博士論文

Pathological studies on canine papillomavirus-associated cutaneous lesions

（イヌパピローマウイルス関連皮膚病変の病理学的研究）

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CONTENTS

GENERAL INTRODUCTION

Papillomavirus (PV) is a non-enveloped, double-stranded DNA virus with a circular genome of about 8,000 base pairs (bp) that includes five or six early (E) open reading frames (ORF), two late (L) regions, and a non-coding long control region (LCR) [Ganguly *et al*., 2009; Munday and Pasavento, 2017a]. PV is classified according to the L1 nucleotide sequence: more than 10% dissimilarity between different genotypes [Bernard *et al*., 2010]. During the process of infection, E6 and E7 oncoproteins play a significant role in keratinocyte differentiation, and promote replication of suprabasal cells, accordingly resulting in amplification of the viral genome. Moreover, the early ORFs will be integrated into genome of host cells by increasing the expression of E6 and E7, and further promote the proliferation of infected cells and tumor development [Scheurer *et al*., 2005; Zheng and Baker 2006].

Papillomavirus infects the squamous epithelium in many mammalian, some avian and reptilian species [Munday 2014]. The virus is associated with benign and malignant neoplasia not only in humans but also in animals, although asymptomatic infections are more common [Antonsson *et al*., 2000; Howley 2013; Lange *et al*., 2011; Munday 2014; Munday *et al*., 2015; Stokking *et al*., 2004]. Human papillomaviruses (HPV) are classified into nearly 200 types, that

include high-risk types, probably high-risk types and low-risk types. Of note, infection of the high-risk HPV types can be a risk factor of developing cervical cancer, which is a common cancer all over the world [Fall *et al*., 2019; Kerge *et al*., 2019; Muñoz *et al*., 2003; Pahud and Ault, 2015].

Canine papillomavirus (CPV) includes 19 genotypes and classified into three genera as Lambda, Tau and Chi [Bernard *et al*., 2010; Lange and Favrot, 2011; Tisza *et al*., 2016]. Infection of CPV may be involved in the onset of canine oral papillomatosis (CPV1 and 6), cutaneous papilloma (CPV1, 2, 6, 7, 13, 17, 18 and 19) and pigmented viral plaque (CPV3, 4, 5, 8, 9, 10, 11, 12, 14, 15 and 16) [Lange *et al*., 2016]. However, most CPV infection causes no symptom [Lange *et al*., 2011; Lange and Favrot 2011; Munday *et al*., 2017a]. There is scarce evidence that CPV infection can lead to squamous cell carcinoma [Munday *et al*., 2013; Sabattini *et al*., 2016; Waropastrakul *et al*., 2012], although malignant transformation from benign papilloma to squamous cell carcinoma has been reported in immunodeficient dogs with CPV2 infection [Thaiwong *et al*., 2018]. However, a study suggested that CPV1 infection induced squamous cell carcinoma from benign papilloma [Thaiwong *et al*., 2018]. Moreover, malignant tumors arising in pigmented viral plaques have been also reported in association

with CPV9, 12 or 16 infection [Munday *et al*., 2017b]. The fact that most pigmented viral plaque cases are found in Pug dogs and Miniature Schnauzers implicates two possibilities in its pathogenesis: host-specific response to the viral infection and/or viral tropism to these canine breeds [Munday and Pasavento 2017a; Tobler *et al*., 2008]. Above all, the malignant transformation cannot be excluded in the CPV associated precancerous cutaneous lesions. And, the identification of CPV in these lesions is also significant.

Information on the localization of CPV nucleic acid in canine precancerous cutaneous lesions according to different CPV types have never been available. Hence, an *in situ* hybridization (ISH) is also needed to detect canine papillomaviral DNA. In human, ISH assay is widely used to test HPV DNA and integrated into standard pathologic assessment of HPV associated infections [Çelebi *et al*., 2018; Keung *et al*., 2019]. It is also being performed as one of the primary methods for the evaluation of HPV stage in head & neck tumors and cervical cancers [Lewis 2012; Witt *et al*., 2015; Manisa *et al*., 2017]. Although immunohistochemistry (IHC) and polymerase chain reaction (PCR) assays have been used to detect viral protein and gene respectively, which cannot illustrate the location of the virus in the skin. The presence of PV within the neoplastic

lesions could not be always confirmed using electron microscopy [Schwegler *et al*., 1997]. In bovine papillomavirus (BPV)-associated lesions, ISH assay has also been used to detect the nucleic acid of BPV and localization of the infected cells [Gaynor *et al*., 2016]. Additionally, as a reliable method, ISH also detected the DNA of *Felis catus* papillomavirus (FcaPV) in cats, and provides more information on the localization of infected cells [Vascellari *et al*., 2019]. However, an ISH study based on viral genotypes of CPV in precancerous lesions are lacking, and there is no information on the localization of infected cells in canine precancerous cutaneous lesions.

In human, p16 is accompanied by strong nuclear and cytoplasmic diffuse overexpression in high-risk HPV infection [Dreyer *et al*., 2017; Plath *et al*., 2018]. P16, as a tumor cell signature, plays a crucial role in cell cycle regulation. It is used as a prognostic biomarker for the tumors which is associated with HPV clinically [Oguejiofor *et al*., 2013]. Human anti-p16 antibody has been used in both dogs and cats until the present, and the results of p16 immunostaining indicated PV infection in the development of some canine and feline malignant tumors [Altamura *et al*., 2016b; Munday and Aberdein 2012; Munday *et al*., 2011a; Munday *et al*., 2015]. Retinoblastoma protein (pRb) is a tumor suppressor protein,

which belongs to the pocket protein family (PPF), bind to and is inactivated by oncogenic HPV proteins. And, it is dysfunctional in several HPV-associated cancers [Dreyer *et al*., 2017]. In addition, low expression or absent of pRb is induced with high-risk HPV (HR-HPV) infection [Castellsague *et al*., 2016]. In human, therefore, oncogenic HPV infection can result in the overexpression of p16 in both the nuclus and cytoplasm of HPV-associated neoplastic cells through inhibiting the pRb expression. Moreover, though FcaPV-2 has been used to degrade the expression of pRb, it is still unknown whether CPV can also represent the similar IHC characteristics within the precancerous skin lesions of dogs, like in those of human and cats [Munday *et al*., 2017b].

The aim of this thesis is to identify CPV-association in canine precancerous lesions in the skin. The expected results may provide more information about the localization of CPV of different genotypes and more details about the pathogenesis in CPV-related precancerous cutaneous lesions. In Chapter 1, to clarify the association of CPV in cutaneous precancerous lesions, CPV was detected by using IHC, PCR and identification of nucleic acid sequence in totally 23 cases including 16 papilloma, 1 papillary hyperplasia and 6 pigmented viral plaque cases. Moreover, IHC features of cytoketatin14, p63 and

Ki-67 in these lesions will be described precisely. In Chapter 2, an *in situ* hybridization (ISH) was performed to detect canine papillomaviral DNA on all CPV-positive tissue sections by PCR. The cases include 2 papilloma and 6 pigmented viral plaque lesions. An adequate information on the specific localization of infected cells and distribution of genotypes of CPVs in a subset of canine precancerous lesions will be provided. In Chapter 3, IHC for p16 and pRb proteins was performed on CPV-infected precancerous cutaneous lesions in dogs. The cases include 16 papilloma, 1 papillary hyperplasia and 6 pigmented viral plaque lesions. In human, oncogenic HPV infection can result in the overexpression of p16 in both the nuclus and cytoplasm of HPV-associated neoplastic cells through inhibiting the pRb expression, which is still unknown in dogs.

CHAPTER 1

Histopathological study on canine papillomavirus-associated cutaneous lesions

INTRODUCTION

Oral papillomatosis and cutaneous viral papilloma associated with canine papillomavirus infection (CPV1, 2, 6, 7, 13, 17, 18 and 19), consist of markedly thickened and folded oral mucosa or epidermis that becomes forming multiple filiform exophytic projections covered by keratin and supported by a fibrovascular stalk [Lange *et al*., 2016]. Papilloma is usually categorized as benign neoplastic lesions on the oral and cutaneous tissues. Papilloma is also called 'warts' or 'verruca vulgaris' [Rath *et al*., 2017]. The term of 'papilloma' is originated from the Latin term 'papilla', which refers to the projection created in the tumor [Goldschmidt and Hendrick, 2002].

Pigmented viral plaque is a proliferative lesion of the skin, and histopathologically characterized by acanthosis and hyperkeratosis of the epidermis with hyperpigmentation. The brownish black pigmentation in this lesion is derived from the abnormal accumulation of melanin pigments within so-called melanophages, rather than a proliferation of melanocytes [Stokking *et al*., 2004]. In human, papillomavirus can cause the similar lesions widespread called epidermodysplasia verruciformis, and the malignant transformation of the lesions is rare [Antonsson *et al*., 2000; Boxman *et al*., 2001]. Canine pigmented viral

plaque is most commonly observed in Pug dogs and Miniature Schnauzers in association with CPV4 infection [Munday, *et al*., 2010; Tobler *et al*., 2008]. Other types of CPV (CPV3, 5, 8, 9, 10, 11, 12, 14, 16 and 18) have also been detected in the lesions of canine pigmented viral plaque [Lange *et al*., 2016]. The fact that Pug dogs and Miniature Schnauzers tend to develop pigmented viral plaque implicates two possibilities in its pathogenesis: host-specific response to the viral infection and/or viral tropism to specific dog breeds [Munday and Pasavento, 2017a; Tobler *et al*., 2008].

There are few reports about neoplastic changes of papilloma and pigmented viral plaque in dogs [Callan *et al*., 2005; Munday *et al*., 2011b; Nagata *et al*., 1995; Stokking *et al*., 2004; Tobler *et al*., 2006]. One of the previous studies suggested that CPV1 infection induced squamous cell carcinoma (SCC) from benign papilloma [Thaiwong *et al*., 2018]. Pigmented viral plaques progressed into SCC have also been reported in Basenji dogs [Luff *et al*., 2016]. Hence, malignant transformation cannot be excluded in the CPV-associated precancerous lesions, and the identification of CPV in these lesions is also significant. The aim of this chapter is to identify CPV association in canine precancerous cutaneous lesions, by using IHC, PCR and nucleic acid sequence

in total 23 cases, including 16 cases of papilloma, 1 case of papillary hyperplasia and 6 cases of pigmented viral plaque.

MATERIALS AND METHODS

Cases and sample collection

Canine tissue samples of papilloma (n=16), papillary hyperplasia (n=1) and pigmented viral plaque (n=6) were examined (total 23 cases). The signalment, clinical presentations, and tumor location and distribution are summarized in **Table 1.1**. Of note, in case 22, malignant trichoepithelioma and pigmented viral plaque were examined in the same region of left forelimb. In case 23, multiple tumor masses between the digits of the left forelimb were also examined. As a negative control, a tissue sample of basosquamous carcinoma (Yorkshire Terrier, 11 years old) was used.

Histopathological and immunohistochemical analyses

Four μm-thick formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized, and stained with hematoxylin and eosin (HE). The neoplastic lesions in all cases were diagnosed through the Classification of Epithelial Tumors of the Skin, Surgical Pathology of Tumors of Domestic Animals [Kiupel, 2018]. Primary antibodies used for immunohistochemistry (IHC) were summarized in **Table 1.2**. Deparaffinized sections were subjected to antigen

retrieval. Namely, non-specific reactions were blocked by immersing the sections in 3% hydrogen peroxide in methanol at room temperature for 5 min, followed by incubation in 8% skimmed milk at 37°C for 40 minutes. The sections were then incubated at 4°C overnight with one of the primary antibodies. After washed 3 times with Tris-buffered saline (TBS), the sections were then incubated with Dako EnVision+System horseradish peroxidase-labedled anti-mouse or anti-rabbit secondary antibody polymers (Dako, Tokyo, Japan) at 37°C for 40 minutes. To visualize reaction products, sections were subjected to chromogen treatment with 0.05% 3,3'-diaminobenzidine and 0.03% hydrogen peroxide in Tris-hydrochloric buffer, and counterstained with hematoxylin. In the IHC detection of canine papillomavirus, a known CPV-associated cutaneous papilloma section was used as a positive control. Normal skin tissues were used as positive controls for all stain. For negative controls, primary antibodies were replaced with TBS.

PCR amplification and sequence assay

Total DNA was extracted from FFPE tissues with QIAamp DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Germany) [Munday *et al*., 2007]. The DNA of CPV2, extracted from a cutaneous papilloma lesion (Dachshund, 14 years old),

was used as a positive control. The L1 gene of papillomavirus (389bp) was amplified using canPVf/FAP64 primer set (5′- CTTCCTGAWCCTAAYMAKTTTG C-3′; 5′-CCWATATCWVHCATNTCNCCATC-3′) [Lange *et al*., 2011; Luff *et al*., 2012]. For CPV18 analysis, two more CPV18- specific primer sets to amplify the L1 gene (1F/1R 5′-TCAACCGCCCTTATTGGCTT-3′; 5′-CCTGGAGCCCGATAT TCCAC-3′ and 2F/2R 5′-ACTTGGTGAGCACTGGTCTG-3′; 5′-AAGCCAATAAG GGCGGTTGA-3′) were used, and the amplified products of which were 300bp and 451bp, respectively. PCR reactions were performed using C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). All amplifications were performed using KOD Fx Neo kit (Toyobo, Tokyo, Japan), according to the instructions. Amplification conditions were 94°C for 2 minutes, 98°C for 10 sec, an appropriate annealing temperature for each primer set for 30 sec and 68°C for 15 sec by 44 cycles with an infinite holding at 20°C. The annealing temperature for canPVf/FAP64 and CPV18- specific primer sets were 50°C and 60°C, respectively. PCR products were electrophoresed on a 2% agarose gel and detected by gel red. The extracted products of cases 3, 18, 19, 20 and 23 from the gel were cloned using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA), and sequenced using BigDye™ Terminator v3.1 Cycle

Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with the known sequences in the GenBank through the BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For sequence analysis, SnapGene software (Version 2.6.2; SnapGene, GSL Biotech, Chicago, IL, USA) was used.

RESULTS

Case information

The 23 dogs examined were with a range of 2 years to 15 years of age (median = 9 years and 1 month), and were comprised of Pug (n=4), Beagle (n=2), Chihuahua (n=2), French Bulldog (n=2), Shiba (n=2), Yorkshire Terrier (n=2), American Stafford (n=1), Boston Terrier (n=1), Dachshund (miniature) (n=1), Labrador Retriever (n=1), Manchester Terrier (Toy) (n=1), Pomeranian (n=1), Poodle (Toy) (n=1), Shih Tzu (n=1) and Standard Dachshund (n=1). As mentioned above, all the samples were divided into papilloma (n=16), papillary hyperplasia (n=1) and pigmented viral plaque (n=6). Papilloma were observed in the head and neck (10 cases), hindlimb (2 cases), tarsal (1 case), palm (1 case), elbow (1 case) and armpit (1 case). Papillary hyperplasia was observed in the forelimb (1 case). Pigmented viral plaque was observed in the forelimb (4 cases), lower abdomen (1 case) and nipple (1 case). The cases and lesions are summarized in **Table 1.1**.

Histopathological findings

Lesions of papilloma (n=16) and papillary hyperplasia (n=1) showed similar histopathological characteristics: papillary projections composed of hyperkeratosis and/or acanthosis with a poor atypia in cases 3 and 7 **(Figs. 1.1A and 1.2)**. The proliferation of squamous epithelium (mainly spinous cell layer) was observed with parakeratosis in case 7 **(Fig. 1.3A)**. Nuclear inclusions were observed in the epithelial cells of cases 3 and 7 **(arrows, inset) (Figs. 1.1B and 1.3B)**. Mitotic figures and koilocytes were rare in these benign lesions.

Lesions of pigmented viral plaque were characterized by acanthosis and hyperkeratosis of the epidermis with hyperpigmentation in case 18 **(Fig. 1.4)**. Koilocytes (cytoplasmic clearing) and nuclear inclusions were observed in case 19 **(Fig. 1.5)**. In one dog (case 23), a black nodule of 2 cm in diameter was found adjacent to the lesion of pigmented viral plaque between the digits of the left forelimb **(Fig. 1.6)**. Histopathologically, the nodule had a distinct margin, and were composed of neoplastic epithelial cells which formed cystic and lobular patterns **(Fig. 1.7)**. Neoplastic cells were round-shaped and had an indistinct cellular margin, giving a basal cell-appearance without showing any adnexal differentiation **(Fig. 1.8A)**. The number of mitotic figures was 3 per 10 high power-

fields, which was higher than papilloma and pigmented viral plaque. Melanin pigments were often observed within the neoplastic cells **(Fig. 1.8B)**. Koilocytes and nuclear inclusions were not observed. Based on these findings, the nodule was diagnosed as basal cell tumor.

Immunohistochemistry

Papillomavirus antigen was immunohistochemically detected in the nucleus of epithelial cells in 2 cases of papilloma **(Fig. 1.9)**, and 5 cases of pigmented viral plaque **(Fig. 1.10A)**. In the lesion of basal cell tumor (case 23), papillomavirus antigen was detected in the nucleus of epithelial tumor cells in the center of the neoplastic lobules **(Fig. 1.10B)**. However, no PV antigen was diagnosed as pigmented viral plaque and trichoepithelioma in case 22 **(Fig. 1.11)**. Thickened basal cell layer of papilloma, and pigmented viral plaque, and neoplastic cells of basal cell tumor were positive for p63 **(Figs. 1.12A, B and C)** and cytokeratin 14 **(Figs. 1.13A, B and C)**. The number of Ki-67-positivite cells was higher in CPV-positive papilloma and basal cell tumor lesions compared to those of CPV-negative papilloma and pigmented viral plaque **(Figs. 1.14A1, A2, B and C)**.

PCR and sequence analyses

By using the primer sets for detecting canine papillomavirus (canPVf/FAP64; L1 gene), which are able to amplify 389 base pairs of the CPV L1 gene, a single positive band was observed through electrophoresis of PCR products from case 3, 7, 18, 19, 20, 21, 22 and 23 as previous reports [Lange *et al*., 2011; Sabattini *et al*., 2016] **(Table 1.1; Fig. 1.15)**. The sequences of the PCR products amplified with the primer set were 99% identical to the reference sequence of CPV2 (GenBank accession number: AY722648.1) in case 3 and 100% identical in case 7. The sequences also amplified with the same primer set were 99% identical to the reference sequence of CPV4 (GenBank accession number: EF584537.1) in case 18, 100% identical in case 19 and 20, 21 and 22, and 100% identical to the reference sequence of CPV18 (GenBank accession number: KT326919.1) in case 23. Moreover, in case 23, the sequencing results were also confirmed with the CPV18- specific primer sets 1F/R and 2F/R, being 99% and 100% identical to the reference sequences of CPV18.

DISCUSSION

In this Chapter, histopathological and IHC profiles of 23 canine cases were examined. The cases were divided into papilloma (n=16), papillary hyperplasia (n=1) and pigmented viral plaque (n=6) **(Table 1.1)**. There is no breed and sex predilection for papilloma [Meuten 2016]. Given the fact that Pug dogs and Miniature Schnauzers tend to develop pigmented viral plaque, two possibilities in its pathogenesis have arisen: host-specific response to the viral infection and viral tropism to specific dog breeds [Tobler *et al*., 2008; Munday and Pasavento, 2017a]. The locations and histopathological findings of the lesions of papilloma and pigmented viral plaque in the present study were consistent with those in the previous studies [Lange *et al*., 2016; Lange *et al*., 2019; Meuten 2016; Tobler *et al*., 2008].

CPV types identified, vary according to the precancerous lesions including pigmented viral plaque, cutaneous papilloma and squamous cell carcinoma [Lange *et al*., 2016]. A novel type of CPV, CPV18, was recently identified from the lesion of pigmented viral plaque of a dog in the United States [Lange *et al*., 2016; Munday and Pasavento, 2017a]. It was the first finding of CPV18 detection in a neoplastic lesion in the dog. In this study, PV antigen was

also detected in papilloma lesions of cases 3 and 7, in pigmented viral plaque lesions of cases 18, 19, 20, 21 and 23. Interestingly, in case 22, no papillomavirus antigen was detected by IHC, whereas the CPV4 nucleic acid was detected by PCR. This suggests that L1 viral protein of papillomavirus cannot be detected on every occasion, and the loss of the L1 viral protein associated with the release of viral gene during transportation [Digiuseppe *et al*., 2017; Lange *et al*., 2019; Longworth *et al*., 2004; Thomson *et al*., 2016].

The morphologic, IHC, PCR and nuclei acid sequencing results suggested PV involvement in the papilloma. Cases 3 and 7 were both immunohistochemically positive for papillomavirus antigen, and also characterized by thickened oral mucosa or epidermis, and the numbers of p63- and cytokeratin 14-positive basal cells were increased **(Figs. 1.12A and 1.13A)**. The number of Ki-67-positive cells was increased in the CPV-positive papilloma compared to that in the CPV-negative papilloma **(Figs. 1.14A1 and A2)**. Based on these results, some viral factors may imply a risk for malignant transformation within CPV2-associated papilloma in dogs. Regarding pigmented viral plaque, though the number and size of the lesions in a dog may increase or decrease spontaneously, malignant transformation of the lesions has been rarely reported

as well. Here, basal cell tumor was found adjacent to the lesion of pigmented viral plaque and the tumor cells were immunohistochemically positive for papillomavirus antigen (case 23). CPV18 gene was detected by PCR in the lesion of pigmented viral plaque. The lesion of pigmented viral plaque was characterized by thickened basal cell layer of the epidermis, small projections consisting of basal cells into the dermis, and the increased number of p63- and cytokeratin 14-positive basal cells **(Figs. 1.12B and 1.13B)**. The proliferating cells in the lesion of case 23 were diffuse positive for p63 and cytokeratin 14, indicating a basal cell phenotype **(Figs. 1.12C and 1.13C)**. Moreover, the number of Ki-67-positive cells was also increased in the tumor tissue compared to that in the pigmented viral plaque **(Figs. 1.14B and C)**. Therefore, basal cells in the pigmented viral plaque may undergone neoplastic change and developed basal cell tumor in case 23. A previous study, proposed a mechanism of papillomavirus infection to the basal cell, ie. viral gene can be maintained in the cell at as low as 100 copies, and cause an asymptomatic infection [Doorbar 2005]. Infected basal cells proliferate and induce the keratinocyte differentiation [Maglennon *et al*., 2011; Munday 2014; Narama *et al*., 2005]. In the present study, papillomavirus antigen was detected by IHC only in the spinous layer and granular layer of the lesion of

papilloma and pigmented viral plaque, and in the center of basal cell tumor lobules.

In conclusion, CPV2 gene was detected in 2 papilloma cases, CPV4 was in 5 cases of pigmented viral plaque, and CPV18 gene were also detected in 1 case of pigmented viral plaque. In the CPV18-positive case, basal cell tumor developed together with pigmented viral plaque. The study of the present chapter shows that CPV18 infection may be associated with neoplastic proliferation of epidermal basal cells. The present information on the identification of CPV indicates a potential risk of malignant transformation in these precancerous cutaneous lesions associated with CPV infection in dogs.

ABSTRACT

In this chapter, to clarify CPV association in cutaneous precancerous lesions, CPV was detected by IHC, PCR and nucleic acid sequence determination in totally 23 cases including 16 cases of papilloma, 1 case of papillary hyperplasia and 6 cases of pigmented viral plaque. Papillomavirus antigen was detected in 2 cases of papilloma (2/16, 12.5%), and 5 cases of pigmented viral plaque (5/6, 83.3%). CPV gene was detected by PCR in 2 cases of papilloma (2/16, 12.5%), and 6 cases of pigmented viral plaque (6/6, 100%). These results illustrated that PCR is a more sensitive method to detect papillomavirus infection in dogs. Moreover, the results of nucleic acid sequence analysis showed that 2 cases of papilloma were infected with CPV2; 5 cases of pigmented viral plaque were infected with CPV4, and 1 case with CPV18. In the case of pigmented viral plaque, CPV18 gene was also detected in the lesion of cytokeratin-14- and P63-positive basal cell tumor that had developed from pigmented viral plaque. This is the first finding of basal cell tumor associated with CPV18-infection in the dog. In addition, the number of Ki-67-positive cells was increased in the CPV-infected papilloma and basal cell tumor compared to that in the CPV-uninfected papilloma and

pigmented viral plaque lesions. The result indicates a potential risk of malignant transformation in the lesions associated with CPV infection.

Table 1.1 Case information and CPV detection

Case no.	Age	Sex	Breed	Location of lesions	Histopathological Diagnosis	PV detection Sequence (Ident. %) IHC	PCR	
1	10y	CM	Beagle	Right Eyelid	Papilloma	-	-	NA
2	8y	CM	French Bulldog	Left forelimb	Papillary hyperplasia	-	-	NA
3	9y	SF	Labrador Retriever	Right Auricle	Papilloma	+	+	CPV2 (99%)
4	12y	SF	American Stafford	Left hindlimb	Papilloma	-	-	NA
5	7y	SF	French Bulldog	Lip	Papilloma	-	-	NA
6	12y	CM	Shih Tzu	Right maxillary	Papilloma	-	-	NA
7	14y	F	Miniature Dachshund	Right Tarsal	Papilloma	+	+	CPV2 (100%)
8	7y	M	Chihuahua	Right Palm	Papilloma	-	-	NA
9	11y	M	Shiba Inu	Left elbow	Papilloma	-	-	NA
10	9y	SF	Pomeranian	Left forelimb	Papilloma	-	-	NA
11	10y	SF	Yorkshire Terrier	Lip	Papilloma	-	-	NA
12	7y	SF	Toy Poodle	Nasal	Papilloma	-	-	NA
13	15y	SF	Shiba Inu	Armpit	Papilloma	-	-	NA
14	2y	SF	Yorkshire Terrier	Mandibular	Papilloma	-	-	NA
15	6y	SF	Beagle	Neck	Papilloma	-	-	NA
16	8y	CM	Boston Terrier	Right Eyelid	Papilloma	-	-	NA
17	8y	F	Chihuahua	Lip	Papilloma	-	-	NA
18	11y	SF	Pug	Left forelimb	PVP	+	+	CPV4 (99%)
19	7y	CM	Pug	Right lower abdomen	PVP	+	+	CPV4 (100%)
20	11y	SF	Pug	Nipple	PVP	+	+	CPV4 (100%)
21	9y	SF	Toy Manchester Terrier	Left forelimb	PVP	+	+	CPV4 (100%)
22	6y	F	Standard Dachshund	Left forelimb	PVP & TE	-	+	CPV4 (100%)
23A	11y	CM	Pug	Left forelimb	PVP	+	+	CPV18 (100%)
23B			Pug	Between digits of left limb	BCT	+	+	CPV18 (100%)
NC	12y	CM	Yorkshire Terrier	Left forelimb	BC	-	-	NA

SF, spayed female; CM, castrated male; PVP, pigmented viral plaque; TE, trichoepithelioma; BCT, basal cell tumor; BC, basosquamous carcinoma; PV, papillomavirus; IHC, immunohistochemistry; PCR, polymerase chain reaction; +, positive; -, negative; NC, negative control; NA, not applicable.

Table 1.2 Primary monoclonal antibodies used for immunohistochemistry

Antibody to	*Monoclonal Type (clone)*	*Dillution*	*Source*
Papillomavirus	Mouse (BPV1/1H8+CAMVIR)	1:80	Abcam, Cambridge, MA, USA
P63	Mouse (BC4A4)	1:100	Biocare Medical, Concord, CA, USA
Cytokeratin14	Mouse (LL002)	1:50	Leica Biosystems, Milton Keynes, UK
Ki-67	Mouse (MIB-1)	Ready to use	Dako, Tokyo, Japan

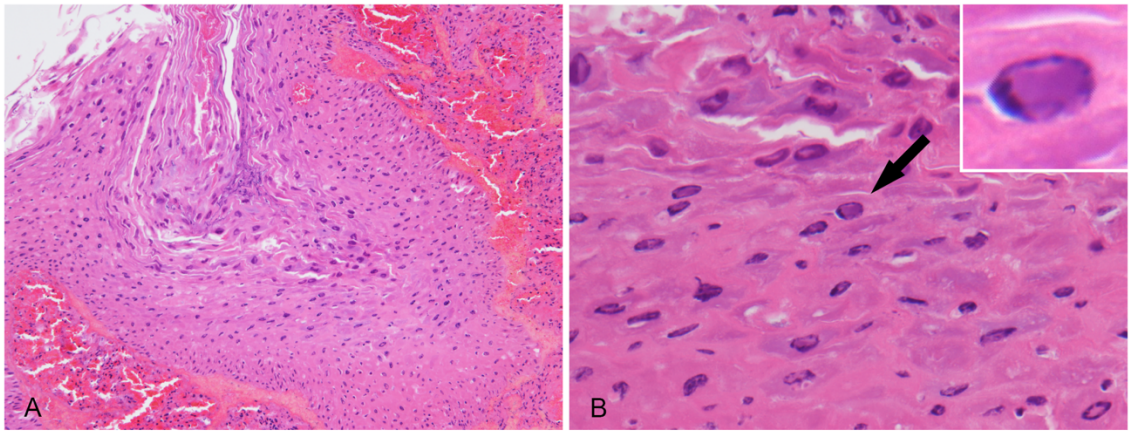

Figure 1.1 Papilloma. Right Auricle. Labrador Retriever. Case 3. **(A)** Proliferation of superficial squamous epithelium with parakeratosis. The lesion is poorly atypical. HE. **(B)** Intranuclear viral inclusions in the epidermis **(arrow, inset)**. HE.

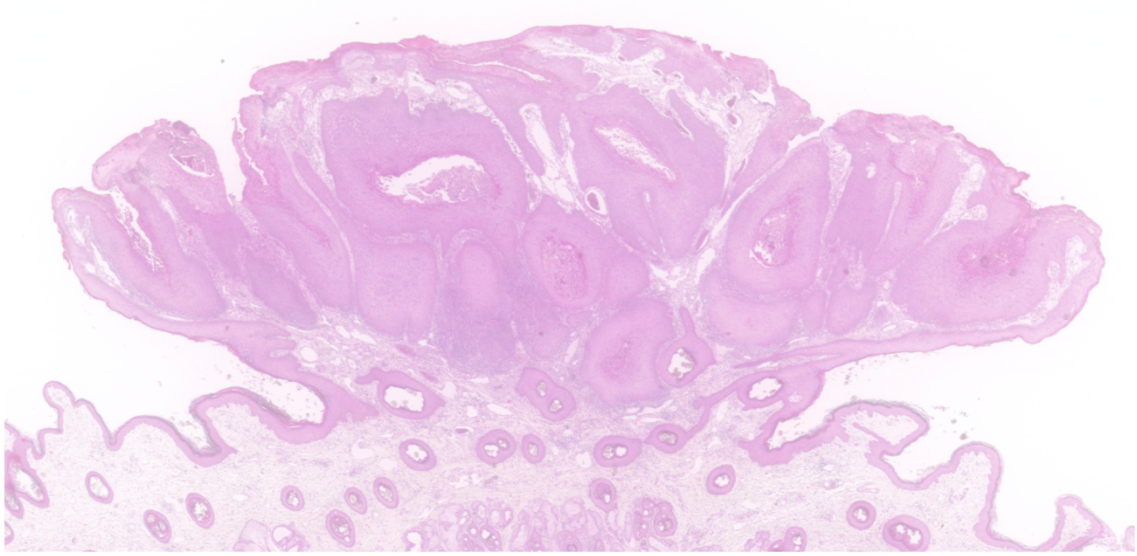

Figure 1.2 Cutaneous papilloma. Right tarsal. Miniature Dachshund. Case 7. A cutaneous papilloma lesion protruded from the skin, which occurred at the junction between the lesion and the adjacent normal skin. HE.

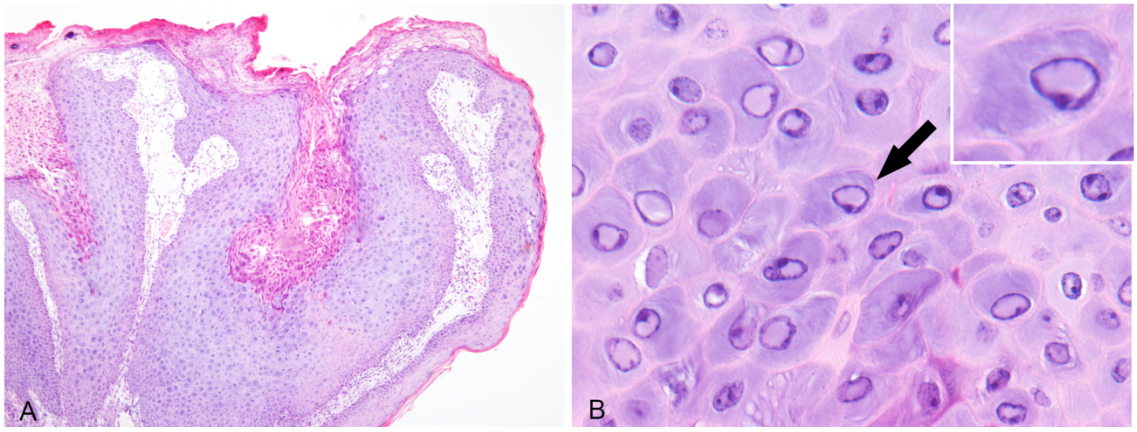

Figure 1.3 Cutaneous papilloma. Right tarsal. Miniature Dachshund. Case 7. **(A)** Papillary proliferation of squamous epithelium with parakeratinosis. HE. **(B)** Intra nuclear viral inclusions. **(arrow, inset)**. HE.

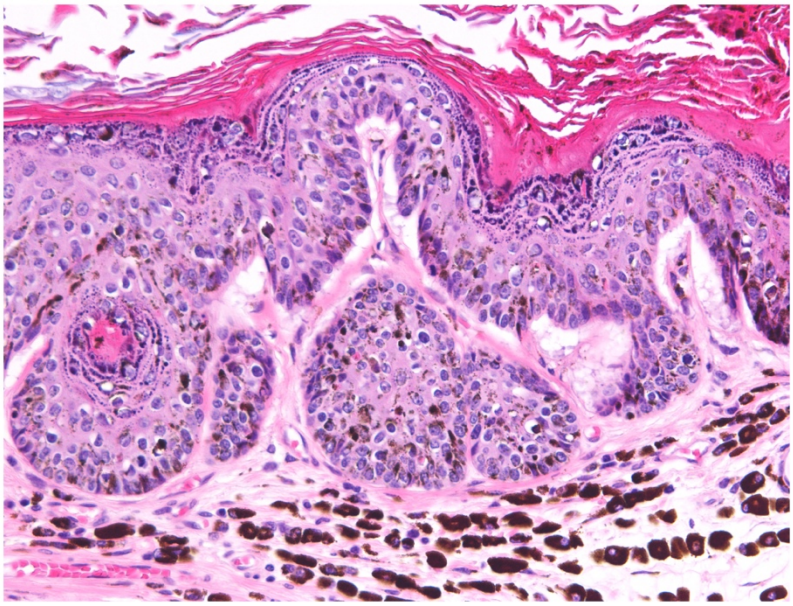

Figure 1.4 Pigmented viral plaque. Left forelimb. Pug. Case 18. Acanthosis and hyperkeratosis of the epidermis with hyperpigmentation. HE.

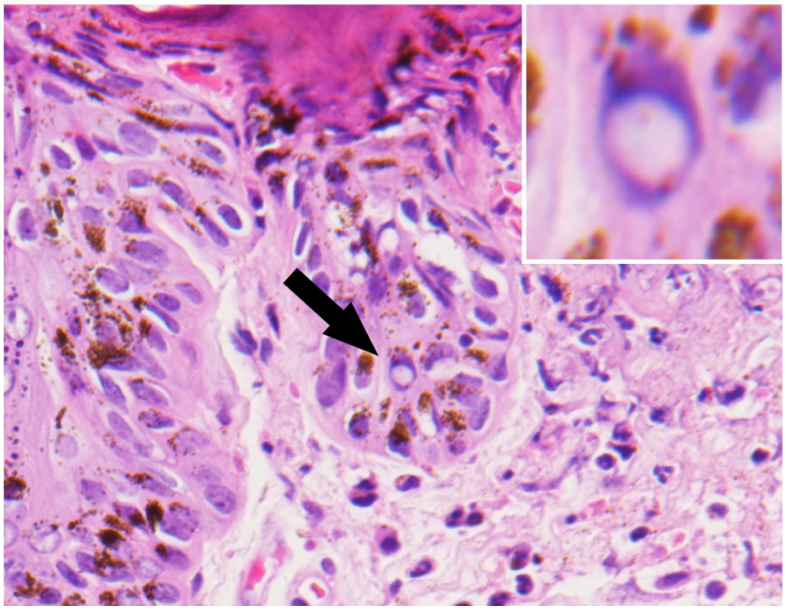

Figure 1.5 Pigmented viral plaque. Right lower abdomen. Pug. Case 19. Acantosis with intranuclear viral inclusion **(arrow, inset)**. HE.

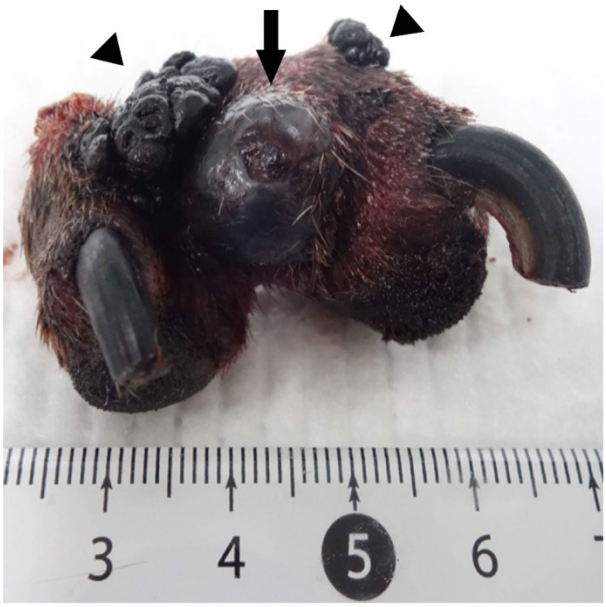

Figure 1.6 Basal cell tumor and pigmented viral plaque. Between digits of the left forelimb. Pug. Case 23. A black nodule **(arrow, inset)** between digits adjacent to the lesion of pigmented viral plaque **(arrowhead, inset)**.

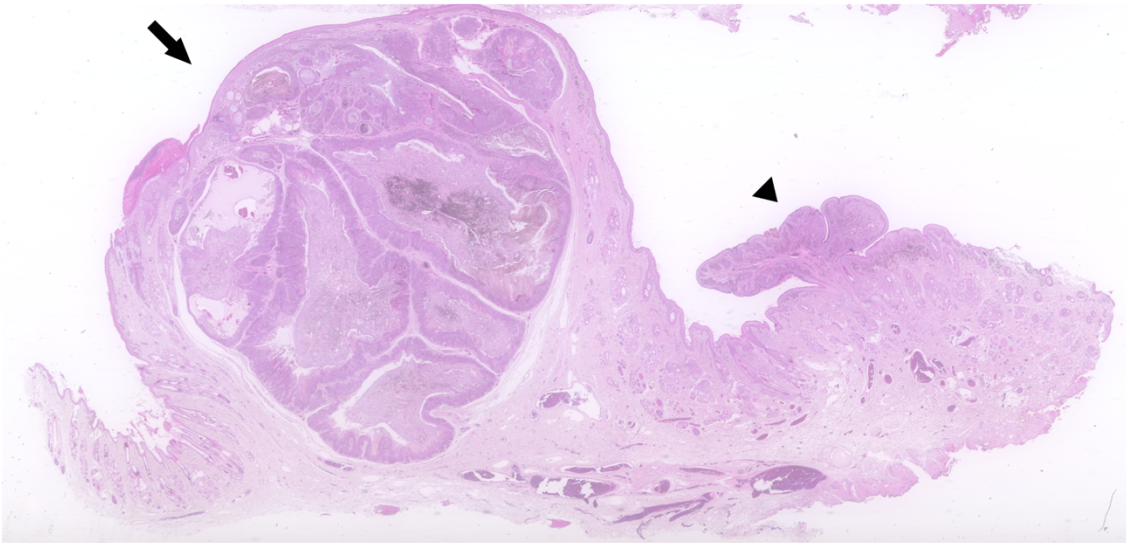

Figure 1.7 Basal cell tumor and pigmented viral plaque. Between digits of the left forelimb. Pug. Case 23. A tumor mass with a distinct margin in the dermis and subcutaneous (basal cell tumor; **arrow, inset**), and adjacent pigmented viral plaque **(arrowhead, inset)**. HE.

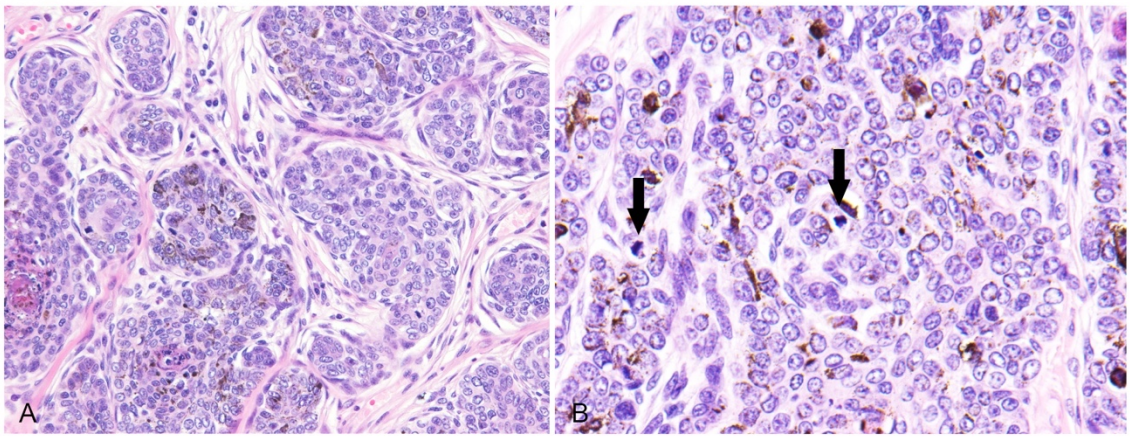

Figure 1.8 Basal cell tumor and pigmented viral plaque. Between digits of the left forelimb. Pug. Case 23. **(A)** Basal cell tumor. Lobules of round basaloid tumor cells separated by fibrous tissues. HE. **(B)** Basal cell tumor. Mitotic figures **(arrows, inset)** and pigments within the neoplastic tissue. HE.

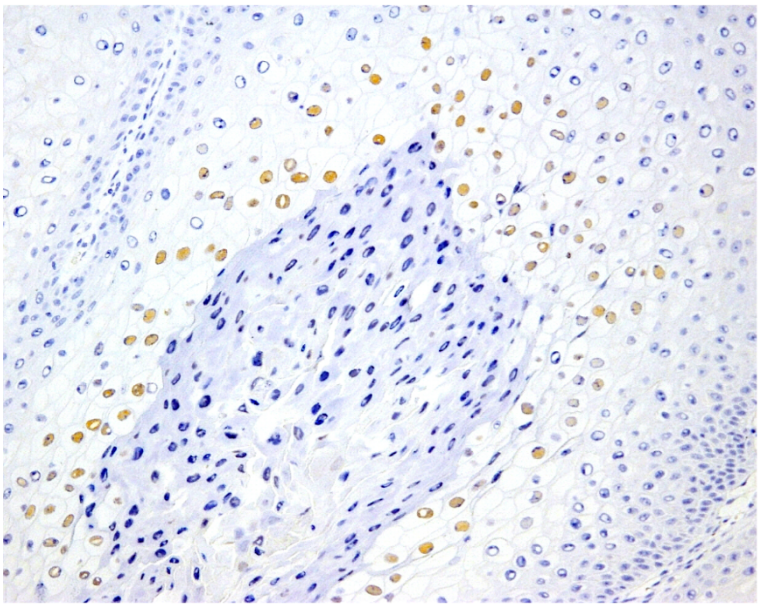

Figure 1.9 Detection of papillomavirus antigen. Cutaneous papilloma. Right tarsal. Miniature Dachshund. Case 7. Papillomavirus antigen is observed in nuclei of epithelial cells. IHC for PV antigen.

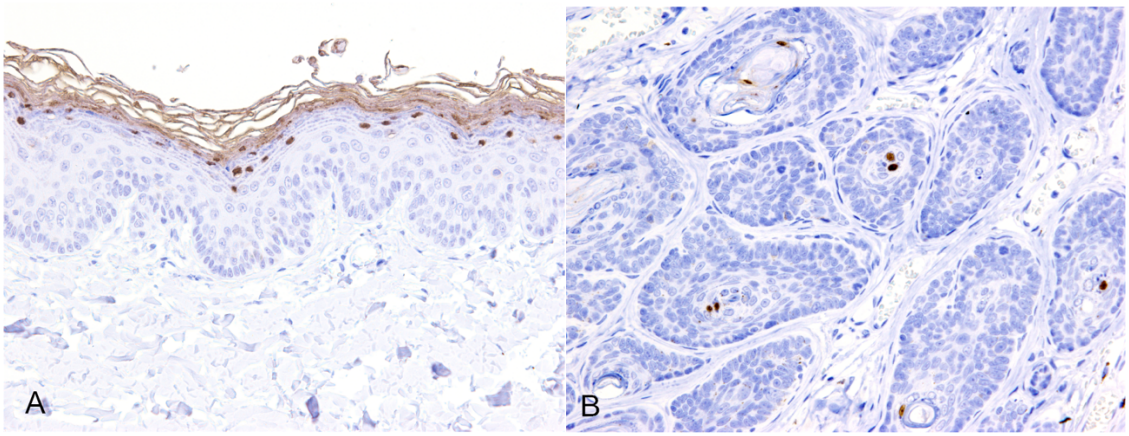

Figure 1.10 (A) Pigmented viral plaque. Right lower abdomen. Pug. Case 19. Nuclei of epithelial cells in the stratum granulosum are positive for papillomavirus antigen. IHC for PV antigen. **(B)** Basal cell tumor. Between digits of the left forelimb. Pug. Case 23. Few tumor cells in the center of neoplastic lobules are positive for papillomavirus antigen. IHC for PV antigen.

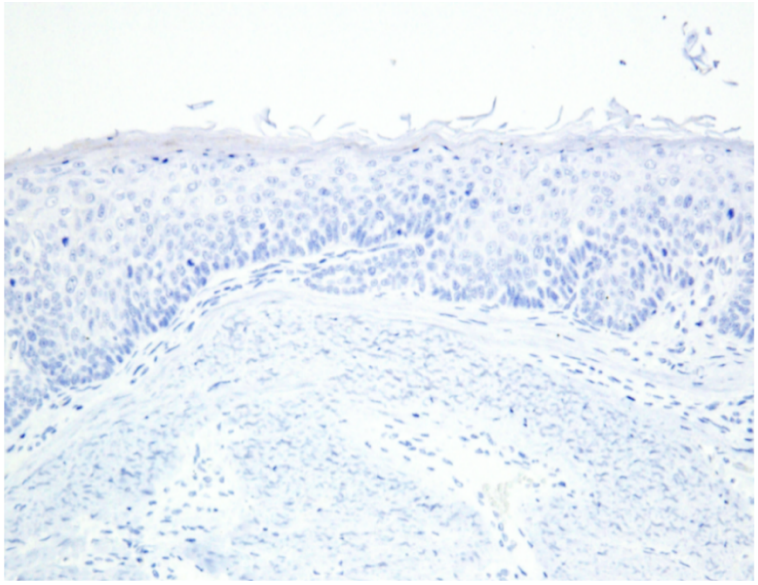

Figure 1.11 Pigmented viral plaque. Left forelimb. Standard Dachshund. Case 22. No papillomavirus antigen is detected. IHC for PV antigen.

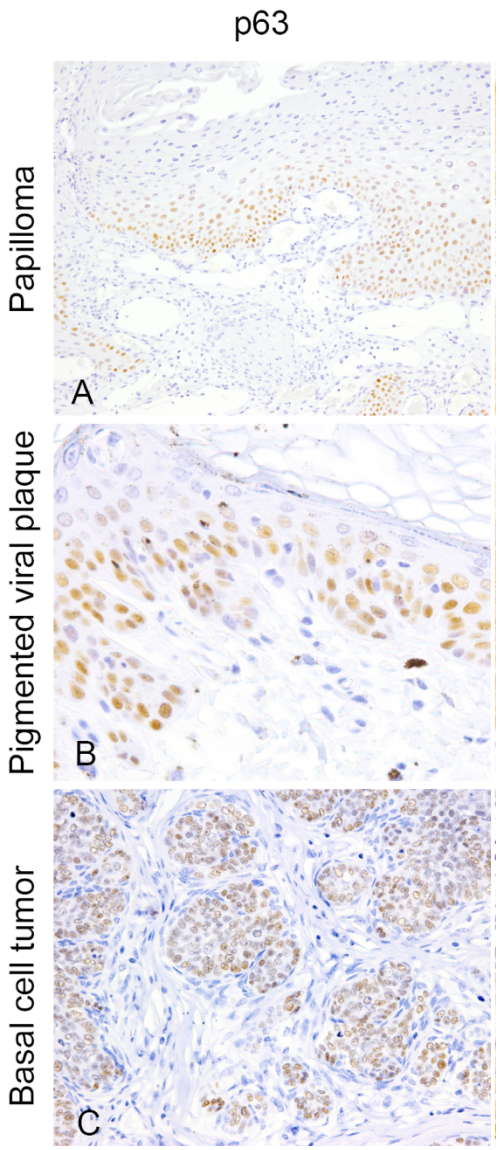

Figure 1.12 Immunohistochemistry for p63 in CPV-associated lesions. A: Papilloma, case 7. B: pigmented viral plaque, case 19. C: basal cell tumor, case 23. Nuclei of proliferating cells are positive for p63. IHC for p63.

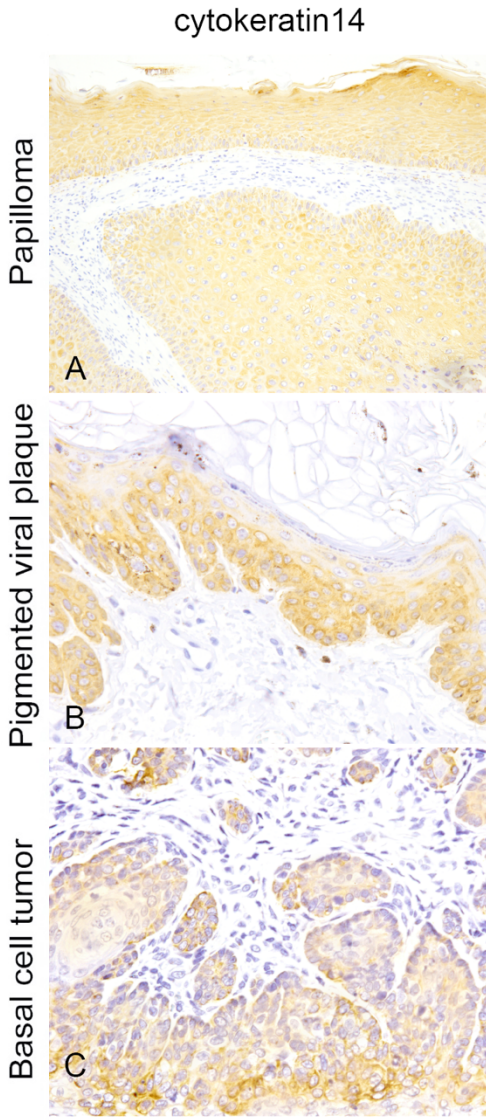

Figure 1.13 Immunohistochemistry for cytokeratin 14 in CPV-associated lesions. A: Papilloma, case 7. B: pigmented viral plaque, case 19. C: basal cell tumor, case 23. Cytoplasm of proliferating cells is positive for p63. IHC for cytokeratin14.

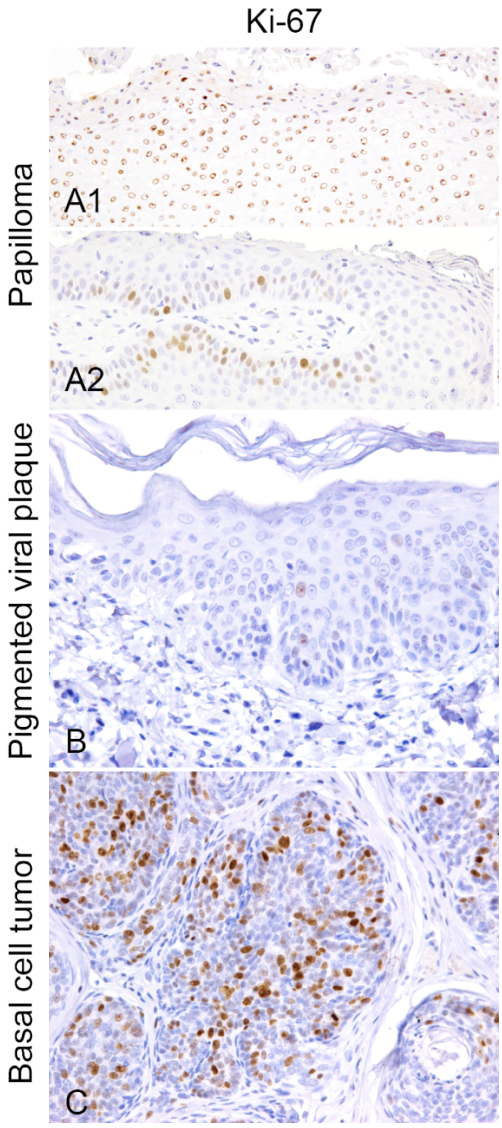

Figure 1.14 Immunohistochemistry for Ki-67. A1: CPV-positive papilloma, case 7. A2: CPV-negative papilloma, case 8. B: pigmented viral plaque, case 19. C: basal cell tumor, case 23. Nuclear expression of Ki-67 in proliferating cells. IHC for Ki-67.

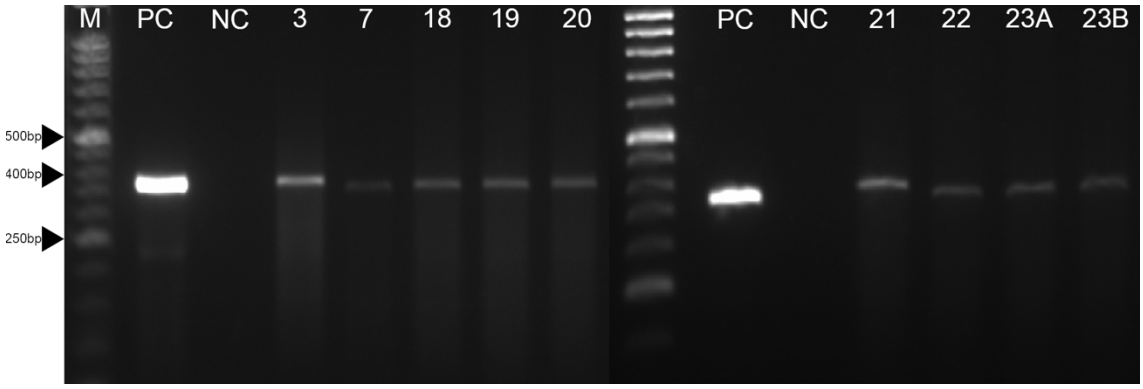

Figure 1.15 Polymerase chain reaction (PCR). M, DNA ladder marker. PC, positive control (CPV2), showing the target amplification of 389bp. NC, negative control. Positive bands are visible on lanes of cases 3, 7, 18, 19, 20, 21, 22, and 23 (lesion A and B) at the similar size position of positive control.

CHAPTER 2

In situ hybridization on canine papillomavirus-associated cutaneous lesions

INTRODUCTION

Immunohistochemistry (IHC) and polymerase chain reaction (PCR) assay have been used to detect viral proteins and genes, respectively, but they cannot illustrate the location of the virus in the lesion. Based on a previous study, virions of CPV within the canine neoplastic lesions could not be always confirmed using transmission electron microscopy [Schwegler *et al*., 1997].

In human, *in situ* hybridization (ISH) assay is widely used for the detection of human papillomavirus (HPV) DNA on a specimen [Çelebi *et al*., 2018; Keung *et al*., 2019; Lewis 2012]. Although PCR assay is highly sensitive, it cannot illustrate the localization of the virus in the precancerous lesions [El-Naggar *et al*., 2012]. Contrast to PCR, ISH targets the signals of HPV DNA directly, and the results are presented similar to those of IHC. ISH signal patterns of HPV DNA have been reported to somehow correlate with the status of HPV in infected cells [Hopman *et al*., 2005]. The ISH signal pattern of HPV can imply the viral integration stage and progression of precancerous lesions [Takamoto *et al*., 2018].

In bovine papillomavirus (BPV)-associated lesions, ISH assay has also been used to detect the nucleic acid of BPV and to confirm localization of the

infected cells. The visualization of virus location could also encourage scientists to take different approaches to determine the viral life cycle [Gaynor *et al*., 2016]. Additionally, PCR assay has been implied to investigate the viral etiology within *Felis catus* papillomavirus (FcaPV)-asscoiated lesions [Lange *et al*., 2009; Munday *et al*., 2013; Munday *et al*., 2011b; Nespeca *et al*., 2006]. However, a positive result of PCR could be ambiguous, since the positive results have also been reported for FcaPV-2 in the normal skin of cats [Thomson *et al*., 2015]. Hence, ISH is also shown as a reliable method to detect the DNA of FcaPV in cats, and provides more information on the viral types and localization in infected cells [Vascellari *et al*., 2019].

In dogs, an ISH study based on viral genotypes of CPV in precancerous cutaneous lesions are also lacking, and there is no information on the localization of infected cells in these lesions. Therefore, it is difficult to understand the progression of CPV-associated precancerous lesions in dogs. To provide effective information on the malignant transformation of the tumors within such lesions is also necessary.

In this chapter, in order to reveal the distribution of viral DNA of different CPV types, an ISH study is conducted on the CPV-positive tissue lesions. The

cases examined include 2 papilloma and 6 pigmented viral plaque lesions. Information on the specific localization of infected cells and distribution of CPVs in canine precancerous lesions is also provided.

MATERIALS AND METHODS

Cases and sample collection

Formalin-fixed CPV-positive biopsy samples confirmed in Chapter 1 were histopathologically examined **(Table 2.1)**. The total 8 samples include 2 cases of papilloma and 6 cases of pigmented viral plaque. As negative control, tissue samples of basosquamous carcinoma that previously revealed to be CPV-negative by IHC and PCR (Yorkshire Terrier, 11 years old).

In situ hybridization

In situ hybridization (ISH) was performed to detect papillomaviral DNA on 4 µm-thick formalin-fixed, paraffin-embedded (FFPE) sections. Digoxigenin DNA Labeling Kit (Roche Applied Science GmbH, Penzberg, Germany) was used to produce hybridization probes by labeling PCR products (primer pair canPVf/FAP64; L1 gene) from each positive case. The primer set of canPVf/FAP64 (5′-CTTCCTGAWCCTAAYMAKTTTGC-3′; 5′CCWATATCWVHC ATNTCNCCATC-3′) amplified the L1 gene of papillomavirus at 389 base pairs [Lange *et al*., 2011; Luff *et al*., 2012]. The ISH HRP detection kit (Biocare medical, Pacheco, CA, USA) was used to detect the probe as follows. After pronase and

autoclave-pretreatment in citrate buffer, DNA on sections was denatured by heating the sections in boiling water for 10 min and quickly chilled at 4°C. Then the sections were hybridized with each of the Digoxigenin-labeled DNA probes at 37°C for 20 hr. The reaction was stopped by keeping the sections at 65°C for 10 minutes. After washing, the sections were incubated with mouse anti-digoxigenin antibody at 4°C overnight, and then incubated with EnVision+System horseradish peroxidase-labeled anti-mouse secondary antibody polymer (Dako) at 37°C for 40 minutes. To visualize the reaction products, sections were subjected to a chromogen treatment with 0.05% 3, 3'-diaminobenzidine and 0.03% hydrogen peroxide in Tris-hydrochloric buffer, and finally counterstained with hematoxylin. The hybridization probes were produced by labeling PCR products (primer pair canPVf/FAP64; L1 gene) of cutaneous papilloma (CPV2) and pigmented viral plaque (CPV4 and CPV18), which did not show any cross reactivity to each other, therefore they were used for detecting CPV2, CPV4 and CPV18, respectively.

RESULTS

Case information

The details of examined cases are summarized in **Table 2.1**. Two cases of papilloma were 9 and 11 years old (median age of 11 years), respectively. Breeds included Labrador Retriever (n=1) and Dachshund (miniature) (n=1). The ages of 6 pigmented viral plaque cases were ranged from 6 to 11 years (median age of 9 years). Breeds included Pug (n=4), Toy Manchester Terrier (n=1) and Standard Dachshund (n=1).

In situ hybridization

By *in situ* hybridization, CPV2 gene was detected in the nuclei of stratified squamous epithelium of the papilloma lesion, as well as those in the adjacent thickened epidermis in cases 3 and 7 **(Figs. 2.1A, B and 2.2A)**. CPV4 and 18 genes were not detected in these cases **(Fig. 2.2B)**.

CPV4 gene was detected in the nuclei of epithelial cells in the spinous layer and granular layer of the pigmented viral plaque lesions in cases 18, 19, 20, 21 and 22 **(Figs. 2.3 and 2.4A)**. Within the perilesional epidermis, nuclear labeling for CPV4 in the nuclei of suparbasal layer was also observed **(Fig. 2.4B)**.

Nevertheless, CPV2 and CPV18 genes were not detected in these cases **(Fig. 2.5)**.

Nuclear labeling for CPV18 gene in the spinous layer and granular layer was observed in the pigmented viral plaque lesions of case 23 **(**23A**, Fig. 2.6)**. In the case, CPV18 gene was also detected in the nucleus of epithelial cells in the spinous layer and granular layer of the adjacent thickened epidermis as well as in that of basal cell tumor lesions **(arrow, inset) (Figs. 2.7A and B)**. Whereas, CPV2 and CPV4 genes were not detected in cases 23 **(Figs. 2.8A and B).** The number of CPV gene-positive cells was comparable between pigmented viral plaque lesions of cases 18, 19, 20, 21, 22 and 23, however it was lower in basal cell tumor lesion (case 23 lesion B) compared to that of pigmented viral plaque.

The results of ISH for CPV2, CPV4 and CPV18 were 100% concordant with the PCR result, which may show no cross reactivity each other **(Tables 2.1 and 2.2)**.

DISCUSSION

The identification of CPV2, CPV4 and CPV18 gene in papilloma and pigmented viral plaque lesions by PCR has suggested the involvement of the virus in the development of the canine precancerous lesions [Lange *et al*., 2016; Meuten 2016]. Whereas, canine healthy skin is also a potential reservoir for CPV [Lange *et al*., 2011]. Thus, the result of PCR assay cannot distinguish between true infection by the virus and virions just existing on the surface of the skin. In addition, IHC for L1 protein of PV may often induce a negative result, because during the process of PV infection, the loss of L1 protein is common [Digiuseppe *et al*., 2017; Lange *et al*., 2019]. Moreover, cumulative data on the distribution of viral DNA of different CPV types are also lacking. Hence, it is necessary to detect CPV gene in the tissues of canine precancerous lesion. Therefore, the purpose of this chapter was not only to confirm the presence of CPVs but also to study their roles in pathogenesis.

In this chapter, the detection and localization of the L1 gene of CPV2, CPV4 and CPV18 within the lesions of papilloma and pigmented viral plaque, were conducted by ISH. Here, CPV gene was detected within the nuclei of epithelial cells by ISH in all CPV-positive cases, including 2 cases of papilloma

and 6 cases of pigmented viral plaque (8/8, 100%). It was 100% concordance to the results of PCR for CPV2, CPV4 and CPV18. The localization of virus gene on a tissue can be confirmedthrough this method more feasible for diagnostic purposes.

In fact, one of the hypothesis models for the progression of CPV infection is that firstly the infection takes place when microtrauma allow CPV to enter the mucosal or cutaneous basal layer of the epidermis. Then, CPV maintain a low copy number of genomes in the basal layer, thus establish a potential infection, and then produce a high number of genomes in the stratified squamous epithelium. Therefore, the nuclei of neoplastic cells in the suprabasal layer were positive for CPV2 gene, which is likely to be associated with the papillary proliferation of epithelial cells. When the virions escape from the innate immune system, the persistent infection of CPV results in the precancerous lesions. Then, the virions are released through the epidermis. When the virus holds in different patterns, CPV may cause different benign lesions or even malignant tumors. Hence, the micro abrasions of skin can allow the infection of basal cells, leading to the production of CPV nuclei acid with a replication activity [Schiller *et al*., 2010]. Moreover, the oncogenic proteins of E6 and E7 also promotes the viral replication

of the postmitotic in the spinous layer and granular layer cells, which cause higher number copies of the viral genome [Doorbar *et al*., 2016] **(Fig. 2.9)**. Thus, the presence of the ISH signal in the nuclei of the epithelial cells of spinous layer and the granular layer illustrates a crucial role in pathogenesis and maintenance by the CPV4 and CPV18 genes in the cases of pigmented viral plaque. Under some circumstances, the numbers of CPV genome may also result in a higher replication in these layers that can lead to basal cell tumor as case 23. These results support the possible involvement of these CPV types in the development of canine precancerous lesions. Moreover, PCR and ISH were all negative in the negative controls (basosquamous carcinoma), implying that ISH can be counted as a reliable method to demonstrate the presence of the PV gene in these precancerous lesions. Given a fact that the signals of ISH were also detected in the adjacent epidermis of papilloma and pigmented viral plaque, the existence of the virions in the perilesional skin may participated in the process of viral replication.

Briefly, PCR assay is a useful method to detect the presence of viral nucleic acid, although it cannot identify viral infection and location in the cutaneous tissue [Mazzei *et al*., 2018]. While, ISH assay can give information on

virus types and their localization in precancerous lesions. In conclusion, this chapter documents the presence of nuclear CPV2, CPV4 and CPV18 genes in lesions of canine cutaneous papilloma, in the adjacent skin, and also in the pigmented viral plaque within tumor lesions, supporting the pathogenesis of CPV2, CPV4 and CPV18 in the precancerous lesions in the skin of dogs. The results also illustrate that ISH is a useful method to confirm CPV infection and provides more information on virus types, and virus localization.

ABSTRACT

Information on the localization of CPV nucleic acid of each CPV types in the precancerous lesions have never been available. Therefore, in Chapter 2, an *in situ* hybridization (ISH) assays were performed to detect canine papillomaviral DNA on all CPV-positive tissue sections. The cases include 2 papilloma and 6 pigmented viral plaque lesions. The hybridization probes were produced by labeling PCR products (primer pair canPVf/FAP64; L1 gene) from cutaneous papilloma (CPV2) and pigmented viral plaque (CPV4 and CPV18), which did not show any cross reactivity each other, therefore they were used for detecting CPV2, CPV4 and CPV18, respectively. The ISH results showed that 2 cases of papilloma were positive for CPV2, 5 cases of pigmented viral plaque were for CPV4 and 1 case of pigmented viral plaque was for CPV18. The results of the *in situ* hybridization for CPV2, CPV4 and CPV18 were 100% concordant with those of the PCR. Moreover, CPV2 signals were observed in the nuclei of neoplastic cells within the perilesional thickened epidermis of 2 cases of papilloma. CPV4 signals were observed in the nuclei of epithelial cells in the spinous layer and granular layer of 5 cases of pigmented viral plaque. In one case of basal cell tumor which had developed adjacent to pigmented viral plaque lesion, nuclei of

tumor cells were positive for the CPV18 gene. The results of this chapter implied that CPV2, CPV4 and CPV18 infection may cause cutaneous papilloma, pigmented vial plaque and basal cell tumor in dogs.

Table 2.1 Case information and CPV detection

Case No.	*Age*	*Sex*	*Breed*	*Histopathological diagnosis*	*PV detection*		
					IHC [a]	*PCR*	*Sequence*
3	9y	SF	Labrador Retriever	Papilloma	+	+	CPV2
7	14y	F	Miniature Dachshund	Papilloma	+	+	CPV2
18	11y	SF	Pug	PVP	+	+	CPV4
19	7y	CM	Pug	PVP	+	+	CPV4
20	11y	SF	Pug	PVP	+	+	CPV4
21	9y	SF	Toy Manchester Terrier	PVP	+	+	CPV4
22	6y	F	Standard Dachshund	PVP	+	+	CPV4
23 A	11y	CM	Pug	PVP	+	+	CPV18
23 B				Basal cell tumor	+	+	CPV18
NC	12y	CM	Yorkshire Terrier	BC	-	-	NA

IHC, immunohistochemistry; PCR, polymerase chain reaction; PV, papillomavirus; CPV, canine papillomavirus; NC, negative control; PVP, Pigmented viral plaque; BC, basosquamous carcinoma; y, year; +, positive; -, negative; NA, not applicable.

[a] For IHC a subset of samples was tested during the initial diagnostic workup.

Table 2.2 Results of ISH

Case No.	*Histopathological diagnosis*	*Papillomavirus detection by ISH*		
		CPV2	*CPV4*	*CPV18*
3	Papilloma	+	-	-
7	Papilloma	+	-	-
18	Pigmented viral plaque	-	+	-
19	Pigmented viral plaque	-	+	-
20	Pigmented viral plaque	-	+	-
21	Pigmented viral plaque	-	+	-
22	Pigmented viral plaque	-	+	-
23A	Pigmented viral plaque	-	-	+
23B	Basal cell tumor	-	-	+
Negative control	Basosquamous carcinoma	NA	NA	NA

CPV, canine papillomavirus; ISH, *in situ* hybridization; PCR, polymerase chain reaction; +, positive; -, negative; NA, not applicable.

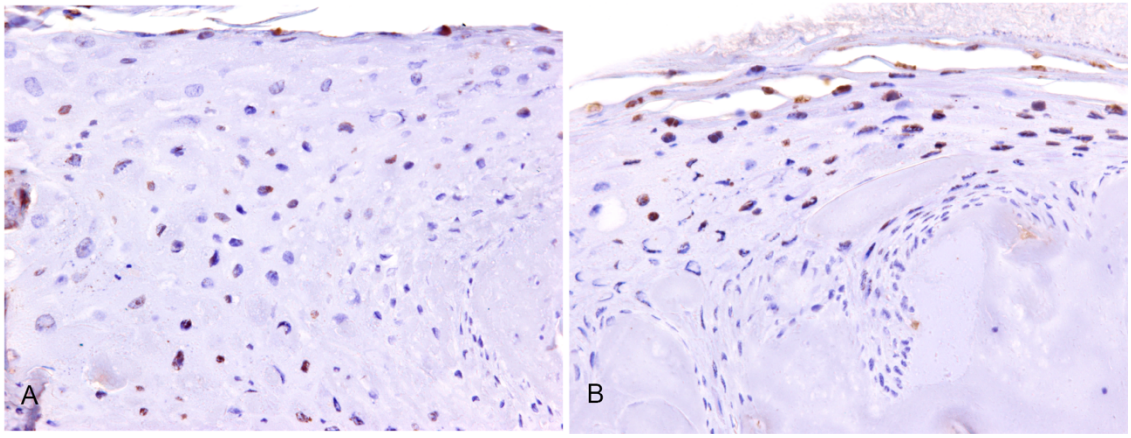

Figure 2.1 Papilloma. Right tarsal. Labrador Retriever. Case 3. **(A)** Nuclei of epithelial cells in the stratified squamous epithelium of a neoplastic lesion are positive for CPV2 gene. ISH. CPV2 probe. **(B)** Nuclear labeling for CPV2 gene in the perilesional thickened skin. ISH. CPV2 probe.

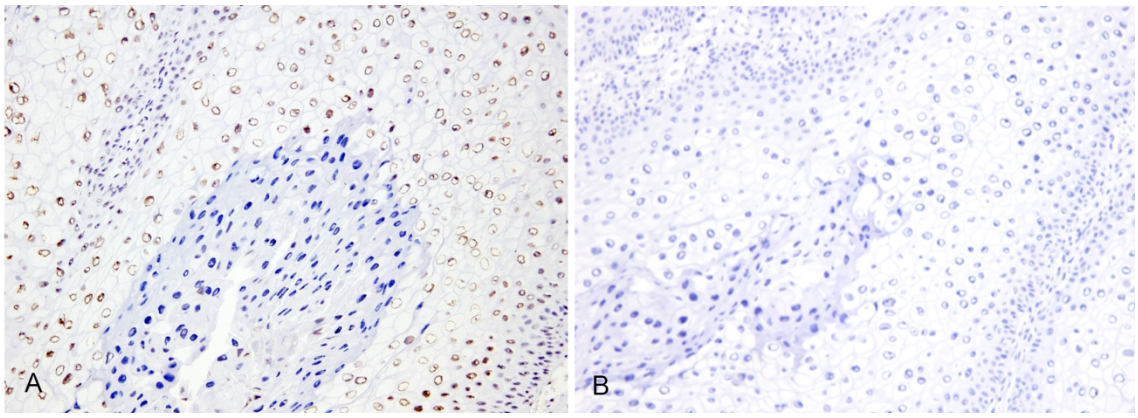

Figure 2.2 Cutaneous papilloma. Right tarsal. Miniature Dachshund. Case 7. **(A)** Nuclear labeling for CPV2 gene in the stratified squamous epithelium. ISH. CPV2 probe. **(B)** Nuclei of the perilesional skin are negative for CPV4 and 18 genes. ISH. CPV4 and CPV18 probes.

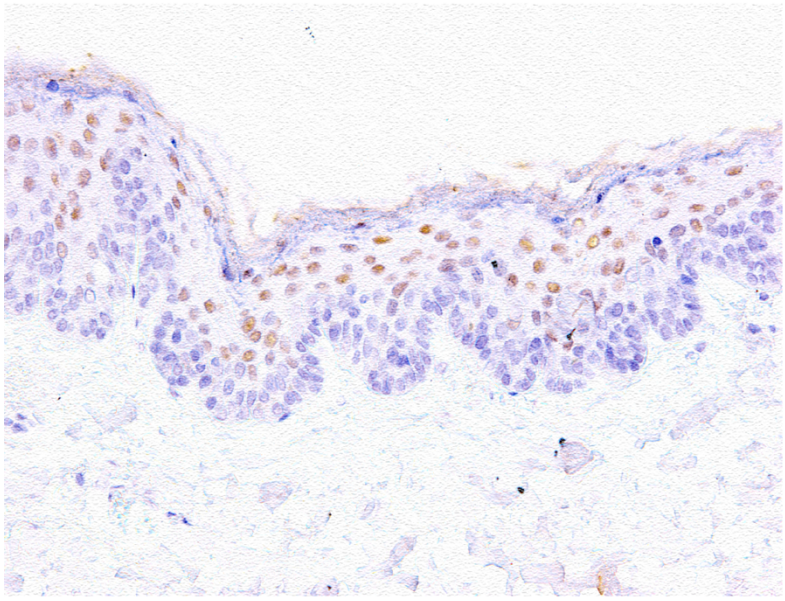

Figure 2.3 Pigmented viral plaque. Right lower abdomen. Pug. Case 19. Nuclei of epithelial cells in the spinous layer and granular layer are positive for CPV4 gene. ISH. CPV4 probe.

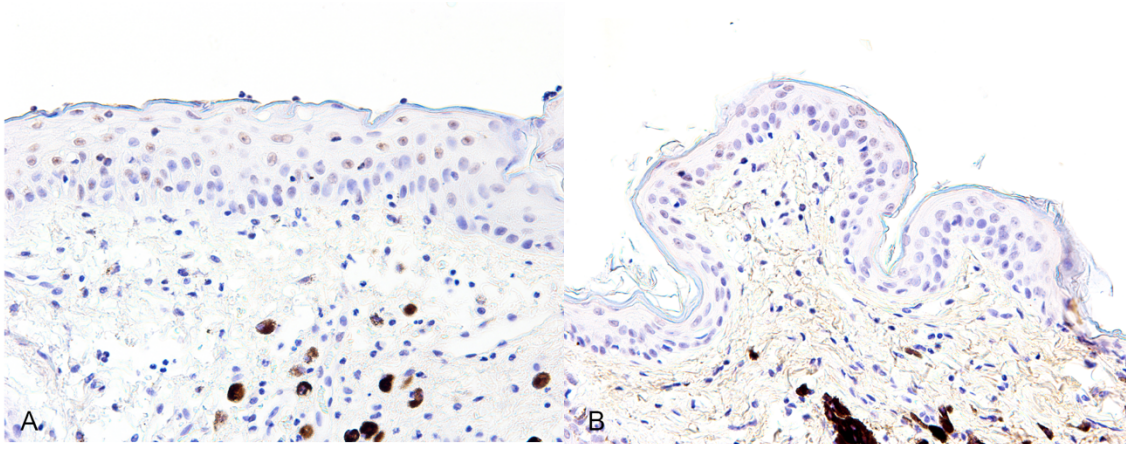

Figure 2.4 Pigmented viral plaque and malignant trichoepithelioma. Left forelimb. Standard Dachshund. Case 22. **(A)** CPV4 nucleic acid is abundant in the spinous layer and granular layer. ISH. CPV4 probe. **(B)** Within the perilesional epidermis, nuclei of epithelial cells in the suprabasal cells are positive for CPV4 gene. ISH. CPV4 probe.

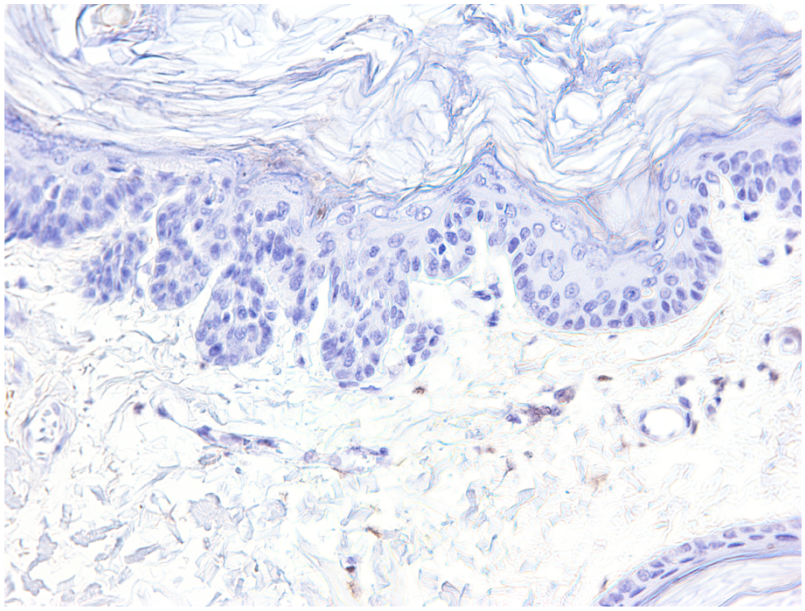

Figure 2.5 Pigmented viral plaque and malignant trichoepithelioma. Left forelimb. Standard Dachshund. Case 22. Nuclei of epithelial cells in thickened skin are negative for CPV2 and 18 genes. ISH. CPV2 and CPV18 probes.

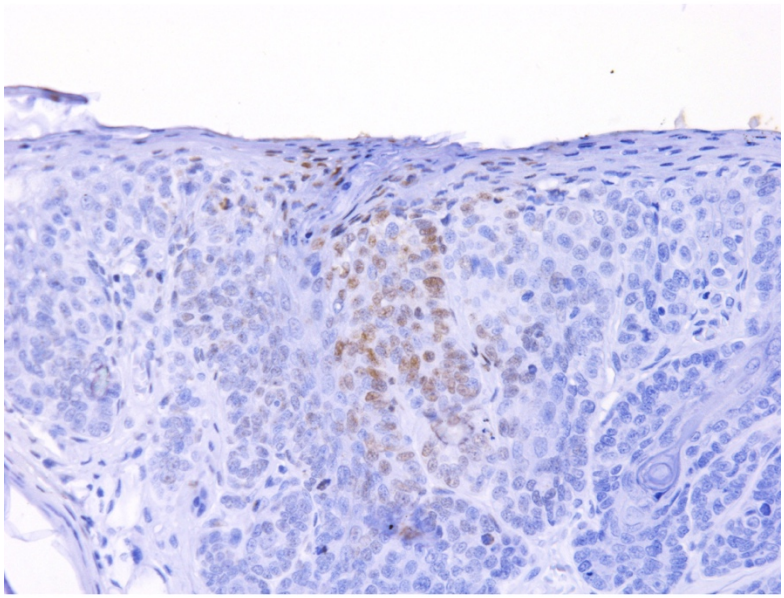

Figure 2.6 Pigmented viral plaque. Left forelimb. Pug. Case 23A. Nuclei of epithelial cells in the spinous layer and granular layer are positive for CPV18 gene. ISH. CPV18 probe.

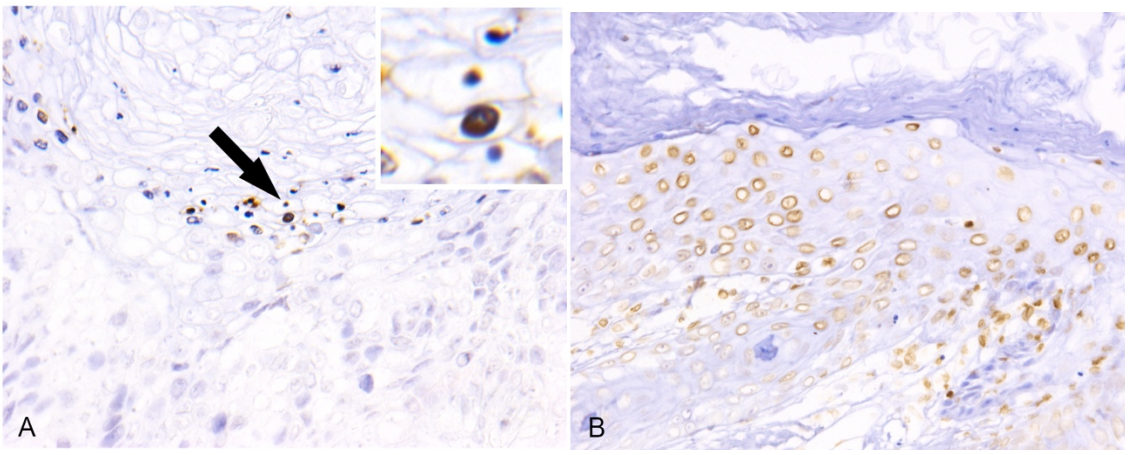

Figure 2.7 Basal cell tumor. Between digits of left limb. Pug. Case 23B. **(A)** Nuclei of tumor cells are positive for CPV18 gene **(arrow)**. ISH. CPV18 probe. **(B)** Nuclei of tumor cells are positive for CPV18 gene in the spinous layer and granular layer within adjacent thickened epidermis. ISH. CPV18 probe.

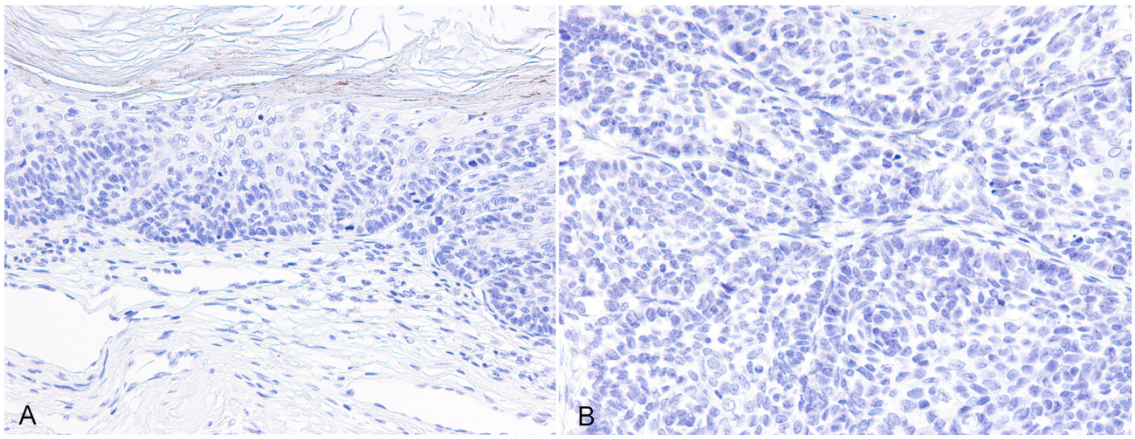

Figure 2.8 Basal cell tumor. Between digits of left limb. Dog. Case 23A and B. **(A-B)** Nuclei of the adjacent thickened epidermis and the tumor cells are negative for CPV2 and CPV4 gene. ISH. CPV2 and CPV4 probes.

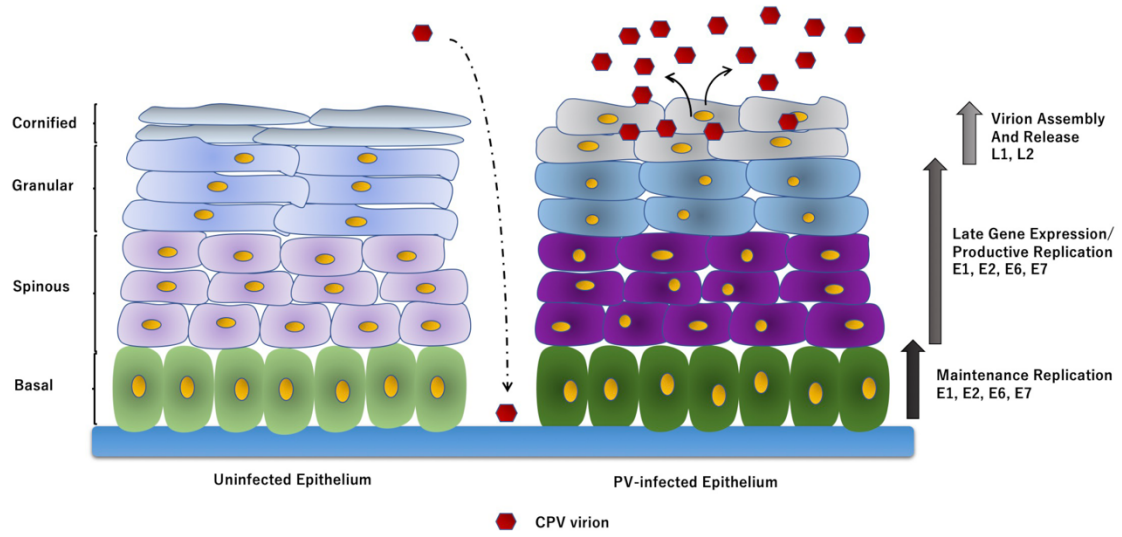

Figure 2.9 A hypothesis model for the progression of canine papillomavirus (CPV) infection Canine papillomavirus infection takes place when microtrauma allow CPV to enter the basal layer of the epidermis. CPV maintains a low copy number of genomes in the basal layer, thus establish a potential infection. When the virus escape from the innate immune system, the persistent infection of CPV can result in papilloma, pigmented viral plaque or other precancerous lesions. When the virus holds a high copy number of genomes in the suprabasal layer, CPV may also cause other benign tumors or even transform into malignant lesions.

CHAPTER 3

p16 and pRb expression on canine papillomavirus-associated cutaneous lesions

INTRODUCTION

In human, HPV can be detected in a proportion of cervical cancer and non-melanoma cutaneous cancers, such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [Bosch *et al*., 2016; Gilbert *et al*., 2019; Martinez *et al*., 2016]. Expression of p16 in neoplastic cells has been associated with these HPV-positive tumors [Dreyer *et al*., 2017; Plath *et al*., 2018]. P16 plays a crucial role in cell cycle regulation, and used as a prognostic biomarker in HPV-associated tumors [Oguejiofor *et al*., 2013]. Overexpression of p16 has been also illustrated in a wide range of human malignancies [Doxtader *et al*., 2012]. HPV infection can result in the overexpression of p16 through expression of E7-oncoprotein and inactivation of pRb [Grobe *et al*., 2013; Rayess *et al*., 2012]. The low or negative expression of pRb is also associated with high-risk HPV (HR-HPV) infection [Castellsague *et al*., 2016]. pRb is a tumor suppressor protein, which belongs to the pocket protein family (PPF), and can inhibit the progression from G1 phase to S phase within the cell cycle, and inactivation of pRb may help to promote cell division [Stricker *et al*., 2010; Munday *et al*., 2016]. In addition, pRb is bound and inactivated by oncogenic p16, and is dysfunctional in several HPV-associated tumors [Boyer *et al*., 1996; Dreyer *et al*., 2017; Dyson *et al*.,

1989]. The loss of pRb is, therefore, considered as one of the indicators of the progression of neoplastic transformation in HPV infection [Andl *et al*., 1998; Lu *et al*., 2003; Wiest *et al*., 2002].

Anti-p16 protein antibody has been applied to immunohistochemistry conducted in canine and feline malignant tumors associated with PV infection [Altamura *et al*., 2016b; Munday and Aberdein, 2012; Munday *et al*., 2011a; Munday *et al*., 2015]. In addition, in human tumors, other conditions can also result in the expression of p16, therefore it is possible that increased p16 expression can be present in some non-PV-induced lesions in dogs and cats, such as merkel cell carcinoma and neuroendocrine carcinoma [Alos *et al*., 2016; Kim 2017; Munday *et al*., 2017b]. Based on a previous report, FcaPV-2 has been shown to degrade pRb [Munday *et al*., 2012]. However, it is uncertain whether all CPVs and FcaPVs can degrade the expression of pRb [Munday *et al*., 2017b]. In this chapter, whether CPV2, CPV4 and CPV18 infection can also lead to this similar immunohistochemical characteristics as in human tumors will be discussed. The identification of an oncogenic mechanism could provide more information on the pathogenesis of CPV.

MATERIALS AND METHODS

Cases and sample collection

Cutaneous biopsy tissue samples from 23 dogs were examined. Sixteen cases were histologically diagnosed as papilloma, 1 case as papillary hyperplasia and 6 cases as pigmented viral plaque. Clinical features of the dogs and tumor location are summarized in **Table 3.1**. Of note, in case 22, malignant trichoepithelioma and pigmented viral plaque were examined in the same region of the left forelimb. In case 23, multiple tumor masses between the digits of the left forelimb were also examined.

Immunohistochemical analyses

Four μm-thick formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized and were subjected to antigen retrieval. Non-specific reactions were blocked by immersing the sections in 3% hydrogen peroxide in methanol at room temperature for 5 minutes, followed by incubation in 8% skimmed milk at 37°C for 40 minutes. The sections were then incubated at 4°C overnight with one of the primary antibodies **(Table 3.2)**. After washed 3 times with Tris-buffered saline (TBS), the sections were incubated with Dako EnVision+System

horseradish peroxidase-labedled anti-mouse secondary antibody polymer (Dako, Tokyo, Japan) at 37°C for 40 minutes. To visualize reaction products, sections were subjected to chromogen treatment with 0.05% 3,3'-diaminobenzidine and 0.03% hydrogen peroxide in Tris-hydrochloric buffer, and counterstained with hematoxylin. The expression of pRb was considered to be reduced within a lesion if less than 25% of the neoplastic cells contained nuclear immunostaining. Cells within the adjacent nonneoplastic epidermis were used as internal positive controls. In the IHC detection of p16, basosquamous cell carcinoma of a dog was used as positive controls. For negative controls, primary antibodies were replaced with TBS.

RESULTS

Immunohistochemical analyses

Among the 16 papilloma cases examined, 2 cases were positive for CPV and other 14 cases were negative for CPV by PCR (Chapter 1). The cytoplasm of the neoplastic cells was positive for p16 in one of the CPV-positive papilloma cases (case no. 7) **(Fig. 3.1)**. In the other 15 papilloma cases, however, neoplastic cells were negative for p16 **(Fig. 3.2A)**. In addition, neoplastic cells in one papillary hyperplasia case was also negative for p16 **(Fig. 3.2B)**.

All six cases of pigmented viral plaque were positive for CPV on PCR examination (Chapter 1). The cytoplasm of basal cells in the pigmented viral plaques were positive for p16 **(Fig. 3.3A)**. In one case of pigmented viral plaque (case no. 23), a basal cell tumor was developed adjacent to the pigmented viral plaque lesion. The cytoplasm of the tumor cells was positive for p16 **(Fig. 3.3B)**. In the normal epidermis adjacent to pigmented viral plaque lesion, cytoplasmic expression of p16 was observed in basal cells. The CPV nucleic acid was also detected in the same region (Chapter 2) **(Fig. 3.4)**.

Regarding pRb, in one CPV-positive papilloma case, scattered nuclear expression of pRb were observed in the center of proliferated foci and the

surrounding nonneoplastic cells **(Fig. 3.5)**. In 15 CPV-negative papilloma cases, nuclear immunoreactivity against pRb was visible throughout the majority of the neoplastic cells **(Fig. 3.6A)**. One papillary hyperplasia case was positive for pRb **(Fig. 3.6B)**.

In six cases of pigmented viral plaque, the nucleus of the epithelial cells was negative for pRb **(Fig. 3.7)**. In the case of pigmented viral plaque and basal cell tumor (case no. 23), scattered nuclear staining of pRb was observed **(arrows, inset) (Figs. 3.8A and 3.8B) (Tables 3.3 and 3.4)**.

DISCUSSION

In Chapter 1 and 2, a potential risk of malignant transformation in canine precancerous cutaneous lesions, which induced by canine papillomavirus (CPV) infection was discussed. However, the pathogenesis of these lesions is still unknown.

In this chapter, within CPV2-infected papilloma, the diffuse cytoplasmic expression of p16 in the neoplastic cells was observed. CPV2 L1 gene detection in this lesion, implied the relation between CPV and p16 expression in CPV2-infectied papilloma. In human, nuclear and cytoplasmic expression of p16 is used as a reliable marker of HPV-induced tumors, which regulates cell division [Cunningham *et al*., 2006; Lu *et al*., 2003; Smeets *et al*., 2007; Stricker *et al*., 2010; Thavaraj *et al*., 2011].

All six cases of pigmented viral plaque examined in this chapter exclusively expressed p16 in the basal layer of thickened epidermis. In one case of basal cell tumor, all the tumor cells were positive for p16 as well. In all the cases of pigmented viral plaque with a diffuse cytoplasmic expression of p16, CPV4 and CPV18 L1 genes were also expressed, which may suggest a relation between the CPV and p16 expressions in CPV4- and CPV18-infected

precancerous cutaneous lesions. As for CPV2-infected papilloma, the cytoplasmic expression of p16 was according to CPV4 and CPV18 infection. Though the association between the nuclear and cytoplasmic expression of p16 and the presence of CPV DNA within canine cutaneous malignant tumors have been reported [Munday *et al*., 2012; Munday *et al*., 2017b], this is the first finding indicating the cytoplasmic expression of p16 in canine precancerous cutaneous lesions with CPV2, CPV4 or CPV18 infection **(Fig. 3.9)**.

The loss of pRb expression was observed in 2 cases of CPV2-infected papilloma, 5 of CPV4-infected pigmented viral plaque and one CPV18-infected pigmented viral plaque. Nonetheless, the nuclear expression of pRb was observed also in the epithelial cells of CPV-negative neoplastic cases. Hence, the absence of pRb in the nucleus would indicate a PV etiology in canine precancerous cutaneous lesions. The cases with a loss of pRb were also accompanied with the cytoplasmic expression of p16, and indicated that the dysfunction of pRb consistently with p16 within the canine precancerous cutaneous lesions. Whereas, in one case of viral papilloma (case 3), the loss of pRb and no expression of p16 may be implied by a fact that the loss of pRb can also present independently of p16 expression, which was revealed in the past

studies on HPV [Boyer *et al*., 1996; Dyson *et al*., 1989]. As previously mentioned, PV could inactivate and degrade pRb in HPV-infected tumors [Dyson *et al*., 1989; McLaughlin-Drubin and Munger 2019]. Tau-papillomavirus, such as CPV2 and CPV17, can degrade pRb after binding to the protein through their C-terminal domain [Munday *et al*., 2016; Wang *et al*., 2010]. Whereas, chi-papillomavirus, such as CPV4 and CPV18 were shown to possess an LXCXE peptide-binding motif (pRb-binding motif) similar to CPV10, CPV12 and HPV16 [Lange *et al*., 2016; Luff *et al*., 2019; Nguyen *et al*., 2007; Guccione *et al*., 2002]. This is in contrast to tau-papillomavirus (CPV2). CPV can also combine with the pRb by releasing the E2F, and the released E2F promotes the expression of p16 as HPV [Chen *et al*., 2019; Grace *et al*., 2017]. Moreover, CPV may use the KDM6A and KDM6B pathways to regulate the expression of p21 and p16, respectively [McLaughlin-Drubin *et al*., 2011; Soto *et al*., 2017] **(Fig. 3.10)**.

In conclusion, the results of this chapter suggested a significant association between the CPV L1 gene and the cytoplasmic expression of p16 and following loss of pRb in canine precancerous cutaneous lesions. Therefore, cytoplasmic expression of p16 and loss of pRb in association may be considered as an indicator of CPV infection in the early stage of canine precancerous

cutaneous lesions. The results provide more information about the pathogenesis of canine cutaneous precancerous lesions, which shows some similarities to HPV-induced tumors.

ABSTRACT

In this chapter, the IHC detection for p16 protein and pRb in CPV-infected skin tumors in dogs was performed. In human, oncogenic HPV-infection can cause the overexpression of p16 protein in both the nucleus and cytoplasm of HPV-associated neoplastic cells through inhibiting the pRb expression. However, it is still unknown whether CPV can also represent the similar immunohistochemical characteristics within the precancerous lesions in dogs. In this chapter, Totally, 23 cases were applied to IHC for the detection of p16 and pRb. Among the cases, 16 were papilloma including, 2 positive for CPV2 and 14 negative for CPV. One was papillary hyperplasia negative for CPV. Other 6 cases were pigmented viral plaque including 5 positive for CPV4, and 1 positive for CPV18 (Chapter 1). Diffuse cytoplasmic expression of p16 protein with faint/no expression of pRb was detected in 1 case of CPV2-associated papilloma, 5 cases of CPV4-associated pigmented viral plaque and 1 case of CPV18-associated pigmented viral plaque. In lesions of the head and neck in a case of CPV2-associated papilloma, tumor cells were negative for p16 protein. Nuclear expression of pRb was observed in 1 case of CPV-negative papillary hyperplasia and 14 cases of CPV-negative papilloma, nonetheless no nuclear and

cytoplasmic expression of p16 protein was observed in all the cases. The diffuse cytoplasmic expression of p16 protein in the neoplastic cells of CPV-positive cases was not consistent with the results of a previous report on papillomavirus pathogenesis in malignant skin tumors of human and dogs, which accompanied with the nuclear and cytoplasmic expression of p16 protein. Moreover, oncogenic CPV can also inhibit the expression of pRb in CPV-associated precancerous lesions, like HPV-associated tumors in human.

Table 3.1 Case information and p16 and pRb expression

Dog no.	Age	Gender	Breed	Location of lesions	Histopathological	PV detection		Sequence	IHC	
					Diagnosis	IHC	PCR	(Ident, %)	p16	pRb [a]
1	10y	CM	Beagle	Right Eyelid	Papilloma	-	-	NA	-	+
2	8y	CM	French Bulldog	Left forelimb	PH	-	-	NA	-	+
3	9y	SF	Labrador Retriever	Right Auricle	Papilloma	+	+	CPV2 (99%)	-	Loss
4	12y	SF	American Stafford	Left hindlimb	Papilloma	-	-	NA	-	+
5	7y	SF	French Bulldog	Lip	Papilloma	-	-	NA	-	+
6	12y	CM	Shih Tzu	Right maxillary	Papilloma	-	-	NA	-	+
7	14y	F	Dachshund (miniature)	Right Tarsal	Papilloma	+	+	CPV2 (100%)	+	Loss
8	7y	M	Chihuahua	Right Palm	Papilloma	-	-	NA	-	+
9	11y	M	Shiba Inu	Left elbow	Papilloma	-	-	NA	-	+
10	9y	SF	Pomeranian	Left forelimb	Papilloma	-	-	NA	-	+
11	10y	SF	Yorkshire Terrier	Lip	Papilloma	-	-	NA	-	+
12	7y	SF	Poodle (Toy)	Nasal	Papilloma	-	-	NA	-	+
13	15y	SF	Shiba Inu	Armpit	Papilloma	-	-	NA	-	+
14	2y	SF	Yorkshire Terrier	Mandibular	Papilloma	-	-	NA	-	+
15	6y	SF	Beagle	Neck	Papilloma	-	-	NA	-	+
16	8y	CM	Boston Terrier	Right Eyelid	Papilloma	-	-	NA	-	+
17	8y	F	Chihuahua	Lip	Papilloma	-	-	NA	-	+
18	11y	SF	Pug	Left forelimb	PVP	+	+	CPV4 (99%)	+	Loss
19	7y	CM	Pug	Right lower abdomen	PVP	+	+	CPV4 (100%)	+	Loss
20	11y	SF	Pug	Nipple	PVP	+	+	CPV4 (100%)	+	Loss
21	9y	SF	Manchester Terrier (Toy)	Left forelimb	PVP	+	+	CPV4 (100%)	+	Loss
22	6y	F	Standard Dachshund	Left forelimb	PVP & TE	-	+	CPV4 (100%)	+	Loss
23A	11y	CM	Pug	Left forelimb	PVP	+	+	CPV 18 (100%)	+	Loss
23B			Pug	Between digits of left limb	BCT	+	+	CPV 18 (100%)	+	Loss
NC	12y	CM	Yorkshire Terrier	Left forelimb	BC	-	-	NA	+	+

SF, spayed female; CM, castrated male; PH, papillary hyperplasia; PVP, pigmented viral plaque; TE, trichoepithelioma; BCT, basal cell tumor; BC, basosquamous carcinoma; PV, papillomavirus; IHC, immunohistochemistry; PCR, polymerase chain reaction; +, positive; -, negative; NC, negative control; NA, not applicable.

[a] Nuclear expression by anti-retinoblastoma protein antibody was present in less than 25% of the neoplastic cells.

Table 3.2 Primary antibodies used for immunohistochemistry

Antibody to	*Host (clone)*	*Dillution*	*Source*
p16	Mouse (G175-405)	1:100	BD Biosciences, San Jose, CA, USA
pRb	Mouse (G3-245)	1:100	BD Biosciences, San Jose, CA, USA

pRb, retinoblastoma protein.

Table 3.3 Papillomaviral DNA and IHC results

Lesions	*CPV DNA*	*p16 expression*	*Loss of pRb expression* [a]
Papilloma	+ (2) [a]	+ (1)	(1)
		- (1)	(1)
	- (14)	- (14)	(0)
Papillary hyperplasia	- (1)	- (1)	(0)
Pigmented viral plaque	+ (6)	+ (6)	(6)

IHC, immunohistochemistry; CPV, canine papillomavirus; pRb, retinoblastoma protein; +, positive; -, negative.

[a] No. of cases in parentheses.

[b] Nuclear expression of pRb was present in less than 25% of the neoplastic cells.

Table 3.4 Presence of papillomaviral DNA within a lesion

Lesion	*Total #*	*Expression of p16*	*Loss of pRb* [b]
With CPV DNA	8	7 (87.5) [a]	8 (100)
Without CPV DNA	15	0 (100)	0 (100)

CPV, canine papillomavirus; pRb, retinoblastoma protein.
[a] No. of cases in parentheses.
[b] Nuclear expression of pRb was present in less than 25% of the neoplastic cells.

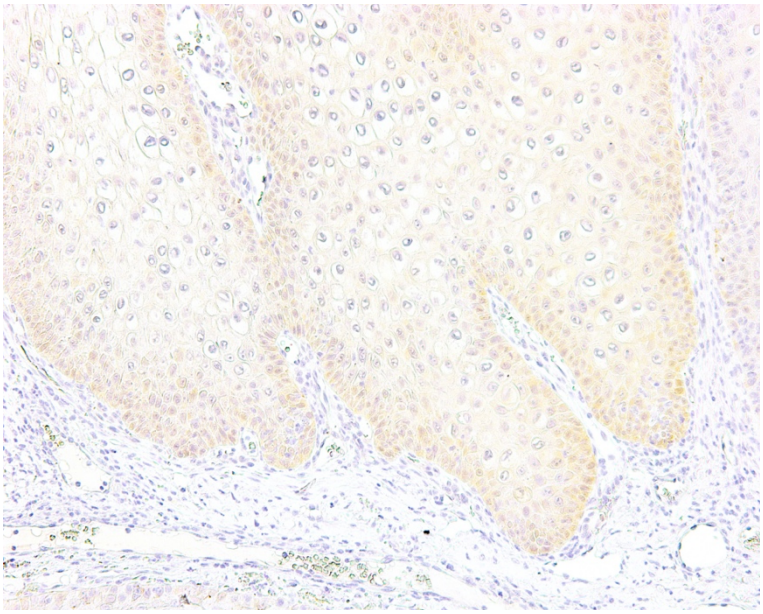

Figure 3.1 Cutaneous papilloma. Right Tarsal. Miniature Dachshund. Case 7. Prominent cytoplasmic expression of p16 within neoplastic cells in the epidermis. IHC for p16.

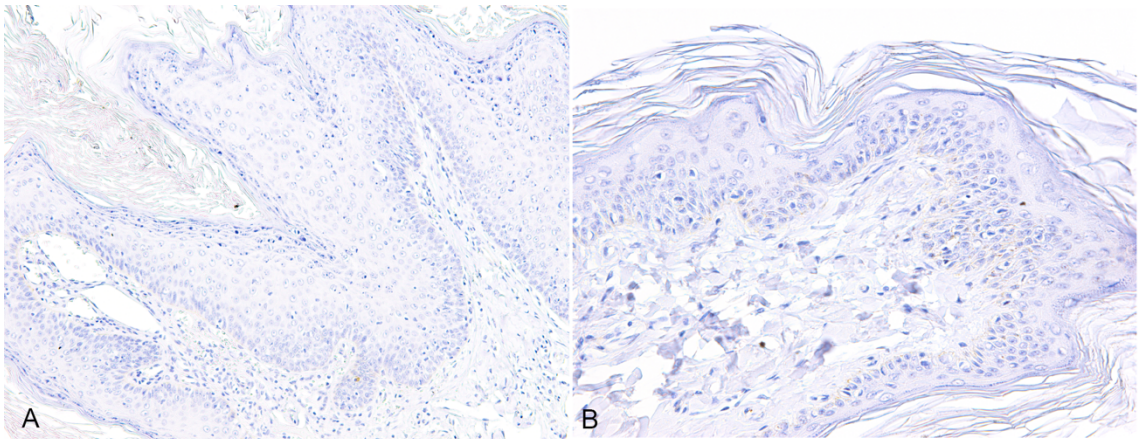

Figure 3.2 (A) Cutaneous papilloma. Right palm. Chihuahua. Case 8. Cytoplasmic immunoexpression of p16 is absent within neoplastic cells in the epidermis. IHC for p16. **(B)** Papillary hyperplasia. Left forelimb. French Bulldog. Case 2. Immunoexpression of p16 is absent in the hyperplastic epidermis. IHC for p16.

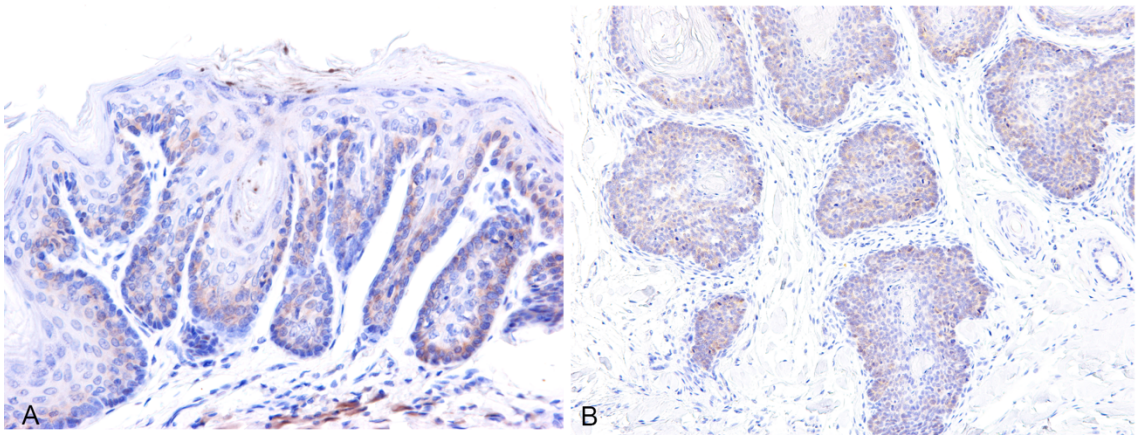

Figure 3.3 (A) Pigmented viral plaque. Left forelimb. Pug. Case 18. Cytoplasmic expression of p16 in basal cells. IHC for p16. **(B)** Basal cell tumor. Between digits of the left forelimb. Pug. Case 23B. Diffuse cytoplasmic expression of p16 in tumor cells. IHC for p16.

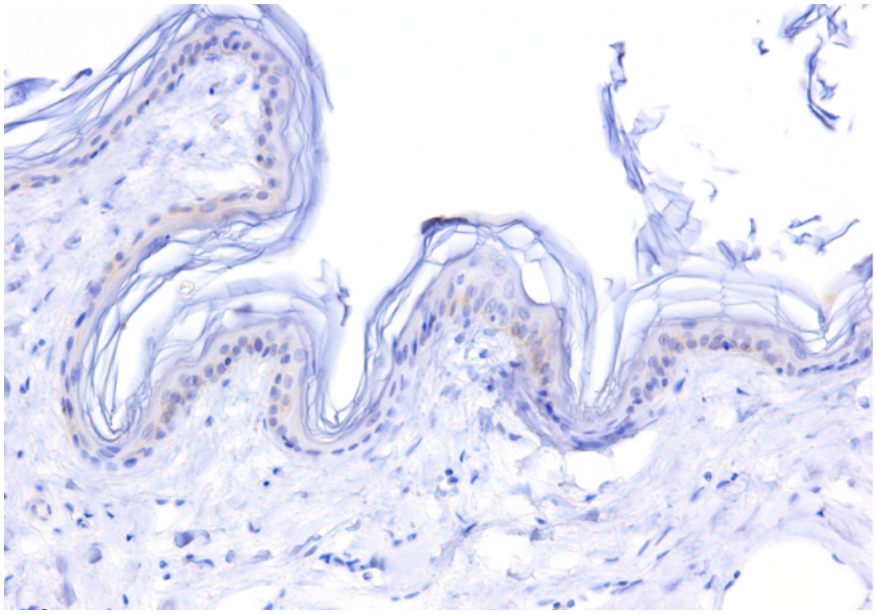

Figure 3.4 Pigmented viral plaque. Left forelimb. Pug. Case 18. Within the perilesional epidermis, cytoplasm of the basal cells is positive for p16. IHC for p16.

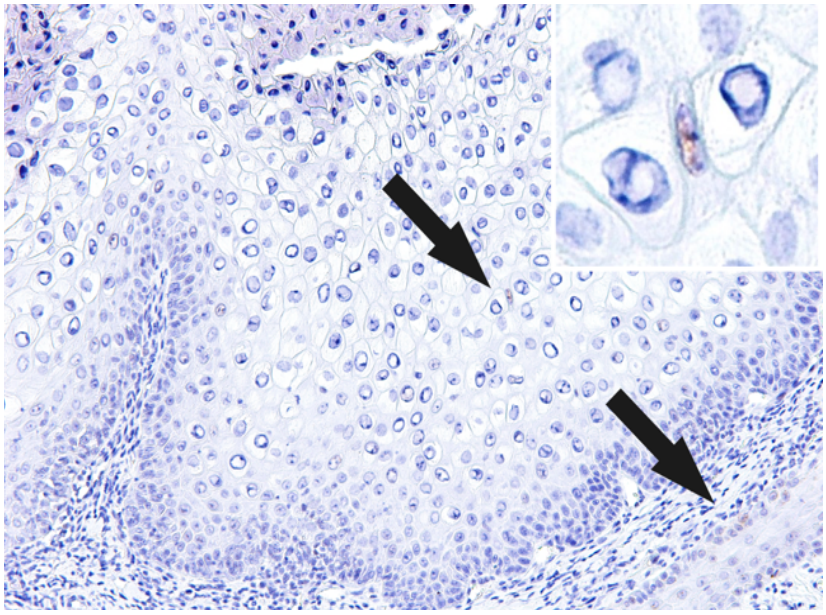

Figure 3.5 Cutaneous papilloma. Right tarsal. Miniature Dachshund. Case 7. Nuclear immunoreactivity against pRb is lost within the neoplastic cells in the epidermis. In contrast, scattered nuclear staining for pRb is observed in the center of proliferated foci and the surrounding nonneoplastic cells **(arrows, inset)**. IHC for pRb.

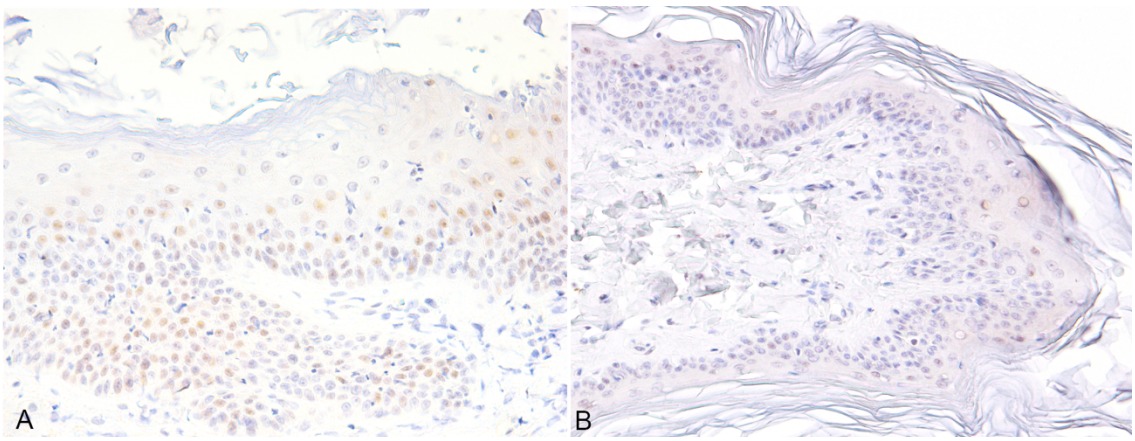

Figure 3.6 (A) Cutaneous papilloma. Right palm. Chihuahua. Case 8. Nuclear immunoreactivity against pRb is visible throughout the epithelial cells. IHC for pRb. **(B)** Papillary hyperplasia. Left forelimb. French Bulldog. Case 2. Nuclear expression of pRb is visible within hyperplastic epidermis. IHC for pRb.

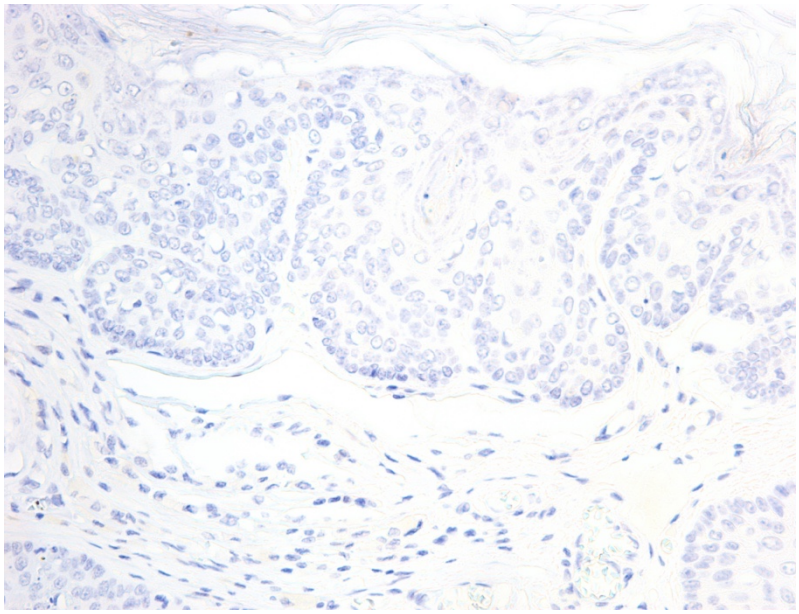

Figure 3.7 Pigmented viral plaque. Left forelimb. Pug. Case 18. Nuclei of epithelial cells are negative for pRb. IHC for pRb.

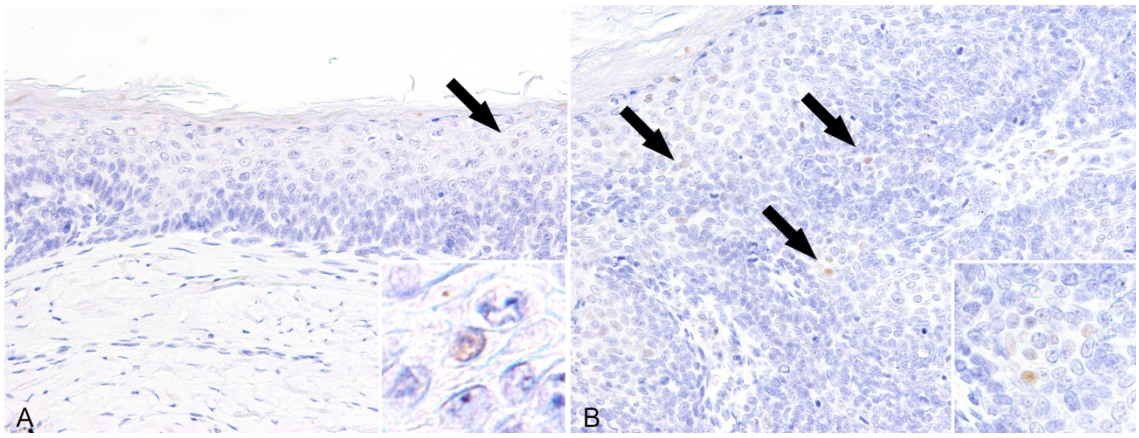

Figure 3.8 (A) Pigmented viral plaque. Left forelimb. Pug. Case 23A. Absence of nuclear expression of pRb within the epithelial cells. In contrast, scattered nuclear staining for pRb is observed in the hyperplastic epidermis **(arrow, inset)**. IHC for pRb. **(B)** Basal cell tumor. Between digits of left forelimb. Pug. Case 23B. Nuclear expression of pRb is lost in the tumor cells. However, scattered nuclear expression of neoplastic cells is observed in the same region **(arrows, inset)**. IHC for pRb.

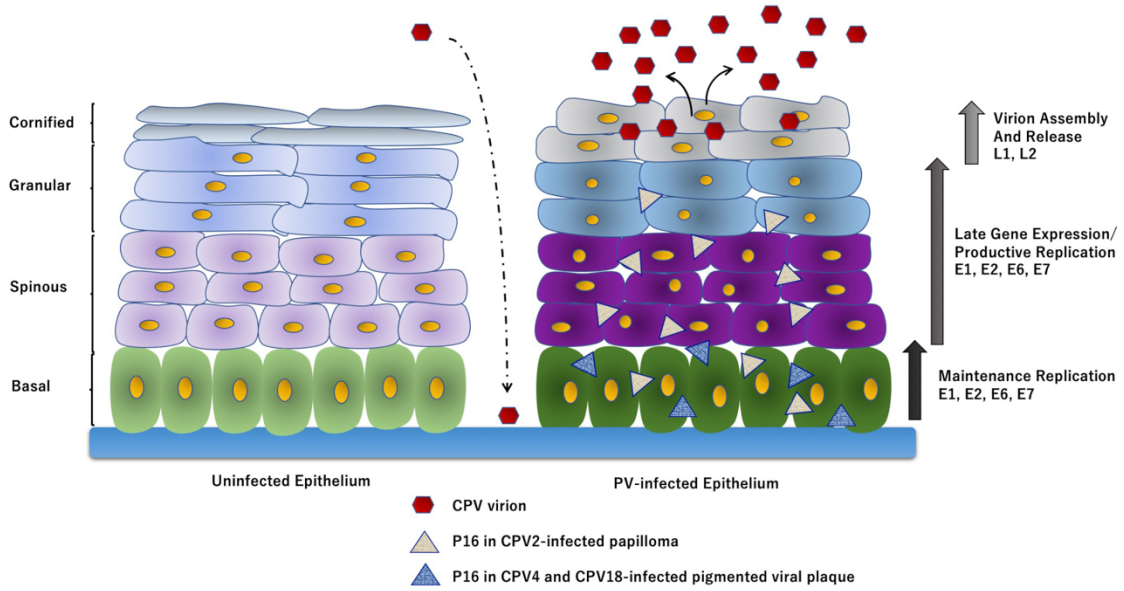

Figure 3.9 A hypothetical model for the pathogenesis of CPV-associated precancerous cutaneous lesions. After infected with PV, tau-PV (CPV2) induces expression of p16 in the cytoplasm of neoplastic cells within all layers of epidermis, that causes papilloma. Meanwhile, chi-PV (CPV4 and CPV18) causes cytoplasmic expression of p16 in basal cells exclusively, thus it results in pigmented viral plaque and basal cell tumor.

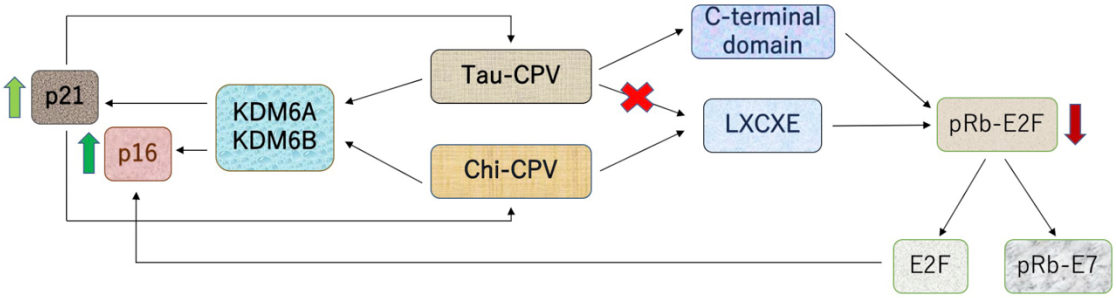

Figure 3.10 Cellular interactions of CPV and their synergy in induction of cell proliferation. Both tau-CPV and chi-CPV activate KDM6A and KDM6B, therefore induce the expression of p21 and p16, respectively. De-repression of DNA replication inhibitor p21 is necessary for survival of CPV. In the meantime, tau-CPV bind pRb, and inhibiting pRb through its C-terminal domain. In contrast to tau-CPV, chi-CPV connect pRb by LXCXE motif, further restrain pRb. Finally, CPV bind with pRb by releasing E2F, which can also regulate the expression of p16.

GENERAL CONCLUSIONS

Some previous studies suggested that CPV-associated precancerous lesions may result in malignant tumors, however a precise study about these changes were not performed. The detection of viral L1 protein and the presence of virions in these lesions were nothing more than the positive results, which cannot illustrate the virus as a causative agent in the lesions. In human, oncogenic HPV can result in the overexpression of p16 within inhibiting pRb in HPV-associated tumors. Whereas, it has not been described within the precancerous lesions of dogs. In Chapter 1, the aim was to identify CPV association in canine precancerous cutaneous lesions. In Chapter 2, providing more information about the localization of CPV nuclei acid of different genotypes. In Chapter 3, giving more details about the pathogenesis in these CPV-related lesions.

In Chapter 1, to clarify CPV association in cutaneous precancerous lesions, CPV was detected by IHC, PCR and nucleic acid sequence determination in all cases. The results illustrated that PCR is a more sensitive method to detect papillomavirus infection in dogs. Moreover, nucleic acid sequence analysis showed that papilloma was infected with CPV2; pigmented viral plaques were infected with CPV4, and 1 case with CPV18. In the case of

pigmented viral plaque, CPV18 gene was also detected in the lesion of cytokeratin-14- and P63-positive basal cell tumor that was developed adjacent to pigmented viral plaque. This is the first finding of basal cell tumor associated with CPV18-infection in the dog. In addition, the number of Ki-67-positive cells was increased in the CPV-infected papilloma and basal cell tumor compared to that in the CPV-uninfected papilloma and pigmented viral plaque lesions. The result indicates a potential risk of malignant transformation in the lesions associated with CPV infection.

Information on the localization of CPV nucleic acid of each CPV types in the precancerous lesions have never been available. Therefore, in Chapter 2, an *in situ* hybridization (ISH) assays were performed to detect canine papillomaviral DNA on all CPV-positive tissue sections. The results of the *in situ* hybridization for CPV2, CPV4 and CPV18 were 100% concordant with those of the PCR. Moreover, CPV2 signals were observed in the nuclei of neoplastic cells within the perilesional thickened epidermis of papilloma. CPV4 signals were observed in the nuclei of epithelial cells in the spinous layer and granular layer of pigmented viral plaque. In one case of basal cell tumor which had developed adjacent to pigmented viral plaque lesion, nuclei of tumor cells were positive for the CPV18

gene. The results of this chapter implied that CPV2, CPV4 and CPV18 infection may cause cutaneous papilloma, pigmented vial plaque and basal cell tumor in dogs.

In Chapter 3, the IHC detection for p16 protein and pRb in CPV-infected skin tumors in dogs was performed. All cases were applied to IHC for the detection of p16 and pRb. Diffuse cytoplasmic expression of p16 protein with faint/no nuclear expression of pRb was detected in CPV-positive cases. However, in lesions of the head and neck in a case of CPV2-associated papilloma, tumor cells were negative for p16 protein. Nuclear expression of pRb was observed in all CPV-negative papilloma, nonetheless no nuclear and cytoplasmic expression of p16 protein was observed in these cases. The diffuse cytoplasmic expression of p16 protein in the neoplastic cells of CPV-positive cases was not consistent with the results of a previous report on papillomavirus pathogenesis in malignant skin tumors of human and dogs, which accompanied with the nuclear and cytoplasmic expression of p16 protein. Moreover, oncogenic CPV can also inhibit the expression of pRb in CPV-associated precancerous lesions, like HPV-associated tumors in human.

This study documents the detection and localization of CPV-infected cells in canine precancerous skin lesions, and may confirm a pathogenic role of CPV2, CPV4 and CPV18 during the development of the lesions. Moreover, the cytoplasmic expression of p16 protein and loss of pRb in CPV-associated lesions indicates the oncogenic potential of the molecules in the precancerous lesions. Therefore, the presence of CPV DNAs within canine precancerous cutaneous lesions was associated with the expression of p16 and the loss of pRb like oncogenic HPV. The findings in the present study may increase the awareness of the oncogenic potential of CPV2, CPV4 and CPV18 in the precancerous skin lesions in dogs.

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