UNIVERSITI PUTRA MALAYSIA

DETECTION OF PAPILLOMAVIRUS IN SQUAMOUS CELL CARCINOMA IN CATS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Detection of Papillomavirus in Squamous Carcinoma Cell in Cats”, by Ng Yen Dee and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

cSCC	Cutaneous squamous cell carcinoma
fSCC	Feline Squamous Cell Carcinoma
H&E	Hematoxylin & Eosin
HPV	Human papillomavirus
IHC	Immunohistochemistry
oSCC	Oral Squamous Cell Carcinoma
PV	Papillomavirus

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 -Projek.

PENGESANAN PAPILLOMAVIRUS DALAM KARSINOMA SEL SKUAMOSA PADA KUCING

Oleh

Ng Yen Dee

2023

Penyelia: Dr. Ong Siew Mei

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Virus papiloma manusia (PV) dikenali menyebabkan karsinoma sel skuamosa (SCC) mulut dan alat kelamin. Pada mamalia bukan manusia, terdapat bukti yang meningkat menunjukkan bahawa PV mungkin memainkan peranan penyebab dalam perkembangan SCC pada spesies seperti arnab, anjing, dan kucing, terutamanya SCC kulit pada kucing. Walau bagaimanapun, peranan tepat PV dalam perkembangan SCC pada kucing masih tidak jelas kerana penyelidikan mengenai mekanisme

karsinogeniknya masih terhad. Selain itu, prevalens PV dalam karsinoma sel skuamosa kucing (fSCC) di Malaysia masih tidak diketahui. Kajian ini bertujuan untuk mendeteksi kehadiran PV dalam fSCC oral dan kulit kucing di mana 35 sampel blok parafin fSCC dikumpulkan dari Makmal Histopatologi Fakulti Perubatan Veterinar, Universiti Putra Malaysia antara 2017 dan 2023. Jantina, umur, dan lokasi bonggol dicatat. Imunohistokimia dijalankan untuk mengesan PV dalam sampel fSCC menggunakan antibodi anti-HPV dan anti-p16CDKN2A (p16) selepas penilaian bahagian haematoxylin dan eosin. Hubungan antara jantina, umur, dan lokasi SCC dengan pengesanan PV dianalisis menggunakan ujian Chi-square atau ujian Fisher's exact dalam SPSS. Imunreaktiviti p16 positif dikesan dalam 16 daripada 35 sampel (45.7%) sementara semua 35 sampel menunjukkan imunreaktiviti negatif terhadap antibodi anti-Virus Papiloma Manusia (anti-HPV) (100%). Terdapat hubungan antara pengekalan p16 dan jantina (p<0.05), dengan imunreaktiviti p16 yang kuat diperhatikan dalam SCC dari kucing jantan. Walau bagaimanapun, walaupun umur (p=0.462) atau lokasi SCC (p=0.082) tidak signifikan dikaitkan dengan pengekalan p16. Kesimpulannya, pengekalan p16 dalam subset fSCCs menunjukkan bahawa mungkin terdapat hubungan antara PV dan perkembangan fSCC walaupun mekanisme molekulnya yang menyumbang kepada tumorigenesis sel skuamosa kucing masih perlu diterangkan.

Kata kunci: Kucing, papilomavirus, karsinoma sel skuamosa, onkogenesis virus, imunohistokimia

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999- Project.

DETECTION OF PAPILLOMAVIRUS IN SQUAMOUS CELL CARCINOMA IN CATS

by

Ng Yen Dee

2023

Supervisor: Dr. Ong Siew Mei

Co-supervisors: Dr. Norfitriah Mohamed Sohaimi, Dr. Kok Mun Keong

Human papillomaviruses (PVs) are known to cause squamous cell carcinoma (SCC) of the mouth and genitalia. In non-human mammals, there is increasing evidence indicating

that PVs may play a causal role in the development of SCC in species like rabbits, dogs, and cats, particularly cutaneous SCC in cats, however, the exact role of PV in the development of SCC in cats remains unclear as investigations into its carcinogenic mechanisms remain scarce. Furthermore, the prevalence of PV in feline SCC (fSCC) in Malaysia is still unknown. This study aims to detect the presence of PV in feline oral and cutaneous SCC where 35 fSCC paraffin block samples were collected from the Histopathology Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia between 2017 and 2023. The gender, age and location of mass were recorded. Immunohistochemistry was conducted to detect the PV in the fSCC samples using anti-HPV and anti-p16CDKN2A (p16) antibodies after evaluation of haematoxylin and eosin sections. The association between gender, age, and location of SCC with the detection of PV was analysed using either Chi-square test or Fisher's exact test in SPSS. Positive p16 immunoreactivity was detected in 16 out of 35 samples (45.7%) whereas all 35 samples showed negative immunoreactivity for anti-Human Papillomavirus (anti-HPV) antibody (100%). There was an association between p16 overexpression and gender (p<0.05), with strong p16 immunoreactivity observed in SCC from male cats. However, neither the age (p=0.462) nor the location of SCC (p=0.082) was significantly associated with p16 overexpression. In conclusion, the overexpression of p16 in a subset of fSCCs suggests that there could be an association between PVs and the development of fSCC although its underlying molecular mechanisms that contribute to feline squamous cell tumourigenesis remains to be elucidated.

Keywords: Cat, papillomavirus, squamous cell carcinoma, viral oncogenesis, immunohistochemistry

1.0 INTRODUCTION

Squamous cell carcinoma is the most common oral cancer and skin cancer in cats. However, the exact definitive cause of SCC in cats can be complex and multifactorial. The common aetiology includes prolonged sunlight exposure, chronic inflammation, and infection of oral or dental tissues, exposure to environmental tobacco smoke and viral infection (Sequeira *et al.*, 2022). The aetiological connection to viral infection such as papillomavirus (PV) is drawing increasing attention to the occurrence of SCC in cats. Similar in domesticated animals such as sheep, dogs, cattle, pigs and horses, PVs have been linked to the occurrence of neoplasia (J. S. Munday and Na, T., 2021).

Felis catus papillomavirus (FcaPV) is another name for PVs in cats, which belong to the family of *Papillomaviridae* and consist of 30 genera (Egberink *et al*., 2013). While PV infection in cats is commonly linked to skin lesions, the virus can also be detected in normal skin. FcaPVs are associated to four distinct types of skin abnormalities: hyperkeratotic plaque that may advance to Bowenoid in situ carcinoma (BISC) and subsequently develop into invasive SCC (ISCC), cutaneous fibropapilloma or feline sarcoids, and cutaneous papilloma (Egberink *et al.*, 2013). In addition, Munday and Na, T. (2021) proposed that besides causing hyperplastic papilloma formation, PV is able to transform cellular regulation indicating that they can also influence the onset of cancer. However, the association between PVs and certain cancers in humans has been extensively investigated, studies focusing on their role in fSCC have been relatively limited.

In the field of medical research, the understanding and study of PV-related SCC demonstrates a significant difference between human and veterinary medicine. Human medicine has made substantial progress with extensive research on PV-associated SCC, while the veterinary counterpart lags behind, marked by limited research initiatives. For example, PV-related cancers in humans, especially cervical cancer, have reached and become an alarming issue among women. Researchers have found that persistent genital high-risk HPV infection accounts for more than 90% of cervical cancer cases (Okunade, 2019). Therefore, regular screening tests and HPV vaccination are the common preventive measures for HPV infection. Vaccination can effectively prevent nearly all cases of cervical cancer, while regular screening enables healthcare providers to detect and remove precancerous cells before they progress into cancer (Okunade, 2019). This signifies that there is limited research of PV-associated SCC among cats.

In the realm of veterinary oncology, particularly in relation to fSCC, the understanding of the underlying PV involvement in the pathogenesis of SCC remains unclear. This study helps to address this knowledge gap by conducting a comprehensive investigation into the presence of PV in fSCC and aims to lay the groundwork for a deeper understanding of the molecular mechanisms driving PV-associated SCC in cats. This study will provide contribution in feline oncology, as well as the field of treatment and diagnosis of fSCC.

1.1 Objective

The study was conducted with the following objective:

1. To detect the presence of PV in fSCC.

1.2 Hypotheses

Null hypothesis 1 : None of the fSCC tumours are positive for PV capsid protein detection.

Alternative hypothesis 1 : A subset of the fSCC tumours have positive for PV capsid protein detection.

Null hypothesis 2: None of the fSCC tumours are positive for p16 immunoreactivity.

Alternative hypothesis 2 : A subset of the fSCC tumours have positive for p16 immunoreactivity .

2.0 LITERATURE REVIEW

2.1 Papillomavirus

PVs are viruses with a circular, non-enveloped and double-stranded DNA structure. FcaPVs are believed to be responsible for oral papillomas, skin papillomas, viral plaques, and BISC, followed by cutaneous squamous cell carcinomas (cSCC) that has been proved by the growing evidences suggested FcaPVs could be linked to the onset of cSCC (Munday *et al*., 2018). There were six types of FcaPVs discovered and it is suggested that the types of PV that are associated with cSCC are FcaPV2, 3 and 6. The type of PV that specifically targets the skin and causes the formation of viral plaques, BISC, cSCC, and basal cell carcinoma is FcaPV2, which is categorised under the *Dyothetapapillomavirus* genus. On the other hand, FcaPVs 3 and 6 belong to the genus *Taupapillomavirus*, both showing association to the formation of cSCC (Munday *et al*., 2018). The amplification of PV DNA was observed in all 20 cases of BISC, 17 out of 20 cases of invasive SCC, and 3 out of 17 non-SCC controls, indicating higher rate of PV detection in feline cSCCs compared to non-SCC lesions and provides confirmation of the association between PV infection and feline cSCCs (Munday *et al*., 2008). Although the connection between PVs and feline skin neoplasia is evident, it does not definitively establish causation as it's plausible that the association may start from cutaneous neoplasms by creating an environment conducive to enhanced PV persistence or replication, meaning PV DNA could be present in neoplastic cells without directly influencing their transformation (Munday and Kiupel, 2009).

2.1.1 Pathogenesis

Infection of PV starts with the contact of viral particles and basal cells of epithelium that enter through a microabrasion wound (Doorbar, 2005). The speed of viral replication can be expressed in slow, subclinical, and rapid, then leading to the formation of lesions (Munday, 2014). Increased basal cell replication and terminal differentiation due to replication of PV cause the thickening and folding of epithelial tissues, leading to formation of papilloma or wart (Chow *et al.*, 2010). When infected cells approach the epithelial surface, they begin to express the L1 and L2 proteins, which are the late genes of viral structural proteins that are responsible for viral replication to promote the assembly of the virion. Viral particles are released into the environment by the shedding of cells from epithelial surface and natural degeneration of normal epithelial cells (Graham, 2017). Multiple studies showed that large quantities of FcaPV-2 was found in the SCC lesion with the expression of E6 and E7 which indicates there was ongoing genetic activities as they are responsible for influencing neoplastic transformation and cell proliferation (Wilczynski *et al.*, 1998; and Thomson *et al*., 2016). According to Isaacson Wechsler *et al.,* (2012), overexpression of PV E6/E7 oncogenes within basal cells leads to irregular cell growth and the accumulation of genetic alterations, consequently it will develop to cancer.

2.2 Squamous Cell Carcinoma

SCC is a cancerous growth originating from the epidermis, characterised by the presence of cells that show differentiation into squamous cells, also known as keratinocytes (Withrow *et al*., 2019). This type of cancer typically affects older animals, with an average age of onset at around 10 years, and it does not display a preference for either gender

(Withrow *et al*., 2019). SCC is the predominant malignant oral tumour found in both dogs and cats, with SCC constituting 70% of malignant oral tumours in feline cases (Withrow *et al*., 2019). On the other hand, cSCC makes up about 15% of skin tumours in cats, predominantly appearing on the head and frequently affecting areas like the pinna, eyelid, and nasal planum (Ludwig *et al*., 2022).

2.2.1 Risk factors

According to Inês *et al* , the data collected regarding the etiological factors associated with fSCC from various studies were viral factors, exposure to environmental tobacco smoke, sunlight exposure and dental pathology problems. Regarding the potential viral cause of the condition, PV was the most frequently screened and detected virus in cats with oral squamous cell carcinoma (oSCC). In addition, Feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) lead to immunosuppression in affected cats, making them more vulnerable to subsequent and persistent infections, as well as the onset of neoplasms. On the other hand, cats that had been exposed to environmental tobacco smoke (ETS) were found to have twice the risk of developing oSCC compared to those who had never been exposed (Sequeira *et al*., 2022). In human research, tobacco, with about 60 substances that can cause epigenetic changes in oral epithelial cells, disrupts immune function and leads to tissue oxidative stress, thereby aids in oSCC formation (Jiang *et al*, 2019).

2.3 Immunohistochemistry

Detection of PV in FSCC can be detected using IHC with both antibodies such as anti-p16INK4a antibody and anti-HPV antibody. PVs consist of a genome that has non-coding upstream, early and late regions, further to know that the early region consists of 8 genes which are E1 to E8 whereas late regions are L1 to L2.

In human PVs, E6 and E7 are oncogenic, leading to the breakdown of tumour suppressor gene protein (p53) and retinoblastoma protein (pRb) throughout the cell cycle. There is an oncogenic mechanism of PVs that is used to disrupt the cell cycle control through affecting pRb function (J. Munday *et al*., 2010). These will interfere the regular function of the pRb leading to heightened levels of cellular p16CDKN2A (p16) protein which is an important tumour-suppressor gene that is involved in the p16/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control. The elevated p16 immunoreactivity observed in human oSCC strongly indicates a papillomavirus-induced origin of the neoplasm. Thus, p16 is used to detect immunohistochemistry. In cats, there's a noted correlation between the existence of FcaPV2 DNA and the presence of p16 and pRb immunostaining in both preneoplastic and neoplastic cSCC lesions, thus the increased p16 with reduced pRb levels being proposed as potential indicators of FcaPV presence in fSCC (Munday *et al,* 2012).

On the other hand, the capsid enveloping the PV viral particle is composed of the L1 protein, which is a focal point for targeting due to its abundant production that only

occurred during viral replication. Thus, PV L1 antigen can be recognised using IHC with anti-HPV antibody. In agreement with Wilhelm *et al*. (2006), PV antigens were detected in feline viral plaques and BISC in cats, indicating that L1 protein is recognisable.

3.0 MATERIALS AND METHODS

3.1 Haematoxylin and Eosin (H&E)

A total of 35 paraffin-embedded blocks of fSCC were collected from the archived samples in Histopathology Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), spanning a period of 2017 to 2023. The diagnosis within the collected samples was confirmed by histological examination of an H&E stained section. The histology of each case was reviewed, and representative tissue sections containing SCC were selected for immunohistochemical staining.

3.2 Immunohistochemistry (IHC)

The tissue samples for IHC were deparaffinised in xylene, rehydrated in graded alcohol for 5 minutes each and rinsed with distilled water for 5 minutes. Antigen retrieval was achieved by incubating the slides in citrate buffer pH 6 solution in an autoclave machine at 105°C temperature for 10 minutes. The slides were allowed to cool down for 20 minutes and washed with distilled water for 3 times before the slides were incubated in 30% hydrogen peroxide in methanol solution for 10 minutes, followed by 2-time washing with Tris-buffered saline (TBS) solution. 8% of skim milk was used to incubate the slides for 1 hour in room temperature to reduce non-specific binding. Following washing three times with TBS solution, two types of monoclonal antibodies which are clone BPV-1/1H8 + CAMVIR was 50-fold diluted with diluent and anti-p16CDKN2A (clone G175-405, BD Pharmigen) was 100-fold diluted in TBS and applied to the slides seperately for incubation overnight at 4°C in a humidified chamber. Primary antibody was substituted with TBS for negative control. The secondary antibody which is horseradish peroxidase (HRP) - (Rabbit/Mouse- Dako REAL EnVision) was used and incubated on the slides for

one hour followed by washing three times with TBS for 30 minutes at 37 °C. The tissues were washed with TBS for three times before the slides were incubated with 3,3′-diaminobenzidine (DAB; Dojindo Laboratories) solution to visualise the immunoreaction, and slides were counterstained with haematoxylin, followed by rehydration of the tissues with 70% of alcohol and 100% of alcohol. Lastly, the tissues were submerged in xylene solution and prepared for mounting.

3.3 Statistical Analysis

The prevalence of fSCC on gender, age and location of lesion was analysed using Microsoft Excel version 2021. The association between detection of p16 overexpression with different factors such as gender, location of SCC lesion and age were investigated by Pearson chisquared or Fisher's exact tests.

4.0 RESULTS

4.1 Histological Descriptions of fSCC and Immunohistochemistry of p16 and HPV Antibodies Staining

The histological features of fSCC on the samples collected included islands of neoplastic squamous epithelial cells that were observed and invaded to the dermal layer. Next, pink laminated materials observed at the centre of neoplastic cells were known as keratin pearls (Figure 1).

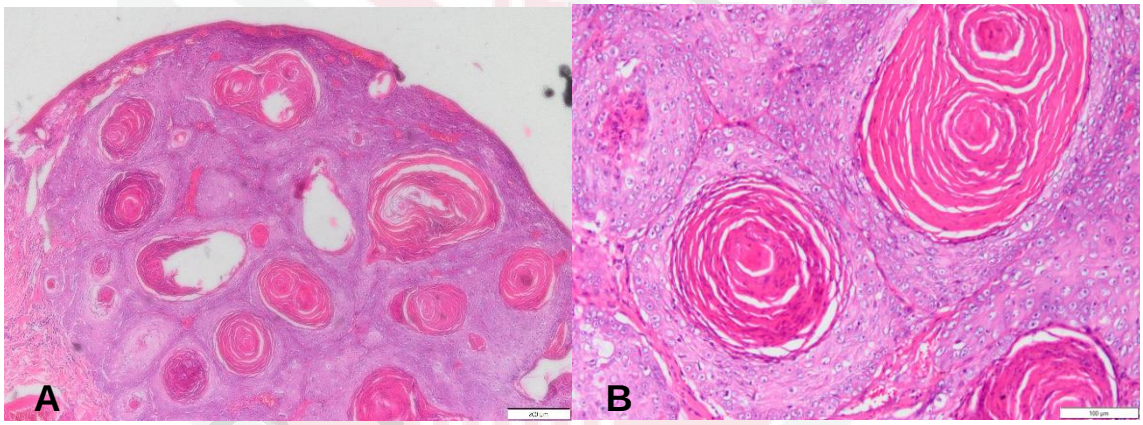
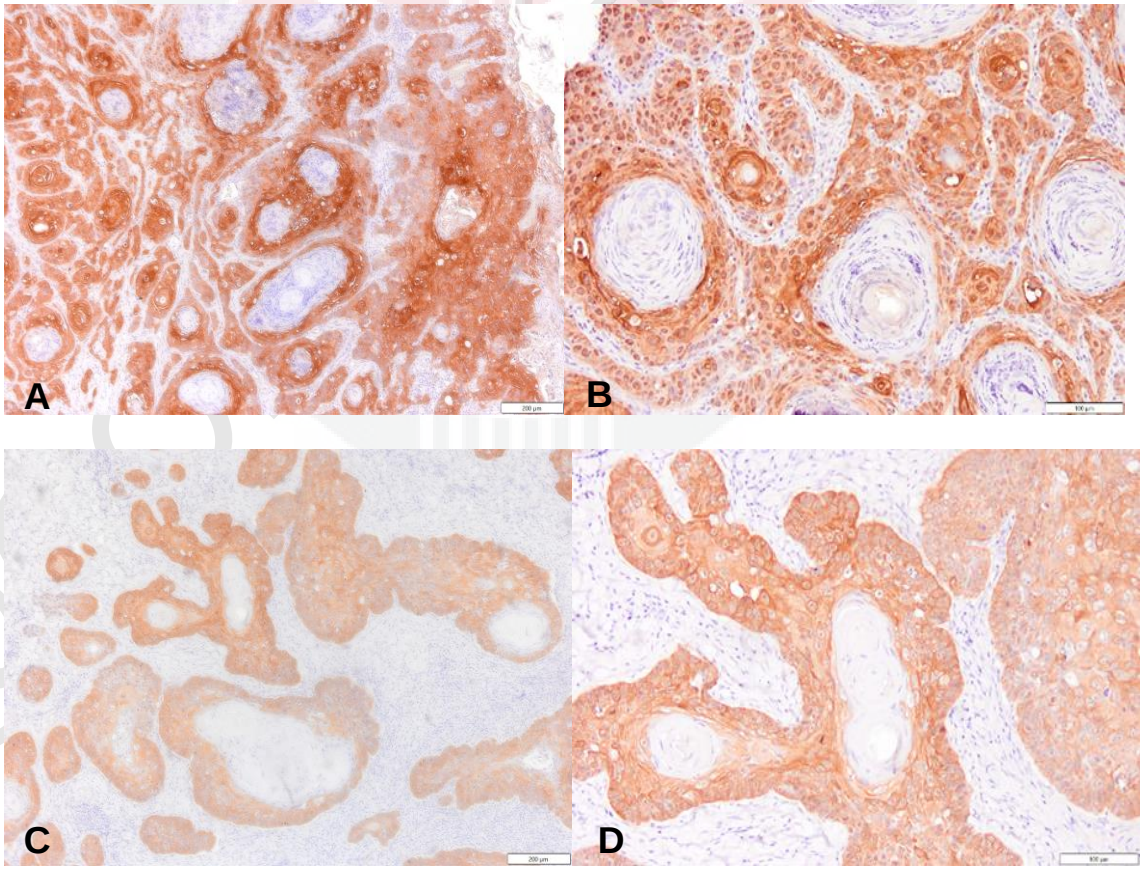


Figure 1: Histological finding of SCC

Histological images of SCC of gum lesions under H&E. Islands of neoplastic squamous epithelial cells were presented with an invasive pattern to the dermal layer. (A) The pink laminated material at the centre of neoplastic cells is the keratin pearl in a higher magnification x10. (B)

The IHC result was categorised into positive and negative for both p16 and HPV antibodies on the SCC samples, followed by the colour intensity of the staining was classified from the IHC positive result. Strong, moderate, and weak intensity were identified from the positive immunoreactivity result of p16 antibody according to the overall colour intensity of the brownish staining, regardless of nuclear or cytoplasmic stain (Figure 2). Positive result of HPV immunoreactivity was only shown in the positive control sample from a canine oral papilloma with intranuclear brownish staining on the epidermal layer (Figure 3).



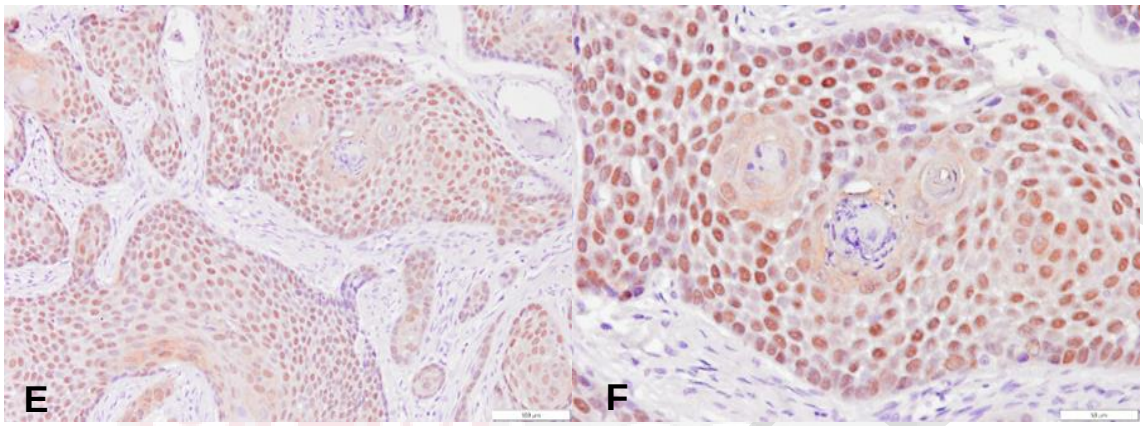


Figure 2 : Different intensity of p16 immunoreactivity on IHC

IHC result for fSCC lesion from mass on thoracic region; Immunoreactivity of p16 is detected and expressed diffusely on the neoplastic cells with strong intensity of brownish staining. Both nuclear and cytoplasmic stains could be seen in the neoplastic cells region. (A,B) FSCC lesion from periorbital lesions; Result of moderate intensity of the positive result for immunoreactivity of p16 with lighter brownish staining could be seen. Mostly cytoplasmic staining on the neoplastic cells region. (C, D) FSCC lesion from oral samples; Weak intensity of the positive result for p16 immunoreactivity is detected on the trabeculae and nests of neoplastic squamous cells. (E) Higher magnification of the nuclear staining on the squamous cells surrounding three keratin pearls in the middle. (F)

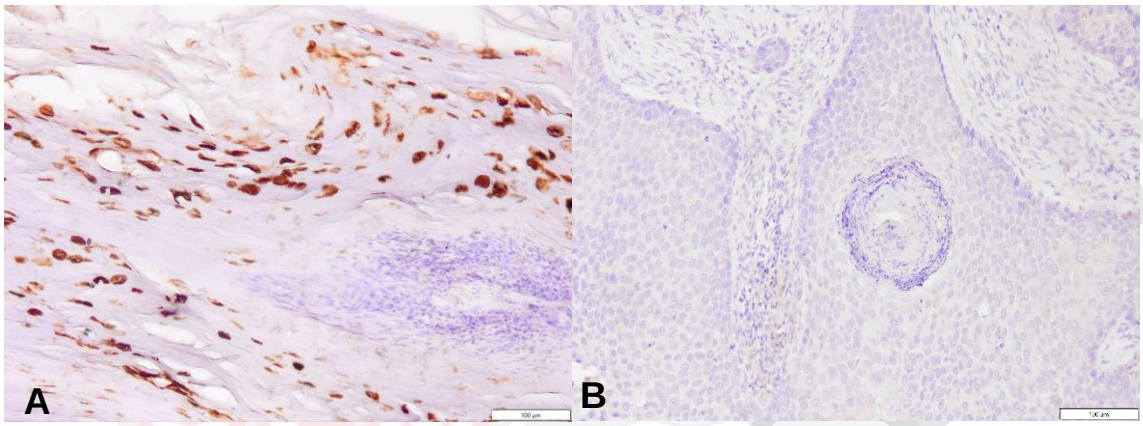


Figure 3: Positive and negative result of anti-HPV antibody immunoreactivity on IHC

Positive control of canine oral papilloma; Intranuclear brownish staining at the epidermal layer indicated positive signal for antigen-specific IHC that was performed using anti-HPV antibodies (A). No intranuclear brownish staining was observed on the sample from a gum SCC lesion (B).

4.2 Result Data Analysis

There were a total of 35 samples of fSCC, subdivided into oSCC (n=16) and cSCC (n=18). Oral SCC samples include gum, tongue, mandible and maxilla whereas cSCC samples include eyelid, ear, lip, nasal, extremity and trunk. The range of the cats' age was from 2 years old to 16 years old with the mean age of 10 years old. Cats that were 8 years old and older appeared to have a higher occurrence rate of fSCC than cats younger than 8 years old. Female cats had a higher occurrence rate of fSCC as compared to male cats.

The total positive cases for p16 overexpression on SCC samples were 16 (45.7%), further categorised into strong intensity (4/16), moderate intensity (4/16) and weak intensity (8/16) among the p16 positive samples. However, none of the samples showed positive results for antigen-specific IHC that was performed using anti-HPV antibodies on all samples.

Overall, it was found that male cats had a higher number of positive p16 immunoreactivity cases (10/34, 29.4%) than that in female cats (5/34, 14.7%) (Figure 5) and there was significant association between the detection of p16 overexpression and gender of SCC cats (p=0.042). Then, result showed that the percentage of positive p16 on cSCC was higher (11/34, 32.4%) as compared to the percentage of positive p16 on oSCC (5/34, 14.7%) among total SCC samples (Figure 6). Lastly, cats that were 8 years old and older had a higher rate of positive PV detection (10/35, 29%) than that in cats with less than 8 years old (5/35, 15%) (Figure 7). However, neither the age (p = 0.426) or the location of SCC (p = 0.082) was significantly associated with the detection of p16 overexpression.

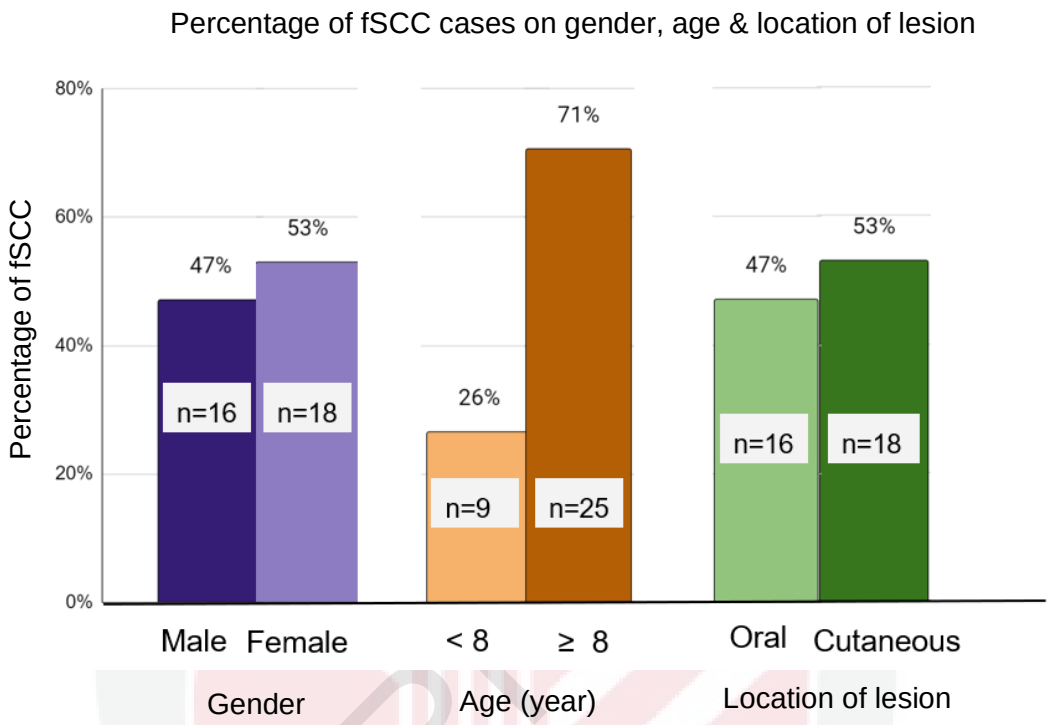


Figure 4

The percentage of fSCC according to the gender, age and location of lesion among the total fSCC samples were shown in the graph.

Figure 5

The graph shown was the result for detection of p16 overexpression with the gender of the cat.

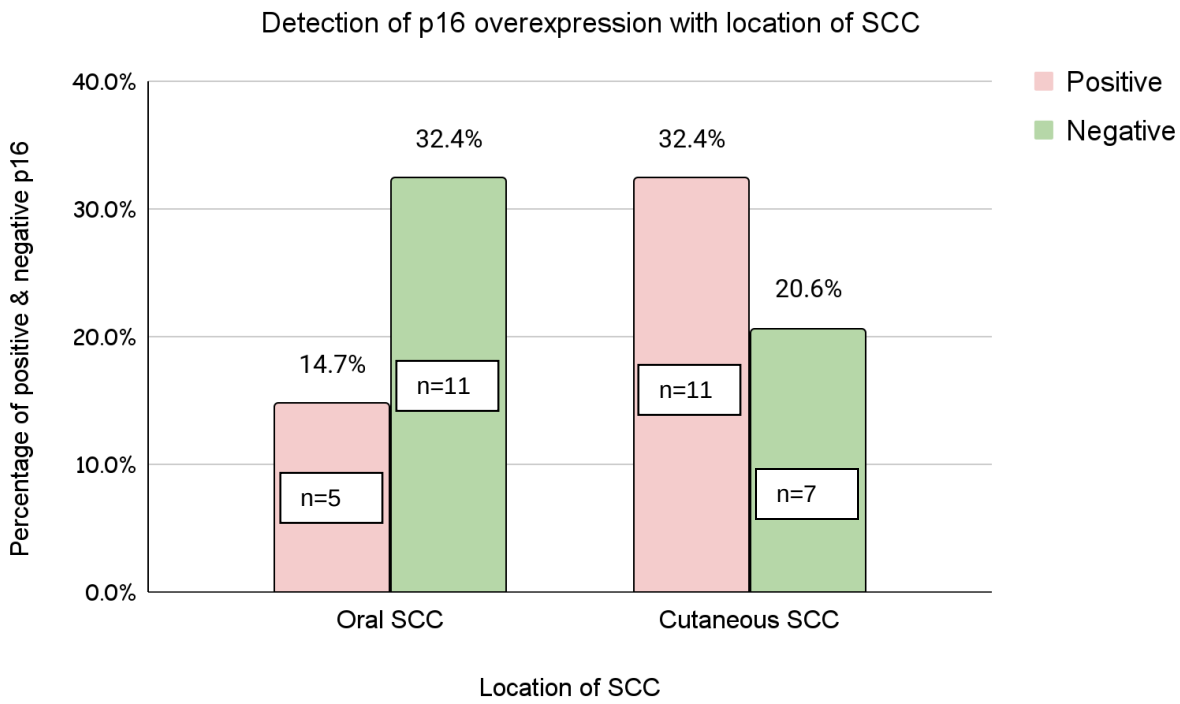


Figure 6

The result of the detection of p16 overexpression with location of SCC in cats.

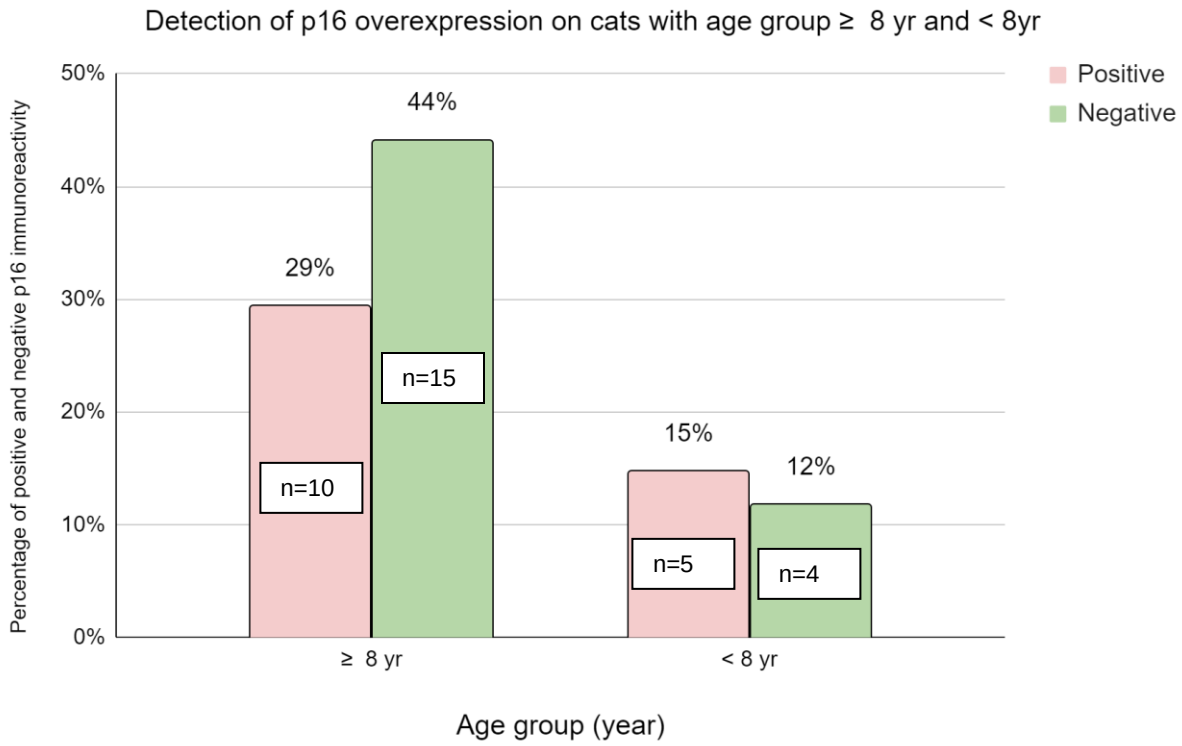


Figure 7

The result of the detection of p16 overexpression with cats age 8 years old or older and younger than 8 years old.

5.0 DISCUSSION

There is limited research reporting the prevalence of fSCC in Malaysia. Based on the study from Pérez-Enriquez *et al.* (2020), female cats had a higher tendency for diagnosing SCC as compared to male cats whereas oSCC had higher prevalence in cats than cSCC which in contrast to this study. In terms of age, both studies from Pérez-Enriquez et al. (2020) and Ho *et al.* (2017) showed that the common age to diagnose SCC in cats was 7 years old until 14 years old, which corresponds to the result in the present study.

In this study, positive immunoreactivity of antibodies against the p16 protein in fSCC was detected in 16 out of 35 samples (46%) whereas none of positive immunoreactivity for anti-HPV antibodies was detected in all 35 samples. In comparison with the study from Supsavhad *et al.* (2016), 58% of positive p16 immunoreactivity was detected in feline oSCC which showed similar results with this study. In humans, detection of p16 by IHC is utilised as an indicator of a PV aetiology in some lesions (Smeets *et al*., 2007). Moreover, p16 immunostaining is used to identify if oSCC was induced by a PV infection in human pathology (Cunningham *et al*., 2006). The p16 protein is a tumour suppressor that plays a crucial role in regulating cell cycle progression. The elevation of this protein occurs primarily because most PVs stimulate cell replication through the degradation of pRb, a process that subsequently leads to an increase in p16 (Parry *et al*., 1995). Multiple studies showed that the association of increasing p16 overexpression was significantly related to the presence of PV DNA in fSCC lesions and the reduction of pRb level in PV-infected cutaneous SCC lesions (Munday & Aberdein,

2012; Ohtani *et al*., 2004). PV oncogenes E6 and E7 can inactivate pRb and lead to p16 overexpression. Studies have shown that hypophosphorylated pRb binds to the PV oncoprotein E7, allowing the overexpression of p16. Therefore, an increase in p16 is associated with functional pRb disturbances, leading to a decrease in pRb level.

On the other hand, there was no immunostaining of PV L1 antigen detected in this present study. The PV L1 protein, which is abundantly generated during viral replication, constitutes the protective capsid enveloping the viral particle (Munday *et al*., 2018). However, L1 protein is usually produced at the late stage of viral replication, thus no immunostaining will be detected if the virus is not replicating. There might be a possibility that the virus in the lesion was not replicating or the virus had caused the infection but left the host cell. Yet, the molecular oncogenesis of PV development in fSCC is still unknown.

PV infection typically occurs via wounds and breaks in the skin. Direct contact is one of the common modes of transmission for PV infection. This can happen during activities such as grooming, playing, or fighting. Contaminated environment is one of the factors of transmission of PV among cats as viruses can survive in the environment for a short period. This occurrence is more prevalent among free-roaming or outdoor cats, given their increased susceptibility to interacting with the contaminated environment as compared to indoor cats. Our result showed that there was an association between the detection of PV and gender of SCC cats. This could be due to male cats tend to roam and engage in territorial disputes or fighting compared to female cats in general.

Nevertheless, the details for housing conditions of the cats for this present study were not gathered.

In agreement with the research from Munday and French (2015), PV-associated SCCs in cats were more common in cSCC than oSCC. The result from Munday *et al*. (2008) showed the amplification of PV DNA from feline cSCC lesions was 37 of 40 (92%) , indicating feline cSCCs are associated with PV infection. In addition, recently, a metagenomic study using 'ViroCap,' which is a tool for targeted capture and next-generation sequencing to detect DNA viruses, has examined 20 cases of feline oSCC and concluded that PV was not frequently linked with feline oSCC (Chu *et al*., 2020). However, this might be due to the exposure of sunlight to the cutaneous body part in cats is more extensive than the oral part. Exposure to ultraviolet (UV) light might cause the impaired DNA and induce neoplasia, indicating the increasing chances of oncogenic mutation to happen. According to Kumar *et al*. (2005), DNA damage in a normal cell cycle will activate the p53, leading to either the restoration of DNA or apoptosis. However, in the cutaneous-PV-infected cells, p53 is inhibited by the oncogene of E6 causing the repair of UV damaged DNA and apoptosis to cease. Therefore, the PV-infected cells might stimulate the growth of epithelial cells and could potentially contribute to the development of cancer by amplifying the replication of cells in the epithelium with UV-damaged DNA (Münger *et al*., 2004). This concluded that the UV light might involve initiation of the tumour, but cutaneous PV infections drive the progression of the SCC development.

For the p16 immunoreactivity intensity, this indication was not further evaluated as the data collection for this present study was not sufficient. Strong intensity of p16-positive oSCCs in cats correlated with significantly prolonged survival compared to moderate intensity of p16-positive SCCs, indicating that p16 immunostaining offers valuable prognostic insights for feline neoplasm (Munday *et al*., 2019). Hence, it's crucial to further explore the clinical outcomes of SCC cases with varying levels of p16 staining intensity. On the other hand, research investigating the factor of age affecting the PV-infected cells in developing SCC in cats is limited and undefined. The limited studies do not provide enough information to determine whether age affects PV infection in cats.

6.0 CONCLUSION

Overall, positive p16 immunoreactivity is detected in fSCC while none of the positive PV capsid protein is detected in fSCC. Moreover, the result shows that male cats of fSCC have a higher likelihood of acquiring papillomavirus infection. In conclusion, there is a positive detection of PV in cats and there could be an association between PVs and development of SCC in cats, yet the fundamental molecular processes that contribute to the development of feline squamous cell tumours are not yet understood. Despite the fact that there is insufficient evidence to conclusive the involvement of PV in fSCC, it should not be dismissed as a potential causative mechanism.

7.0 LIMITATIONS AND RECOMMENDATIONS

In this study, the clinical data of the patients were limited as it was not sufficient to conduct further investigations on the survival rate of the PV associated fSCC and the prognosis of the PV infected SCC cats. Thus, the clinical data such as survival rate and the prognosis of the patient cats should be gathered for further improvement. Moreover, the accuracy of the data such as the sunlight exposure to the lesion could be determined by collecting the information of the housing condition and the lifestyle behaviour of the cats, whether they are indoor, outdoor or semi-roamer cats. Next, IHC that was used in this study was only to detect the presence of the L1 gene of the virus as it only detected when virus is replicating and the presence of tumour suppressor gene produced by the host as it could be due to other agents that trigger the production. Thus, more reliable or accurate tests should be conducted such as PCR to assess the presence and gene expression of FcaPV in fSCC samples. From this, a new type or existing type of FcaPV could be discovered in Malaysia and it provides the information for the study of the prevalence of PV infection in Malaysia. To obtain more accurate findings, markers such as the tumour suppressor gene of the host could be added to detect their expression. The expression of oncogenes such as E7 or E6 and interaction with host tumour suppressor genes such as p16, pRb, or p53 could become one of the methods to investigate PV as a cause to development of fSCC.

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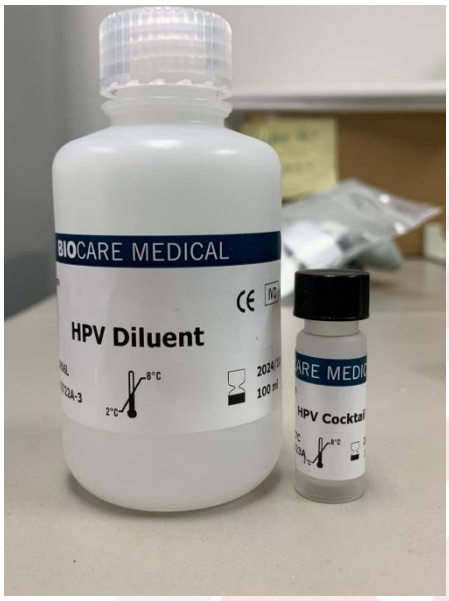
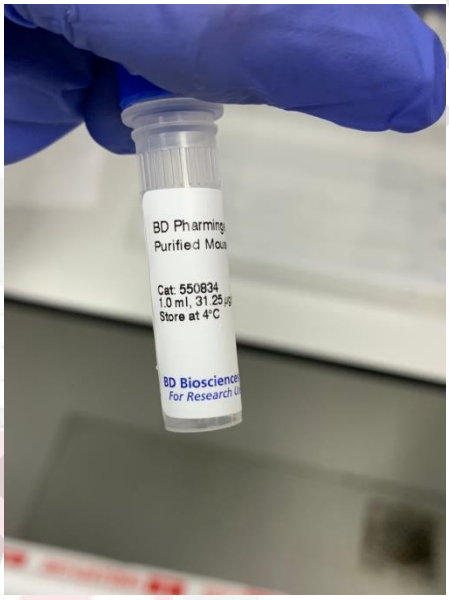
9.0 APPENDICES

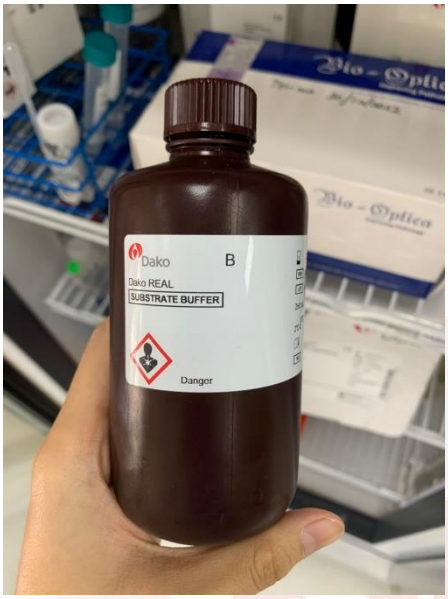


Anti-HPV antibody (clone BPV-1/1H8 + CAMVIR



Anti-p16CDKN2A (clone G175-405, BD Pharmigen)

Horseradish peroxidase (HRP) - (Rabbit/Mouse- Dako REAL EnVision)



3,3′-dia-minobenzidine (DAB; Dojindo Laboratories) solution