites of for reproduction and assembling of PV (18). Only when the infected cells differentiated to hornification did the late protein L1 of COPV began to appear and complete the final assemblage (19, 20). During this period, stratum corneum cells full of virus particles can exfoliate easily and infect other healthy dogs thus starting a new round of infection.

As mentioned above, the distribution of L1 protein positive signal and long fine cosh-like positive particle sedimentation area detected in parakeratotic areas have been previously reported in HPV research (21). Therefore, we concluded that COPV and HPV had a few similarities in their pathogenic processes.

The comparison of gene sequences showed no variation among different strains of COPV, indicating that L1 gene is quite conserved. L1 gene of COPV had a relatively higher homology with FPV (22), HPV-1a and HPV-63 (23, 24). The phylogenetic tree indicated further that COPV has quite close evolutionary relationship with HPV. We also successfully detected COPV L1 protein using rabbit HPV L1 antibody in an immunohistochemical test. Therefore, it is possible that canine infected by COPV can be used as an animal model for human infection by HPV.

Based on the coherence of molecular mass measurement, identity of restriction enzyme patterns between sequenced cloned COPV and different wild-type COPV (10, 25), we may conclude that NCR2 was essential DNA part of wild-type COPV rather than artificial cloned product. Retrieval in GenBank and EMBL database revealed no similarity between NCR2 and known DNA sequence. Therefore, we considered the detection of NCR2 as basis for identification of COPV.

As opposed to the conservative L1 gene, NCR2 sequence has a relatively stronger variability. Compared with the isolate Y62 (26), 3 of the 5 samples demonstrated point mutation in different degrees (Table 1). The significance of these mutations needs further study.

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TABLES

Table 1 - Information of dogs

No.	Breed	Age (Month)	Sex	Weight (kg)	Wart existence (week)
1	German Shepherd Dog	6	Male	20	6
2	Beagle	4	Female	8	3
3	Siberian Husky	6	Female	13	5
4	Great Pyrenees	4	Male	18	4
5	Caucasian Sheepdog	5	Female	20	5

Table 2 - The primers used in the study

Target gene	Primers (5'-3')	Predicted product
L1	P1:CACAGCCCAGCACCAAG P2:TGCGTTTGCGTTTCACA	1464 bp including nt 6879-8343
NCR2	P1:GACAAGTCCGACAGTCCAAC P2:GGTCAGATAAGCGGGTAGG	916 bp including nt 4218-5134

Table 3 - Mutations in specific NCR2 sequence of COPV

Sample No.	Mutation sites	Changes
COPV T2	nt 4254	"T" was replaced by "C"
COPV T3	nt 4252-4253	"AT" were replaced by "ACT"
COPV T4	nt 4379	"A" was replaced by "T"
	nt 4937-4938	"TG" were replaced by "TTG"
	nt 4992	"A" was replaced by "G"

FIGURES

Figure 1 - Clinical and histopathological examination of canine oral papillomatosis. (A) Appearance of dog's oral mucosa (German Shepherd Dog). (B) Transverse section of canine oral papillomatosis, H.E staining, 100 \times . This picture displays each layer of epithelium thickened around vascular connective tissue axis, hyperkeratosis. Koilocytes in different sizes distribute in prickle cell layer.

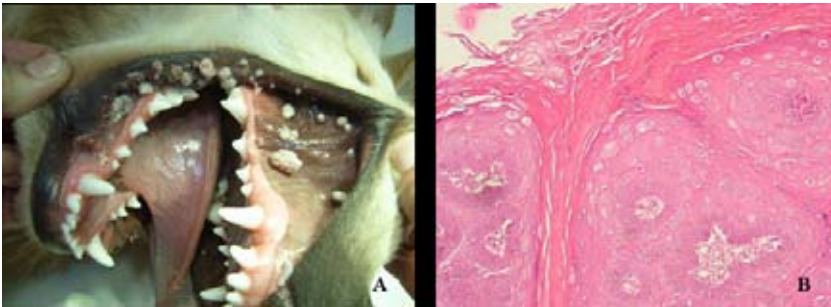


Figure 2 - Koilocytes. (A) Panorama of a typical koilocyte. Cell nucleus is extruded to side, the nuclear membrane is wrinkled, the chromatin is margined and the cytoplasm is filled with rarefied substance (Scale=2000nm). Inset: small particles in cell nucleus (Scale bar=500nm). (B) Mitochondria in koilocyte are swollen with vacuolization. (Scale bar=500nm). (C) Microfilament-like substances aggregated in koilocyte; with slight swelling mitochondria. (Scale bar=500nm).

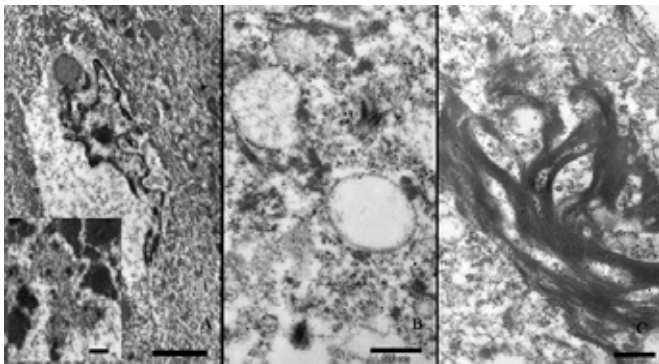


Figure 3 - Results of PCNA and COPV L1 protein immunohistochemical staining (100 \times). (A) PCNA positive cells distributed widely from stratum basale to granular layer of the epithelium. (B) Strong positive signal of COPV L1 protein mainly distributed in stratum corneum while weak positive signal could be seen in granular and prickle cell layer. (C) The control group of PCNA presented no positive signal. (D) The control group of L1 protein immunohistochemical staining presented no positive signal in any layer.

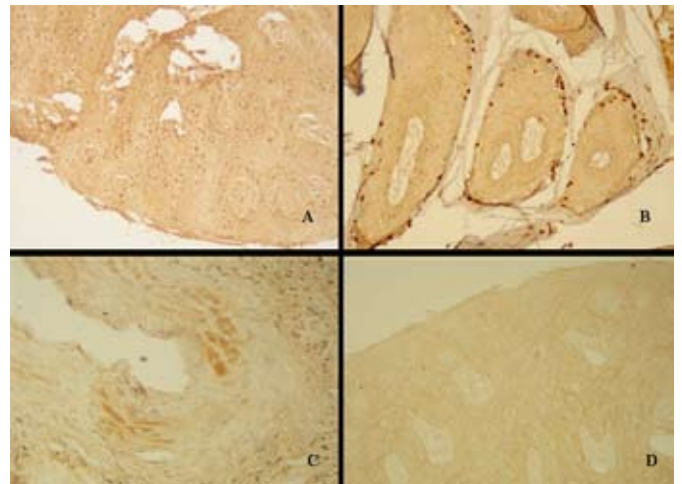


Figure 4 - Electrophoretogram of COPV L1 and NCR2. (A) Amplified products of COPV L1. (B) Amplified products of NCR2. 1-5: Sample No. of amplification. M: DNA Marker III. The length of L1 gene amplification is about 1386bp and the length of NCR2 amplification is about 849bp.

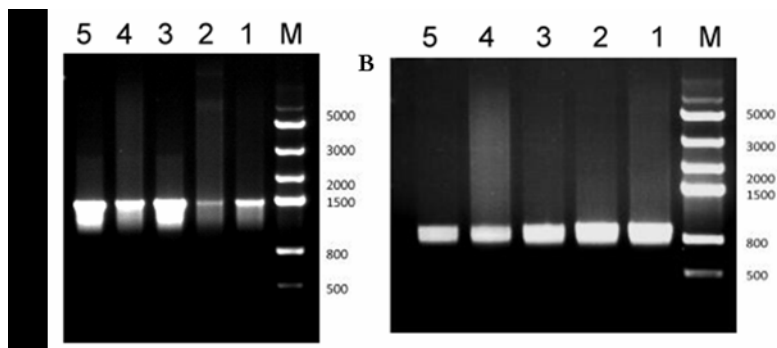


Figure 5 - Phylogenetic tree of PVs' L1 gene. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only >50% is shown). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood model as described in the methods section, and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). Phylogenetic analyses were conducted in MEGA4. COPV's Accession No. are D55633, L22695, NC_001619 and D26115, the other 6 kinds of PVs were taken from their standard strain.

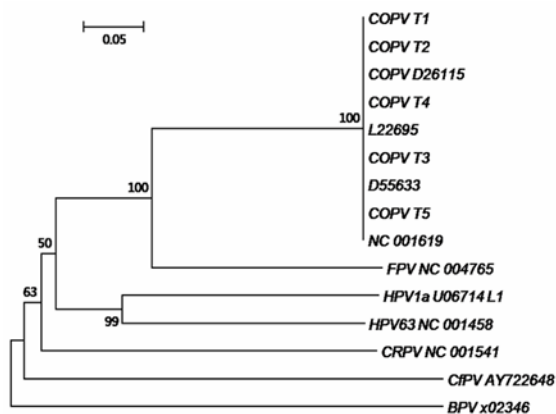


Figure 6 - Intercellular bridge and unknown multi-layer keratinized structure. (A) Elongated step-type intercellular bridges between cells with intermediates desmosomes. (Scale bar=1000nm). (B) Concentric circular multi-layer hornification structure in mature papilloma prickly cell layer, about ten times the size of koilocytes. Several organelles are scattered in the center while a few completely vacuolized cells (K) are present in the surrounding around (Scale bar=5000nm).

