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Felis catus Papillomavirus Type 2 Infection and Skin Cancer in Domestic Cats

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Abstract

Felis catus papillomavirus type 2 (FcaPV-2) is a virus which commonly infects the skin of domestic cats. While most infections are asymptomatic, there is growing evidence that FcaPV-2 may play a role in the development of a subset of feline cutaneous squamous cell carcinomas (SCCs).

In the first part of this thesis, the natural history of FcaPV-2 infection was investigated with the aim of determining when cats become infected with the virus. A real-time PCR assay was developed to quantify FcaPV-2 DNA in feline skin swabs. This assay was then used to measure the FcaPV-2 DNA load in serial samples from two populations of cats. Results from these studies showed that most kittens are exposed to FcaPV-2 in the first few days of life. Additionally, the primary source of exposure is likely to be direct contact with other cats in the household, particularly their queen, as some of the queens appeared to be shedding large amounts of virus. FcaPV-2 mRNA was also detected in some of the kittens, confirming that they had become infected with FcaPV-2 soon after birth.

The aim of the second part of this thesis was to determine the quantity and transcriptional activity of the FcaPV-2 DNA present in feline cutaneous SCCs in order to determine if the virus was involved in cancer development or just present as an innocent bystander. Real-time PCR assays were developed to measure FcaPV-2 gene expression in SCCs and the results clearly distinguished two subsets of feline cutaneous SCCs. The majority of the SCCs had low copy numbers of FcaPV-2 DNA and no FcaPV-2 gene expression, suggesting the virus was an incidental finding. In contrast, around a third of the SCCs had detectable FcaPV-2 gene expression and high copy numbers of FcaPV-2 DNA, similar to that found in the FcaPV-2-induced premalignant lesions. There was also a significant association between FcaPV-2 gene expression and alterations in a host cell cycle regulatory protein (p16). Taken together, these results strongly suggest that FcaPV-2 played a role in the development of around a third of the feline cutaneous SCCs.

The results from the studies reported in this thesis support a causative role of FcaPV-2 in a proportion of feline cutaneous SCCs. However, as infection of cats is common and appears to occur early in life, there may be little opportunity to prevent SCC development by preventing FcaPV-2 infection.

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Abbreviations

Common Abbreviations

28Sr	Reference gene coding for the 28s ribosomal sub-unit
ABL2	Abelson proto-oncogene 2 non-receptor tyrosine kinase RNA reference gene
ACTB	Beta actin RNA reference gene
ANOVA	Analysis of variance statistical method
B2M	Beta-2 microglobulin RNA reference gene
BISC	Bowenoid in situ carcinoma
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
Cq	Number of PCR cycles when threshold reached
CV	Coefficient of variation
DSH	Domestic short hair
EV	Epidermodysplasia verruciformis
FFPE	Formalin fixed paraffin embedded
FIV	Feline immunodeficiency virus
FVP	Feline viral plaque
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase RNA reference gene
GUSB	Beta glucuronidase RNA reference gene
H&E	Haematoxylin and eosin stain
IgG	Immunoglobulin G
MHC	Major histocompatibility molecules
NRQ	Normalised relative quantity
ORF	Open reading frame
p16	Cyclin dependant kinase inhibitor $p16^{INK4A}$
p53	Tumour suppressor p53 protein
pRb	Retinoblastoma protein
PCR	Polymerase chain reaction
PV	Papillomavirus
qPCR	Quantitative PCR
RPL17	Ribosomal protein L17 RNA reference gene
RPS7	Ribosomal protein S7 RNA reference gene
RPS19	Ribosomal protein S19 RNA reference gene
RT	Reverse transcriptase
SCC	Squamous cell carcinoma
SNP	Single nucleotide polymorphism
VLP	Virus-like particle
YWHAZ	Tyrosine 3-monooxygenase/ 5 tryptophan 5-monooxygenase activation protein zeta

Papillomavirus Abbreviations

Domestic cats		**Phylogeny- genus**	**Tissue infected**
FcaPV-1	*Felis catus* papillomavirus type 1 Formerly Felis domesticus papillomavirus 1	Lambdapapillomavirus	oral mucosa
FcaPV-2	*Felis catus* papillomavirus type 2 Formerly Felis domesticus papillomavirus 2	Dyothetapapillomavirus	skin
FcaPV-3	*Felis catus* papillomavirus type 3	Taupapillomavirus	skin
FcaPV-4	*Felis catus* papillomavirus type 4	Taupapillomavirus	unknown
Humans			
HPV-1	Human papillomavirus type 1	Chipapillomavirus	skin
HPV-2	Human papillomavirus type 2	Alphapapillomavirus	skin
HPV-4	Human papillomavirus type 4	Gammapapillomavirus	skin
HPV-5	Human papillomavirus type 5	Betapapillomavirus	skin
HPV-6	Human papillomavirus type 6	Alphapapillomavirus	genital mucosa
HPV-8	Human papillomavirus type 8	Betapapillomavirus	skin
HPV-9	Human papillomavirus type 9	Betapapillomavirus	skin
HPV-11	Human papillomavirus type 11	Alphapapillomavirus	genital mucosa
HPV-16	Human papillomavirus type 16*	Alphapapillomavirus	genital mucosa
HPV-17	Human papillomavirus type 17	Betapapillomavirus	skin
HPV-18	Human papillomavirus type 18*	Alphapapillomavirus	genital mucosa
HPV-27	Human papillomavirus type 27	Alphapapillomavirus	skin
HPV-38	Human papillomavirus type 38	Betapapillomavirus	skin
HPV-57	Human papillomavirus type 57	Alphapapillomavirus	skin
HPV-76	Human papillomavirus type 76	Betapapillomavirus	skin
HPV-93	Human papillomavirus type 93	Betapapillomavirus	skin
Domestic dogs			
CPV-1	*Canis familiaris* oral papillomavirus Formerly COVP	Lambdapapillomavirus	oral mucosa
CPV-2	*Canis familiaris* papillomavirus type 2	Taupapillomavirus	skin
Domestic cattle			
BPV-1	*Bos taurus* papillomavirus type 1	Deltapapillomavirus	skin
BPV-2	*Bos taurus* papillomavirus type 2	Deltapapillomavirus	skin
BPV-3	*Bos taurus* papillomavirus type 3	Xipapillomavirus	skin
BPV-4	*Bos taurus* papillomavirus type 4	Xipapillomavirus	oral/ oesophageal mucosa
BPV-13	*Bos taurus* papillomavirus type 13	Deltapapillomavirus	skin
BPV-14	*Bos taurus* papillomavirus type 14	Deltapapillomavirus	skin
Horses			
EcPV-2	*Equus caballus* papillomavirus type 2	Dyoiotapapillomavirus	genital mucosa
Rabbits			
SfPV-1	*Sylvilagus floridanus* papillomavirus type 1 Formerly cottontail rabbit papillomavirus	Kappapapillomavirus	skin
OcPV-1	*Oryctolagus cuniculus* papillomavirus type 1	Kappapapillomavirus	oral mucosa
Mice			
MnPV-1	*Mastomys natalensis p*apillomavirus type 1	Iotapapillomavirus	skin
MmuPV-1	*Mus musculus* papillomavirus type 1	Pipapillomavirus	skin

Chapter 1

Literature Review

Papillomaviruses (PVs) are double-stranded DNA viruses, belonging to the *Papillomaviridae* family, which usually infect stratified squamous epithelia. Over 300 PV types have been identified, each infecting a specific anatomic site in a particular species.[1,2] While many PV infections are asymptomatic some PV types can cause benign skin lesions, such as the self-resolving papillomas (warts) that commonly occur on the hands and feet during childhood. Additionally, a small number of PV types can cause cancer, including the well-known examples of cervical cancer in women caused by human papillomavirus type 16 (HPV-16) and skin cancer in rabbits induced by Sylvilagus floridanus PV type 1.[3,4]

There is also some evidence to suggest that *Felis catus* papillomavirus type 2 (FcaPV-2) may play a role in the development of feline cutaneous squamous cell carcinomas (SCCs). However FcaPV-2 has also been found in samples of normal skin from cats, so the possibility that the virus is just an incidental finding cannot be excluded.[5] Feline SCCs are the most common malignant skin cancer of cats, frequently occurring on the nasal planum and pinnae.[6,7] The cancers are locally invasive and advanced cases require radical surgical treatment, such as complete resection of the nasal planum, or euthanasia.[8] Prophylactic vaccination against PV capsid proteins has been shown to be highly effective at preventing infection and cancer development in people. Therefore, if FcaPV-2 does play a role in cancer development it may be possible to prevent some of these cancers by vaccination.[9] However, it is currently unknown when cats become exposed to the virus or how it is transmitted through the cat population.

The overall objective of this thesis was to investigate FcaPV-2 infection in cats, with the ultimate goal of reducing the number of cats that die due to skin cancer. To achieve this, two specific aspects of FcaPV-2 infection were addressed. Firstly, the natural history of FcaPV-2 infection in cats was investigated with the aim of determining when cats are likely to become infected with the virus. This was important to determine if there is an opportunity for prophylactic vaccination. The second aim was to investigate the role of FcaPV-2 in cutaneous SCCs, particularly the transcriptional activity of the virus and whether this correlated to changes in host cell-cycle regulatory proteins. This information was important to determine if the virus was

involved in cancer development or just present as an innocent bystander. In addition, if FcaPV-2 was transcriptionally active, this would provide insight into the mechanism by which the virus causes cancer; information which is important for future development of therapeutic targets.

This chapter will introduce PVs and then review what is known about these viruses: how they are acquired and transmitted within populations; the response of the host immune system; and the molecular mechanism of their normal lifecycle and of cancer development. Following this, the current knowledge of feline PVs will be reviewed, particularly in regards to a possible role in cancer development, and the likely similarities and differences to PV-induced cancer in other species.

1.1 Introduction to papillomaviruses

1.1.1 Papillomavirus structure and taxonomy

Papillomaviruses are small, roughly spherical viruses with an icosahedral capsid measuring 55-60 nm in diameter.[10] The capsid is formed from 72 star-shaped capsomeres, each of which is composed of 5 L1 proteins (Fig. 1.1).[11] An internal minor capsid protein (L2) is also associated with each capsomere.[12] Enclosed within the capsid, the PV genome consists of approximately 8 kbp of circular, double stranded DNA packaged as a minichromosome by cellular nucleosomes.[13]

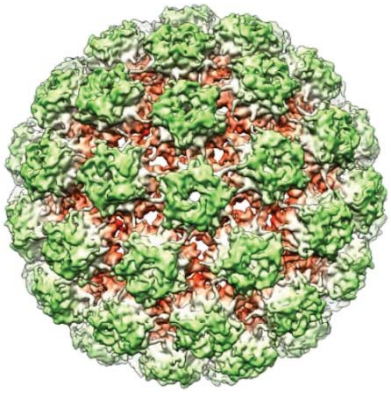

Figure 1.1. Bovine Papillomavirus Capsid. By Vossman, CC BY-SA 3.0 https://commons.wikimedia.org/w/index.php?curid=11506441

The PV genome can be broadly divided into three regions: a region encoding up to six early genes (E1, E2, E4, E5, E6 and E7) which are involved in viral genome maintenance and replication; a region encoding the late genes (L1 and L2) which code for structural proteins; and an upstream regulatory sequence called the long control region (Fig. 1.2).[10] Both early and late coding regions produce polycistronic RNAs, transcribed from one strand, which then undergo extensive alternative splicing.[10]

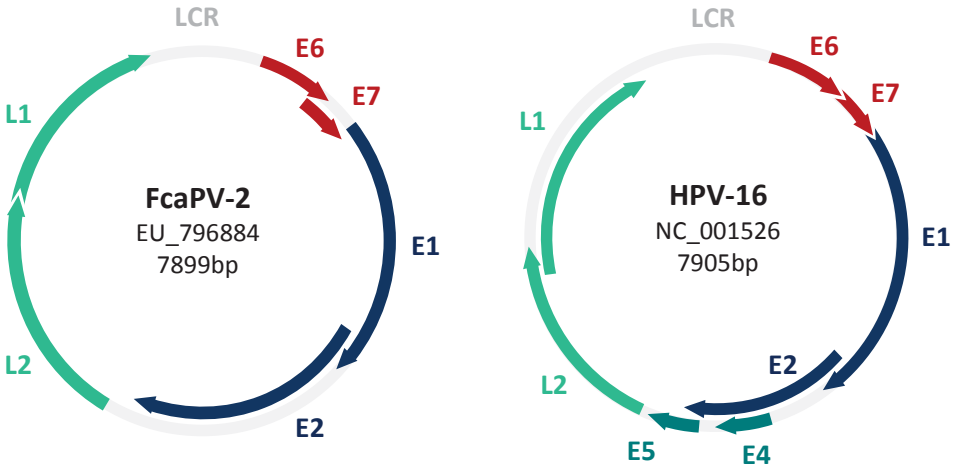

Figure 1.2. Genome organisation of *Felis catus* papillomavirus type 2 (FcaPV-2) and human papillomavirus type 16 (HPV-16), with accession numbers and genome size.

Classification of PVs is based on the sequence homology of the L1 open reading frame (ORF). The *Papillomaviridae* family includes 35 genera, with PVs in different genera having less than 60% similarity within the L1 ORF.[1,14] PV genera are named by letters from the Greek alphabet and the majority of human papillomaviruses (HPVs) are contained in the alpha, beta and gamma genera (Fig. 1.3). Different PV species within a genus share between 60 and 70% similarity.[14] The widely used classification of PV type represents a taxonomic classification below species level in which different PV types have less than 90% homology in the L1 ORF.[15] To date, 198 PV types have been identified from humans, over 100 PV types from a variety of non-human mammals, and a few types have been found on birds and reptiles.[2,16]

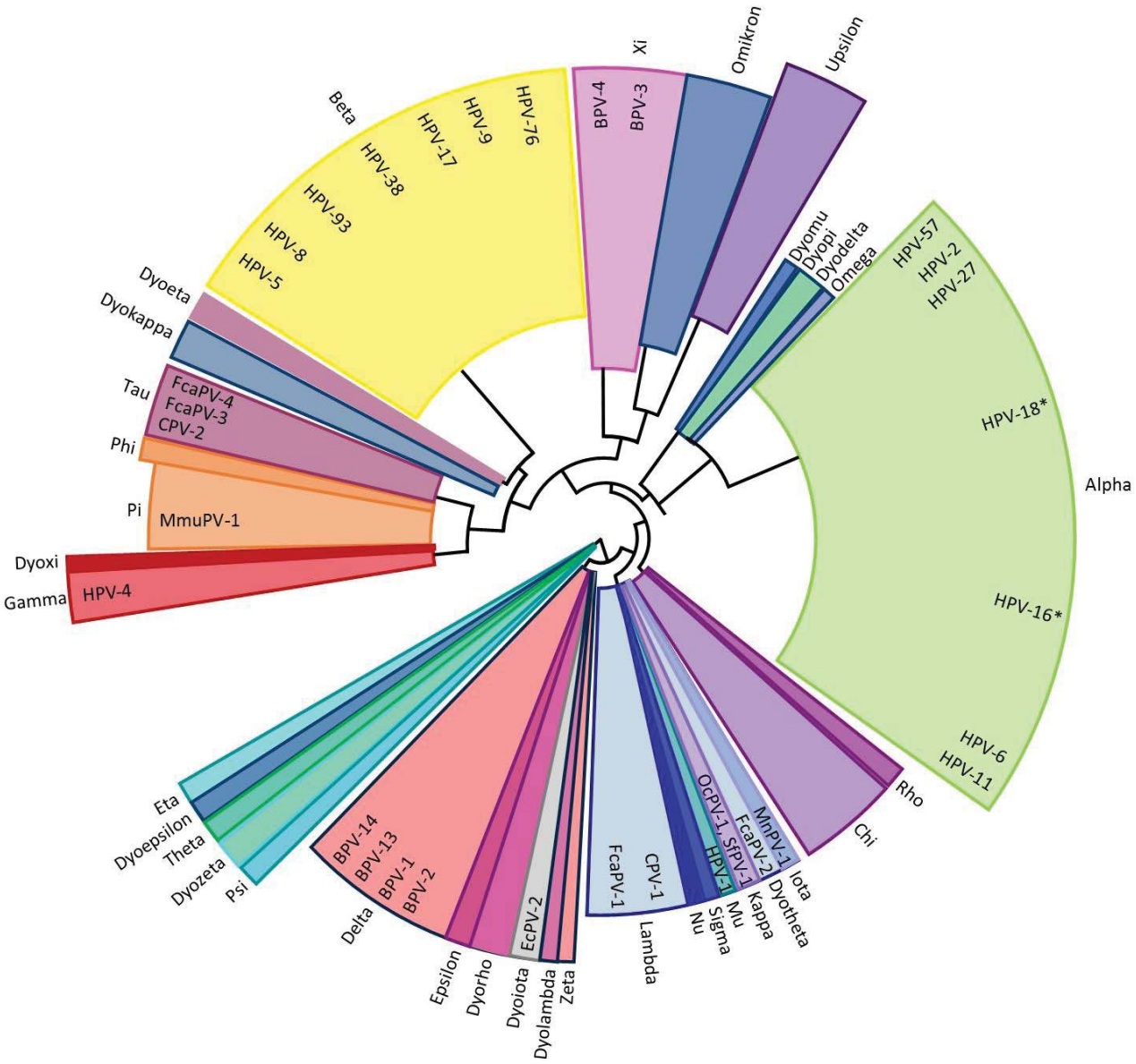

Figure 1.3. Schematic representation of PV genera. Adapted from the phylogenetic analysis by Rector A, Van Ranst M. Animal papillomaviruses. Virology. 2013;445(1–2):213-223. PV types mentioned in this chapter have been added in their respective genera.*High-risk HPVs.

The great multitude of different PV types is thought to be due to ancient papillomavirus-host co-speciation; as the host animals have evolved and differentiated, so too have their PVs.[17] In addition, many PV types can exist in different niches on a single individual. For instance *Sylvilagus floridanus* PV type 1 (SfPV-1) causes papillomas on the skin of rabbits but not on mucous membranes, whereas *Oryctolagus cuniculus* papillomavirus type 1 (OcPv-1) induces papillomas on the oral mucosa of rabbits but not on the skin.[18]

1.1.2 The spectrum of PV-induced disease

Papillomaviruses were first identified in cutaneous and genital warts in people in the mid twentieth century.[19] Papillomas, which are commonly referred to as warts, are the typical PV lesion, histologically described as a discrete exophytic mass composed of fronds of hyperplastic squamous epithelium supported by a thin fibrovascular stroma. The hyperplasia is mostly of the intermediate and superficial epithelial layers and the cells retain their normal orderly pattern of maturation (Fig. 1.4). Many papillomas contain individual or small clusters of atypical epithelial cells with large dark nuclei and prominent peri-nuclear clear zones. These atypical keratinocytes, often called koilocytes, produce large quantities of viral protein and are virtually pathognomonic for papillomavirus infection. Clumped keratohyalin granules in the stratum granulosum is another PV-induced change that is sometimes present.

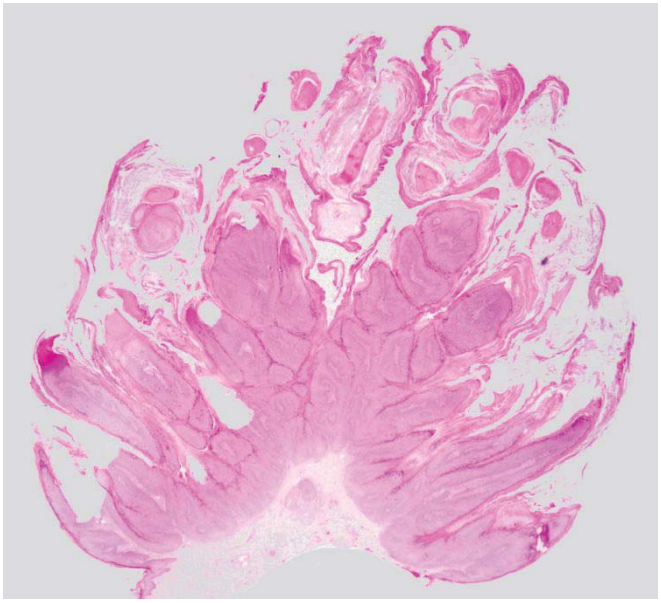

Figure 1.4. Cutaneous papilloma from a dog. Histology section stained with haemotoxylin and eosin.

In people, HPV-6 and -11 are responsible for the majority of genital warts (condyloma acuminatum) and HPV-1, -2, -27, and -57 cause common warts that often occur on the hands and feet of children.[20] As well as the typical warts, PVs can also induce flat plaque-like lesions on the face and hands, and inverted warts, such as those that occur on the soles of feet. Some PVs, particularly those from the beta genus which infect skin, complete their lifecycle without causing any gross or histologic lesion at all.[21] In addition to genital and cutaneous warts, HPVs rarely cause oral and respiratory lesions such as recurrent respiratory papillomatosis and focal epithelial hyperplasia of the lips and oral mucosa.[22,23]

Several types of HPVs are also capable of causing dysplastic epithelial lesions, some of which can progress to invasive cancer. A subset of alpha HPVs have been associated with various anogenital cancers, particularly cervical cancer but also cancers of the vagina, vulva, penis, and anus.[24] In the cervix, HPVs can induce cancer of both the ectocervix, which is lined by stratified squamous epithelium leading to squamous cell carcinomas (SCCs), and cancer of the glandular tissue of the endocervix, leading to adenocarcinomas. HPV-induced cancers from other genital locations are generally SCCs, and these can be basaloid or keratinising depending on the degree of differentiation of the neoplastic cells; less well differentiated cancers have a more basaloid appearance.[25] Alpha HPVs have also been linked to some head and neck SCCs, particularly those of the tonsils and some beta HPVs are linked to cutaneous SCCs in immunosuppressed people.[26,27]

The progression from dysplasia to invasive cancer is best described in squamous cervical lesions, where a number of intermediate stages have been characterised.[25] These are briefly described here as the names for the various stages will be used subsequently.

- Cervical intraepithelial neoplasia grade 1 (CIN1) represents mild dysplasia characterised by disorganised proliferation of the basal cells that is restricted to the lower epithelium. The upper epithelium retains its normal maturation and frequently exhibits viral cytopathic changes (koliocytes).
- CIN2 represents moderate dysplasia characterised by basal cell hyperplasia with mitoses and abnormal nuclei present in the lower half of the epithelium. Cell maturation and keratinisation in the upper layers is present but reduced. Koliocytes are present infrequently.
- CIN3 represents severe dysplasia sometimes progressing to carcinoma *in situ*. Squamous maturation is absent or restricted to small areas. Cells exhibit numerous features of

malignancy and mitoses are present throughout the epithelium but the cells remain confined by the basement membrane. Koliocytes are present infrequently.

- SCCs are invasive cancers, characterised by nests and cords of neoplastic squamous epithelial cells invading deep into the surrounding tissue, often inciting a surrounding fibrous reaction. These tumours are often ulcerated and inflamed. Koliocytes are not present.

Most animal species also appear to be susceptible to PV infection and many animals develop similar lesions to those seen in people. Over 100 animal PV types have been identified so far, most from cutaneous papillomas in a range of species including: dogs, rabbits, horses, cattle, mice, porcupines, racoons, a brush-tail bettong (marsupial), primates, manatees, turtles, a chaffinch and an African grey parrot.[28-40] A smaller number of PV types have been identified from other lesions such as pigmented skin plaques on dogs, cats, horses (aural plaques) and a python.[41-44] Additionally PVs have been detected on normal skin from goats, pigs, hedgehogs and mice.[45-48] The delta genera of PVs that infect ungulates have a unique ability to induce proliferation of the underlying connective tissue as well as epithelium. Thirteen delta PV types have been isolated from cutaneous fibromas and fibropapillomas from cattle, deer, elk, sheep, and camels.[49,50] Interestingly the delta PVs also seem to have a wider host range than other PV genera: bovine PV types -1, -2 and -13 can cause fibropapillomas (sarcoids) on horses and the recently discovered bovine PV type 14 has been associated with fibropapillomas on domestic and exotic felids.[50] Oral and genital PV-induced lesions have also been documented in animals. Oral papillomas containing PV DNA have been described in dogs, hyenas, exotic felids (lion, cougar, bobcat), rabbits and a polar bear.[17,29,51-53] Oral epithelial hyperplasia associated with PVs has also been described in pygmy chimpanzee.[54] PV-induced genital papillomas have been described in cetaceans and horses.[55,56]

PV-induced cancer also occurs in non-primate animals and is often associated with infection of an aberrant tissue or host species, immunosuppression or the presence of co-carcinogens. There are also some examples of spontaneously occurring PV-induced cancers in the normal host species. The earliest known example of PV-induced cancer was in cotton-tail rabbits infected with *Sylvilagus floridanus* PV type 1 (SfPV-1). First described in the 1930s, SfPV-1 causes cutaneous papillomas, often with extensive keratin horns, on cottontail rabbits in the midwestern United States of America.[57] In a study of domestic rabbits with experimentally induced papillomas and wild cottontail rabbits with pre-existing papillomas that were followed for 6 months or longer, 75% of the papillomas on the domestic rabbits progressed to

carcinomas, whereas only 25% of the papillomas on the wild cottontail rabbits progressed to carcinomas.[58] The higher rate of cancer development in the different species of rabbit is consistent with the hypothesis that aberrant infection of a non-host species causes disruption of the normal PV lifecycle which can lead to cancer development. Similarly sarcoids in horses are associated with bovine PVs -1, -2 and -13 and some of these lesions can be locally aggressive.[49] In addition to an aberrant host, infection in an aberrant location on the normal host species may also predispose to PV-induced cancer. In a vaccine trial of 4500 colony beagles, nine developed SCCs at the site where they had previously been vaccinated with a live, unattenuated, canine oral papillomavirus (CPV-1) vaccine.[59] CPV-1 usually infects oral mucosa and although PV structural proteins were identified in 5 cases, no PVs were seen on electron microscopy and extracts of the tumours were not infectious, suggesting the cancers did not support completion of the PV lifecycle.[59] PV-induced cancer in dogs has also been linked to immunosuppression as there have been several reports of high rates of cutaneous papillomas and pigmented viral plaques in dogs on long-term immunosuppressive therapy or with defects in cell mediated immunity, and some of these lesions have progressed to SCCs.[60,61] Rarely, progression from benign PV-induced lesions to SCCs has been reported in dogs with no known immunosuppression.[62] In cattle, immunosuppressive and carcinogenic compounds in bracken fern have been implicated in the neoplastic transformation of *Bos taurus* papillomavirus (BPV) type 4-induced alimentary papillomas, and in conjunction with BPV-2 in the development of bladder cancers.[63] Spontaneously occurring PV-induced cancer appears to occur in horses with recent studies implicating *Equus caballus* papillomavirus type 2 (EcPV-2) in the development of penile SCCs, the most common cancer of the equine penis.[64,65] There is also growing evidence that PV-induced lesions on domestic cats can also progress to invasive cancer, which will be discussed in more detail in the fourth part of this chapter. PV DNA has also been found in cancers from bats, ferrets, sheep and mice, and a papilloma-polyoma virus hybrid is responsible for a progressively debilitating papillomatosis and carcinomatosis syndrome in endangered western barred bandicoots.[66,38,67-69]

1.2 Epidemiology of PV infection

1.2.1 The high-risk HPVs

As with most aspects of PV biology, the current understanding of the epidemiology of PV infection is mostly derived from studies of the high-risk mucosal HPVs. Less is known about cutaneous HPVs and the epidemiology of PV infection in animals.

HPV infection is common. Worldwide, the point prevalence of HPV in cervical swab samples from women with normal cervical cytology has been estimated to be 11.7%, although this varies by regions with the highest prevalences tending to occur in developing nations.[70] The highest prevalence of HPV in cervical swab samples occurs in women less than 25 years of age and decreases thereafter, with some but not all populations exhibiting a second peak after 40 years of age.[70,71] HPV-16 was the most frequently detected PV type, accounting for 22.5% of HPV positive cervical swab samples from women with normal cervical cytology.[70] The particularly high prevalence of HPV in young women is supported by studies demonstrating a rapid rise in HPV antibody titres from near baseline in the early teens to peak in the twenties, and a slow decline thereafter.[72,73] First exposure of most women to high-risk HPV therefore coincides with the onset of sexual activity and longitudinal studies around this time have confirmed that HPV is primarily a sexually transmitted disease.[74] Prospective studies measuring the incidence of HPV infection suggest that the majority of young women become infected with a high-risk HPV, via sexual transmission, at some stage during their lifetime.[75-79]

In most women high-risk HPV infections are rapidly cleared, with approximately 55% cleared by 6 months, 70% by 12 months, and 80% by 24 months post infection, although there is some variation between different HPV types and HPV-16 may be cleared more slowly.[76,80] Clearance of HPV-induced lesions requires the development of a cell-mediated immune response and regressing lesions are frequently infiltrated by lymphocytes and macrophages.[81] Around half of the women infected with HPV-16 develop antibodies against the virus in the 12 months following the initial infection, and while these do not influence the regression of existing lesions, they are thought to prevent reinfection.[82] Several large prospective studies have found that repeated detection of high-risk HPVs was associated with an increased risk of developing high grade intra-epithelial cervical lesions (CIN2+).[80,83,84] To determine whether repeated detection of HPV DNA represents a persistent infection or clearance and reinfection, Xi *et al.* (1995) analyzed

HPV-16 variants in serial samples from 70 women. No change in the predominant variant was detected in any subject, regardless of whether the variant was common or rare and despite occasional transient detection of additional variants, therefore confirming viral persistance rather than reinfection.[85] Thus it appears that some women are less able to clear high-risk HPV infections and are therefore predisposed to the development of cervical cancer.

While high-risk HPV is primarily a sexually transmitted disease, there is also evidence for other forms of transmission. Vertical transmission from mother to baby has been documented, and in a recent meta-analysis HPV DNA was detected in samples from 8% of newborns, including samples from nasopharyngeal aspirates, oral or genital swabs, and cord blood.[86] Babies born to HPV DNA positive women frequently have the same type and variant of HPV detectable, confirming the mother as the likely source of infection.[86,87] However, it has not been conclusively shown that the HPV DNA found on infants represents a true infection rather than contamination by HPV shed from the mother. Repeated type-specific HPV detection up to 2 years of age, and the detection of HPV RNA in a small number of infants suggests that some infants truly do become infected.[87-90] However, larger studies demonstrating the presence of transcriptionally active HPV and evidence for seroconversion are needed. Perinatal infection is usually presumed to be acquired during birth through an infected birth canal however HPV DNA has been detected on babies born by caesarian section to HPV DNA postive mothers.[91] HPV DNA has been occasionally detected in amniotic fluid, placental biopsies, and cord blood but as the virus is not believed to cause viraemia the significance of these findings remains unknown.[92,93]

Horizontal, non sexual transmission of high-risk HPVs has also been documented: Rice et al (2000) found HPV-16 DNA in 51.7% of buccal samples from 3-11 year old children in London, approximately 11% of which was transcriptionally active.[16] A subset of positive and negative children were retested 30 months later at which time 40% of the positive group had become negative and 63% of the negative group had become positive.[94] Samples from the environment and the parents of a subset of children were all negative, leading the authors to speculate that the likely source of exposure was direct contact between the children.[94] However, despite the relatively frequent detection of high-risk HPV DNA in oral swabs from children, it would appear that few children mount an immune response: a Swedish study of 0-13 year old children found a seroprevalence of 3% for HPV-16 and 0.6% for HPV-18, whereas a study of Australian children found no seropositivity in children less than 10 years of age.[87,95] This suggests that much of the high-risk HPV DNA found in oral swab samples from children is contamination from a small

number of infected individuals, or alternatively, infection is not associated with the development of a humoral immune response.

A key question that still exists in regards to the clearance of high-risk HPV infection is whether a DNA-negative state represents complete virologic clearance or immunologic control of infection below detection limits of current HPV assays (i.e., viral latency).[96] In an analysis of 16-23 year old women who were enrolled in the placebo arm of a large clinical trial of a HPV vaccine, 8% of the women had reappearance of a specific HPV type after being negative for a period of 8 months or greater over the 3 year follow up period.[97] Interestingly, 83.7% of the women reported no new sexual partners in the 12 months preceeding reappearance of a specific HPV type which suggests reactivation of a latent infection rather than acquisition of a new infection of the same type. Similarly, low rates of incident PV infection were found in women who had not been sexually active for 18 months (5%) but higher rates were found (22%) in HIV positive, sexually abstinant women with low CD4+ T cell counts, again supporting the existence of latent PV infections.[98]

In summary, the current understanding of the natural history of high-risk HPV infection in women is that, despite possibly frequent exposure to high-risk HPVs during childhood, most girls do not become infected or do not mount an immune response to these viruses until the onset of sexual activity. Subsequent to this, most women will become infected at some point by at least one high-risk HPV, usually in their early twenties, and will mount an immune response that clears this infection within 1-2 years. Reinfection with the same HPV type appears to be uncommon and this is likely due to acquired immunity and changes in sexual behaviour.

High-risk HPV infection has been less well studied in men but the overall prevalence of detectable high-risk HPV DNA appears to be similar to that in women.[99] In contrast, seroprevalences are generally lower and peak later in life.[95,99] In men HPV infections often involve keratinized epithelium that may be less likely than mucosal epithelium to induce a humoral immune response.[100]

1.2.2 Cutaneous HPVs

Cutaneous warts are a frequent occurrence on the hands and feet of children. Although many HPV types have been detected in warts, common warts on the hands and feet are most often associated with HPV-2, -27 and -57 from the alpha genus, and plantar warts are often associated with HPV-1 and -4 from the mu and gamma genera respectively.[20,101] A large survey collecting information on over 9000 American children found an overall prevalence for cutaneous warts of 3.3%, although a much higher prevalence of cutaneous warts was found in a study of Dutch children.[102] The peak prevalence of cutaneous warts occurs in school-aged children followed by a gradual reduction with increasing age.[103,104] The high frequency of warts in school-aged children has classically been attributed to barefoot activities and shared showering facitilites. However more recent studies have found no evidence for this, instead they have shown that children with classmates or family members with warts are at higher risk of developing warts themselves.[105] Cutaneous PV infection in other species requires access of an infectious dose of PVs to the basement membrane.[106] Therefore, the high prevalence of cutaneous warts in school-aged children may be due to the frequent occurrence of abrasions, particularly on the hands and feet, combined with close contact with other children with warts in the school environment. Resolution of cutaneous warts usually takes around a year, which is similar to clearance rates of mucosal HPVs.[107]

HPVs from the beta genus also infect skin although infections with these HPV types are generally asymptomatic. Several studies have investigated the natural history of infection with beta HPV types, due to their possible involvement in cutaneous SCCs in immunosuppressed people. Cutaneous beta HPVs are thought to be acquired soon after birth. Antonsson *et al.* (2003) swabbed the foreheads of 16 babies within one hour of birth and then daily until they left hospital.[108] HPV DNA was found on 2 of 16 babies at the first sampling and 8 of 16 babies by their second day of life. In total 11 of 16 babies were positive for HPV DNA at some point during the study. In 4 of 11 cases the same PV type was detected on the baby and the mother. Swabs were also taken from the bedside table but none of the HPV types found in the environmental samples were also detected in the corresponding samples from the baby or the mother. This suggests that direct contact between mother and child was more important for PV acquisition than transfer from virus-laden inanimate objects or surfaces (fomites) in the surrounding environment.

Intrafamilial transmission was also a feature of a study by Weissenborn *et al.* (2009) who analysed serial skin swabs from parents and children from ten families. Family members typically displayed a similar profile of HPV types, whereas the HPV profiles differed markedly between families.[109] A limitation acknowledged in both this study and the previous study by Antonsson *et al*. (2003) was the inability to distinguish whether the HPV DNA detected on the swab samples represented an established infection or contamination with cornified squames from another individual. Repeated detection of the same HPV type in a single location was thought to be more suggestive of a true infection rather than contamination. Accordingly, Weissenborn *et al.* defined a PV infection as persistent when DNA from a specific HPV type was detected in more than two-thirds of the samples at a single body site, or if the specific HPV type was consistently detected in any sample from the individual for a period of 9 months or longer. Using this criterion 92% of adults and 66% of children had at least one, and up to six beta HPV types with which they were persistently infected. A type-specific persistent infection in one parent led to that HPV type being frequently detected on other members of the family; however both parents could maintain persistent infections with different HPV types that were less frequently found on the corresponding parent. Similarly, some children became persistently infected with HPV types from their parents whereas others only occasionally harboured types their parents were persistently infected with. Interestingly, none of the children developed a persistent infection with a HPV type that did not also infect one of their parents. Type-specific persistent beta HPV infections were also documented in a Dutch study which analysed eyebrow hairs from 23 adults over a two year period.[110] Over the eight consecutive samplings individual participants maintained unique HPV profiles, ranging from never having detectable HPV to consistently having the same five HPV types detected, with additional types detected only occasionally. Type-specific beta PV DNA was detected over 6 months or longer in 74% of the individuals. Interestingly five of the participants shared a household but, unlike the families studied by Weissenborn *et al.*, HPV types persistently infecting individuals were not found on other members of the household. This observation argues against HPV contamination as a main source of HPV detected in the plucked eyebrow hairs although a disadvantage, compared to swabs, is that such samples represent a very small area of skin and some infections may have been missed. Furthermore the maintenance of unique HPV profiles in members of a household suggests that the infectivity of beta HPVs is low in such a household setting or, alternatively, that the majority of available sites for infection are already taken by the beta HPVs that make up an individual's beta HPV profile.

Despite the difficulties in distinguishing true infection from contamination, these studies have shown that exposure to beta HPVs occurs soon after birth, probably from close contact with parents, and that most adults are asymptomatically infected with a unique set of beta HPV types that persist for several years.

While the overall prevalence of beta HPVs is high, around 60%, the prevalence for any specific type is much more variable. The seroprevalence for HPV-8, which is often the most prevalent beta HPV type in serological surveys, is around 11- 35% and this appears to increase with increasing age.[111,112] In comparison the seroprevalence of the less common HPV-93 is less than 1%.[111] As with high-risk mucosal HPV types, it is thought that around half of people with HPV on their skin generate antibodies, although it is not known why some people infected with cutaneous HPVs generate antibodies and others do not.[113]

1.2.3 Epidemiology of PV infection in animals

PV infection is thought to be common in non-primate mammals as PV DNA has been found in swab samples from a large proportion of domestic and zoo animals.[114] Numerous PV types have been found in the species which have been studied in more detail: 17 different canine PV types and 14 different bovine PV types have been identified so far.[1,50,115] Asymptomatic PV infections also appear to be common. A study of clinically normal dogs found that more than half of the dogs were positive for at least one canine PV type on cytobrush samples from the interdigital skin and oral mucosa.[116] Another study of dogs without any clincial signs of PV-induced disease found a seroprevalence for CPV-1 that ranged from 10.5% to 21.9%.[117] Similarly, clinically normal horses are frequently infected with BPV-1 and -2.[118] However not all animal PVs are as ubiquitous, for example EcPV-2, which causes penile papillomas and SCCs, is rarely found in mucosal swabs from clinically normal horses.[119]

Few studies have investigated the transmission of animal PVs. Early experimental infections of rabbits with SfPV-1 required innoculation of scarified skin so it was presumed that natural transmission similarly required direct innoculation of PVs into a wound.[57] Later it was shown that biting arthropods such as mosquitoes and assassin bugs could mechanically transmit SfPV-1, leading to the development of papillomas in cottontail and domestic rabbits.[120] Similarly BPV-1 has been found on flies, supporting the hypothesis that they may transmit the virus.[121] BPV-1 has also been found in blood, including blood from a neonatal calf born to a dam with cutaneous

papillomatosis, although the clinical significance of this is currently unknown.[122,123] In a reseach colony of African multimammate mice (Mastomys coucha) naturally infected with *Mastomys natalensis* papillomavirus type 1 (MnPV-1), no evidence of MnPV-1 infection could be found in fetuses or newborn mice but virtually all mice were infected by 4 weeks of age.[124] The authors considered the most likely mode of transmission to be either close skin contact between the mothers and their offspring during nursing or via virus-loaded dust particles.[124] It was also suggested that fighting wounds may have allowed access of the virus to the epithelial basal cells, as fighting was far more common among male mice, and males had a higher incidence of PV-induced tumours (papillomas and keratoacanthomas) than female mice.[124] Interestingly, the offspring from dams with a high incidence of MnPV-1-induced tumours also had a high incidence of MnPV-1-induced tumours, despite the presence of antibodies (possibly maternally derived) against MnPV-1 E2 protein in the offspring, which suggests that close skin contact is an important mode of transmission.[125]

Many PV-induced lesions in animals, particularly those that occur on young animals, resolve spontaneously after a period of several months to a year. The immune responses to CPV-1 and SfPV-1 have been extensively studied as models for human PV-induced disease.[126] Early studies demonstrated that maturation of SfPV-1 papillomas induced an antibody response that could protect against new infections by SfPV-1 but had no effect on existing papillomas.[127] Antibodies against the L1 capsid proteins were later shown to prevent PV binding and internalisation, and virus-like particle (VLP) vaccines were highly effective at preventing PV-induced disease if given prophylactically.[128] The importance of cell-mediated immunity for papilloma regression was investigated using explants of SfPV-1 infected keratinocytes autografted onto rabbits before and after regression of existing papillomas. In rabbits with existing papillomas the explants also developed into papillomas, whereas in regressor rabbits that had already developed a cell-mediated immune response the explants failed to develop papillomas.[127] Regression of the exisiting papillomas was associated with an influx of CD8+ lymphocytes, and in CPV-1-induced oral papillomas in dogs, an influx of both CD4+ and CD8+ lymphocytes.[129,130] Antibody responses to naturally acquired PV infection, rather than experimentally induced PV infection, have been studied in an African multimammate mouse colony. Strong antibody responses were detected against the MnPV-1 E2 and L1 proteins whereas only weak responses were found against the E6 and E7 proteins.[125] Significant antibody responses against MnPV-1 L1 protein were first detected when the mice were 4.5 months of age and then increased markedly at 8.5 months of age, which correlated with the initial appearance of skin papillomas.[125] Interestingly a signficantly stronger

antibody response was seen in the mice that developed papillomas compared to those that remained tumour-free.[125] This again highlights the ineffectiveness of the humoral immune response at resolving PV-induced lesions. Despite this, vaccination of infected mice with a MnPV-1 VLP vaccine at 8 weeks of age significantly lowered the MnPV-1 DNA load and prevented the development of papillomas in the vaccinated mice.[131] Therefore, the current model of the immune response to natural PV infection is that initial infection stimulates a humoral response, generating antibodies directed against the PV capsid protein which prevent the subsequent re-infection of neighbouring epithelium. These antibodies are ineffective at resolving current infection but the subsequent development of a cell-mediated immune response eventually resolves the current infection. The degree to which antibodies protect against new infection, and the efficacy of the cell-mediated response at clearing/suppressing current infection varies according to the individual animal, and the PV type involved.

Another aspect of the natural history of PV infection that has been well studied in animals is the occurrence of latent PV infections. A latent PV infection is the presence of PV DNA in the basal cells but without the production of progeny virions in the more superficial epithelial layers. The existence of latent PV infections in people has been suggested but has not been directly demonstrated so studies on rabbits have sought to establish a model of PV latency that may be applicable to people. Latent PV infections have been most clearly demonstrated in rabbits experimentally infected with either SfPV-1, or *Oryctolagus cuniculus* Papillomavirus 1 (OcPV-1), a mucosal PV.[106,132,133] An early study demonstrated that innoculation of scarified skin of domestic rabbits with low concentrations of SfPV-1 did not induce papillomas but did result in the detection of SfPV-1 DNA at the site for 18 weeks post innoculation.[133] Furthermore, skin irritation with photodynamic therapy increased the rate of papilloma formation from these sites demonstrating that the SfPV-1 DNA present latently was capable of reactivation.[133] Similar latent infections were demonstrated in another study and E1 (but not E6 or E7) transcription was found at sites of latent SfPV-1 infection.[106] UV irradiation of these sites induced PV gene expression and caused the development of papillomas in 26% of infected sites.[106] A more recent study experimentally induced papillomas on the tongue of rabbits by tattooing the underside with a suspension containing OcPV-1.[132] These papillomas spontaneously regressed after around 8 weeks, but the OcPV-1 DNA remained detectable for up to a year post infection.[132] The existence of latent PV infections is important as it may explain the frequent amplification of PV DNA from clinically normal animals in cases where productive infections are usually associated with the development of papillomas. Additionally, reactivation of latent PV infections may explain the development of lesions in people and animals with no recent PV exposure.

1.3 Molecular mechanisms

1.3.1 The normal PV lifecycle

The current understanding of the normal PV lifecycle is predominantly based on research of the high-risk mucosal HPVs, particularly HPV-16. PV infection is thought to require entry into a mitotically active basal stem cell and this first requires access of the PV to the basement membrane through micro-wounds. This has been supported by experiments in mice where HPV-16 virus-like particles (VLPs) were found to infect cervical epithelium only after mechanical or chemical disruption exposed the basement membrane.[134] VLPs are empty PV capsids that are created by self-assembly of recombinantly expressed PV L1 capsid proteins. In the skin, basal stem cells reside in both follicular and interfollicular locations. Access of PVs to the interfollicular basal stem cells requires micro-wounds whereas access to the stem cells in the bulge of the follicular epidermis may occur through the hair follicle.[135,136] Binding of VLPs to the basement membrane requires heparan sulfate proteoglycans.[137] Following binding, the capsids undergo a series of conformational changes that lead to protease digestion of L2 protein and exposure of its N terminus allowing transfer of the VLP to a receptor on the basal epithelial cell and internalisation of the virus.[138] Following internalisation, the L2 protein facilitates transport of the genome to the cell nucleus, a process which appears to require infection of a mitotically active cell, while the remaining capsid undergoes lysosomal degradation.[139] It has been suggested that many PVs have adapted their life cycle to the wound-healing process, binding initially to the basement membrane during the open wound phase and subsequently transferring to basal epithelial cells during the wound healing phase.[138]

Following entry into a suitable cell, there is an initial period of genome amplification, and cell proliferation, creating a foci of infected basal cells each harbouring around 50 copies of PV DNA.[132] Subsequent to this initial genome amplification, PV copy numbers are maintained at a relatively constant level in the basal stem cells, replicating in synchrony with these cells which have effectively become a reservoir for the virus. When one of these basal stem cell divides, a daughter cell is induced to differentiate and begins to move vertically through the epithelial layers. In the suprabasal epithelial layers, PV proteins in these daughter cells create an environment permissible for large-scale replication of the PV genome which occurs subsequently in the intermediate epithelial layers. Production of the viral capsid proteins and virion assembly occurs in the terminally differentiated superficial cells and the virions are released in the dead

cells that desquamate from the surface. The PV lifecycle can therefore be approximately divided into five phases (Fig. 1.5): establishment and maintenance of a foci of infected basal cells, cell-cycle re-entry in the suprabasal layer, PV genome amplification in the intermediate layers, production of PV structural proteins and the assembly of virions in the superficial epithelium, and finally shedding of infectious virions.

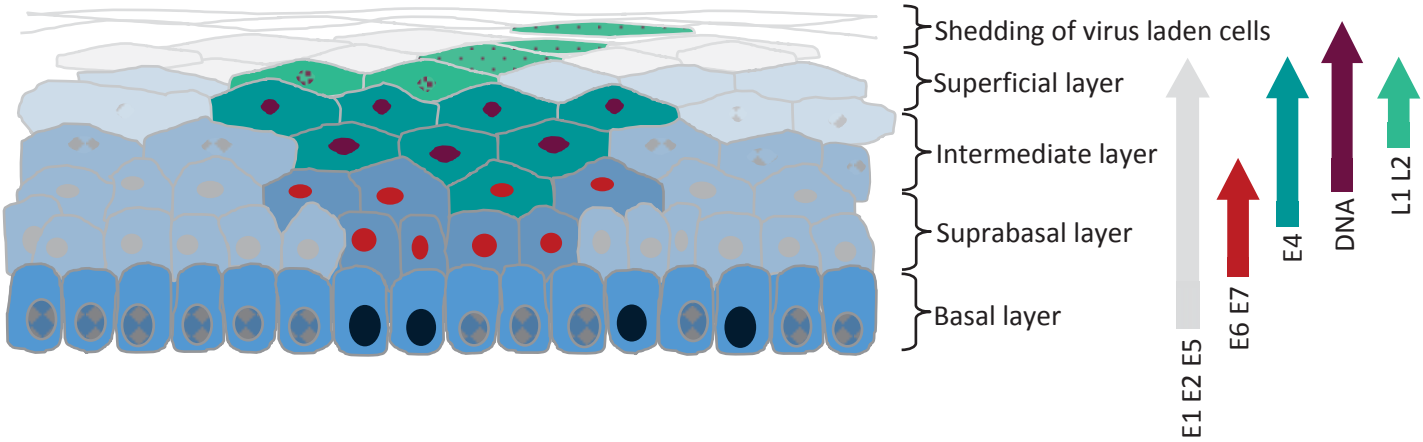

Figure 1.5. Patterns of PV gene expression in the normal PV lifecycle. The arrows show the level of epithelium at which FcaPV-2 mRNA and FcaPV-2 DNA can be readily detected.

In the basal epithelial layer, PV gene expression is driven by transcription from an early promoter in the long control region that generates polycistronic mRNAs encoding all of the early genes, which are polyadenylated at the early polyadenylation signal. These are then alternatively spliced to code for the exclusively early genes (E6 and E7) and those expressed throughout the PV lifecycle (E1, E2, E4 and E5).[140] PV protein expression at the basal cell level has not been detected, but it is presumed that at least E1 and E2 proteins are essential for the initial establishment of PV copy number.[141,142] The E1 protein is a viral DNA helicase which prises apart and unwinds the viral DNA, and facilitates DNA replication by cellular replication proteins.[143] The E2 protein is a transcription factor, both activating and inhibiting PV transcription to co-ordinate the PV lifecycle with epithelial differentiation.[144] E2 is also needed for viral DNA replication and is important for partitioning PV genomes between daughter cells during basal cell division.[143]

In the suprabasal epithelial layers, there is increased expression of the PV E6 and E7 genes (Fig. 1.5).[145] The function of the E6 and E7 proteins is to create a cellular environment that is conducive to amplification of the PV genome. During the normal slow cycle of basal cell replication, the host replication proteins needed for viral genome replication are readily available. However, as the cells begin to differentiate and enter the suprabasal layer they exit

the cell cycle meaning the necessary replication proteins are no longer available. To overcome this problem, the PV E6 and E7 proteins interact with numerous host proteins and keep the cell in a synthesis phase-like state, allowing the PV to continue to utilise the host replication machinery.[146,147] The numerous cellular interactions of the PV E6 and E7 proteins have been recently reviewed by Vande Pol and Klingelhutz (2013), and Roman and Munger (2013). Significant differences exist in the nature of these interactions between different PV types.[148-150] One of the most well-known roles of E6 is to reduce (or bypass) the function of p53 which occurs through multiple different routes including, in the high-risk HPV types, ubiquitin-ligase mediated degradation of p53.[151] This prevents p53 directed cell cycle arrest or apoptosis of the PV infected cells. Another function of high-risk HPV E6 is binding and degradation of several PDZ-containing proteins which are thought to be associated with the cellular membrane and affect such processes as cell polarity, maintaining cell-to-cell interactions, and various cell-signalling pathways.[152] While the role of this interaction *in vivo* is unknown, *in vitro* binding to PDZ-containing proteins appears to be necessary for maintenance of PV genomes in infected cells.[153] Other E6 functions include degradation of the pro-apoptotic protein Bak and activation of cellular telomerase, the enzyme that adds nucleotides to the ends of chromosomes to prevent the shortening during replication which eventually results in cellular senescence.[154,155] A major function of the PV E7 protein is binding, and in some cases degradation, of retinoblastoma family proteins: p105 (pRb), p107, and p130. As part of the cell cycle G_1/S checkpoint, these proteins form a complex with the E2F set of transcription factors. Disruption of this complex by the PV E7 proteins releases inhibition of E2F-responsive genes (such as cyclin A and E, minichromosome maintenance protein, and proliferating cell nuclear antigen) which drives the cells into a synthesis phase-like state, allowing the cells to retain their proliferative capacity while continuing to differentiate.[156,157] While both high- and low-risk HPVs can bind and degrade p130, causing cell cycle re-entry in the intermediate and upper epithelial layers, only the high-risk types appear capable of degrading pRb and p107, causing proliferation of cells in the basal and suprabasal layers.[158]

Many PVs in the alpha genera contain an E5 ORF but this ORF is not essential to the PV lifecycle and is missing in a number of PV types, including beta PVs and FcaPV-2.[1,42] E5 is a short, transmembrane protein that localises to membranes of the Golgi apparatus and endoplasmic reticulum.[159] The bovine PV E5 protein has the ability to transform cultured fibroblasts but the role of the E5 protein in normal productive infection is less well understood.[160] Similar to the E6 and E7 proteins, E5 appears to be important for retaining the proliferative capabilities of suprabasal epithelial cells.[161,162] A likely mechanism involves alterations in the cycling and

activation of growth factor receptors.[163] The PV E5, E6, and E7 proteins have also been shown to have immunomodulatory functions resulting in downregulation of an array of proinflammatory and chemotactic cytokines as well as antigen processing and presenting molecules, allowing the virus to remain undetected by the immune system, often for many months.[164,165]

In the intermediate epithelial layers, a change in PV transcription is triggered via activation of a late promoter which favours expression of E1, E2, and E4 mRNAs.[140] Correspondingly, immunofluorescence for PV E7 protein wanes and is replaced with strong immunofluorescence for the PV E4 protein.[142] This change is associated with at least a 100-fold increase in PV genome amplification.[132] It has been suggested that this vegetative genome amplification represents a switch to a different mode of PV DNA replication: from bidirectional theta replication in basal cells to rolling circle amplification in the intermediate epithelial cells.[166] The epithelial layer at which vegetative PV genome amplification occurs differs somewhat between different PV types.[167] Some, such as CPV-1, trigger vegetative genome amplification as soon as the host cells leave the basal layer while others, such as HPV-16, postpone vegetative genome amplification until later stages of differentiation.[167] It has been shown for HPV-16 that E4 is necessary for genome amplification in differentiated cells and this protein appears to induce cell cycle arrest in the G2 phase, which may allow sustained replication of viral DNA.[168] However this may not be the case for all PV types and E4 may have other functions that are important for genome amplification but which are currently less well understood.[169,170]

Terminal differentiation of the host cell in the superficial epithelial layers down-regulates the activity of the early polyadenylation signal resulting in transcription of the capsid protein genes L1 and L2.[140] L1 expression follows that of E4, occurring in a proportion of E4 expressing cells in the superficial epithelial layers.[142] Assembly of the PV virions occurs in the nucleus, and while L1 proteins can undergo self-assembly into capsids *in vitro*, L2 and possibly also E2 are necessary for efficient encapsidation of PV DNA.[171-173] Once assembled, the PV capsids undergo a relatively slow maturation process involving the formation of disulphide bonds between L1 molecules.[174] This maturation process is driven by the change from a reducing to an oxidising environment in the cornified cells prior to exfoliation.[174] Expression of the E4 protein in differentiated cells induces morphologic abnormalities in the cornified envelope which may make the cells more fragile and facilitate release of the infectious virions.[175] A study utilising an athymic mouse xenograft model showed that desquamated cornified cells containing HPV-11 were infectious to human foreskin fragments when intact but infectivity was increased following mechanical disruption of the cells, whereas free virus on its own was less infectious.[176] It has been suggested

that during sexual activity, desquamated cells would be deposited onto epithelial surfaces and that some of these would break open and release a large number of PVs onto a small focal area of genital epithelium.[176] An implication of this is that fewer infectious virions may be needed when intimate physical contact is likely. This may explain why genome amplification occurs relatively late in the lifecycle of high-risk HPVs (resulting in fewer infectious virions being produced) compared to other PVs, such as HPV-1 which infects human feet and CPV-1 which infects oral mucosa of dogs.[167] HPV-16 virions have been shown to maintain infectivity for 7 days at room temperature and although transfer via fomites is unlikely to be important for HPV-16, it is likely that other PV types are similarly resistant to dessication in the environment.[177]

The PV lifecycle, from infection of a permissive basal cell to production of infectious virions, is thought to take a minimum of three weeks and may take several months.[178] The PV does not cause cell lysis and virus amplification occurs in the terminally differentiated keratinocytes, which have little immune surveillance. Additionally, there is no viraemia and the PVs downregulate innate immune sensors so that there is little to no inflammation or antigen presentation. Thus the PV lifecycle is very effective at evading the innate immune response and delaying the adaptive immune response.[178]

1.3.2 High-risk HPV-induced cancer

For a small number of HPV types deregulation of the normal lifecycle can lead to the development of cancer. The World Health Organization International Agency for Research on Cancer currently recognises 12 HPV types as being carcinogenic to people, all of which belong to the alpha genera and infect mucosal tissues.[179] These are commonly referred to as the high-risk HPV types and the most well-known are HPV-16 and HPV-18. A key event in high-risk HPV-induced cancer is deregulation of the PV oncogenes, E6 and E7. In the normal PV lifecycle these genes are expressed in the suprabasal layers of the epithelium and the E6 and E7 proteins interact with multiple host targets to keep the cell in a synthesis-like phase of the cell cycle, allowing the virus to utilize host cell replication machinery to replicate its own genome.[147,180] In contrast, high-risk HPV-induced cancers are characterised by the expression of E6 and E7 throughout the full thickness of the epithelium (Fig. 1.6).[145] The pattern of immunofluorescent staining for the PV E6 and E7 proteins mirrors this change in gene expression: in low grade lesions the E6 and E7 proteins are restricted to the suprabasal cells whereas in high grade *in-situ* cancers there is strong immunostaining for E6 and E7 throughout the epithelium.[142]

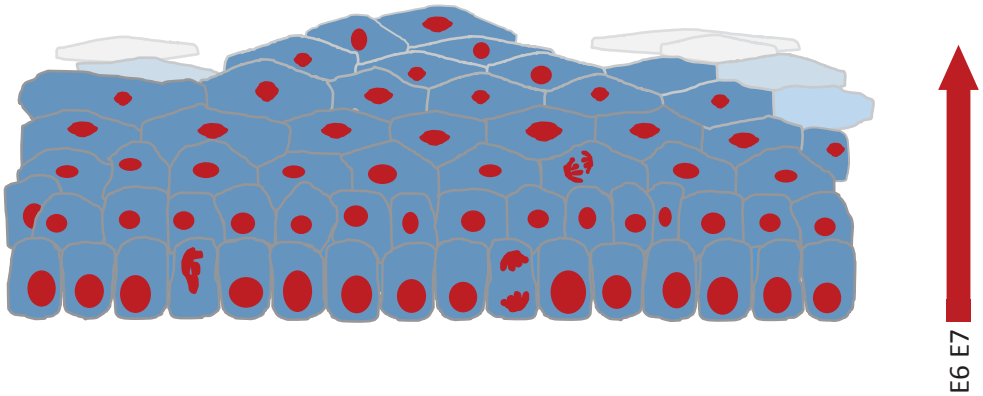

Figure 1.6. High-risk HPV gene expression in an *in situ* cancer (CIN3).

The consequences of increased levels of E6 and E7 include deregulated cell proliferation, cell immortalisation, and increased chromosomal instability. Deregulated cell proliferation is largely a consequence of the E7 mediated bypass of the G_1/S cell-cycle checkpoint, which is mediated by interactions between E7 and various host proteins, including pRb.[181] While the high-risk E7 protein alone is capable of inducing suprabasal cell proliferation, cell immortalisation requires E6-mediated activation of cellular telomerase as well as E7-mediated inactivation of the pRb pathway.[13,182] Transformation of the immortalized cells also requires the acquisition of genetic mutations, a process facilitated by the chromosomal instability induced by the PV proteins during mitosis. The effects of E6 and E7 on mitosis include: the formation of supernumerary centrosomes leading to cells with one or multiple spindle poles instead of the normal two; chromosomes misaligning or lagging during metaphase; and chromosome breakage.[183] Often such defects lead to the production of non-viable daughter cells but occasionally the acquisition of additional or rearranged chromosomes may confer a growth advantage.[184] Normally, cells with such genetic abnormalities would not be able to proliferate and would be either repaired or induced to undergo apoptosis. However E6-mediated degradation of p53 and inhibition of a range of apoptosis signalling molecules, which act independently of p53, interfere with this process and allow proliferation of these cells resulting in the development of cancer.[185]

A common mechanism of deregulation of E6 and E7 transcription is integration of the PV DNA into the host genome. The E6/E7 mRNA transcripts from integrated HPV-16 DNA are constitutively expressed and appear to be more stable, with a longer half-life, than E6/E7 transcripts from episomal HPV-16 DNA.[186] Around 40% of HPV-16-induced cancers contain only integrated HPV DNA, and a further 30% contain both integrated and episomal HPV DNA.[187] In comparison, all HPV-18- induced cancers contain exclusively integrated HPV DNA.[188] Integration appears to occur randomly in regards to the host genome and is not associated with a specific

nucleotide sequence. All human chromosomes can harbour integrated PV DNA and there is a predilection for transcriptionally active DNA and commonly fragile sites, although the biological significance of this is uncertain.[189,190] The events leading to integration of HPV DNA into the host genome are not well understood. It was initially thought to be a chance event, predisposed to by an ineffective immune response leading to persistent infections with high copy numbers of episomal HPV DNA. More recent studies however have shown that PV-induced chromosomal instability may lead to integration, implying that at least some degree of E6/E7 deregulation occurs before integration, although the mechanism of this remains unknown.[180] This has also been supported by studies showing that integration appears to be a relatively late event in cancer development with just 5% of CIN2 and 16% of CIN3 having transcriptionally active integrated HPV DNA compared to 88% of cervical cancers.[191] Further, it has been shown that integration does not always lead to the production of unusually stable E6/E7 mRNAs; some cell-lines contain hundreds of copies of integrated PV DNA, and the majority of these are transcriptionally silent. A study detecting PV RNA by *in-situ* hybridisation found that most HPV-induced cancers contained only one site of transcriptionally active HPV DNA, and when multiple sites were present there was selection for cells with only one active integration site, with other sites silenced by DNA methylation.[192] The factors that determine whether integrated HPV DNA is silenced or transcriptionally active are still being determined. Sites of integrated PV DNA commonly have disruption of the E2 ORF and it is thought that if intact, the E2 protein inhibits transcription of integrated E6 and E7.[193,194] Episomally derived E2 protein may also repress transcription from integrated PV DNA. Supporting this, interferon beta treatment of a HPV-16 infected cell line caused a rapid loss of HPV-16 episomes and emergence of cells bearing previously latent integrants, in which there was increased expression of E6 and E7.[195] Various host factors, such as the cytokine tissue necrosis factor-α (TNFα), have also been shown to be capable of repressing HPV E6/E7 transcription from integrated HPV DNA.[196] The proposed interplay of events from high-risk HPV infection to cervical cancer is summarized diagrammatically Fig. 1.7 which has been adapted from Pett and Coleman (2007).

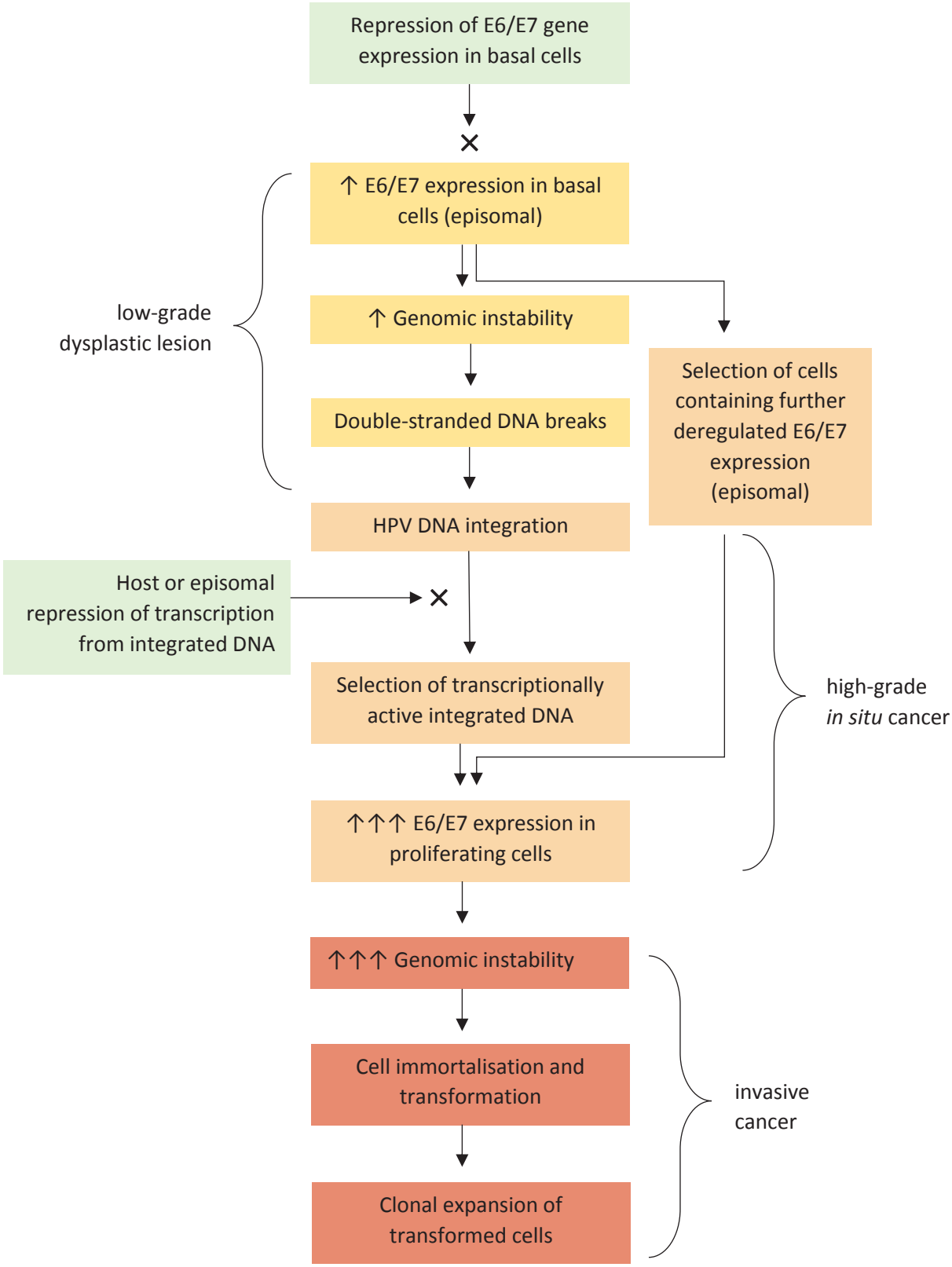

Figure 1.7. Proposed mechanism of PV-induced cancer. Adapted from: Pett M, Coleman N. Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? *J Pathol.* 2007;212:356-367.

The key differences that account for the oncogenic potential of the high-risk HPVs compared to the low-risk HPVs are still being determined and have been the focus of numerous studies. The oncogenic potential of a particular HPV type is probably determined by the properties of its E6 and E7 proteins, as it has been shown that E6 and E7 proteins from high-risk, but not low-risk, HPV types can immortalize primary cell lines *in vitro.*[197] Classically, the difference has been most attributed to interactions of E6 and E7 with the cell-cycle regulatory proteins pRb, p53, selected PDZ domain containing proteins, and telomerase. For example, it has been shown that E6 from the high-risk HPVs (HPV-16 and -18) but not the E6 from the low-risk HPVs (HPV-6 and -11) promotes ubiquitin-mediated proteasomal degradation of p53.[151] Similarly while all HPV types bind to pRb, only HPV16 E7 has been shown to mediate proteasomal degradation of pRb via a cullin 2 ubiquitin ligase complex.[198] Activation of telomerase and the ability to bind to PDZ domains of certain host proteins are also features exclusive to the high-risk HPVs.[154,199] However, more recent studies have suggested that expression of the host cyclin dependant kinase inhibitor $p16^{INK4A}$ (p16) in response to high-risk E7 expression is the main carcinogenic determinant, and degradation of pRb by high-risk HPV E7 proteins has evolved from the need to negate p16 induced cellular senescence.[200] Evidence for this includes markedly reduced cell viability in HPV-16 E7 expressing carcinoma cell lines after depletion of p16, or KMD6B (the histone demethylase which appears to be responsible for the increased p16 in HPV-induced cancers), and then rescue of the cells by re-expression of p16.[201]

1.3.3 Host factors in high-risk HPV-induced cancer

While infection with high-risk HPV is necessary for the development of cervical cancer, less than 1% of these infections eventually progress to invasive cervical cancer, suggesting that other host or environmental factors are also involved.[80,202,203] Viral persistence, the type of cells infected and hormonal effects are some of the factors thought to determine whether an infection will progress to cancer. Epidemiologic studies suggest that the longer a high-risk HPV infection persists, the greater the chances of cancer development. Most HPV cervical infections resolve within 12 months and those that persist for longer have an approximately 5 fold greater risk of developing cancer by 30 months than newly acquired infections.[80] Resolution of HPV-16 infections is associated with a cytotoxic T-cell response directed again the PV early proteins, particularly E6.[204] Immunosuppression, particularly of the cell-mediated immune response, reduces the rate of HPV clearance; HIV positive adolescent girls had a longer time till clearance

of initial infection (689 days) than HIV-negative girls (403 days) and the rate of clearance in the HIV-positive girls increased with increasing $CD4^+$ T cell counts.[205] The development of cervical cancer has also been shown to have a significant inherited component leading to the speculation that there may be genetic differences in the effectiveness of cell-mediated immunity at clearing high-risk HPV infections.[206] The strongest candidate genes appear to be those located in the human leukocyte antigen (HLA) region of chromosome 6 which code for the major histocompatibility molecules (MHC). MHC class II molecules are present on the surface of antigen presenting cells and present peptides derived from extracellular proteins to T-helper lymphocytes. It has been shown that carriers of two specific haplotypes (DRB1*1501 and DQB1*0602) for the beta subunits that make up the peptide binding groove of MHC class II molecules confer a reduced ability to clear HPV-16 infections.[207] The association between these haplotypes and cervical cancer has been recently confirmed by a large study of Swedish women using single nucleotide polymorphism (SNP) analysis of over 700,000 known SNPs in the human genome.[208] This study identified 55 SNPs significantly associated with the development of cervical cancer, all of which were located in the genes coding for major histocompatibility molecules confirming the importance of antigen presentation in the development of cervical cancer. As well as genes coding for MHC class II beta subunits, a significant association was found with a particular MHC class I haplotype and a variant of the gene coding for MHC class I polypeptide-related sequence A (MICA), a cell surface glycoprotein that is expressed in response to cellular stress and activates anti-tumour immune responses.[208] While the associations between cervical cancer and the aforementioned genes are significant, they are not particularly strong and it remains to be determined whether they account for the strong heritability of cervical cancer or whether there are additional genes involved.

Further clues about the host factors associated with HPV-induced cancer come from studies investigating the particular susceptibility of the cervix to HPV-induced cancer. Although the high-risk HPVs commonly infect mucosa of the vulva, vagina, cervix, anus, penis, and oral mucosa, cancer of the cervix is far more common than cancer of any of the other sites; globally in the year 2002 cervical cancer accounted for 4.5% of the total number of cancer cases, compared to cancers of the vulva and vagina which made up just 0.2%.[209] Both the presence of a particularly susceptible population of cells and hormonal effects have been identified as important host factors for the development of cervical cancers. Establishment of a productive PV infection is thought to require access to the basement membrane and transfer to a mitotically active stem cell. At the cervix, reserve (stem) cells are located in an area called the transformation zone where they can proliferate to populate the stratified squamous epithelium of the lower genital

tract.[210] Herf et al (2012) investigated 120 cervical lesions and found that all of the high grade and cancerous lesions (CIN2/3, SCCs, adenocarcinomas) came from a discrete population of embryonal cells adjacent to the transformation zone at the squamo-columnar junction, whereas the lower grade lesions came from both the stratified squamous epithelium of the ectocervix and the junctional cells.[211] Thus it may be that cervical cancer develops primarily at the squamo-columnar junction because these cells are exposed and susceptible to infection but fail to regulate viral gene expression, leading to a non-productive or abortive infection.[135] The phase of the cell cycle or differentiation state of the cell at the time of virus entry may also be important in determining whether HPV gene expression can be appropriately regulated. A recent study attempted to reconstruct the early events of HPV-induced cancer using an immortalised neonatal foreskin keratinocyte cell line transfected with HPV-16.[180] Interestingly, when cultured on a fibroblast and collagen plug at an air-liquid interface (an organotypic raft culture), some cell line clones developed high-grade dysplasia associated with E6 and E7 deregulation and loss of normal contact inhibition whereas other clones maintained a low grade dysplasia with more normal patterns of HPV gene expression. There were no differences in HPV genome methylation patterns or chromosomal instability between the clones and in all cases the HPV-16 DNA remained episomal.[180] Therefore, the different outcomes in this model could only be explained by differences in cell cycle or differentiation states of the infected cells, or possibly the copy number of HPV DNA at initial establishment.

Chronic exposure to oestrogen has also been implicated as a factor causing deregulation of HPV gene expression in the early stages of cancer development in the cervix, and long-term use of oestrogen containing oral contraceptives is a risk factor for the development of cancer at this site.[212] The developing squamous cells in the transformation zone of the cervix have a particularly high concentration of oestrogen receptors.[213] Binding of these receptors stimulates cell proliferation and oestrogens have also been shown to promote HPV early gene transcription, change the local immune environment, inhibit apoptosis, induce chromosomal aberrations, and produce carcinogenic metabolites; any or all of which may contribute to its synergistic effects with HPV in inducing cervical cancer.[214,215] Several other host factors increase the risk of developing cervical cancer, such as smoking and nutrient deficiencies, but rather than influencing regulation of HPV gene transcription these factors probably reduce the effectiveness of cell-mediated immunity at clearing HPV infections and would be common to all anatomical locations.

1.3.4 Beta HPVs and skin cancer

The cutaneous beta PV types HPV-5 and -8 are capable of causing skin cancer in people with the condition epidermodysplasia verruciformis (EV).[216] EV is a rare autosomal recessive skin disease where mutations in transmembrane channel-like 6 and -8 genes (EVER1/2) confer increased susceptibility to infection with a group of so-called EV type beta PVs.[217] EV patients develop numerous flat warts and macules during childhood and frequently develop skin cancers as adults, particularly on areas of skin exposed to ultraviolet radiation (UV).[216] The mechanism of cancer development is different to that of the high-risk PVs as the cancers contain episomal, rather than integrated, HPV DNA and retain the ability to produce the PV E4 and L1 proteins, features which are lost in high-risk PV-induced cancers.[218] A recently proposed model suggests that the EVER1 and EVER2 proteins form a complex with a zinc transporter (ZnT1) to limit the concentration of available zinc cations in the nucleolus, reducing the activities of several transcription factors including activator protein 1 (AP-1).[217] The mutated EVER1 or EVER2 proteins in EV patients cannot perform this constitutive repression of AP-1 and other transcription factors, consequently there is increased transcription of genes that promote cell proliferation and HPV gene expression, which presumably accounts for the increased susceptibility to beta PV-induced lesions.[219] Interestingly, EV patients are uniquely susceptible to PVs from the beta genus, whereas their susceptibility to the alpha PVs is the same as immunocompetent people. One explanation for this may be the lack of an E5 ORF in the EV-type beta PVs as the HPV-16 E5 protein has been shown to interact with the EVER1/EVER2/ZnT1 complex, inhibiting its repression of transcription.[219] EV type beta PVs may therefore be unable to release the repressive effects of the EVER1/EVER2/ZnT1 complex in immunocompetent hosts providing a barrier to replication of beta PVs that is readily bypassed by alpha PVs. As well as explaining the unique susceptibility of EV patients to the beta PVs, this model could also potentially explain the limited ability of beta PVs to induce cell proliferation in immunocompetent people.

The beta PVs have also been implicated in the development of skin cancers in chronically immunosuppressed people, particularly organ-transplant recipients who have a high incidence of warts and a 65 fold greater risk of developing SCCs than immunocompetent people.[220] The role of beta PVs in these cancers is still contentious. Active beta PV infections have been demonstrated in premalignant skin lesions and also in dysplastic skin surrounding invasive cancers but not in the cancers themselves.[27,221] If the beta PVs are involved in the development of these cancers, it may be that they are a cofactor, along with UV-induced DNA damage, in

initiating cancer development but are not required for maintenance of the malignant phenotype. Evidence for a co-operative role between UV radiation and beta PVs in the development of non-melanoma skin cancer includes the observation that expression of beta PV E6 protein protects cells from UV-induced apoptosis.[222] In response to UV exposure, p53 was upregulated in beta PV E6 expressing cell lines (whereas the high-risk HPV cells lines had reduced p53 due to E6-mediated degradation), but the E6 expressing cells were still protected from p53-mediated apoptosis through degradation of the pro-apoptotic protein Bak.[222] Thus, as with the high-risk alpha PVs, the beta PV E6 and E7 proteins appear to be key for the development of beta PV-induced cancer, but the mechanism is different and less well understood. Although the beta-PVs generally do not cause degradation of p53, the E6 protein has been shown to degrade the histone deacetylase p300, which may bypass the function of p53.[223] Additionally the E6 protein of beta HPV-38 and -8, and to a lesser extent HPV-5, can activate telomerase and prolong the lifespan of primary keratinocyte cell cultures.[224] Another function of E6 from HPV-5 and -8 is degradation of the transcription factor mothers against decapentaplegic homolog 3 (SMAD3), which inhibits transcription of transforming growth factor β (TGF-β) induced genes, including those that code for the cyclin-dependant kinase inhibitors p16, p17, p21 and p27.[217,225] This might promote progression through the G_1/S checkpoint and may explain the increased oncogenic potential of these two beta PV types, as another beta PV type (HPV-9), that has not been associated with skin cancer, was not able to degrade SMAD3. The beta E7 proteins interact with pRb, however E7 from beta HPV-5 and -8 did not cause cellular proliferation in the suprabasal layers of organotypic raft cultures.[198,226] Interestingly in the same study, E7 from HPV-5 and -8 caused upregulation of p16 which co-localized with cyclin E, indicating that although the suprabasal cells were not proliferating, they did appear able to overcome the effects of p16 and progress through the G_1/S checkpoint.[226] More recent studies have found an interaction between beta PV E6 and mastermind-like protein 1 (MAML1), a transcriptional coactivator in the Notch signalling pathway which promotes keratinocyte differentiation and withdrawal from the cell cycle, but overall the mechanism of cancer development is still largely unknown for the beta PVs.[152]

1.3.5 PV-induced cancer in animals

Investigations into the mechanism of cancer development of several animal PVs have revealed similarities to both high-risk HPV-induced cancer and beta HPV-induced cancer in people, as well as novel mechanisms of cancer development unique to animal PVs.

The mechanism by which SfPV-1 causes cutaneous SCCs in rabbits appears to be similar to the mechanism of high-risk HPV-induced cancer, although there are some differences. In both instances the E6 and E7 proteins play a central role. Similar to the high-risk HPVs, constitutive expression of SfPV-1 E7 *in vitro* can immortalise primary rabbit keratinocytes and the effect is enhanced by co-expression of E6 and E7.[227] The SfPV-1 E7 protein also degrades pRb and promotes cell proliferation in a similar manner to the high-risk HPVs.[227] However unlike the high-risk HPV E6 protein, the SfPV-1 E6 protein does not target p53 for degradation. This was demonstrated in E6/E7 expressing rabbit keratinocyte cells lines which contained elevated levels of p53, and this was shown to be functional by further inducing p53 expression with chemical DNA damage.[227] However, despite the presence of functional p53, the E6/E7 expressing rabbit keratinocyte cell lines continued to proliferate suggesting a bypass of p53-mediated growth arrest, although the mechanism of this remains unknown. The physical state of the PV DNA also differs between high-risk HPV-induced cancers, which frequently contain integrated PV DNA, and rabbit SCCs in which the SfPV-1 DNA is predominantly episomal.[228] Transcription patterns also differ: progression of HPV-induced dysplastic cervical lesions (CIN2) to *in situ* carcinomas (CIN3) has been associated with an 8-10 fold increase in the abundance of E7 transcripts and a marked reduction in E2-specific transcripts whereas there appears to be little difference in the quantity or type of mRNA present between SfPV-1-induced papillomas and carcinomas in rabbits.[229,230] Additionally, transcripts coding for the PV capsid proteins were absent in HPV-induced cervical SCCs and rare in CIN3 lesions even when using a highly sensitive RNA sequencing technique, whereas several rabbit SCCs have been shown to contain SfPV-1 L1 transcripts by *in-situ* hybridisation, a much less sensitive technique.[229,231] The presence of low levels of L1 transcripts in rabbit SCCs may reflect the predominance of episomal rather than integrated viral DNA.

Mastomys natalensis papillomavirus type 1 (MnPV-1) infected mice have been advocated as a model for beta HPV-induced cancer in people. Spontaneously occurring papillomas and keratoacanthomas (a benign skin tumour originating in the hair follicle) in the multimammate mouse *Mastomys coucha* (formerly *Mastomys natalensis*) contain large quantities of MnPV-1

DNA in both the lesions and adjacent normal skin.[124] The MnPV-1 DNA does not appear to be integrated in any of these lesions, and the lesions do not spontaneously regress but may progress to SCCs with additional stimulus.[232] This is similar to the skin lesions on patients with EV, which contain large quantities of episomal beta HPV DNA and readily progress to SCCs in areas exposed to UV.[216] Constitutive expression of MnPV-1 E6 in the basal cells of transgenic mice did not result in any histologic changes relative to the wild-type MnPV-1 infected mice, however it did increase the development of SCCs after topical carcinogen and tumour promoter treatment.[233] MnPV-1-induced SCCs frequently contain mutations in HRas proto-oncogene GTPase (H-ras), an enzyme involved in regulating cell division in response to growth factor stimulation.[233] However, the SCCs from the E6 transgenic mice had predominantly wild-type H-ras which suggests that the MnPV-1 E6 protein facilitates cancer development but requires the acquisition of additional mutations including, but not limited to, H-ras activation.[233]

Another mouse PV, *Mus musculus* papillomavirus type 1 (MmuPV-1), causes papillomas and benign skin cancers, called trichoblastomas, in several inbred laboratory mouse strains with deficient T-cell immune responses.[234] These lesions appeared to arise from the follicular infundibulum, supporting previous studies suggesting the existence of a susceptible cell population at this site.[136] Transcriptome analysis showed 26 host genes that were dysregulated in the trichoblastomas, providing targets for future investigation.[234] However, there were no clear insights into the mechanism of cancer development as the changes were small and there was considerable overlap in the genes dysregulated between the normal skin and papillomas, and the papillomas and trichoblastomas.[233]

Several bovine PVs display a unique mechanism of cancer development. A key mechanism in BPV-induced cancer appears to be binding of the BPV E5 protein to cellular platelet-derived growth factor beta (PDGFβ) receptors, causing continued activation of this receptor which leads to increased cell proliferation and loss of contact inhibition in primary bovine fibroblasts.[160,235] In contrast, the BPV E6 and E7 proteins have a much weaker transforming ability.[236] *In vivo* evidence for a role of the BPV-2 in the development of bovine bladder cancer includes the co-localisation of BPV E5 protein and PDGFβ receptors in naturally occurring cancers.[237] However, although BPVs can transform cells, they have not been shown to be capable of immortalizing primary cell lines *in vitro* and BPV-induced bladder cancer does not occur in regions where cattle do not graze bracken fern (which contains immunosuppressive and carcinogenic compounds). Therefore, the relative contribution of the virus compared to these exogenous carcinogens in bovine bladder cancer is currently unknown.[63]

Interaction of the BPV E5 protein with PDGFβ receptors is also thought to be important in the development of equine sarcoids. BPV-1, -2 and -13 are thought to cause equine sarcoids, fibroblastic skin tumours of horses that occasionally display locally aggressive behaviour.[49] High copy numbers of BPV DNA and immunostaining for the BPV E5 and E7 proteins has been demonstrated in equine sarcoids, in both the neoplastic fibroblasts and the overlying epidermis.[238] Additionally, immunostaining for PDGFβ receptors was present in the neoplastic fibroblasts but not adjacent normal fibroblasts.[239] More recently, it has been shown that BAG family molecular chaperone regulator 3 (BAG3) is frequently increased in equine sarcoids and this appears to protect the cells from apoptosis.[240] BAG3 is a co-chaperone protein that can bind to anti-apoptotic proteins, protecting them from proteosomal degradation. BAG3 has been shown to facilitate cancer cell survival in a human colon cancer cell line and it has been hypothesized that BAG3 may protect PV E5 and E7 proteins from degradation in equine sarcoids.[240,241] Interestingly, there appears to be a breed predisposition for equine sarcoids and several MHC class I and II haplotypes are associated with a significantly increased risk of developing sarcoids.[242] This suggests that the ability of the host to present BPV peptides to initiate an adaptive immune response may be important for determining the outcome of infection.

Another bovine PV, BPV-4, causes benign fibropapillomas in the alimentary tract of cattle, which can progress to cancer in cattle grazing bracken fern. In contrast to E5 from BPV-1 and -2, the BPV-4 E5 protein does not appear to activate PDGFβ receptors, although it does alter the transcription of cell-cycle factors, including cyclin A.[243] The role of the BPV-4 proteins in neoplastic transformation of alimentary papillomas is uncertain. A number of other functions of BPV E5 have been recently reviewed by DiMaio (2013) and these may contribute to the transforming ability of the BPV-4 E5 protein. These functions include inhibiting acidification of intracellular organelles, inhibiting cell-cell communication, and reducing surface expression of MHC class I molecules.[244]

Recent studies have implicated *Equus caballus* papillomavirus type 2 (EcPV-2) in the development of equine penile SCCs, although the mechanism has not yet been determined.[64,65] The most compelling evidence for a role of EcPV-2 in equine penile SCCs was the frequent detection of large quantities of EcPV-2 DNA in the SCCs, whereas little to no EcPV-2 DNA was present in a large number of other equine samples.[65] However, both pRb and p53 immunostaining were frequently present in equine penile SCCs suggesting that, if EcPV-2 does induce penile SCCs, the mechanism is very different from that of the high-risk HPVs.[245]

PVs have also been implicated in the development of multiple viral plaques and *in situ* carcinomas on the skin of dogs, a condition the authors likened to EV.[62,246] A breed predisposition has been suggested but this has not been investigated further and an inherited defect in EVER1/2 seems unlikely as at least one case presented in an old dog.[62] CPV-2 has also been associated with skin cancer, particularly the development of aggressive SCCs on the footpads of immunosuppressed dogs.[61] The CPV-2 E6 protein has zinc- and PDZ-domain binding motifs suggesting similar cellular interactions to the high-risk HPVs. Interestingly, the CPV-2 E7 protein does not have the conventional pRb binding site yet still manages to degrade pRb through an alternative binding domain.[247]

Overall, it appears that PVs have evolved many different mechanisms of manipulating cell-cycle regulation which has led to multiple mechanisms of cancer development. Many of the mechanisms are still being determined but a key factor in all cases so far has been the interactions of the PV E6, E7, and sometimes E5 proteins, with host cell-cycle regulatory proteins. Therefore, it appears that alterations in the normal levels, distribution, and activity of these proteins fundamentally underlies all PV-induced cancer.

1.3.6 Summary of the natural history of PV infection

The current understanding of the epidemiology and molecular mechanism of PV infection in animals and people can be combined in the following model of the natural history of PV infection. Infection arises when a PV gains access to basal epithelial cells at a site of microtrauma, in an immunologically naïve animal. The source of infectious virions is usually direct contact with another infected animal or less commonly via fomites. The consequence of this initial infection depends on the type of cell infected and the stage of the cell-cycle. If the PV infects a basal cell that is proliferating, for example during wound-healing, this will facilitate expression of viral proteins creating a productive PV infection that may result in lesion development after several weeks. Alternatively the PV may infect a basal cell that is not mitotically active and will not support viral gene expression resulting in persistence of the PV DNA but without genome amplification or the production of progeny virions. If the PV infects a cell in the suprabasal layer, the cell will have already begun the process of differentiating so PV replication will not be supported and the PV will be lost when the cell sloughs from the surface. Once a suitable basal cell is infected, the virus will replicate as the cell divides creating a small focus of infected basal cells. Some of these cells will exit the cell cycle and differentiate, moving

progressively upwards through the epithelium to the surface. PV gene expression is tightly co-ordinated to this differentiation process. Expression of the early genes, particularly E6 and E7, in the suprabasal layers creates an environment that allows the virus to utilise host cell DNA replication machinery. Vegetative replication of the PV genome occurs in the intermediate epithelial layers and infectious virions are assembled in the superficial layers, then remain in the cornified squames as they are sloughed off into the environment. Such productive lesions are initially 'hidden' from the immune system for several weeks to months. Development of a type-specific humoral immune response occurs first and prevents subsequent infection by preventing PVs from binding to the basement membrane and entering cells. Following this, development of a cell-mediated immune response will either clear the existing infection completely, or surpress viral protein expression but allow the PV genome to persist in a few infected basal stem cells. The latter situation has been described as a latent PV infection and can be reactivated by stimuli such as wound repair or UV irradiation. Due to the presence of tissue-resident memory T cells, any such reactivation will be rapidly recognized and brought under control. In rare instances, deregulation of PV gene expression in a productive infection can lead to unregulated cell proliferation, cell immortalisation and chromosomal instability which leads to the acquisition of additional genetic mutations and the development of invasive cancer. A number of host, viral and environmental factors are associated with the development of invasive cancer. These include specific PV types, such as the high-risk HPVs, and the effectiveness of the cell-mediated immune response at clearing productive PV infections. Certain populations of cells also seem particularly susceptible, for instance the junctional cells of the cervical transformation zone, although the stage of the cell cycle and the infective dose is probably also important. Environmental factors may also be important, either directly or indirectly. An example of a direct effect is exogenous carcinogens which may speed up the acquisition of additional genetic mutations in the final stages of the progression from highly dysplasic PV-induced lesions to invasive cancers. Environmental factors can also alter the effectiveness of the cell-mediated immune response at clearing PV infection, thereby indirectly contributing to cancer development.

1.4 PV infections of domestic cats

1.4.1 The discovery of PV-induced lesions in cats

By the early 1980s PV-induced papillomas had been described in many domestic animals including rabbits, cattle, horses, dogs and sheep.[248] It had also been observed that some of these PV-induced papillomas could progress to squamous cell carcinomas (SCCs), a common and aggressive type of skin cancer.[4] Reviews of feline skin tumours during this time noted the rare occurrence of squamous papillomas, but PV-induced papillomas were not described.[249-251] Two studies using immunohistochemistry to detect PV capsid proteins in a variety of epithelial lesions failed to detect any PV capsid proteins in a number of feline lesions.[252,253] The first report of PV-induced lesions in domestic cats came from Carney *et al.* (1990) who described multiple, sessile, hyperkeratotic plaques on two unrelated geriatric Persian cats, both of whom had been receiving long term immunosuppressive therapy.[254] Grossly these plaques were not typical of PV-induced papillomas seen in other species but the cellular changes noted microscopically were considered to be indicative of a productive PV infection. Immunohistochemistry, electron microscopy, and cross-hybridisation with other PV DNA confirmed the presence of PV in these lesions, which are now referred to as feline viral plaques (FVPs).[254,255] A similar case was reported soon afterwards in a 6 year old non-purebred cat that was infected with feline immunodeficiency virus (FIV).[256] The authors speculated that cats may be commonly infected with PVs but clinical manifestations only occur in cats that are immunosuppressed. A potential role of immunosuppression in PV-induced lesion development has been suggested several times subsequently but currently there is no conclusive evidence. A more typical PV-induced papilloma was later found on the eyelid of a 4 year old domestic short hair cat (DSH) and noted to have intranuclear inclusions in keratinocytes of the stratum spinosum and stratum granulosum, a feature often seen in PV infection in other species.[257] Papillomavirus capsid antigens were detected within these keratinocytes, providing the first evidence of a typical viral papilloma in domestic cats. Few such lesions have been reported since.

1.4.2 Early studies on feline viral plaques and Bowenoid *in situ* carcinomas

Not long after Carney's description of FVPs, several case series were published describing another lesion with microscopic features suggestive of PV infection.[254,258-260] These were initially referred to as feline multicentric squamous cell carcinoma *in situ* and resembled Bowen's disease in people. However they were later renamed Bowenoid *in situ* carcinomas (BISCs) reflecting several small but significant differences to the human condition.[261] Clinically the lesions were often indistinguishable from FVPs usually presenting as multiple, pigmented, occasionally ulcerated, flat plaques on the face, limbs and trunk.[262] Similar to FVPs, BISCs consist of well-demarcated epidermal hyperplasia with irregular acanthosis, hyperpigmentation, hypergranulosis with clumped keratohyalin granules and koilocytes. Both lesions may also contain abnormal keratinocytes in the stratum spinosum with swollen blue-grey fibrillar cytoplasm, large perinuclear vacuoles, and vesicular or shrunken nuclei. These latter cells have been variably reported as koilocytes, clear cells or simply described as abnormal keratinocytes. The most noticeable difference between FVPs and BISCs is that FVPs usually retain the normal organisation of the epidermis whereas in BISCs the epidermis is disorganised with loss of normal stratification. Additionally cellular atypia including anisocytosis, multinucleated keratinocytes, and rare mitoses three or more cell layers above the basal layer are present only in BISCs.[262] While follicular involvement is sometimes seen in FVPs, it is a consistent feature of BISCs.[262] In 1992 Miller *et al.* reported five cases of BISC diagnosed by the Dermatology Service at the College of Veterinary Medicine at Cornell University. Prior to 1990 the Dermatology Service had not diagnosed the condition in over 550 biopsies. This was consistent with the lack of any published report of the lesion prior to 1990.[258] This relatively recent increase in the incidence of the disease has not been explained. Most human patients with Bowen's disease have a solitary squamous cell carcinoma *in situ* on sun-exposed skin. UV exposure, arsenic compounds and PVs have been implicated.[263] Several authors have noted that BISCs in cats are usually multiple and occur in haired or pigmented skin thus UV light is not likely to be an inciting factor. These authors also ruled out exposure to arsenic compounds leaving PV infection as a plausible aetiology.[258,260] The presence of histologic changes typical of a productive PV infection, including swollen keratinocytes in the stratum spinosum and clumped keratohyalin granules, also supported a PV aetiology although these changes were not always present.[264,265] Some of the cats that had BISCs were FIV positive or may have had local immunosuppression due to *Demodex cati* mites, suggesting that immunosuppression may be a contributing factor in the

development of BISCs, as well as FVPs.[258,266] However many of the affected cats had no evidence of immunosuppression.[258,264]

The similarities between FVPs and BISCs led to the hypothesis that BISCs develop from FVPs. This idea was investigated by Wilhelm *et al.* (2006), who evaluated cutaneous plaques from 26 cats and differentiated them by histology as well as doing immunohistochemistry for the presence of PV capsid protein.[262] Of the 26 cases, seven were diagnosed as FVPs and six of these were positive for PV capsid protein. Ten cats had both FVPs and BISCs, sometimes in the same skin sample, and transitional lesions between the two were occasionally observed supporting a common aetiology. Similar transitional lesions have been reported by other authors.[267] All of these cases were positive for PV capsid protein whereas only one out of the nine exclusively BISC cases was positive.[262] This may suggest that FVPs and BISCs have a different aetiology or it may be that the neoplastic transformation disrupted viral replication, a phenomena previously noted in SCCs arising from viral papillomas in cottontail rabbits.[58] Interestingly one author also reported cases with lesions that contained areas of SCC surrounded by areas more characteristic of BISCs.[260] A higher proportion of BISCs were positive for PV capsid protein by immunohistochemistry in another study which reported 11 positive cases out of 23.[268] Interestingly in that study, seven cases were tentatively diagnosed as advanced actinic keratosis but had some features of BISCs and one was positive for PV capsid protein by immunohistochemistry. This indicates that there may be some ambiguity between the two diagnoses in a small proportion of advanced lesions.[268]

1.4.3 PCR and sequencing of the first feline PV

The first studies using polymerase chain reaction (PCR) to detect feline PV DNA were based on consensus primers for the conserved L1 gene that codes for the major viral capsid protein. Kidney *et al.* (2001) designed consensus primers by aligning various ungulate PV L1 sequences as well as those from a mouse PV and CPV-1. Their study did not detect any PV DNA by PCR in 50 vaccine site associated sarcomas.[269] Another study used primers that had been previously designed to detect a segment of the L1 gene of a range of human PVs: FAP59 and FAP64. These also failed to amplify any PV DNA in skin swab samples from five clinically normal cats.[114] Just over a decade after Carney *et al.* (1990) identified FVPs on two Persian cats, the first feline PV was fully sequenced from PV DNA that had been isolated from these lesions.[270] This new virus had an 8300 bp genome with a typical PV genome structure and was designated *Felis domesticus*

papillomavirus type 1 (FdPV-1).[270] FdPV-1 was most closely related to canine oral papillomavirus (CPV-1) and both FdPV-1 and CPV-1 have been subsequently reclassified to the Lambda genus with FdPV-1 also updated to reflect the correct taxonomic classification of the host species, now *Felis catus* papillomavirus type 1 (FcaPV-1).[271,272]

1.4.4 PCR of papillomas, FVPs and BISCs

Subsequent to the publication of the genome sequence of FcaPV-1, Nespeca *et al.* (2006) designed consensus primers to detect the highly conserved PV E1 gene in a series of feline BISCs and SCCs. Seven novel PVs were amplified from 5 of 21 BISCs and 4 of 22 SCCs. These new PVs had a relative amino acid identity of 56% and 71% with FcaPV-1 and CPV-1 respectively. Because the classification of PVs is based on the sequence of the L1 gene, the taxonomic classification of these novel PVs could not be determined. Shortly following, Munday *et al.* (2007) also amplified two novel PVs from formalin fixed paraffin embedded (FFPE) BISCs initially using the FAP59/FAP64 primers and then designing a new primer set based on the sequences amplified. With these more specific primers (JMPF and JMPR) PV DNA was amplified from 11 of the 18 BISCs and six samples were sequenced. Five of these contained an identical novel PV which was most closely related to HPV-17 and was quite phylogenetically distant from both CPV-1 and FcaPV-1.[273] This PV later turned out to be the same as one of the novel PVs amplified by Nespeca *et al.* The other novel papillomavirus amplified by Munday *et al.* was 97% homologous to an already identified human PV. Interestingly, none of the seven novel PVs amplified from feline BISCs were FcaPV-1.

In 2007, Munday *et al.* published the second confirmed case of a typical PV-induced papilloma in a cat.[274] This exophytic mass from the dorsal nasal surface of a 12 year old DSH cat had histologic features of PV infection and PV DNA was amplified by PCR using the FAP59/FAP64 primer set. Sequencing of the amplicon revealed 98% similarity to HPV-9.[274] This was surprising given the high host specificity of PVs. As HPVs have only rarely been detected in feline lesions it remains to be seen whether these represent a true infection.[274,275] Another study, the first study to use PCR for PVs on FVPs, utilised 3 different primer sets on samples from cotton swabs taken from both the plaques and normal skin of an affected cat.[276] The JMPF/JMPR primers amplified the same PV that had been previously amplified from BISCs and the MY09/MY11 primers amplified a novel PV.[265,273,276] Some interesting observations from this study included the successful amplification of PV DNA from skin swabs, a non-invasive sampling method, and the presence of multiple PV

types in both lesional and non lesional skin swabs complicating the perceived relationship between the viruses and the development of the lesions.

The studies published from 1990 to 2008 therefore led to the following conclusions: most FVPs had evidence of productive PV infection as did a small proportion of BISCs. Furthermore, some BISCs may have developed from FVPs and this transformation may have disrupted the normal PV lifecycle, as occurs in other species.[58] Consistent with the hypothesis that PVs caused BISCs was the frequent detection of PV DNA in these lesions. However several different PVs had been found in both lesional and non-lesional skin, and the previously classified FcaPV-1 had only been found in a small number of FVPs and no BISCs. Therefore, it was generally accepted that a feline PV caused FVPs although it was not known which PV type was responsible and whether immunosuppression was also required. Conversely, while an association between BISCs and PVs was certain, it was not clear whether the PVs actually caused BISCs or whether they were merely present in the lesions.

1.4.5 Sequencing of the second feline PV

In 2009, Lange *et al.* extracted DNA from frozen and paraffin-embedded tissue of three BISCs and used rolling-circle amplification to amplify, clone, and then sequence a 7899 bp PV designated *Felis domesticus* Papillomavirus type 2 (FdPV-2), which was present in all three BISCs.[42] Interestingly the sequence had a binding motif for pRb (LXCXE), a feature generally restricted to the high-risk mucosal HPVs.[277] Unlike the alpha PVs however, a potential E5 ORF could not be identified in FdPV-2. Pairwise alignment of the FdPV-2 L1 ORF sequence to that of the closest related PV revealed 57.1-58.1% identity and comparison with FcaPV-1 revealed only 51.1% identity.[42] Thus FdPV-2 appeared to be a new genus of PV. This was later confirmed with FdPV-2 classified as the first PV in the Dyotheta genus, species dyothetapapillomavirus 1, and the PV type was updated to *Felis catus* papillomavirus type 2 (FcaPV-2).[14]

Subsequent to the complete sequencing of FcaPV-2 it was determined to be the most consistently present PV in FVPs, BISCs and SCCs.[42,265,276] For instance, Munday *et al.* (2010) amplified FcaPV-2 from 14 of 14 viral plaques, with primers specific for FcaPV-2 (JMPF/JMPR), but only 1 of 14 trichoblastomas, a benign follicular skin tumour.[278] The study also used two sets of consensus primers (MY09/MY11 and FAP59/FAP64) which amplified PV DNA from 8 of the 14 viral plaques including FcaPV-2 and two novel PVs designated FdPV-MY1 and FdPV-MY2.[276,278]

The greater sensitivity of the JMPF/JMPR primers compared to the consensus primers indicated that previous studies using consensus primers may have under-reported the prevalence of PV DNA in feline lesions. The presence of FcaPV-2 in all of the viral plaques compared to a very low proportion of another feline skin tumour provided strong support for FcaPV-2 being the aetiological agent of FVPs. None of the cats in the study were noted to have immunosuppression or concurrent disease in the brief case histories submitted with the samples.[278]

FcaPV-2 was also found in 100% of BISCs in a series of studies primarily aimed at investigating the more common SCCs.[267,279,280] In the first of two studies, using a nested PCR protocol, all 20 BISCs had amplifiable FcaPV-2 DNA compared to 3 of 17 non-SCC samples, including two fibrosarcomas and an apocrine gland cyst.[279] The second study amplified FcaPV-2 from 8 of 8 FVPs and 14 of 14 BISCs, compared to just 1 of 14 trichoblastomas.[267] Rather than using a nested PCR, a 2012 study used four sets of primers.[280] These were MY09/MY11 and JMPF/JMPR as used previously and 2 new primer sets designed to specifically amplify two novel PVs identified in previous studies: JMY2F/JMY2R for FdPV-MY2, and JMY3F/JMY3R for the FdPV-MY3, which had been recently identified in two SCCs.[281] All five FVPs contained FcaPV-2 DNA and one also contained FdPV-MY1 DNA. All ten BISCs contained FcaPV-2 DNA and one also had FdPV-MY2 DNA. Another study by a separate group detected both FcaPV-2 DNA and DNA from several HPVs in 7 of 22 feline BISCs.[275] The lower rate of detection may be explained by the use of consensus primers and a nested reaction using primers designed to amplify beta HPVs as these primers could not bind as efficiently to the FcaPV-2 sequence as the JMPF/JMPR primers. This study and those by Munday *et al.* showed that FcaPV-2 DNA was consistently present in almost all FVPs and BISCs whereas other PV types were detected only occasionally, supporting a role of FcaPV-2 in the development of FVPs and BISCs.

1.4.6 FcaPV-2 in squamous cell carcinomas

The detection of PV DNA in a small proportion of SCCs by Nespeca *et al.* raised the interesting possibility that PVs might also play role in the development of these cancers.[265] Cutaneous SCCs are the most common malignant skin cancer of cats, accounting for 15-49% of all feline skin tumours.[6,282] Clinically, the lesions generally start as single or multiple plaques that progress to large ulcerative lesions which are often highly invasive and locally destructive, requiring either extensive surgical resection or euthanasia.[261] Although common, the exact incidence of cutaneous SCCs in most cat populations is unknown, as most countries do not have obligatory

cat registration and records from individual veterinary practices are not collated at a national level. An analysis based on submissions to veterinary pathology laboratories in Switzerland estimated that cutaneous SCCs accounted for around 5% of all feline cancer.[283] However, this may have underestimated the true incidence as cutaneous SCCs tend to have a characteristic gross appearance and so may not be routinely submitted for histopathology.

Cutaneous SCCs tend to arise on the nasal planum, pinnae and eyelids, particularly in cats with lightly pigmented skin.[261] The predilection for UV-exposed skin and the demonstration of high rates of TP53 mutation (which prevent the normal upregulation of p53 in response to UV-exposure), led to the widely accepted notion that feline SCCs are primarily caused by the accumulation of UV-induced genetic damage.[284,285] However, it was hypothesized that PVs might be a co-factor in the development of these cancers and/or induce a subset of SCCs that occur in densely haired areas that are protected from UV light. A study using nested PCR with primers specific for FcaPV-2 amplified FcaPV-2 from 17 of 20 formalin fixed paraffin embedded (FFPE) SCCs.[279] These SCCs were predominantly from UV exposed sites whereas subsequent studies analysed SCCs from both UV exposed and protected sites. The results from these studies are summarised in Table 1.1.

	SCC UV protected	SCC UV exposed	SCC UV status unknown	Non-SCC skin samples	Ref
	3/13[a]	6/26[a]		-	275
	7/7	5/11		1/14	267
	9/11	6/19		-	280
	19/25	19/45		-	281
		33/51		-	286
			4/22[a]	0/11[a]	265
			17/20	3/17	279
Mean	68%	45%			
		51%		9.5%	

Table 1.1. Proportion of SCCs containing amplifiable PV DNA according to site (PV positive/ total tested). [a] These studies used consensus primers whereas the other studies used primers specific for FcaPV-2.

From these results it can be seen that the rate of FcaPV-2 DNA detection was considerably higher in SCCs than in non-SCC samples, particularly in SCCs from UV protected areas. This demonstrates a strong association between FcaPV-2 and SCCs however it does not prove that

FcaPV-2 influences the development of SCCs as the cancers may simply provide a suitable environment to harbour the virus.

FcaPV-2 has also been found in normal skin and sporadically in other lesions. Four studies in particular have included FFPE non-SCC skin lesions such as: fibrosarcomas, hypersensitivity dermatitis, eosinophilic plaques, apocrine gland cysts and cystadenomas, melanomas, mast cell tumours, trichoblastomas, granulomatous dermatitis, a case of dermatophytosis and a follicular cyst.[265,267,273,279] From a total of 60 such lesions, two fibrosarcomas and an apocrine gland cyst were positive for FcaPV-2. As a previous series of 50 fibrosarcomas were negative for PV DNA this result probably represents an incidental infection.[269] Supporting this, FcaPV-2 was found in 39% of skin swabs from normal skin of 44 adult cats, with 52% of the cats having at least one positive sample.[5] The much higher rate of asymptomatic infection detected by skin swabs can be explained by several factors. Firstly formalin fixation can damage and fragment DNA so some of the PV DNA in the formalin fixed samples may not have been amplifiable. Secondly cutaneous PV infections can be restricted to focal areas of skin so are more likely to be detected by swab samples because they encompass a larger surface area.[287] Finally, if there has been any viral replication, the virus is in the highest concentration in the surface squames which are easily collected by swabs but may be lost in the fixation process for paraffin embedded samples. Thus the results indicate that at least 52% of adult cats are probably asymptomatically infected with FdPV-2.[5] Higher rates of PV infection were not observed in cats asymptomatically infected with FIV, although it is unknown whether these cats were immunosuppressed so the contribution of immunosuppression to cutaneous PV infection remains poorly understood. Another interesting observation from this study was the failure of the consensus primers to amplify any PV infection from swab samples. This indicates that the consensus primers were not sensitive enough to amplify the small amount of FcaPV-2 DNA present so the presence of other PV types in normal feline skin cannot be confirmed or excluded.[5]

1.4.7 Sequencing of the third and fourth feline PVs

Although studies looking for PV DNA in SCCs have mostly found FcaPV-2, several studies using consensus primers have also detected other PV types. Nespeca *et al.* (2006) detected a novel PV in four SCCs and O'Neill *et al.* (2011) found FcaPV-2, HPV-21, and a novel PV that was 84% similar to HPV-21, in a series of SCCs. [18, 275] The novel PV identified by Nespeca *et al.* was subsequently found in three separate studies, where it was designated FdPV-MY2.[278,281,286] Eventually the

complete genome was amplified from a BISC, fully sequenced and classified as *Felis catus* papillomavirus type 3 (FcaPV-3) in the Tau genus.[288] FcaPV-3 was the first non-canine PV in this genus and unlike FcaPV-2, it did not contain a pRb binding motif in the E7 ORF but did contain an ORF encoding an E5 protein. Following this, another closely related feline PV was sequenced and classified as *Felis catus* papillomavirus type 4 (FcaPV-4), also in the Tau genus.[289] The L1 nucleotide sequences of the two viruses shared 65.1% identity. Interestingly, FcaPV-4 was detected in a sample from the oral cavity of a cat with severe gingivitis, despite being previously amplified from three nasal planum SCCs in which it was the only PV type detected (FdPV-MY3).[281,286] As with the other Tau PVs, the FcaPV-4 E7 ORF did not contain a pRb binding motif. However unlike FcaPV-3, FcaPV-4 did not have an identifiable E5 ORF.

1.4.8 Feline PVs in oral lesions

The presence of PV DNA in oral lesions has been investigated by several studies. Sundberg *et al.* (2000) first reported two cases of feline oral papillomas that were positive for PV capsid proteins by immunohistochemistry.[255] Munday et al (2009) used PCR with three different sets of consensus primers to screen 20 oral SCCs for the presence of PV DNA. PV DNA was detected in only one SCC and this was a novel PV most closely related to HPV-76.[290] Another human PV, HPV-38, was found in a study by a different group which analysed 35 mucosal lesions.[275] A further study by Munday *et al.* (2011) failed to detect any PV DNA in another 30 oral SCCs, suggesting that PVs are unlikely to be involved in the development of feline oral SCCs.[291] Several years later FcaPV-4 was detected in a sample of oral mucosa with microscopic signs of PV infection, although there was no grossly visible lesion.[289] Soon afterwards, two cats presented with multiple papillomas on the ventral surface of the tongue which in both cases had been an incidental finding. The CP4/5 consensus primers amplified FcaPV-1, but not FcaPV-4, from both cases.[292] As PVs tend to infect either skin or mucosal epithelia, this finding may suggest that FcaPV-1 primarily infects the oral mucosa. This supports previous evidence that, although FcaPV-1 was first detected in a FVP, these lesions are usually caused by FcaPV-2. In light of the discovery of these two potential oral feline PVs, Munday *et al.* (2015) analysed another series of 36 oral SCCs and 16 oral inflammatory lesions, this time with primers specific for FcaPV-1 and -4.[293] FcaPV-1 was amplified from one SCC and one inflammatory lesion, which also had histologic features of PV infection. No PV DNA was amplified by the primers specific for FcaPV-4. These results did not support a role of PVs in feline oral SCCs but showed that FcaPV-1 can

asymptomatically infect the mouth of cats, as well as causing oral papillomas. Overall, there is little evidence to support a significant role of PVs in oral disease in cats.

1.4.9 Alterations in host cell cycle regulatory proteins p16, pRb and p53

In normal basal epithelial cells the hypophosphorylated form of the retinoblastoma protein (pRb) forms a complex with the E2F transcription factors preventing the cell from progressing from the gap 1 (G_1) phase to the synthesis (S) phase of the cell cycle. When the cell is ready to divide, there is increased activity of the cyclin dependant kinases -4 and -6 (CDK4 and CDK6). These phosphorylate pRb causing it to release the E2F transcription factors, allowing the transcription of the genes required for the cell to progress into the S phase of the cell cycle. The activity of CDK4 and CDK6 is tightly regulated by several cyclin-dependant kinase inhibitors including p16.[294] In human mucosal epithelial cells infected with high-risk HPV there is upregulation of p16 as part of the innate immune response to the virus.[201] To overcome this p16-mediated cellular senescence the PV E7 protein interacts with, and in some cases degrades, pRb which releases E2F transcription factors allowing the cell to progress through the G_1/S checkpoint, creating an environment that is suitable for viral replication.[157,198] The upregulation of p16 can be detected by immunohistochemistry and serves as a highly sensitive and specific biomarker of high-risk HPV-induced dysplasia or cancer in cervical epithelial samples from women.[295]

Studies in cats have documented a similar increase in p16 in FVPs, BISCs and a proportion of SCCs.[267,280,281,286] The first study evaluated p16 immunostaining in 14 FVPs, 14 BISCs, 7 SCCs from UV protected areas, 11 SCCs from UV exposed areas, and 14 trichoblastomas.[267] A marked increase in p16 immunostaining was present in all of the FVPs, BISCs and SCCs from UV protected areas, and this change was restricted to the histologically abnormal epithelium. Three of the SCCs from UV exposed sites also had increased p16 as did one trichoblastoma but the immunostaining was less intense in these cases. FcaPV-2 DNA was present in all of the FVPs, BISCs and SCCs from UV protected areas, as well as five SCCs from UV exposed sites, and one trichoblastoma.[267] These results suggested an attractive hypothesis: SCCs from UV protected sites develop as an undetected viral plaque that subsequently progresses through a BISC to a SCC. Additionally FcaPV-2 can also infect normal skin and be present in lesions without disrupting host cell cycle regulatory proteins and therefore not cause an increase in p16 or contribute to carcinogenesis. While the results of the aforementioned study supported this

hypothesis, it was also acknowledged that p16 can be increased in non PV-induced cancers, so the mechanisms for the increase in p16 in these tumours needed to be investigated before it could be conclusively used as evidence of PV infection.[267] A similar study was subsequently published containing a larger number of SCCs: 25 from UV protected sites and 45 from UV exposed sites.[281] Consistent with the previous study, increased p16 was detected in 84% of the SCCs from UV protected sites, whereas significantly fewer (40%) of the SCCs from UV exposed sites contained increased p16 immunostaining. An association between the presence of PV DNA and increased p16 immunostaining was also confirmed by the detection of PV DNA significantly more frequently in SCCs with increased p16 than in SCCs without increased p16.[281] This association between p16 and FcaPV-2 DNA was also demonstrated in a case study of a Devon rex cat that initially presented with a BISC and was eventually euthanized due to SCC metastasis to the thorax and spinal cord. Fluorescent *in situ* hybridisation of skin biopsies revealed FcaPV-2 DNA in the neoplastic keratinocytes and at post mortem both the skin cancers and the internal metastases had strong p16 immunostaining.[296]

Another host protein that is often increased in cancers is the tumour suppressor protein p53. In many cutaneous cancers in people, UV-induced mutations of the TP53 gene are associated with impaired degradation and abnormal intracellular accumulation of the dysfunctional protein and consequently with positive p53 immunostaining.[297] In contrast, the viral E6 protein from the high-risk HPVs tags p53 for ubiquitin-mediated degradation and these lesions are usually characterized by an absence of TP53 mutations and absent p53 immunostaining.[298-300] It is worth noting however that the chromosomal instability induced by the high-risk HPV oncogenes can cause secondary TP53 mutations, and this mutated p53 can be resistant to E6-mediated degradation.[301] Thus PV-induced mucosal lesions in people usually, but not always, contain no p53 immunostaining. Favrot *et al.* (2009) hypothesized that, in cats, a difference in p53 immunostaining might help to distinguish between UV-induced actinic keratosis and PV-induced BISCs.[268] The results showed that, as expected, the majority of actinic keratosis (11 of 14) had increased p53 immunostaining but so did 4 of 22 BISCs, with focal areas of p53 immunostaining also visible in the remaining BISCs. Therefore, p53 immunostaining was not useful to differentiate between actinic keratosis and BISCs. Interestingly, two BISCs had PV antigen immunostaining around the periphery and p53 immunostaining in the centre of the lesion suggesting that a secondary TP53 mutation may have arisen within the PV-induced lesion.

The association between the presence of FcaPV-2 DNA and changes in the pattern of immunostaining for p16, pRb and p53, was explored in a series of FVPs, BISCs and SCCs by

Munday and Aberdein (2012).[280] A pattern of reduced pRb immunostaining and increased p16 immunostaining was frequently identified in lesions containing FcaPV-2 DNA: 9 of 11 SCCs from UV protected areas had both amplifiable PV DNA and reduced pRb, and 10 of 11 had increased p16. A similar pattern of reduced pRb, increased p16, and amplifiable FcaPV-2 DNA was present in most of the FVPs and BISCS. In comparison few of the SCCs from UV exposed sites contained FcaPV-2 DNA, reduced pRb, or increased p16. Overall, of the 26 lesions that had reduced pRb, all had increased p16 and PV DNA was amplified from 24. The authors speculated that the remaining two lesions may have had reduced pRb due to another cause or that PV DNA was present but not amplifiable. No association was observed between p53 immunostaining and the presence of PV DNA or p16/pRb immunostaining. While most of the FVPs and BISCs had reduced p53 immunostaining, increased p53 immunostaining was present in some of the BISCs and SCCs. Therefore, the results of this study supported the hypothesis that the increased p16 immunostaining was the result of FcaPV-2 infection and that the virus also caused a reduction in pRb.[280]

The association between the presence of FcaPV-2 DNA and a pattern of reduced pRb immunostaining and increased p16 immunostaining may suggest that FcaPV-2 contributes to carcinogenesis in a similar manner to the high-risk HPVs. An assumption in this hypothesis is that the increased p16 and reduced pRb are important in carcinogenesis and not simply an indicator of an incidental PV infection. In PV-induced tonsillar SCCs in people, the presence of immunostaining for p16 is indicative of a PV aetiology and these cancers have a favourable prognosis compared to tobacco or alcohol-induced SCCs.[302,303] The reason for this is thought to be that the cells in the PV-induced SCCs are more likely to have functional apoptosis pathways and are thus more likely to undergo apoptosis in response to chemotherapy or radiotherapy.[304] To assess whether the p16 positive, PV containing feline SCCs had a different clinical behaviour to those without any evidence of PV involvement, Munday *et al.* (2013) did a retrospective study of 30 cases of nasal planum SCCs where a non-excisional biopsy had been taken.[286] The 18 p16 positive SCCs had a mean survival time of 643 days which was significantly longer than the mean survival time of the 12 p16 negative cases which was 217 days. This supported the existence of two sub groups of SCCs with different behaviours and aetiologies, the most plausible scenario being a UV-induced subset with a poorer prognosis and a PV-induced subset with a more favourable prognosis. Interestingly the difference between survival times for the SCCs with amplifiable PV and those without was not significantly different. This result was explained by the presence of incidental PV infection in a small proportion of UV-induced SCCs and formalin damage rendering PV DNA non-amplifiable in a small number of p16 positive SCCs. These

occurrences, when combined with a modest sample size, suggest that increased p16 may be a more reliable indicator of a PV aetiology than the presence of amplifiable PV DNA. An additional interesting point from this study was the presence of PV DNA from FcaPV-3 exclusively in four p16 positive SCCs, although the majority of the PV positive lesions contained FcaPV-2 (27 of 33). This finding tentatively implicates FcaPV-3, as well as FcaPV-2, as having carcinogenic potential.

1.4.10 Feline sarcoids

In 1993, Yager and Wilcock published a histologic description of fibropapillomas in dogs and cats.[305] These skin tumours grossly and histologically resembled fibropapillomas in cattle and equine sarcoids. They described the tumours as consisting of tightly interwoven plump spindle cells growing in intimate association with a hyperplastic overlying epithelium. Minimal cellular atypia and few mitotic figures were seen. Despite being very similar to those seen in cattle and horses, the feline and canine lesions were much less common with only 14 such diagnoses out of 13,000 biopsies.[305] Equine sarcoids are thought to be caused by BPV-1, -2 and possibly also BPV-13, and given the similarity to the equine lesions the authors proposed a PV aetiology for feline fibropapillomas.[49,306] Subsequently there were scattered reports of feline lesions histologically consistent with fibropapillomas. Grossly these were described as firm, fleshy, alopecic, sometimes ulcerated skin masses on the nose, lips, pinna or digits.[307,308] The typical histological findings of PV infection, as seen in FVPs and some BISCs have not been seen in feline fibropapillomas.

In 2001 a group from Wisconsin collected 23 feline fibropapillomas and designed consensus primers for the E1 gene by aligning sequences from 11 diverse PVs.[309] Of the 17 tumours for which tissue was available and amplifiable DNA was present, all were positive for PV DNA. Sequencing of one case revealed a novel PV that was most similar to the delta PVs, particularly BPV-1 and BPV-2, but dissimilar to the other feline PVs. This study also confirmed the fibroblastic nature of the neoplastic cells by electron microscopy. Other interesting observations included a moderate rate of recurrence following surgical excision and that the majority of the cases were in young cats from rural areas. The presence of PV DNA and the histologic and epidemiologic similarities to equine sarcoids led the authors to speculate that this novel PV may be a ruminant PV causing neoplasia in an aberrant host.[309] The results of this study were confirmed and expanded by another group who amplified the same, or a very similar, novel PV from 9 of 12 feline fibropapillomas.[310] *In situ* hybridisation localised the PV DNA to the neoplastic

mesenchymal cells rather than the overlying hyperplastic epithelium. Immunohistochemistry for PV capsid protein was negative in all cases indicating a non-productive infection. The presence of a non-productive infection with novel PV in mesenchymal cells underneath the hyperplastic epidermis prompted the authors to suggest the term feline sarcoids to describe the lesions and feline-sarcoid associated PV (FeSarPV) to describe the novel PV.[310] This group also used primers specific for BPV-1, BPV-2, and CPV-1 which failed to amplify these PVs excluding their involvement in feline sarcoids. Subsequentlu FeSarPV has been amplified from 6 of 7 feline sarcoids, none of 120 non-sarcoid feline samples, 5 of 16 bovine fibropapillomas, and 5 of 18 biopsies of inflammatory skin disease from cattle.[306,311,312] These studies demonstrated that FeSarPV can infect both cats and cattle but because the virus was only found in sarcoids on cats, cattle were thought to be the likely reservoir host of FeSarPV. This has been recently confirmed with full sequencing of FeSarPV; phylogenetic analysis of the 7966 bp genome classified it as a delta PV, closely related to BPV-1, -2 and -13, and the authors proposed FeSarPV be renamed bovine papillomavirus type 14.[50]

1.5 Conclusion

In summary, there is considerable evidence that FcaPV-2 causes feline viral plaques and BISCs. Additionally FcaPV-1 appears to cause oral papillomas and other PVs have been isolated from cutaneous papillomas, although these lesions are very rare in cats. BISCs and feline sarcoids are examples of PV-induced cancer in cats, although both lesions are rare. There is also some evidence to suggest that FcaPV-2 plays a role in a subset of the very common cutaneous SCCs, particularly those that occur in UV protected areas. Early evidence of this was the observation of transitional lesions between PV-induced premalignant lesions and SCCs.[260] Subsequently FcaPV-2 DNA was detected in around half of all feline cutaneous SCCs, and even more commonly in SCCs from densely haired skin, compared to less than 10% of non-SCC skin lesions.[281, 267] SCCs that harbour FcaPV-2 DNA also frequently had increased levels of the host cyclin dependant kinase inhibitor $p16^{INK4A}$ (p16).[281] Increased p16 is used as a biomarker of high-risk HPV-induced cancers in people and p16 positive oropharyngeal SCCs tend to have a better prognosis than p16 negative SCCs.[313] Similarly, p16 positive nasal planum SCCs in cats had a better prognosis than p16 negative SCCs, and the positive SCCs were more likely to harbour PV DNA, suggesting that the PV may be important in development and continued growth of these cancers.[286] However, p16 can be upregulated independent of PV infection, and FcaPV-2 DNA has been detected in skin swabs from a high proportion of healthy cats, so the possibility remains that the virus is merely an innocent bystander. A key event in PV-induced cancer in people is deregulation of PV early gene expression. Therefore, the finding of increased E6 and E7 gene expression in feline cutaneous SCCs could provide the missing link between the presence of FcaPV-2 DNA and alterations in the host cell cycle regulatory proteins, thus confirming or rejecting a role of FcaPV-2 in the aetiology of feline cutaneous SCCs.

Aside from their role in the various skin lesions, little is known about the epidemiology of feline papillomaviruses. Around half of the adult cats in one study were positive for FcaPV-2 DNA by PCR on skin swabs.[5] When or from where this infection was acquired is unknown. The frequent presence of asymptomatic FcaPV-2 infections may suggest that FcaPV-2 behaves similarly to beta HPVs in people, where infants are exposed to the virus by close contact with infected family members soon after birth.[314] Infection may additionally require access of the virus to the basement membrane through micro-wounds. With growing evidence supporting a role of FcaPV-2 in the development of cutaneous SCCs, it is important to determine when most cats will

become infected with FcaPV-2, as prophylactic vaccination presents the best opportunity to prevent this common and aggressive disease.

The following chapters of this thesis will present research aimed at addressing these gaps in the understanding of FcaPV-2 infection in cats and the role of the virus in the development of cutaneous SCCs. Chapter 2 will address the question of when cats become exposed to the virus. Chapter 3 will follow on from this to establish when infection occurs and whether there is an opportunity for prophylactic vaccination. Chapters 4 and 5 will then present some findings in relation to the detection of FcaPV-2 gene expression in feline cutaneous SCCs and what this means in regards to a role of the virus in these cancers. Chapter 6 will discuss efforts to determine the physical state of FcaPV-2 in feline cutaneous SCCs and Chapter 7 will provide a general discussion and directions for future research.

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Chapter 2

Timing and Source of Exposure to FcaPV-2

2.1 Introduction

Evidence is accumulating for a role of FcaPV-2 in feline skin cancer, but there has only been one study in which the epidemiology of infection has been investigated.[1,2] In this study, Munday and Witham (2010) detected FcaPV-2 DNA in skin swabs from 23 of 44 adult cats using conventional PCR, suggesting that asymptomatic FcaPV-2 infection is common among cats. However, there have been no investigations to determine the age at which kittens become infected with FcaPV-2 or the source of this infection. This information is crucial to determine whether preventing infection with FcaPV-2 could be a feasible method of reducing the incidence of SCCs in cats.

In rabbits, dogs, and cattle, vaccination with PV-type specific virus-like particles (VLPs), which are essentially empty PV capsids, has been highly effective at preventing PV-induced lesions when given prior to experimental challenge but has little effect on existing lesions.[3-5] Similarly, in people prophylactic HPV vaccination is highly effective at preventing type specific HPV infection and the subsequent development of intraepithelial cervical cancer (CIN3).[6] However, vaccination must be given prior to the first exposure to HPV as, once the PV has entered the basal cells, vaccination is of little use to prevent lesion progression. Other PV-directed therapies against existing infections have not yet demonstrated efficacy at clearing PV-induced lesions.[7] Therefore, vaccination against FcaPV-2 potentially presents the best opportunity to reduce the incidence of PV-induced lesions in cats but would only be feasible if the majority of kittens are not infected until several weeks after birth.

The first aim of this study was to develop a real-time PCR assay to detect and quantify FcaPV-2 DNA in feline skin swabs and hair plucks. Skin swabs are a highly sensitive method of detecting PV infections as they sample a large surface area and collect mature skin cells (cornified squames) which contain large quantities of progeny virions ready to be released into the environment. However, skin swabs may also collect virus-laden squames present on the skin surface that came from the environment, after being shed from other cats. Therefore, while skin swabs are useful for assessing exposure to PVs, the detection of PV DNA in skin swabs from a certain cat does not necessarily indicate that individual is infected with PV. In contrast, tissue

samples and hair plucks contain cells from a greater thickness of epidermis and are less prone to environmental contamination. However these samples are taken from a small area of skin and so may miss focal infections. In people, hair follicles are thought to be the reservoir of beta HPVs and the presence of PV DNA in hair plucks has been suggested to indicate a true infection.[8,9] Additionally, all previous studies detecting FcaPV-2 DNA have used conventional PCR so the quantity of viral DNA has not been determined. The quantity of PV DNA is likely to be higher in samples that are supporting PV replication, so real-time PCR was used to quantify the FcaPV-2 DNA load in both swab and hair pluck samples, from multiple locations, to try to assess both exposure to FcaPV-2 and the timing of infection.

Following the creation and validation of an assay to quantify FcaPV-2 DNA in skin swabs and hair pluck samples, the second aim of this study was to apply this assay to samples from client owned cats and their kittens. More specifically, the aim was to determine the proportion of pregnant queens that were infected, the range of viral DNA loads and, most importantly, the age at which kittens were first exposed to the virus. Furthermore, by quantifying the FcaPV-2 DNA present on cats the viral loads could be compared between queens and kittens, and between the different litters and households; it was hoped that this would provide an insight into the likely mode of transmission of FcaPV-2. This study was the first time that FcaPV-2 has been quantified and the first time that the presence of this virus has been investigated in very young cats.

2.2 Methods

2.2.1 Sample collection

Eleven pregnant queens from five cat breeders, from the Manawatū region of New Zealand, were recruited for the study. Each queen was sampled on three separate occasions: once within the 3 weeks prior to kittening, at 7 days after kittening, and at 28 days after kittening. Samples were also collected from two kittens from each litter when the kittens were 2, 7 and 28 days of age. At each timepoint four samples were collected from each cat: hair plucks from the head, dorsal midline and umbilical region, and a pooled sample of skin swabs taken from the same regions. This ensured that the majority of the skin surface of the 2 day old kittens was sampled. Each hair pluck consisted of 10- 20 hairs which were placed into a 1.5 mL microtube containing 500 µL sterile saline (0.9% NaCl, pH 5.4). The cotton tipped swabs (Protec Solutions Ltd, Wellington, New Zealand) were first moistened in sterile saline then drawn across a 3 x 3 cm area of skin five times. The three swab heads were collected into 500 µL sterile saline in one 5 mL universal container (Sarstedt AG and Co, Nümbrecht, Germany). Gloves and instruments were changed between kittens, and containers were pre-filled with saline to minimise contamination during sampling. This study was approved by the Massey University Animal Ethics Committee (protocol 12/101).

The samples were transported to the laboratory within 1 hour of collection. The hair pluck samples were then centrifuged at 10,000 × g for 1 minute and 400 µL of supernatant removed, leaving 100 µL of saline with the hairs. The pooled swab samples were vortexed, and the swab heads were squeezed out and removed. The remaining solution and the hair plucks were kept at -20°C until DNA extraction.

2.2.2 DNA extraction

Seven different DNA extraction protocols were trialled on excess hairs from the hair pluck samples. Three of these were standard protocols: phenol-chloroform-isoamyl alcohol extraction, NaOH extraction and Tris-KCl extraction.[10-12] Four commercially available kits were also tried, according to the manufacturer's recommended protocol, including: Ambion MagMax

Viral RNA isolation kit (Life Technologies, Carlsbad, CA, USA) which isolates total nucleic acids with magnetic beads; InstaGene Matrix (Bio-rad, Hercules, CA) which utilises a chelex resin; prepGEM Tissue (ZyGem, Hamilton, NZ), a closed-tube protease method designed for small samples; and Roche High Pure PCR Template Preparation kit (Roche Applied Science, Mannheim, Germany), a spin-column based system. The Roche High Pure PCR template preparation kit was the only method that yielded amplifiable reference gene DNA from all of the hair pluck samples and so this method was chosen for use on the study samples. The kit was used according to the recommended protocol, apart from the proteinase K digestion step, which was extended from the recommended 1 hour to overnight to ensure complete digestion of the hair pluck samples. The presence of amplifiable DNA in the study samples was assessed by qPCR for *Felis catus* 28s ribosomal RNA gene.[13]

2.2.3 Generation of recombinant plasmids as standards for qPCR

Forward E7SF (5'TGGCACGACACCTACCATTAAAGACA) and reverse E7SR (5'GCCTCCACCTCAACCTCGATCTC) primers were designed to amplify a 111 bp fragment of the FcaPV-2 E7 gene, in a region where the corresponding amino acid sequence is highly conserved among many PV types.[14] Conventional PCR was used to amplify this fragment from a swab sample of a viral plaque previously shown to be FcaPV-2 positive.[15] The PCR was performed using TopTaq Master Mix (Qiagen GmbH, Hilden, Germany), a final concentration of 0.6 µM of each primer and 4 µL of template DNA in a total volume of 50 µL. The cycling conditions consisted of an initial 3 minute denaturation step at 94°C, followed by 35 cycles of denaturation (94°C for 20 seconds), annealing (60°C for 20 seconds) and elongation (72°C for 90 seconds), with a final extension step (72°C for 7 minutes). The gel-purified PCR product was cloned into a commercial plasmid vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA) using One Shot Chemically competent *E.coli*, according to the manufacturer's recommendations (Invitrogen). Six clones were analysed by PCR and all contained the 111 bp insert on gel electrophoresis. Plasmids were isolated from two clones using the commercially available Roche High Pure Plasmid Isolation Kit (Roche Applied Science) according to the manufacturer's recommendations and sequenced to confirm the identity of the insert. The quantity and quality of isolated plasmid DNA was assessed using a Nanodrop spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). The E7 plasmid was then linearized with Pst I restriction enzyme (Thermo Scientific Inc.) in a total volume of 100 µL for 2 hours at 37°C. The reaction was stopped with 4 µL of 0.5 M EDTA.

The resulting preparation comprised a stock solution containing the equivalent of 10^9 copies/µL of the target DNA sequence. The same protocol was used to create a recombinant plasmid for the *F. catus* 28S ribosomal RNA gene using previously published primers (forward: 5'CGCTAATAGGGAATGTGAGCTAGG, reverse: 5'TGTCTGAACCTCCAGTTTCTCTGG).[13] Serial dilutions in water of these recombinant plasmids were used to derive standard curves for absolute quantification.

2.2.4 Quantitative PCR assay

Quantitative PCR assays were run using an Eco real time instrument (Illumina Inc., San Diego, CA, USA). The PCR was performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), a final concentration of 0.4 µM of E7SF primer, 0.3 µM of E7SR primer, and either 2 µL of E7 plasmid DNA or 4.5 µL of sample DNA in a total volume of 10 µL. The optimal combination of primer concentrations were selected from a checkerboard assay. The cycling conditions consisted of an initial 5 minute denaturation step at 95°C, followed by 45 cycles of denaturation (95°C for 5 seconds), annealing (60°C for 20 seconds) and elongation (72°C for 15 seconds). The cycling was followed by a melting step from 55°C to 95°C. The number of viral DNA copies per reaction was calculated from quantification cycle (Cq) values using either Eco (single run) or EcoStudy (multiple runs) software (Illumina Inc.) based on the standard curve generated from seven, ten-fold serial dilutions of the E7 recombinant plasmid. The same protocol was followed for the reference gene but with final primer concentrations of 0.5 µM (forward primer) and 0.4 µM (reverse primer). Values lower than 2 copies per reaction were extrapolated. The cycle threshold for detection was set in the exponential phase of the amplification curve, at a level which excluded any non-specific amplification from reaching the threshold; this was 0.119 for the E7 assay and 0.035 for the reference gene assay. Samples were considered positive if they amplified in an exponential manner, crossed the threshold, and had a similar melting peak to the recombinant plasmid standards. All standards and samples were run in duplicate for all assays, with the exception of the reference gene assay for the swab samples, in which the samples were run singly to confirm the presence of amplifiable DNA. A non-template control (water) was included in each run to check for the presence of contamination. A sample of liver, taken from a cat being necropsied at Massey University was used as a positive control for the reference gene and as a negative extraction control for FcaPV-2 assay. For analysis of samples from multiple runs, the master standard curve was used within the EcoStudy

software. One standard (2×10^6 copies/µL) was designated as a plate control and included on each contributing plate. Plates that showed a Cq variation between a master standard and a control standard of ≤ 0.5 were considered valid.

2.2.5 Quantitative PCR assay validation

Validation was performed by determining the sensitivity, linearity, precision and reproducibility of the assay. The sensitivity of the assay was determined by testing serial dilutions of the recombinant plasmid stock solutions. The calculated efficiency and r^2 values of the curves generated from these dilutions assessed the linearity of the assay. Precision was evaluated by calculating intra-assay variability based on the distribution of linearized Cq values for five replicates of each standard in a single PCR run. Reproducibility was evaluated by calculating inter-assay variability by comparison of the linearized Cq values obtained for the same standards in three separate PCR runs. The intra- and inter-assay variability was expressed as a coefficient of variation (CV), calculated as the ratio of the standard deviation to the mean of the linearized Cq values for a particular standard, expressed as a percentage.

2.2.6 Normalisation

The hair pluck samples were normalised to copies of FcaPV-2 DNA per copy of reference gene DNA. This was done by dividing the mean number of copies of FcaPV-2 per reaction by the mean number of copies of *F. catus* 28S DNA per reaction for each sample. In contrast to the cells within the plucks, the swab samples contained predominantly cornified squames that lack a nucleus. As the majority of cells within the swabs would not be expected to contain host DNA, the copies of FcaPV-2 DNA were not normalised to copies of reference gene DNA, but determined per swab instead. This was done by estimating the number of copies in the total elution volume of 70 µL, from the mean of the qPCR duplicates which each used 4.5 µL of template DNA. As swabs from three different areas were pooled together prior to DNA extraction, the total number of copies of FcaPV-2 DNA was then divided by 3 to get the estimated mean copy number of FcaPV-2 DNA per swab.

2.2.7 Statistical analysis

Data was analysed using R 2.15.2 software (R Foundation for Statistical Computing, Vienna, Austria). Log transformed data was used for statistical analysis. Samples that had no detectable virus were set at half the minimum detectable level of the assay prior to log transformation. Pearson correlation coefficients and ANOVA models were used.

2.3 Results

2.3.1 Sample collection

All eleven queens were purebred: four were Burmese, three Persians, two Norwegian Forest Cats, one Mandalay, and one British Blue. The eleven cats came from cat breeders in the Manawatū region of New Zealand and were housed in five households. In four of these households (households 1-4) the breeding queens and their kittens were not confined and so had direct contact with other queens and kittens. Household 5 was different in that the queens were isolated after kittening and had contact with only their own kittens for the duration of the study. All of the queens had at least two kittens; however one litter of two kittens in household 5 was delivered by caesarean section and both kittens died at 3 days of age. In total 372 samples were collected from 33 cats.

2.3.2 Performance of the q*P*CR assay

The qPCR assay demonstrated good analytical sensitivity as it was able to detect the equivalent of 2 copies of FcaPV-2 DNA (Fig. 2.1). In all runs, a similar melting peak was observed between 81.4°C and 82.6°C for the E7 assay, or between 79.0°C and 80.2°C for the reference gene assay. Sequencing of samples with melting peaks at the upper and lower end of these ranges demonstrated that they were the expected FcaPV-2 E7 amplicons, but the sequence varied by 1-4 base pairs, presumably due to DNA polymerase errors. The assay was linear within the tested range from 2 to 2×10^7 target copies (efficiencies and r^2 values: 99.59%, r^2=0.998 and 91.28%, r^2=0.992 for the E7 and reference gene assays respectively). For the newly developed FcaPV-2 E7 assay the coefficient of variation (CV) of the mean linearized Cq values obtained with five replicates of different dilutions of the standard in a single test run ranged from 8.9% to 20.9% (Table 2.1). Raw Cq values were converted to a linear form prior to calculating the CV values to avoid underestimating the degree of variation.[16] The CV of the mean linearized Cq values obtained in three separate runs of the test ranged from 5.4% to 22.6%. This indicates adequate precision and reproducibility of the assay.[17]

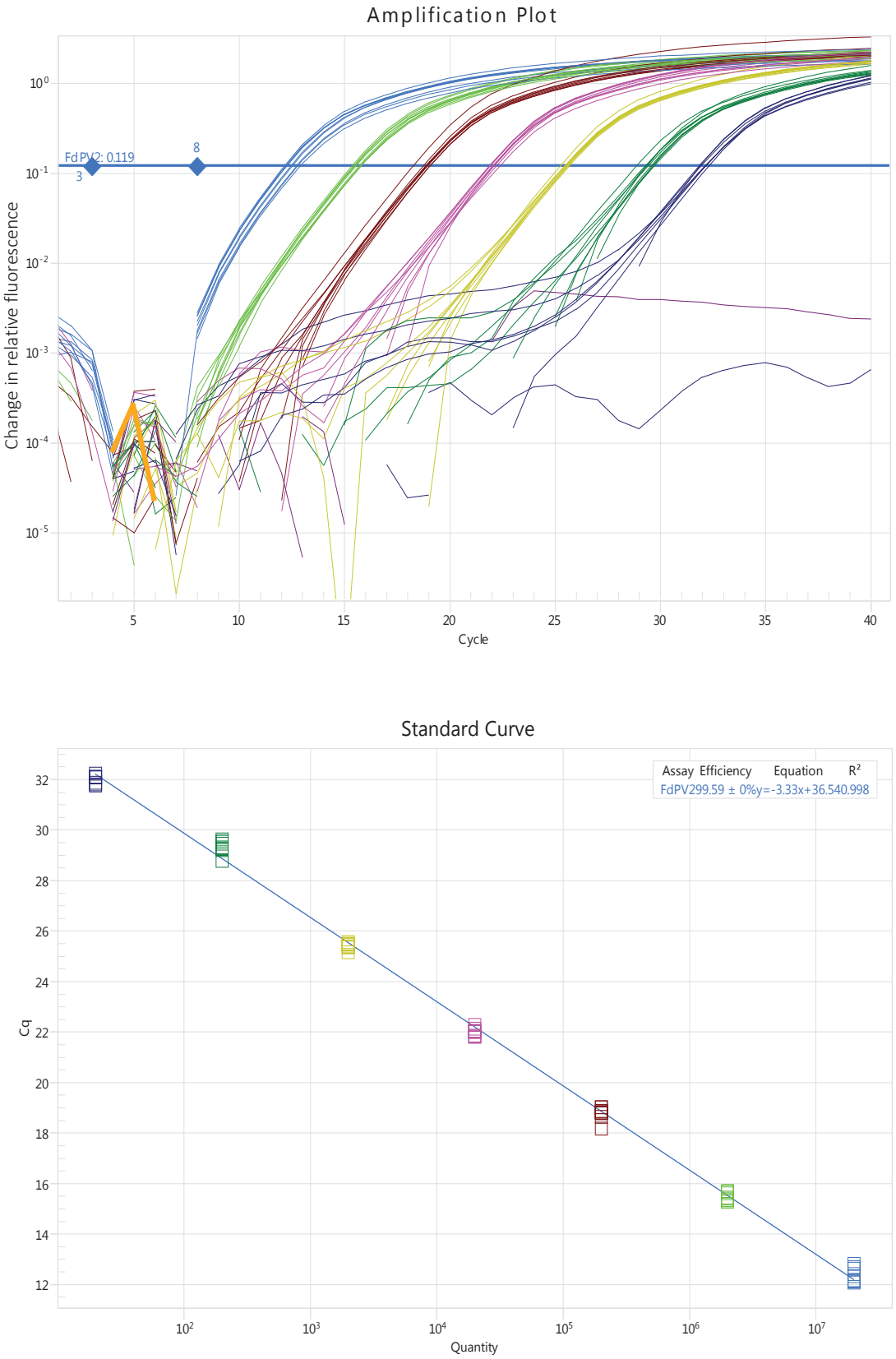

Figure 2.1. Amplification plot and standard curve for the newly developed qPCR FcaPV-2 assay. E = 99.59%, r^2 = 0.998.

Plasmid dilution	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Intra-assay CV	13.8	13.5	20.9	12.1	8.9	11.5	11.7
Inter-assay CV	8.9	10.0	6.8	7.5	5.4	22.6	13.8

Table 2.1. Coefficient of variation (%) as a measure of precision (intra-assay) and reproducibility (inter-assay) of the newly developed FcaPV-2 assay.

2.3.3 Testing *o*f the samples

Sixteen of the 372 samples did not contain amplifiable DNA and were excluded from the study. Prior to kittening, FcaPV-2 DNA was detected in either swabs or hair plucks from 10 of 11 (91%) queens (Table 2.2). However, FcaPV-2 DNA was subsequently amplified from the one queen that was initially negative 7 days after kittening so that FcaPV-2 DNA was detected from all 11 queens during the study.

FcaPV-2 DNA was detected in at least one sample type from 20 of 22 kittens (91%) at 2 days of age, from 14 of 20 kittens (70%) at 7 days of age, and from 15 of 20 kittens (75%) at 28 days of age (two of the kittens died at 3 days of age). Of the two kittens that were negative at 2 days of age, FcaPV-2 was subsequently amplified from one at 7 days of age and the other at 28 days of age so that FcaPV-2 was detected from all 22 kittens, in either a swab or a hair pluck sample, at some point during the study.

	Pregnant Queens	**Kittens 2 days**	**Kittens 7 days**	**Kittens 28 days**	**Queens all time points**	**Kittens all time points**
Both sample types: cats positive for FcaPV-2 (%)	10/11 (91)	20/22 (91)	14/20 (70)	15/20 (75)	11/11 (100)	22/22 (100)
Swabs: cats positive for FcaPV-2 (%)	8/10 (80)	19/22 (86)	12/17 (71)	13/20 (65)	11/11 (100)	20/22 (91)
Swabs: mean FcaPV-2 DNA load (range)[a]	1330 (6-5700)	3510 (1-24200)	9400 (8-72000)	2100 (3-18700)	9400 (6-92500)	4700 (1-72000)
Hair plucks: cats positive for FcaPV-2 (%)	10/11 (91)	12/22 (55)	12/20 (60)	13/20 (65)	11/11 (100)	19/22 (86)
Hair plucks: mean FcaPV-2 DNA load (range)[b]	3.4 (0.01-37)	0.21 (0.02-4.4)	4.7 (0.01-234)	0.09 (0.01-1.6)	2.5 (0.01-45)	1.62 (0.01-234)

Table 2.2. FcaPV-2 DNA detected in swab and hair pluck samples at the different time points. [a] Mean FcaPV-2 DNA load in copies of FcaPV-2 DNA per swab. [b] Mean FcaPV-2 DNA load in copies of FcaPV-2 DNA per copy of reference gene DNA. Note: 2 kittens died at 3 days of age. Only samples confirmed to have amplifiable DNA were included in this table. Range reported for positive samples only.

Quantitative PCR revealed large variations in the viral DNA load between cats, ranging from 1 to 92,520 copies per swab, and from 0.01 to 234 copies per copy of reference gene DNA in the hair plucks. The mean viral loads for the individual animals are shown in Table 2.3. The load of FcaPV-2 DNA detected in samples from each queen was significantly correlated to the load of FcaPV-2 DNA in samples from her kittens ($r = 0.79$; $p < 0.01$).

All of the cats from the four litters in households 1 and 2 had low viral DNA loads in their swab samples (Table 2.3). Out of 32 swab samples tested, none had greater than 23 copies of FcaPV-2 DNA. An ANOVA model was used to generate 95% confidence intervals for the mean viral DNA loads in the swab samples from the different litters and households. Mean viral DNA loads were considered significantly different if the 95% confidence intervals did not overlap ($p < 0.05$). There were no significant differences in the mean viral DNA loads between the four litters in households 1 and 2, or between the two households. In contrast, the cats from both litters in household 3 had high viral DNA loads with 17 of the 18 swab samples having greater than 1000 copies of FcaPV-2 DNA. There was no significant difference between the mean viral DNA loads from these two litters, but there was a significant difference between the mean viral DNA load in household 3 compared to households 1 and 2. Cats from the two litters in household 4 had significantly lower viral DNA loads than those of household 3, greater than those of household 1, but not significantly different from those in household 2. Household 5 was the only household where there were significant differences in the mean viral DNA load between different litters within the same household. Litters one and three had significantly lower viral DNA loads than litter 2.

It was not possible to statistically compare the viral DNA loads between the different groups based on the hair pluck samples as, unlike the log transformed swab data, the hair pluck data was not normally distributed so the necessary statistical assumptions were not considered reasonable. There was however a good correlation between the load of viral DNA in swab samples and the mean load of viral DNA in the three corresponding pluck samples ($r = 0.78$; $p < 0.01$). So both sample types supported a similar pattern of viral DNA loads between households.

The mean viral DNA load detected on cats within each litter did not change significantly between the three time-points measured ($p = 0.16$). Thus the cats maintained a relatively constant FcaPV-2 DNA load over the course of the study.

	Hair pluck samples (copies/ref gene)[a]			**Swab samples (**copies/swab)[b]		
	PP/ 2 days[c]	7 days	28 days	PP/ 2 days[c]	7 days	28 days
Household 1						
Litter 1 (BM)						
Queen 1	0.02	0	0	5.74	0	0
Kitten 1	0.02	0.02	0.01	0	NA	0
Kitten 2	0	0	0	1.06	0	5.68
Litter 2 (M)						
Queen 2	0	0	0	NA	0	0
Kitten 1	0.25	0	0	12.7	0	0
Kitten 2	0	0	0	4.01	NA	0
Litter 3 (BM)						
Queen 3	0	0.07	0.10	0	0	0
Kitten 1	0	0	0.07	2.80	0	2.59
Kitten 2	0	0	0	0	0	5.30
Household 2						
Litter 1 (BB)						
Queen 1	7.65	0.31	0	22.5	16.1	13.0
Kitten 1	0	0	0	2.07	7.71	0
Kitten 2	0	0.01	0	0	NA	0
Household 3						
Litter 1 (BM)						
Queen 1	0.44	0.66	1.65	57.2	9573	11461
Kitten 1	0.42	0.38	0.11	14314	1872	1748
Kitten 2	1.63	79	0.10	9928	5915	3237
Litter 2 (BM)						
Queen 2	6.2	9.2	3.14	3500	9964	55439
Kitten 1	0.32	6.97	0.07	6155	34959	18714
Kitten 2	0.32	3.41	0.26	4923	37482	8636
Household 4						
Litter 1 (NF)						
Queen 1	2.78	0.13	1.31	390	20.7	309
Kitten 1	0.05	0	0	50.7	14.6	10.3
Kitten 2	0	0	0	54.2	0	11.1
Litter 2 (NF)						
Queen 2	0.31	0	0.26	271	160	318
Kitten 1	0.05	0.01	0.02	24.2	77.4	0
Kitten 2	0.04	0.13	0.03	51.0	28.4	0
Household 5						
Litter 1 (P)						
Queen 1	4.93	1	0	5705	176	304
Kitten 1	0	0.07	0	27.3	25.9	0
Kitten 2	0	0.01	0.00	30.2	62.0	18.4
Litter 2[d] (P)						
Queen 2	12.5	15.57	5.7	NA	92520	81315
Kitten 1	0.77	2.14	0.90	16773	71952	4143
Kitten 2	0.70	1.39	0.33	24225	7094	5059
Litter 3 (P)						
Queen 3	2			2020		
Kitten 1	0			560		
Kitten 2	0			90.4		

Table 2.3. FcaPV-2 DNA loads in swabs and hair pluck samples from individual cats.
[a] Mean FcaPV-2 DNA load (copies of FcaPV-2 DNA per copy of reference gene DNA) in hair plucks (*n* = 3). [b] FcaPV-2 DNA load per swab from a sample of three pooled swabs. [c] Each queen was sampled on three separate occasions: once within the 3 weeks prior to kittening, and then 7 and 28 days after kittening. Samples were also collected from two kittens from each litter when the kittens were 2, 7 and 28 days of age. [d] In household 5, Litter 2 had a significantly higher mean FcaPV-2 DNA load than Litters 1 or 3. In households 1-4, there was no significantly difference between the litters within each household. NA: no amplifiable reference gene DNA. BM: Burmese, M: Mandalay, BB: British Blue, NF: Norwegian Forest Cat, P: Persian

2.4 Discussion

This is the first time that quantitative PCR has been used to detect FcaPV-2 infection in cats and the assay demonstrated good sensitivity, linearity, precision and reproducibility. FcaPV-2 DNA was detected in samples from a high proportion of kittens, with 91% of kittens testing positive at 2 days of age when the results from both sample types were considered. Additionally, there was a strong correlation between the FcaPV-2 DNA load detected in samples from each queen and her kittens. This suggests that kittens are often exposed to FcaPV-2 early in life, and the queen or the immediate environment is the likely source of this exposure. These results are consistent with the epidemiology of human cutaneous beta PVs which can be detected on babies as early as 1 day of age and this early transmission is thought to result mainly from direct contact with the parents.[18,19] Therefore, it appears likely that FcaPV-2 shed from the queens would similarly be the primary source of exposure of kittens to FcaPV-2.

Infection by many PV types requires micro-trauma to the epithelium and exposure of the basement membrane to an infectious dose of PVs.[20] Such micro-trauma seems likely in newborn kittens given the fragility of their skin combined with the birthing process and mouthing of the kittens by the queen. Considering the high level of exposure of most kittens to FcaPV-2, it appears likely that infection would occur soon after birth. In addition to the skin swabs, FcaPV-2 DNA was quantified in hair pluck samples as high loads of FcaPV-2 DNA in hair plucks were expected to indicate that the kitten itself was infected. Large quantities of FcaPV-2 DNA were indeed found in the hair pluck samples from several kittens as early as 2 days of age but it seems unlikely that this would be from an infection of the kitten's skin as, even if infection had occurred immediately after birth, there had not been enough time for the virus to complete its lifecycle.[21] Therefore, the results of this study are useful for assessing exposure to FcaPV-2 but do not indicate when infection occurs.

FcaPV-2 DNA was also detected in samples from a high proportion of the pregnant queens which supports the results of an earlier study that FcaPV-2 is frequently amplifiable from clinically normal cats.[2] It appears that asymptomatic PV infections of the skin are common in both humans and animals.[9,22] FcaPV-2 has been associated with pre-neoplastic and neoplastic skin lesions in cats but the frequent detection of this virus from healthy queens and kittens in the current study suggests that the presence of FcaPV-2 alone is not sufficient for cancer development, and that other viral, host or environmental factors may be important for the

outcome of FcaPV-2 infection. This is similar to human beta-PVs which are ubiquitous on the skin of people, and only cause neoplasia when the body fails to mount or maintain an effective immune response.[23,24]

Wide variations in the quantity of FcaPV-2 DNA was found between cats, although for each cat the FcaPV-2 DNA load remained relatively constant for the duration of the study. The range of viral loads spanned six orders of magnitude and was similar to what has been reported for cutaneous beta PV infections in people.[25] The cause of this variation in cats is unknown, however in people there is significant variation in the effectiveness of cell-mediated immunity at limiting viral replication for specific PV types.[26] By extrapolation, it is possible that the consistently high viral DNA loads detected in some cats, particularly some of the queens, may indicate reduced effectiveness of cell mediated immunity. It remains to be established whether these cats may be at an increased risk of developing cancer due to PV infection.

As well as variations in the viral load between cats, there was also a pattern in the viral loads detected on cats in different households. Cats within each of the four households where the different litters were allowed to mix had similar FcaPV-2 DNA loads, but there were significant differences in the mean viral DNA loads detected from cats between the different households. In the 5th household, significant differences also existed between the FcaPV-2 DNA loads of the three litters. This was the only household where the queens were isolated after kittening, albeit with frequent handling of all cats by the breeder. Despite considerable opportunity for fomite transfer of the virus, the viral DNA loads in each litter of kittens was similar to those detected in their queens, and different to the other litters, suggesting that prolonged direct contact may be more important for spreading the virus between kittens than transfer on fomites. This is similar to the findings of Weissenborn *et al.* (2009), who swabbed children and parents from ten German families. Family members typically displayed a similar profile of beta HPV types, whereas the profiles of PV types detected differed markedly between families.[19]

Interpretation of the difference in viral DNA loads between the households in this study is difficult. It may represent different abilities of the kittens to limit FcaPV-2 replication following infection although a hereditary basis for this seems unlikely as both high and low FcaPV-2 DNA loads were seen in related litters of Burmese and Persian kittens. Similarly, physical characteristics of the skin and haircoat could potentially influence the kittens' susceptibility to FcaPV-2 infection, however this was not evident in the present study as both high and low FcaPV-2 DNA loads were seen on kittens from short and long-coated breeds. Alternatively, high

levels of FcaPV-2 shed from the queens may be detectable on their kittens but the kittens themselves may not necessarily be shedding large amounts of the virus, thus the differences may reflect differences in viral shedding by the queens rather than inherent differences in the kittens.

The timing of FcaPV-2 infection is important because PV VLP vaccines need to be administered prior to first infection to be effective. It is possible to prevent infection of people with the high-risk HPVs by vaccination as these PVs are spread by sexual activity and people are not infected prior to puberty.[27] The results of this study suggest that kittens are exposed to FcaPV-2 at a very early age, although there was no temporal increase in the viral load on the kittens to indicate the timing of infection. However it seems likely that infection would occur soon after the initial exposure, so there may not be a practical opportunity to vaccinate cats prior to first infection. A factor that may need to be considered in the relationship between exposure and infection is the presence of maternal antibodies. In people, IgG antibodies to HPV-16, -18, -6, -11 and -31 have been detected in umbilical blood from 33% of newborns and the presence of IgG in the newborn was strongly correlated to the presence of the same IgG in the mother.[28] Follow-up serology of eight children that had a strong IgG response to a single HPV type as a newborn, showed that only one child had detectable anti-HPV antibodies at 4 years of age, suggesting the IgG was from transfer of maternal antibodies.[28] The prevalence of antibodies to cutaneous beta PVs in people ranges from less than 1% to over 30% depending on the particular PV type. Therefore, it is plausible that some kittens may acquire maternally derived antibodies to FcaPV-2, although whether these would prevent infection is unknown. Currently there is no serological assay to detect anti-FcaPV-2 antibodies in cats, so the development of such an assay would facilitate similar studies to investigate the humoral immune response to FcaPV-2. If present, maternal antibodies could potentially prevent a kitten from mounting a humoral immune response to FcaPV-2 VLP vaccination but, even if this was the case, there may be a small window of opportunity for vaccination between waning of maternal antibody and the kittens becoming infected with FcaPV-2.

In conclusion, a robust and highly repeatable assay was developed for quantifying FcaPV-2 DNA in feline skin swabs and hair plucks. This assay was used to detect FcaPV-2 DNA on the skin of all eleven clinically asymptomatic adult cats. Additionally, it appears that most kittens are exposed to FcaPV-2 in the first few days of life and the primary source of exposure is likely to be direct contact with other cats in the household, particularly their queen. The ubiquitous nature and early exposure of kittens to FcaPV-2 suggests that prophylactic vaccination is unlikely to be a

practical way of reducing the incidence of FcaPV-2-induced lesions, although the timing of infection needs to be further established before ruling out this possibility. Additionally, results of this study showed large variations in the quantity of FcaPV-2 DNA detected on different groups of cats. This warrants further investigation as it may suggest differing susceptibilities to FcaPV-2 which may be one reason why only a proportion of infected cats develop disease.

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Chapter 3

Timing of FcaPV-2 Infection in a Cat Colony

3.1 Introduction

The detection of FcaPV-2 DNA in skin swabs from 91% of 2-day-old kittens, reported in the previous chapter, suggested that kittens are exposed to FcaPV-2 early in life. However it was not clear whether the kittens were truly infected with the virus, or whether the virus that was found on the kittens was shed from their queen and so represented an environmental contaminant. In other species, vaccination with papillomavirus (PV) virus-like particles (VLPs), which are recombinant capsid proteins that self-assemble into pseudo-capsids, has been shown to be highly effective at preventing PV infection and the development of cancer.[1,2] However, these vaccines cannot resolve existing PV infections and so must be given prior to infection.[3] Therefore, the aim of this study was to determine whether the FcaPV-2 DNA present on young kittens represented a true infection, or whether the detected DNA was an environmental contaminant from virus shed by the queens. This study carries on from chapter 2 with the aim of determining when kittens are infected with FcaPV-2 to assess whether there is an opportunity for prophylactic vaccination.

In the previous chapter it was found that determining the quantity of FcaPV-2 DNA present in hair plucks and skin swabs did not allow for differentiation of true infection versus environmental contamination. In this chapter, two new approaches were used to try to determine whether the FcaPV-2 DNA detected represented a true infection. Firstly, it was hypothesized that if the viral DNA detected in the kittens was simply due to environmental contamination then the amount of DNA detected would be similar on all of the kittens in the litter and on the queen. To test this hypothesis, pregnant cats that had either a high or low FcaPV-2 viral DNA load were identified and the viral DNA load on them and all of their kittens was followed, from birth, through weaning and until establishment in a cat colony at around 8 months of age. Kittens in high or low FcaPV-2 DNA load litters were also cross-fostered onto queens with the opposite viral DNA load to see the effect on the viral DNA load of each kitten.

The second approach was to look for FcaPV-2 RNA in skin swabs from kittens. Because mature squames shed into the environment are anucleate, they do not support gene expression and

were not expected to contain detectible host or viral RNA. However firm swab samples pick up a small number of less mature nucleated skin cells which contain host RNA and may potentially contain viral RNA. Therefore, the detection of viral RNA would suggest that the PV has truly infected the epidermis. This is the first time that skin swabs have been investigated for the presence of FcaPV-2 RNA.

3.2 Methods

3.2.1 Selection of cats

Skin swabs were taken from five pregnant queens at the Centre for Feline Nutrition at Massey University, Palmerston North, New Zealand. Two queens were identified with a high FcaPV-2 DNA load (Queens 1 and 3) and two with a low FcaPV-2 DNA load (Queens 2 and 4). The four queens that were selected had a total of 17 kittens, all of which were included in the study. All queens and kittens were domestic short hair breed. This study was approved by the Massey University Animal Ethics Committee (protocol 13/91).

3.2.2 Swab collection for DNA analysis

Cotton tipped swabs (Protec Solutions Ltd, Wellington, New Zealand) were first moistened in sterile saline then firmly drawn across a 3 x 3 cm area of skin five times. Two swabs were collected from each cat, one from the dorsum between the shoulder blades and the other from the abdomen cranial to the umbilicus. The two swab heads were collected into 500 µL sterile saline in one 5 mL universal container (Sarstedt AG and Co, Nümbrecht, Germany). The samples were transported to the laboratory within 30 minutes of collection where they were vortexed and the swab heads were squeezed out and removed. The remaining solution was stored at -20°C until DNA extraction.

3.2.3 Sampling time points

Swabs were collected from all queens 2 and 4 weeks prior to parturition. Following parturition, the queens and kittens were swabbed when the kittens were 1 day old, then before and after cross-fostering, and then weekly until the kittens were weaned and established in an outdoor run at 12-15 weeks of age. Once they were in the outdoor run, samples were taken at 4 weekly intervals until the kittens were approximately 32 weeks of age. Queen 1 was the first to deliver, giving birth to five kittens. Four days later, Queen 2 gave birth to three kittens. The next day one kitten was removed from Queen 1 (high viral DNA load) and cross-fostered onto Queen 2 (low

viral DNA load). All cats were swabbed again two and five days later. The kittens from these two litters were gradually mixed and weaned over a six week period before being moved to an outdoor run at 15 weeks of age. Three weeks after the first two litters were weaned Queen 3 gave birth to four live kittens although two died several days later due to complications at birth. Four days later Queen 4 gave birth to five kittens and these were swabbed the following day, along with Queen 3 and her two remaining kittens. The next day, one kitten was cross-fostered from Queen 4 (low viral DNA load) onto Queen 3 (high viral DNA load). All kittens were swabbed again two and five days later. The kitten cross-fostered onto Queen 3 died due to misadventure at 9 days of age. At around 6 weeks old the kittens were weaned and moved to an outdoor run. In total, 342 samples from 21 cats were collected over a 12 month period for analysis of the FcaPV-2 DNA load.

Control swabs of room air were taken at five time points during the study. These swabs were soaked in saline, waved around inside the room that contained the cat pens, without touching any surfaces, and then collected and processed with the test samples for DNA analysis. The purpose of these samples was to assess the degree of contamination occurring during the process of collecting the skin swabs from the cats.

3.2.4 Swabs for RNA analysis

In addition to the swab samples collected for DNA analysis, swabs were also collected from two litters for RNA analysis. The swabs for RNA analysis were taken in a similar manner to the DNA swabs. Initial efforts to detect RNA in the skin swabs were unsuccessful, however part-way through the kittening season it was discovered that collecting the swabs into Ambion RNAlater solution (Life Technologies, Carlsbad, CA, USA), rather than saline, yielded small quantities of extractible RNA. This method was used to collect swabs from Queens 3 and 4 and their kittens when the kittens were 1, 3, 8 and 15 days of age. A total of 35 swabs were collected from the two queens and seven kittens. Skin biopsies were also collected at post-mortem examination from the three kittens that died. These were taken from the dorsal area of skin that had been previously swabbed and were kept at -70°C prior to RNA extraction.

3.2.5 DNA and RNA extraction

DNA was extracted from the samples using the Roche High Pure PCR Template Preparation kit (Roche Applied Science, Mannheim, Germany) as reported in chapter 2. RNA was extracted from the swab and tissue samples using the ReliaPrep RNA Tissue Miniprep system (Promega, Madison, WI, USA) according to the manufacturer's recommendations. The tissue samples required an additional step of homogenizing 40 mg of each sample with Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) prior to DNA/RNA extraction. RNA was reverse transcribed into cDNA with the Roche Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) using 0.6 µg total RNA, and both random hexamer and oligo-dT primers according to the manufacturer's recommendations.

3.2.6 Real-time PCR

The real-time PCR assay developed and validated in chapter 2 was used for the swab samples in this study. This assay amplifies a 111 bp section of the FcaPV-2 E7 gene and the quantity of FcaPV-2 DNA is determined by comparing the Cq values from the test sample with a known concentration of a recombinant plasmid containing the same sequence. Quantities were normalised to copies per swab as normalising to feline genomic DNA was meaningless given the virus-containing squames were anuclear. To detect FcaPV-2 E6/E7 mRNA, the same real-time PCR assay was used, but with a cDNA template. This was possible because PV DNA is transcribed as a polycistronic RNA that is then alternatively spliced to create protein-coding transcripts.[4] By comparing splicing patterns of several different PV types, the 111 bp region of E7 was expected to be present only in the spliced transcripts coding for the FcaPV-2 E6 and E7 proteins.[5] Because the same sequence was present in viral DNA and cDNA, a negative RT control was included for every sample to exclude the presence of genomic DNA in the cDNA samples. Assays were also designed to detect mRNA coding for the FcaPV-2 capsid (L1) protein, and two *Felis catus* RNA reference genes (Beta actin and Abelson proto-oncogene 2 non-receptor tyrosine kinase), which are described in detail in the next chapter.

3.2.7 Statistical analysis

Data was analysed using Minitab 17.2.1 (Minitab Inc, State College, PA, USA). Log base 10 transformed data was used for statistical analyses. Samples that had no detectable virus were set at half the minimum detectable level of the assay prior to log transformation. Pearson correlation coefficients, Student's *t*-test, and ANOVA models were used. Results were back transformed and reported as geometric means and 95% confidence intervals (CI).

3.3 Results

3.3.1 FcaPV-2 DNA load in the swab samples

The quantity of FcaPV-2 DNA in the swab samples pre- and post-weaning is shown in Fig. 3.1. Queens 1 and 3 had consistently high FcaPV-2 DNA loads over the course of the study, with mean FcaPV-2 DNA copy numbers of 10,234 (95% CI 3890–26,915) and 15,849 (5623–44,668) copies of viral DNA per swab. In comparison, the viral load was significantly lower on Queens 2 and 4, with mean viral loads of 457 (170–1259) and 282 (100–832) copies respectively ($p < 0.001$). The quantity of FcaPV-2 DNA found on the kittens prior to weaning was strongly correlated to the quantity on their queen ($r = 0.833$; $p < 0.05$). High viral loads were found on the kittens from Queens 1 and 3 from as early as one day of age with mean FcaPV-2 copy numbers of 11,749 (3548–39811) and 1995 (417–9550) copies per swab. These queens and their kittens were designated the high viral DNA load litters. In comparison, the kittens from Queens 2 and 4 had significantly lower viral DNA loads, with means of 83 (22–324) and 69 (20–229) copies of FcaPV-2 DNA per swab, at one day of age ($p < 0.001$). These queens and kittens were designated the low viral DNA load litters. Cross-fostering a kitten from a litter with a high viral DNA load to a litter with a low viral DNA load resulted in a marked decrease in the quantity of FcaPV-2 DNA in swabs from that kitten: from 16,103 copies per swab to 14 copies per swab over 3 days. In reverse, cross-fostering a kitten from a low viral DNA load litter to a high viral DNA load litter, resulted in a marked increase in the quantity of FcaPV-2 DNA detected: from 54 copies to 6064 copies after 2 days and 158,966 copies after 7 days. Interestingly, although Queen 2 and her kittens generally maintained a low viral DNA low, a marked increase in the quantity of FcaPV-2 DNA detected on these cats occurred 1-2 weeks after the birth of the kittens, and 5 days after the addition of a cross-fostered kitten to the litter. The FcaPV-2 DNA load peaked at over 10,000 copies per swab for around 2 weeks before returning to under 100 copies per swab.

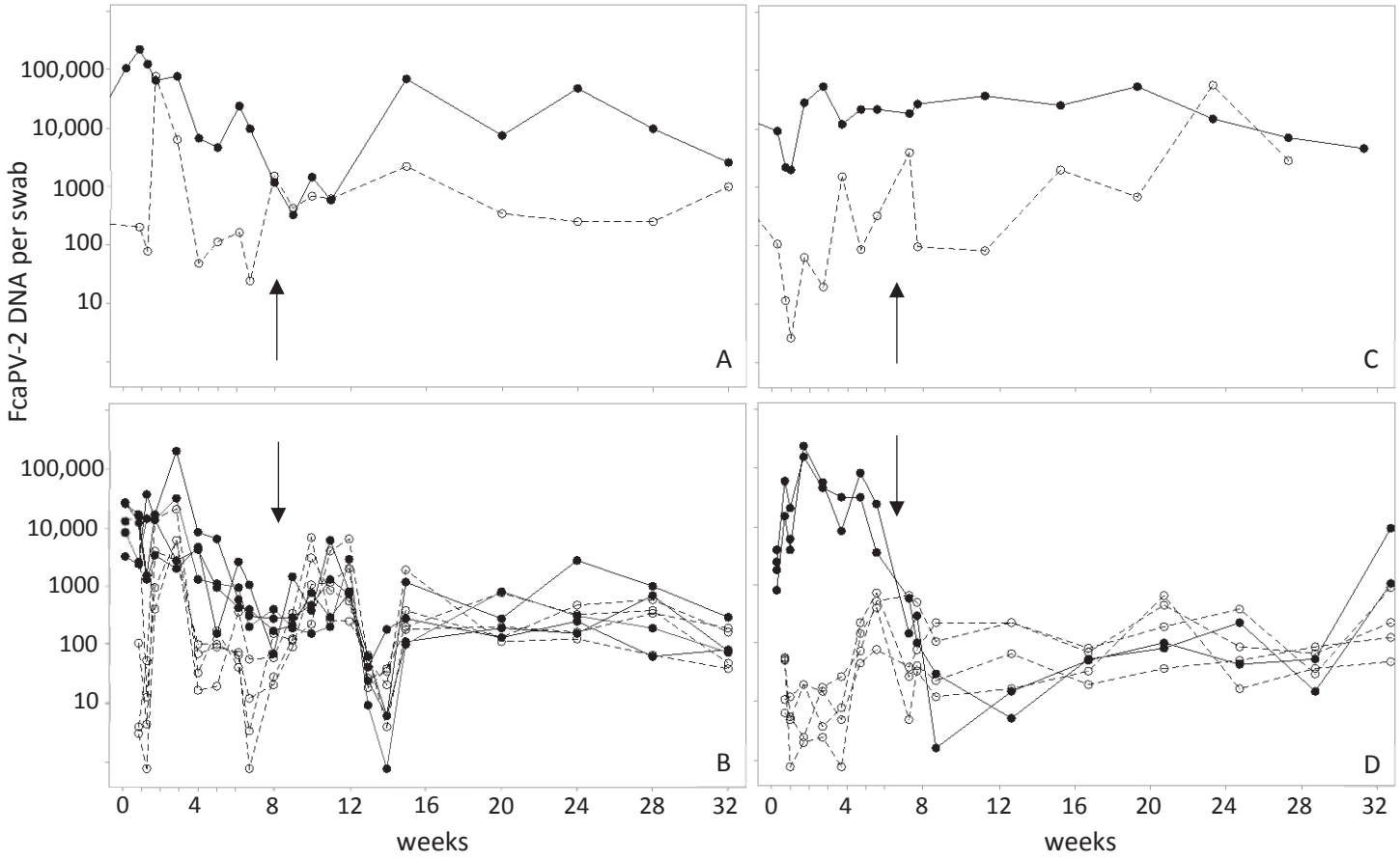

Figure 3.1. Copies of FcaPV-2 DNA per swab over time. Arrows indicate when the kittens were weaned from the queens. **A.** Queen 1 (solid circles) and Queen 2 (open circles). **B.** Queen 1's kittens (solid circles) and Queen 2's kittens (open circles). **C**. Queen 3 (solid circles) and Queen 4 (open circles). **D.** Queen 3's kittens (solid circles) and Queen 4's kittens (open circles). Note the similar pattern in viral load between each queen and her kittens pre-weaning but not post-weaning.

At weaning the kittens were separated from their queen and later moved into a large outdoor run. Following this separation, there was no longer any correlation in the viral DNA load between each queen and her kittens. Additionally, significant correlations in the viral DNA load between kittens within a litter, which were present pre-weaning, were also lost after weaning despite the kittens still being in contact with each other. In general, after weaning the viral DNA load on the kittens from the high viral DNA load litters dropped and the viral DNA load on the kittens from the low viral DNA load litters increased, so that all of the kittens had a similar viral DNA load that was relatively stable for the remainder of the study, at around 93 copies of FcaPV-2 DNA per swab (95% CI 69−123). Two deviations from this general pattern were noted. Firstly, there was a drop in the viral DNA load on all of the kittens from the first two litters when they initially entered the outdoor run. At this stage the kittens had been weaned for several weeks.

The viral DNA load remained low for 1-2 weeks before increasing again to levels similar to the weeks prior to entering the outdoor run. Secondly, when the last two litters joined the outdoor run, the FcaPV-2 DNA load on these kittens initially stabilised at a level mid-way between the low and high viral DNA load litters but then increased slightly towards the end of the study.

After weaning the Queens were returned to different outdoor runs, with other adult cats. The viral DNA load on Queens 1 and 3 remained high with mean FcaPV-2 DNA copy numbers of 4467 (95% CI 891−22,909) and 15,849 (9120−35,481) copies per swab. In contrast, the viral loads on Queens 2 and 4 were signifcantly lower, at 550 (295−1023) and 1096 (295−1023) copies of FcaPV-2 DNA per swab ($p < 0.001$).

Viral DNA was also found in four out of five room air swabs, which contained between 9 and 116 copies of FcaPV-2 DNA. As all of the non-template controls in the lab were negative, it is likely that the FcaPV-2 DNA on these swabs came from dust particles that stuck to the saline-soaked swabs as they were waved around in the room containing the cat pens.

3.3.2 FcaPV-2 RNA in swab and tissue samples

The results from the additional swabs collected for RNA analysis are shown in Table 3.1. Amplifiable RNA was present in 26 of 35 samples, although typically only the more abundant reference gene (Beta actin) was amplifiable. This meant that although the presence or absence of viral gene expression could be demonstrated, it was not possible to determine the relative quantity of viral gene expression. Late viral gene expression was found in three samples, and all RT controls were negative, confirming this was indeed viral mRNA and not contaminating viral DNA. No FcaPV-2 early gene expression was detected in the swab samples.

	Day 1		Day 3		Day 8		Day 15	
	E7	L1	E7	L1	E7	L1	E7	L1
Queen 3	–	–	–	–	–	–	–	–
Kitten 10	–	–	–	+	–	–	–	–
Kitten 11	–	–	–	–	–	–	NA	NA
Kitten 16[a]			–	–	NA	NA		
Queen 4	–	–	–	–	–	–	–	–
Kitten 13	–	+	NA	NA	–	+	NA	NA
Kitten 14	–	–	NA	NA	NA	NA	NA	NA
Kitten 15	–	–	–	–	NA	NA	–	–
Kitten 16[a]	–	–						
Kitten 17	–	–	NA	NA	–	–	–	–

Table 3.1. FcaPV-2 early (E7) and late (L1) gene expression in swab samples. + : amplifiable FcaPV-2 and reference gene mRNA, – : no amplification of FcaPV-2 mRNA but amplifiable reference gene mRNA, NA: no amplifiable mRNA. [a] Kitten 16 was cross-fostered from Queen 4 to Queen 3 after the first sampling.

Skin biopsies were collected from three kittens that died during the first few weeks of life. All three samples contained good quality RNA as both reference genes amplified. FcaPV-2 early gene expression was detected in one of these biopsies but no late viral gene expression was detected (Table 3.2).

	E7	L1
Kitten 9	–	–
Kitten 12	+	–
Kitten 16	–	–

Table 3.2. FcaPV-2 early (E7) and late (L1) gene expression in post-mortem tissue samples. +: amplifiable FcaPV-2 mRNA, –: no amplifiable FcaPV-2 mRNA.

3.4 Discussion

In the previous chapter FcaPV-2 DNA was detected in skin swabs from kittens as young as 2 days of age. The aim of the study presented in this chapter was to try to determine if the FcaPV-2 DNA detected in swab samples from young kittens was the result of a true infection, with the virus replicating in the kittens' skin, or whether it was just a contaminant from virus-laden squames shed from the queen and deposited on the kittens' skin. The close correlation between the viral load on the queens and kittens while they were in close contact pre-weaning, and then the loss of this correlation post-weaning, suggested that the FcaPV-2 DNA found on the kittens pre-weaning was a contaminant shed from the queens. Further supporting this, when a kitten was moved from a litter with a high viral DNA load to a litter with a low viral DNA load, the FcaPV-2 DNA load on the cross-fostered kitten dropped dramatically over 3 days. Conversely, when a kitten was moved from a litter with a low viral DNA load to a litter with a high viral DNA load, the quantity of FcaPV-2 DNA on the cross-fostered kitten rose dramatically over a similarly short time period. These changes occurred much faster than the complete PV lifecycle, which is tightly linked to the differentiation process of the epidermis and generally takes weeks to months.[6] Therefore, the results of this study suggest that the source of FcaPV-2 DNA on young kittens was not a true infection of the kittens but rather an environmental contaminant.

Small quantities of FcaPV-2 DNA were also found in swabs of the air in the room that contained the cat pens. As cornified squames make up a large component of dust, the presence of FcaPV-2 DNA in the environment is consistent with shedding of FcaPV-2 from the queens.[7] It is also possible that the immediate environment, rather than the queens, might be the source of FcaPV-2 DNA found on the kittens. However this is unlikely as the quantity of FcaPV-2 DNA in the swabs from the queens and the kittens fluctuated in a similar pattern while their environment remained constant. To the authors' knowledge this is the first time that PV DNA has been detected in samples of air and the detection illustrates the difficulty in the interpretation of small quantities of PV DNA present in skin swab samples.

While these results suggest that the FcaPV-2 DNA in the swabs of the young kittens probably came from the queens, there was also evidence that some of the kittens became infected with FcaPV-2 over the course of the study. Firstly, FcaPV-2 DNA loads increased in both groups of kittens post-weaning. At this stage the kittens had been in the same environment without any contact with other cats for many weeks, so the most plausible explanation for this rise was a

new infection with viral replication occurring in one or several of the kittens. Additional evidence for FcaPV-2 infection in the kittens was the detection of FcaPV-2 early gene expression in a skin biopsy from one of the kittens that died 4 days after birth and low levels of late viral gene expression in skin swabs from two other kittens in their first 8 days of life. Skin biopsies from two other kittens were negative but due to the focal nature of PV infections, FcaPV-2 infection could easily have been missed. The detection of only late viral gene expression in the skin swabs is consistent with the expected pattern of viral gene transcription where early gene expression is confined to the lower epidermal layers which are unlikely to be reached by superficial skin swabs.

The presence of both late viral gene expression and feline reference gene expression in the swab samples suggests that the sampled cells were from the mid- upper epidermal layers where the cells still have nuclei and are transcribing genes. In contrast, cornified squames shed from another animal but present on the kitten's skin would be anuclear and unlikely to contain either viral or host mRNA. Therefore, the detection of FcaPV-2 mRNA was considered evidence of a true FcaPV-2 infection, and was found in samples from 3 of 9 (33%) kittens in this study (although there were 17 kittens, samples from only nine kittens were suitable for RNA analysis). However this probably underestimates the proportion of kittens that became infected with FcaPV-2. The quantity of mRNA in the swab samples was low due to the small number of nucleated cells collected by this method. Therefore, some true FcaPV-2 infections may not have been detected because too few infected cells were collected. By comparison, the biopsies collected at post-mortem contained large numbers of nucleated cells and lots of mRNA was extracted. However the biopsies were also an insensitive measure of true FcaPV-2 infection because they represented only a small area of skin and may have missed focal FcaPV-2 infections. While FcaPV-2 DNA is shed from the skin surface and distributed widely in cornified squames, FcaPV-2 mRNA is restricted to the focal area of epidermis overlying the infected basal cells. Therefore, the chance of detecting FcaPV-2 mRNA in a skin biopsy from an infected cat is likely to be very low. Due to the lack of a sensitive measure of true FcaPV-2 infection it was not possible to determine the prevalence of FcaPV-2 infection in young kittens, but the presence of FcaPV-2 gene expression in a tissue biopsy and several skin swabs is strongly suggestive that some of the kittens were indeed infected with FcaPV-2.

The presence of FcaPV-2 late viral gene expression in swabs taken from kittens that were only 1-8 days old was surprising. The timing of PV gene expression immediately following viral entry is not well understood. Late viral gene expression is thought to coincide with genome replication,

which occurs in terminally differentiating cells in the intermediate epidermal layers. The time taken for basal cells to enter this layer varies between body sites and species but is usually 2-4 weeks.[7] However, transit times as short as 3 days have been demonstrated in neonatal rats, and late viral gene expression has been reported as early as 5 days after transfection of a human osteosarcoma cell line with human PV type 18 (HPV-18).[8,9] Therefore, the early detection of FcaPV-2 late gene expression in kittens may be plausible if they were infected soon after birth. Alternatively it may be possible that FcaPV-2 infection can be acquired intrapartum as there is some evidence for this route of transmission for HPVs.[10]

The presence of infection with FcaPV-2 in such young kittens suggests that maternal antibodies to FcaPV-2, if present, did not confer protection against infection. Around half of people infected with cutaneous HPVs develop type specific anti-HPV antibodies, and maternal antibodies against several different mucosal HPV types have been demonstrated in umbilical cord blood from around a third of newborn babies.[11,12] Antibodies against a cutaneous PV (*Canis familiaris* papillomavirus 3) have also been found in sera from dogs.[13] Therefore, it is likely that cats would similarly produce antibodies against FcaPV-2. A possible reason for the lack of a protective effect in this study could be an insufficient quantity of passively transferred anti-FcaPV-2 antibodies. Passive transfer of serum from a rabbit immunized with HPV-16 VLPs to naïve mice protected the latter against a HPV-16 VLP challenge by preventing the VLPs from binding to the basement membrane.[14] This mechanism was only effective at high antibody concentrations, such as those produced following vaccination with VLPs, although different mechanisms may exist at lower antibody titres.[14] Therefore, a weak humoral immune response or waning of antibodies over time could explain the lack of protection from passive transfer of maternal antibodies in this study. The different mechanisms of passive transfer of antibodies may also be important; in people passive transfer is entirely transplacental, whereas in cats passive transfer of antibodies occurs both transplacentally and via colostrum.[15]

In the present study, the quantity of FcaPV-2 DNA detected on all of the cats in an enclosure was, on several occasions, uniformly high one day and then uniformly low a few days later. This suggests that FcaPV-2 DNA may not persist for very long on the skin of cats which may be due to continued removal of dead skin cells by grooming. It follows that for continued detection of high quantities of FcaPV-2 DNA there needs to be a continued source of the virus. In this study, Queens 1 and 3 had consistently high FcaPV-2 DNA loads, even when they were isolated for the four weeks prior to parturition and after they had been returned to the large outdoor runs. This suggests that these cats were infected with FcaPV-2 and shedding large quantities of virus that

translocated to the kittens and to the environment. This supports the findings of chapter 2, which also found large quantities of FcaPV-2 DNA on adult cats. The presence of FcaPV-2 infections in adult cats may suggest that some cats fail to clear FcaPV-2 infections acquired as kittens or that adult cats frequently become re-infected with the virus. However it is possible that the FcaPV-2 DNA detected on the adult cats was due to environmental contamination therefore the presence of infection needs to be confirmed by the detection of FcaPV-2 RNA in swabs or tissue biopsies from these cats.

As FcaPV-2 infection was confirmed in 33% of a subset of kittens in this study within the first 8 days of life, there appears to be little opportunity to prophylactically vaccinate kittens against FcaPV-2. Vaccination of pregnant queens may be a viable way to prevent FcaPV-2 infection in kittens as vaccination of pregnant women with HPV-6 and -11 VLPs has been advocated as a way to prevent juvenile-onset recurrent respiratory papillomatosis, a rare PV-induced disease of children.[16] Although naturally acquired maternal antibodies to FcaPV-2, if present, did not confer protection against infection in this study, vaccination with FcaPV-2 VLPs would be expected to induce a stronger humoral immune response that may be capable of preventing FcaPV-2 binding and entry into basal skin cells in the kittens.[17] However, with the exception of cat breeders and research colonies, many feline matings are unplanned which could limit the feasibility of this option. Another approach could be vaccination to prevent new PV infection, regardless of past or current infection. This approach has been tried in people and did not protect against high-risk HPV-induced cancer, however it has been shown to prevent PV-induced cutaneous tumours in rodents.[2,18] Whether vaccination against new infections would reduce the incidence of FcaPV-2-induced skin disease in cats is currently unknown.

In summary, the FcaPV-2 DNA detected on young kittens in this study appeared to come from their queens rather than represent a true infection of the kittens with FcaPV-2. However, the finding of FcaPV-2 mRNA in swab and tissue samples from three kittens confirmed that some kittens do become infected with FcaPV-2 soon after birth. This is the first time that FcaPV-2 RNA has been detected in skin swabs. Additionally two adult cats in the study appeared to be consistently shedding large quantities of virus, which may suggest that cats are susceptible to persistent infections with FcaPV-2, or readily become re-infected.

3.5 References

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Chapter 4

FcaPV-2 Gene Expression in SCCs

4.1 Introduction

The overall objective of this thesis was to investigate FcaPV-2 infection in cats, with the ultimate goal of reducing the number of cats that die due to skin cancer. The first research aim was to investigate the natural history of FcaPV-2 infection in cats, to determine when cats are likely to become infected with the virus. This was addressed in chapters 2 and 3. The focus of the remainder of this thesis will be the second research aim, which was to investigate the involvement of FcaPV-2 in feline cutaneous SCCs, to differentiate between FcaPV-2 being a cause of skin cancer in cats and FcaPV-2 being present in skin cancers simply as an innocent bystander.

Although many infections with FcaPV-2 are asymptomatic, FcaPV-2 is thought to cause feline viral plaques (FVPs) and Bowenoid in situ carcinomas (BISCs).[1] There is also some evidence that FcaPV-2 causes cutaneous SCCs, which are the most common malignant skin cancer of cats.[2] Early evidence of this was the observation of transitional lesions between FVPs, BISCs, and SCCs.[3] Subsequently, FcaPV-2 DNA was detected in around 50% of all feline cutaneous SCCs, and 76% of those on densely haired skin, compared to less than 10% of non-SCC skin lesions.[1,4] Additionally, the SCCs that harboured FcaPV-2 DNA frequently had increased levels of the host cyclin dependent kinase inhibitor p16INK4A (p16) and reduced levels of retinoblastoma protein (pRb).[4,5] Increased levels of p16 are frequently seen in human PV-induced SCCs and are thought to be a cellular defence response triggered by expression of the high-risk HPV E7 protein.[6] The response is ineffective however as it is bypassed by HPV E7-induced degradation of retinoblastoma protein (pRb), an important mechanism by which PVs influence cell-cycle regulation.[6,7] Increased levels of p16 in both feline cutaneous SCCs and human oropharyngeal SCCs have been associated with a more favourable prognosis compared to p16 negative SCCs, which lends further support for an association between FcaPV-2 and feline skin cancer.[8,9]

However, despite the evidence supporting an association between FcaPV-2 and feline cutaneous SCCs, FcaPV-2 DNA is also frequently present on the skin of clinically normal cats which makes it difficult to exclude the possibility that it is merely an incidental finding in SCCs. Previous studies,

using end-point PCR to detect FcaPV-2 DNA, did not determine the amount of virus present nor its transcriptional activity. Latent PV infections are common in other species so it is plausible that the detection of FcaPV-2 DNA may indicate a latent infection that is unrelated to cancer development.[10] Also, while increased levels of p16 in feline cutaneous SCC appears to have prognostic significance, it has not been conclusively demonstrated that the increased p16 expression is the result of an active infection with FcaPV-2. Alternatively, increased p16 expression in these SCCs may be due to genetic or epigenetic changes which are unrelated to PV infection. Although p16 is down-regulated in most non-HPV-induced cancers, it is upregulated in some distinct subsets including triple-negative breast cancers, treatment-resistant prostate carcinomas, and non-small cell lung cancers.[11-13]

To distinguish between an incidental latent infection with FcaPV-2 and involvement of the virus in cancer development, it is necessary to determine the quantity and transcriptional activity of the FcaPV-2 present in feline cutaneous SCCs. A key event in high-risk HPV-induced cancer in people is deregulation of HPV E6 and E7 gene expression, which is normally restricted to the suprabasal layers of the epidermis. The PV E6 and E7 proteins interact with multiple host targets to keep the cell in the synthesis phase of the cell cycle, allowing the virus to utilize host cell replication machinery to replicate its own genome.[14] Overexpression of the PV E6 and E7 genes in basal cells leads to abnormal cell proliferation and the acquisition of additional mutations, which can progress to invasive cancer.[15] Therefore, if FcaPV-2 is involved in the development of the cancer, it would be expected to be present in high copy numbers and actively transcribing genes, especially E6 and E7.[16] Alternatively, if the FcaPV-2 DNA present in feline cutaneous SCCs is a latent infection, low viral copy numbers (less than one per cell) and minimal gene expression would be expected as has been described in other species.[10]

Therefore, the aim of this chapter was to develop real-time PCR assays to detect FcaPV-2 early and late gene expression and then use these assays, along with the previously designed quantitative real-time PCR, to determine the quantity and transcriptional activity of the FcaPV-2 DNA present in feline cutaneous SCCs. The presence of p16 protein within the SCCs was also investigated to determine whether increased levels of p16 correlated with E6/E7 gene expression.

4.2 Methods

4.2.1 Sample Collection

To minimise RNA degradation, samples of cutaneous SCCs were collected as they were being excised by a veterinarian as part of routine treatment. A portion of the biopsy was collected into Ambion RNAlater solution (Life Technologies, Carlsbad, CA, USA) within 30 minutes of removal, chilled for 12 hours to allow tissue penetration and then stored at -20°C. The remaining tissue was collected into 10% neutral buffered formalin, processed for histology and embedded in paraffin wax. Sections (3 µM) were cut and stained with haematoxylin and eosin (H&E). The resulting sections were viewed to confirm the diagnosis of SCC, based on the presence of islands and trabeculae of neoplastic squamous cells originating in the epidermis and extending through the basement membrane into the dermis.[17] Eight SCCs had ample tissue and so were also used in a reference gene validation study. Control samples of normal skin were collected from eight cats that had been euthanized for unrelated reasons. This study was exempt from requiring animal ethics approval (Massey University Code of Ethical Conduct for the Use of Live Animals for Research, Testing and Teaching, 2013, section 5.1) as the samples were removed from cats as part of the normal course of veterinary treatment, as recommended and carried out by a registered veterinarian not involved in the study.

4.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from the skin samples using the ReliaPrep RNA Tissue Miniprep system (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Nucleic acid concentrations were measured with a Qubit 2.0 fluorometer, using Qubit dsDNA High Sensitivity, and Qubit RNA Broad Range assay kits (Life Technologies). To remove any residual genomic DNA an additional post-extraction DNAse digestion was performed using Ambion Turbo DNA-free DNAse according to the manufacturer's protocol (Life Technologies). To check for complete removal of genomic or viral DNA, RNA from all of the samples was included in real-time PCR runs as minus reverse transcriptase (minus-RT) controls. Complementary DNA synthesis was performed with the Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) using 0.6 µg total RNA, and both random hexamer and oligo-dT primers according to the manufacturer's recommendations. For reference gene validation, the cDNA

concentration was measured with the Qubit 2.0 fluorometer (Life Technologies) and diluted with DNAse-free, RNAse-free water to a final concentration of 5 ng/µL.

4.2.3 Development of real-time PCR assays for feline reference genes

To compare the quantity of FcaPV-2 gene expression between samples, viral gene expression needed to be normalised against the expression of two or more stably expressed reference genes. Although reference gene stability has not been previously assessed in feline skin samples, it has been assessed in other feline tissues.[18,19] Real-time PCR assays were established for nine of the previously used candidate reference genes, using either previously published primers, or new primers designed using Primer3 and MFold software, shown in Table 4.1.[20,21] All real-time PCR assays were performed using an Eco real-time instrument (Illumina Inc., San Diego, CA). The reference gene assay reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD), primer concentrations as listed in Table 4.1, and 10 ng of cDNA, in a total volume of 10 µL. The cycling conditions consisted of an initial 5 minute denaturation step at 95°C, followed by 40 cycles of denaturation (95 °C for 5 seconds), annealing (60°C for 20 seconds), and elongation (72°C for 15 seconds). The cycling was followed by a melting step from 55°C to 95°C. Assay efficiencies were calculated from standard curves derived from PCR products. Reactions were run in triplicate and each plate included a positive control and no template control.

Gene [a]	Primer (5'→3') F: forward, R: reverse	Primer Conc [b]	bp	Exon	Gbp	Efficiency	r^2
ACTB	F: CAACCGTGAGAAGATGACTCAGA	0.9	127	3/4	410	90.3%	0.994
	R: CCCAGAGTCCATGACAATACCA	0.9		4			
YWHAZ	F: GAAGAGTCCTACAAAGACAGCACGC	0.5	115	4	> 2800	96.2%	0.996
	R: AATTTTCCCCTCCTTCTCCTGC	0.5		5			
GUSB	F: CTACATCGATGACATCACCATCAG	0.9	80	4	532	90.4%	0.995
	R: CGCCTTCAACAAAAATCTGGTAA	0.9		5			
GAPDH	F: AGTATGATTCCACCCACGGCA	0.9	102	3	173	92.0%	0.999
	R: GATCTCGCTCCTGGAAGATGGT	0.5		3/4			
RPS7	F: ACGAGTTCGAGTCTGGCATC	0.7	74	1	836	93.3%	0.998
	R: AACTTCGATCTCCTTGGCCG	0.7		1/2			
RPL17	F: AGAACACACGGGAAACTGCC	0.9	118	4	118	92.2%	0.996
	R: CCACCATTGTAGCGTCGGAA	0.9		4			
RPS19	F: CGGAGGACGTCAGAGAAATGG	0.9	135	4	665	95.7%	0.994
	R: GTCAGTTTGCGTCCCCCATC	0.9		4/5			
ABL2	F: AGACTGATGACTGGAGACACCT	0.5	135	8	1433	98.3%	0.997
	R: CCACAGCAGTACCCCAAAAG	0.5		9			
B2M	F: GCCGTCCAGCATTCTCCAAAG	0.9	159	1/2	> 3600	99.3%	0.994
	R: ATCTGTCTGTTCCGCTTCCA	0.9		2			

Table 4.1. Reference gene primers and assay performance. [a] ACTB: Beta actin, primers from Kessler *et al.* (2009); YWHAZ: Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta, primers from Penning *et al.* (2007); GUSB: Beta glucuronidase, primers from Kessler *et al.*; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, primers from Penning *et al.*; RPS7: Ribosomal protein S7, accession number NM_001009832.1; RPL17: Ribosomal protein L17, accession number NM_001128842.1; RPS19: Ribosomal protein S19, accession number XM_003997752.3; ABL2: Abelson proto-oncogene 2 non-receptor tyrosine kinase, accession number XM_003999233.1; B2M: Beta-2 microglobulin, accession number NM_001009876.1. [b] Final primer concentration in µM.

4.2.4 Reference gene validation study

Each of the nine reference gene assays were run on a subset of samples: eight SCCs that had ample tissue and the eight normal skin samples, according to the reaction protocols described above. The template concentration was standardised by using 10 ng cDNA. All reactions were performed in triplicate and each plate included a positive control and no template control. Residual genomic DNA was excluded on the basis of the melting temperature and/or minus-RT controls. Reference gene stability was analysed with two statistical algorithms GeNorm and NormFinder. The GeNorm algorithm is a pairwise comparison model included in the qbase+ software (Biogazelle, Zwijnaarde, Belgium) and NormFinder is an excel add-in which uses an ANOVA-based model. [22,23]

4.2.5 Development of real-time PCR assays for FcaPV-2 gene expression

In the HPV life cycle, all early transcripts are synthesized as polycistronic mRNAs and then alternatively spliced. The E6 protein is translated from unspliced transcripts whereas the E7 protein is translated from a region of E6 spliced onto E7.[24] The E7SF/R primers (chapter 2) were designed to amplify a 111 bp fragment in the E7 region which was expected to be present in mRNA transcripts coding for both E6 and E7 proteins but not for any other early gene proteins. The use of the same primers to amplify both DNA and RNA was possible because the PV genome does not have conventional introns and extrons, relying instead on alternative splicing of polycistronic RNA. However, because it was not possible to distinguish contaminating viral DNA from cDNA, RNA from every sample was included as a minus-RT control to exclude the presence of residual viral DNA. Real-time PCR reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences), 0.4 µM of E7SF primer, 0.3 µM of E7SR primer, and 1 µL of template cDNA. To detect FcaPV-2 late gene expression, L1SF (5'ACGCGGTACCAATTTCACCCTGT) and L1SR (5'GGGGGTAAGAGTGACCACGCACA) primers were designed to amplify a 145 bp fragment from the FcaPV-2 L1 gene. Reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences), 0.5 µM of each primer, and 1 µL of template cDNA. All reactions were run in duplicate. The minus-RT controls as well as positive and no-template controls were included on each plate. Assay linearity for the newly developed L1 assay was assessed from standard curves of diluted PCR product. FcaPV-2 gene expression for each sample was reported as a normalised relative quantity (NRQ), which was the level of FcaPV-2 gene expression relative to the average of all positive samples, normalised to reference gene expression. Normalisation was done using ABL2 and ACTB reference genes, which were selected from the reference gene validation study. These assays were performed as for reference gene validation but with 1 µL of the same cDNA as used for the FcaPV-2 E6/E7 assay.

4.2.6 Quantification of FcaPV-2 DNA

The real-time PCR assay developed in chapter 2 was used to quantify the FcaPV-2 DNA. The DNA extraction method was also the same but with the additional step of homogenizing 40 mg of each sample with Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) prior to DNA extraction. As previously described, FcaPV-2 copy number was normalized to copies of *F. catus* 28S ribosomal DNA, which was more appropriate than per cell as the neoplastic cells may have been polyploid.

4.2.7 Immunohistochemistry for p16

Immunohistochemistry for p16 was performed at BioMed Central Medical Testing Laboratory (BioMed Central, Palmerston North, NZ), using a mouse antihuman p16 monoclonal antibody (BD Biosciences, San Jose, CA) as has been previously reported.[1] SCCs with ≥ 75% of the neoplastic cells exhibiting strong nuclear and cytoplasmic immunostaining were considered positive.[25]

4.2.8 Analysis of real-time PCR data

Real-time PCR data was analysed using EcoStudy software (Illumina Inc, San Diego, CA). Plates that showed a Cq variation of < 0.5 for the positive control were considered valid. The presence of FcaPV-2 E6/E7 expression was compared to p16 status and FcaPV-2 DNA copy number using Fisher's exact tests; the skewness of the data precluded the use of tests that assume a normal distribution. Statistics were done with SPSS statistics 22 (IBM, Armonk, NY).

4.3 Results

4.3.1 Sample collection

A total of 17 SCC samples were collected, of which 16 were confirmed to be SCCs by histology. Histologically, the SCCs were erosive, plaque-like lesions comprised of islands and trabeculae of neoplastic squamous cells that originated in the epidermis and extended into the dermis. Squamous cells towards the periphery of the trabeculae had a more basaloid appearance with small dark nuclei and scant cytoplasm, while the more central squamous cells were larger with glassy eosinophilic cytoplasm and large vesicular nuclei and prominent nucleoli. The mitotic index ranged from moderate to high. Some islands of neoplastic cells had keratinised centres and were infiltrated by neutrophils. Stromal fibroplasia was moderate to abundant, and the stroma often contained small cords of partially-keratinized neoplastic cells. The one remaining sample had marked full thickness epidermal dysplasia with involvement of the follicular infundibula, which did not extend through the basement membrane, typical of a Bowenoid *in situ* carcinoma (BISC). Additionally, the lesion contained swollen keratinocytes with grey foamy cytoplasm, characteristic of PV infection. BISCs are a rare type of *in-situ* SCC which are thought to be caused by FcaPV-2.[26] No histological evidence of PV infection was visible in the 16 SCCs. Eight of the cases came from the nasal planum, seven from the ears, one from the conjunctiva of the medial canthus, and the BISC came from left temporal skin.

4.3.2 Performance of the real-time PCR assays

The *Felis catus* reference gene real-time PCR assays and the FcaPV-2 E6/E7 assay were linear within the tested range, with efficiencies ranging from 90.3% to 99.3% and r^2 values ≥ 0.994 (Table 4.1). In all assays a single melt-peak was observed, and all no template controls were negative. All minus-RT controls were also negative excluding the presence of residual DNA. The sensitivity of the ACTB and ABL2 assays was good as they were able to detect the equivalent of 100 and 1000 copies of ACTB and ABL2 cDNA respectively. Amplicons from the ACTB and ABL2 reference gene assays were sequenced which confirmed the identity of the product. The FcaPV-2 L1 assay could not be sufficiently optimised for quantification so was performed as an end-point PCR. PCR products were visualised on a UV transilluminator after gel electrophoresis in 1.5% agarose and expression of L1 was reported as present or absent. The 145 bp band was

sequenced and confirmed to be the expected L1 amplicon. The reason for the poor performance of this assay was later determined to be inefficient primer binding, possibly due to secondary structures forming in the template DNA and obscuring the primer binding sites. The assay was re-developed in chapter 5 with a different set of primers, which improved the performance of the assay and allowed quantification of late viral gene expression.

4.3.3 Reference gene selection

The results from the NormFinder analysis are shown in Fig. 4.1. The size of each line represents the intra-group variation and its location on the *y* axis represents the inter-group variation. ACTB and ALB2 appeared to be good candidates as they showed little change in relative gene expression between normal and neoplastic skin, as indicated by their position near zero on the *y* axis, and they had a relatively small amount of variation within each group as shown by the short vertical line. YWHAZ also had little variation within each group of samples but had a slightly reduced relative gene expression in the neoplastic samples. The other six genes did not appear to be suitable.

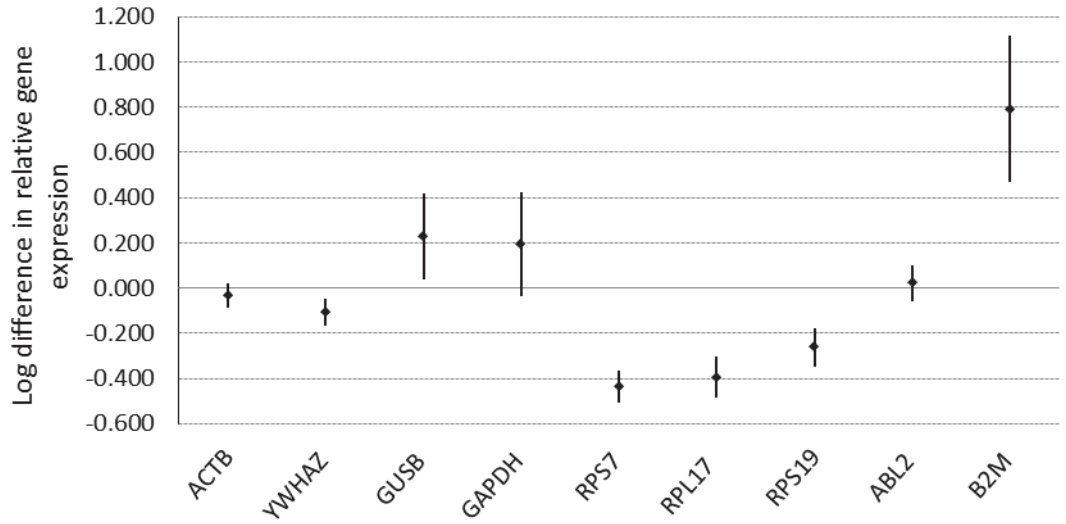

Figure 4.1. Reference gene stability in NormFinder. Point: inter-group variation, Line: intra-group variation.

The Normfinder algorithm combines both inter- and intra-group variation into a stability value. Suitable reference genes should have a stability value of < 0.20.[22] For this set of reference genes only ACTB and ABL2 were suitable, with stability values of 0.15 and 0.16 respectively or 0.098 when used together. YWHAZ had a stability value of 0.22. NormFinder therefore suggested that ACTB and ABL2 were the best choice for reference gene normalisation.

The GeNorm statistical algorithm ranks candidate reference genes by their GeNorm M value which is a gene stability measure defined as the average pairwise variation between a particular gene and all other candidate reference genes.[23] Stably expressed reference genes typically have M values < 0.5 and a coefficient of variation (CV) < 0.25.[27] An important assumption is that the candidate reference genes are not co-regulated, as co-regulated genes will have a similar ratio in all samples but are not necessarily stably expressed. The initial GeNorm analysis ranked the three ribosomal protein genes best. They had considerably lower M values than the other candidate reference genes, however this was highly suspicious for co-regulation. Removal of any one of RPS7, RPS19 or RPL17 increased the M value of the remaining ribosomal protein genes, and changed the ranking order quite significantly thus confirming co-regulation of these genes. The analysis was therefore repeated using only one ribosomal protein gene RPS19, shown in Fig. 4.2. In this second analysis three candidate genes had M-values < 0.5. These were YWHAZ, ABL2 and ACTB with M-values of 0.38, 0.40 and 0.43 respectively.

In conclusion, after removal of co-regulated genes from the GeNorm analysis, both Normfinder and GeNorm ranked the candidate reference genes similarly. Both algorithms identified ACTB, ABL2 and YWHAZ as potentially suitable reference genes for this sample set. For normalisation of the 17 SCCs and 8 control samples in this study ABL2 and ACTB were used; the stability of these genes over the test samples was good with a combined M-value of 0.518 and CV values of 0.175 and 0.184 respectively.

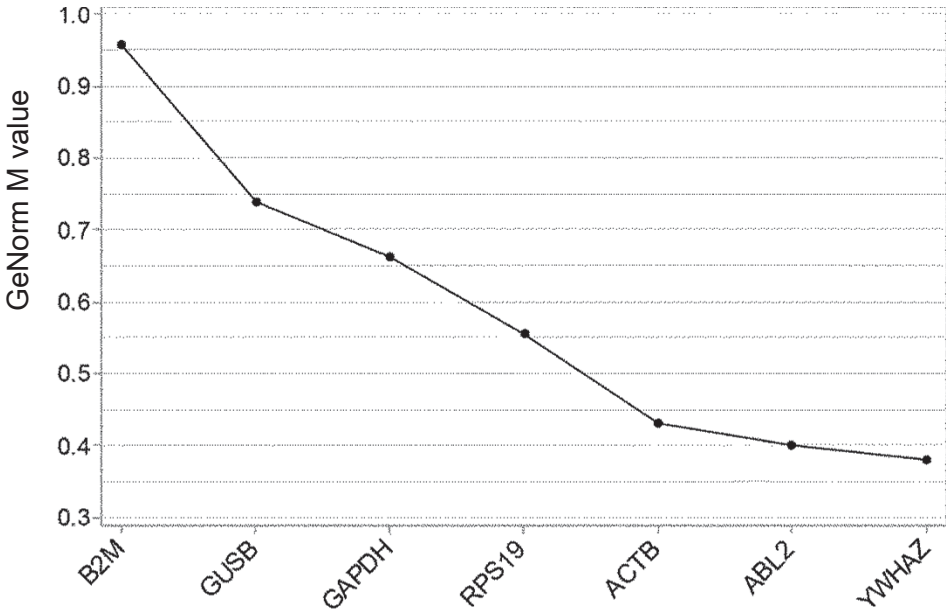

Figure 4.2. Candidate reference genes ranked according to their GeNorm M values after the removal of RPS7 and RPL17 (lower values indicate higher stability).

4.3.4 FcaPV-2 gene expression

FcaPV-2 E6/E7 gene expression was detected in six samples: the BISC, two nasal planum SCCs, a conjunctival SCC, and two normal skin control samples (Table 4.2). When normalized to reference gene expression, the relative quantity of E6/E7 expression in the two nasal planum SCCs and the BISC was > 100 fold higher than in the conjunctival SCC and the control samples. The three SCCs with high levels of E6/E7 expression also had high FcaPV-2 DNA loads and increased p16 immunostaining. Therefore, the presence of high levels of E6/E7 expression was significantly associated with both increased p16 immunostaining and high FcaPV-2 DNA loads ($p < 0.01$). Expression of the FcaPV-2 L1 gene was only detected in the three SCCs that had high levels of E6/E7 expression.

Tumour description	FcaPV-2 DNA	FcaPV-2 load [a]	Increased p16	E6/E7 mRNA	E6/E7 NRQ [b]	L1 mRNA
temporal skin BISC	+	10248	+	+	122.0	+
nasal planum SCC	+	464	+	+	57.7	+
nasal planum SCC	+	1131	+	+	27.6	+
nasal planum SCC	+	49.0	+	-	-	-
nasal planum SCC	+	0.01	+	-	-	-
nasal planum SCC	-	-	-	-	-	-
nasal planum SCC	-	-	-	-	-	-
nasal planum SCC	-	-	-	-	-	-
nasal planum SCC	+	< 0.005	-	-	-	-
conjunctival SCC	-	-	-	+	< 0.005	-
right pinna SCC	+	0.05	-	-	-	-
right pinna SCC	+	0.01	-	-	-	-
right pinna SCC	+	< 0.005	-	-	-	-
right pinna SCC	+	0.10	+	-	-	-
left pinna SCC	+	< 0.005	-	-	-	-
left pinna SCC	+	0.02	-	-	-	-
left pinna SCC	+	0.04	-	-	-	-
control	+	< 0.005	-	-	-	-
control	+	< 0.005	-	+	0.01	-
control	+	0.01	-	-	-	-
control	-	-	-	+	0.01	-
control	+	< 0.005	-	-	-	-
control	+	< 0.005	-	-	-	-
control	+	< 0.005	-	-	-	-
control	-	-	-	-	-	-

Table 4.2. FcaPV-2 DNA load, p16 immunostaining and FcaPV-2 gene expression in SCCs and controls. [a] Copies of FcaPV-2 DNA per copy of reference gene DNA. [b] NRQ: Normalised relative quantity of E6/E7 gene expression, see methods section 4.2.5.

4.3.5 Quantification of FcaPV-2 DNA

FcaPV-2 DNA was detected in the BISC, 12 of 16 SCCs and 6 of 8 controls (Table 4.2). However, when normalized to copies of FcaPV-2 DNA per copy of reference gene DNA, the 19 positive samples could be divided into four samples that had over 48 copies, and the remaining 15 samples that all had less than 0.1 copies per copy of reference gene DNA. The four SCCs with high FcaPV-2 DNA loads included three nasal planum SCCs and the BISC. The BISC had the highest copy number with 10,248 copies of FcaPV-2 DNA per copy of reference DNA, whereas the three SCCs had between 49 and 1131 copies of FcaPV-2 DNA per copy of reference DNA.

4.3.6 Immunohistochemistry for p16

The four SCCs with high FcaPV-2 DNA loads all had increased immunostaining for p16 (Fig. 4.3). Two other SCCs also had increased p16 immunostaining but had negligible quantities of FcaPV-2 DNA. No p16 immunostaining was seen in the control samples and they had little to no FcaPV-2 DNA. Increased p16 immunostaining was significantly associated with the presence of a high FcaPV-2 DNA load, defined as having > 0.1 copies of FcaPV-2 DNA per copy of reference gene DNA ($p < 0.01$). FcaPV-2 DNA copy number and gene expression are shown in relation to p16 status in Table 4.3.

		DNA copy number [a]		**E6/E7 expression** [b]		**L1 expression** [c]
	n	high (%)	mean (range)	high (%)	mean (range)	pos (%)
p16 positive SCCs	6	4 (67)	1982 (0-10248)	3 (50)	34.6 (0-122)	3 (50)
p16 negative SCCs	11	0	1.1×10^{-2} (0-0.05)	0	2.1×10^{-4} (0-0.002)	0
control normal skin	8	0	5.1×10^{-4} (0-0.01)	0	1.9×10^{-3} (0-0.01)	0

Table 4.3. FcaPV-2 DNA copy number and E6/E7 expression compared to p16 status.
[a] FcaPV-2 DNA copy number per copy of reference gene DNA, high: number of samples which had > 0.1 copies/ copy of reference gene DNA. [b] Relative FcaPV-2 E6/E7 expression, high: number of samples which had a relative gene expression value of > 0.1. [c] FcaPV-2 L1 expression, pos: number of samples which had a visible 145 bp band corresponding to the L1 amplicon.

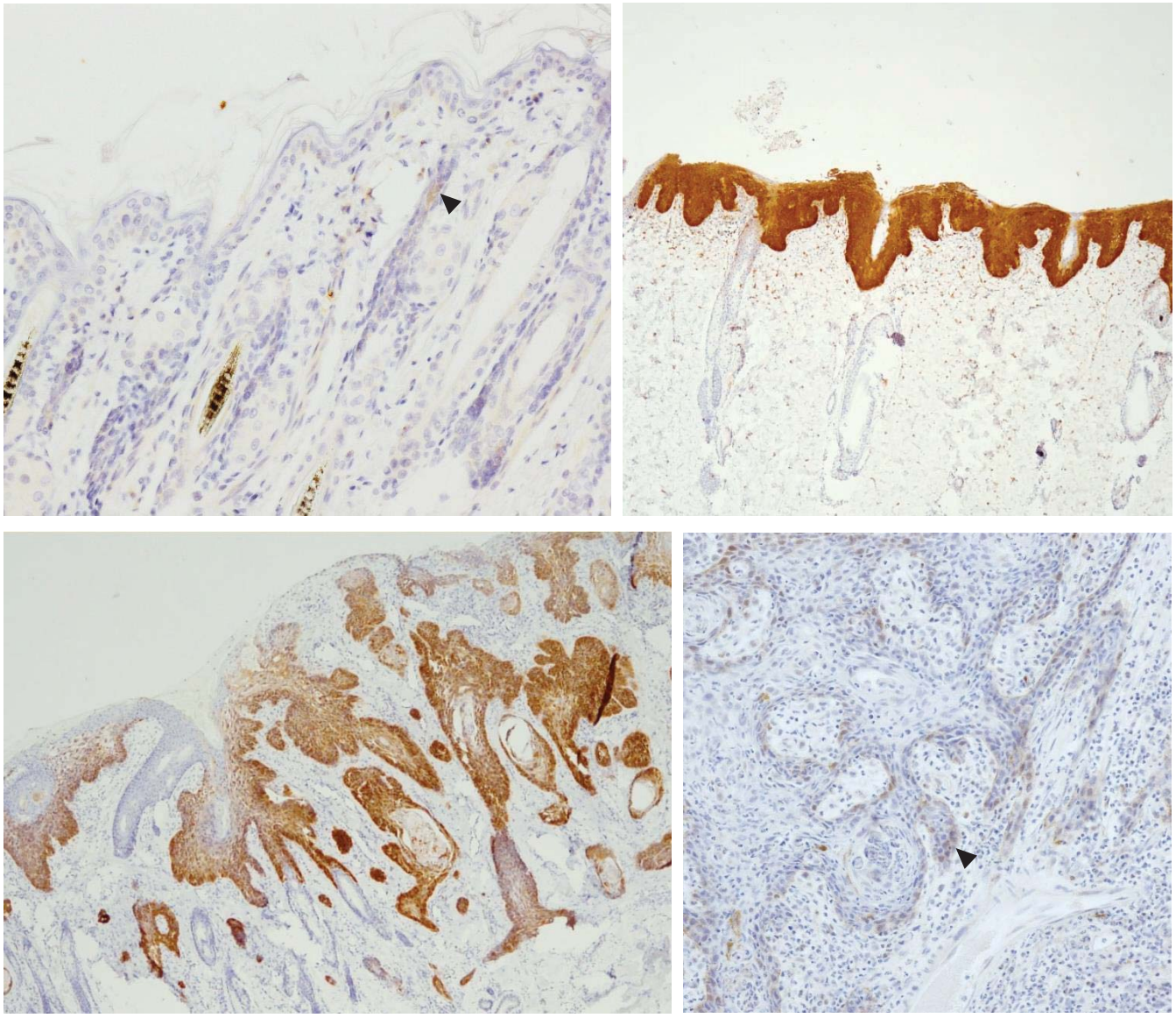

Figure 4.3. p16 immunohistochemistry. Top left: normal skin control with negative p16 immunostaining. Note occasional cells have weak p16 immunostaining (arrowhead) which is normal in basal cells and melanocytes undergoing senescence. 200x magnification. Top right: BISC with intense brown colouration in both the nuclei and cytoplasm of greater than 75% of the dysplastic cells, indicative of positive p16 immunostaining.[25] 100x magnification. Bottom left: nasal planum SCC with positive p16 immunostaining. 100x magnification. Bottom right: pinna SCC with negative p16 immunostaining. Weak p16 immunostaining is present in some cells at the edge of neoplasm (arrowhead), however as it was present in less than 75% of the neoplastic cells the sample was p16 negative overall. 200x magnification.

4.4 Discussion

In previous studies, FcaPV-2 DNA has been consistently detected in a proportion of feline cutaneous SCCs, suggesting that the virus may be involved in cancer development.[2] However, as skin infections with FcaPV-2 are common, it is hard to determine the significance of detecting FcaPV-2 DNA in these cancers.[28] The results of the study in this chapter demonstrated, for the first time, the presence of FcaPV-2 E6/E7 expression in a proportion of feline cutaneous SCCs. While it cannot be definitively determined whether or not this E6/E7 expression was responsible for cancer development, the detection of FcaPV-2 E6/E7 expression shows that the FcaPV-2 DNA was transcriptionally active in these cancers and not merely present due to a latent PV infection. Overall the detection of FcaPV-2 E6/E7 expression adds further evidence for a role of FcaPV-2 in the development of some feline cutaneous SCCs.

To further define the role of FcaPV-2 gene expression in these cancers, changes in the levels of p16 protein were also investigated. In high-risk HPV-induced cancer p16 is up-regulated in response to PV E7 protein, and degradation of pRb by the PV E7 protein is thought to be an important mechanism by which PVs influence cell-cycle regulation.[6,7] Previously it has been shown that feline cutaneous SCCs that contain PV DNA have reduced immunostaining for pRb and increased immunostaining for p16.[5] In the present study, the presence of FcaPV-2 E6/E7 gene expression was significantly associated with a marked increase in immunostaining for p16 within the neoplastic cells. While this does not prove a causative association it does suggest that expression of the FcaPV-2 E6 and E7 genes influenced cell-cycle regulation within the cancers.

Increased p16 immunostaining was identified in all three SCCs that had high levels of E6/E7 expression but was also found in three SCCs that did not have detectable E6/E7 expression. The presence of increased p16 immunostaining without apparent PV involvement has been reported in several types of human cancers, and genetic and epigenetic mechanisms have been suggested.[12,29] Alternatively, some evidence suggests that feline cutaneous SCCs could be caused by more than one PV type.[9] Other PV types would not be detected by the FcaPV-2-specific primers used in the present study so infection with another PV type could explain the increased levels of p16 in absence of FcaPV-2 E6/E7 expression. Therefore, while the presence of increased p16 immunostaining in all of the SCCs that contained high levels of FcaPV-2 E6/E7 expression supports a role of FcaPV-2 in cancer development, p16 alone is unlikely to be specific for FcaPV-2-induced cancer. Conversely, FcaPV-2 E6/E7 expression was also detected in three p16 negative

samples, including two control samples. However the relative quantity of E6/E7 expression in these cases was very small and probably represented a background infection of a small number of cells.

In the present study, 13 of 17 SCCs and 6 of 8 control samples contained detectable FcaPV-2 DNA. This is consistent with previous studies, and the results in chapters 2 and 3, that reported frequent detection of FcaPV-2 DNA in feline skin.[28] Some of this FcaPV-2 DNA may have been due to latent PV infections, which are thought to occur when PV DNA persists in the basal stem cells subsequent to immune clearance of a productive infection.[30,31] Copy numbers of PV DNA reported in latent infections are usually less than 1 per cell.[32] In this study, six control samples and nine SCCs had less than 0.1 copies of FcaPV-2 DNA per copy of reference gene DNA which is suggestive of a latent infection. In contrast, the two nasal planum SCCs with detectable FcaPV-2 E6/E7 expression had over 450 copies of FcaPV-2 DNA per copy of reference gene DNA, and the BISC had over 10,000 copies. This is the first time that the copy number of FcaPV-2 DNA has been determined in feline cutaneous SCCs and the results demonstrate a wide variation, spanning six orders of magnitude. It is not known whether the detection of high copy numbers within a lesion indicates a causative association. However, the highest copy number in this study was detected in a BISC, which are generally accepted to be caused by PV infection.[26,33] In addition, all of the lesions that showed FcaPV-2 E6/E7 expression also contained high FcaPV-2 copy numbers and increased p16 immunostaining. In contrast, one SCC in the present study contained 49 copies of FcaPV-2 DNA per copy of reference gene DNA and increased p16 immunostaining, but no E6/E7 expression. The role of FcaPV-2 in this lesion is uncertain.

The SCCs in the present study came from a variety of sites on the body. Previously, SCCs from the nasal planum were found to contain FcaPV-2 DNA more frequently than SCCs from the pinna or conjunctiva.[4] Consistent with this, the three SCCs with high FcaPV-2 DNA loads in the present study all came from the nasal planum. In comparison, little or no FcaPV-2 DNA was detected in the SCCs from the pinna or conjunctiva.

Overall, FcaPV-2 E6/E7 gene expression was detected in 3 of 17 (18%) cutaneous SCCs. Results of previous studies have suggested that up to 50% of feline cutaneous SCCs could be associated with PV infection.[4] The lower numbers present in this study could be due to only looking for one PV type. Alternatively, the requirement of this study to collect biopsies into RNAlater solution within 30 minutes limited the sample size and meant that only neoplasms that were diagnosed

as SCCs prior to excision were included. Additional larger studies are required to more accurately determine the proportion of feline cutaneous SCCs that have FcaPV-2 E6/E7 gene expression. In the present study, expression of the late viral L1 gene was detected in all of the SCCs with FcaPV-2 E6/E7 gene expression. It is not known whether these transcripts were being translated into the L1 protein but if they were this would suggest that there was a productive FcaPV-2 infection. This raises the possibility that the cancers may provide a permissive environment for high levels of FcaPV-2 replication, which could result in E6/E7 gene expression and high FcaPV-2 DNA copy numbers. Therefore, if a productive infection is present in a proportion of SCCs, it would be difficult to differentiate between E6/E7 gene expression influencing cancer development and E6/E7 gene expression being due to the presence of a productive infection in a pre-existing SCC. However this is not supported by previous studies which have found that other skin tumours, that would also be expected to provide a suitable environment, rarely contain PV DNA.[1]

The detection of L1 gene expression also suggests that the FcaPV-2 DNA was present episomally rather than integrated into the host genome. In people, high-risk HPV-induced cervical cancers contain one or more copies of PV DNA that is integrated into the host genome.[34] This integration causes persistent high-level expression of E6 and E7 leading to cancer development, but usually prevents transcription of L1, and no production of progeny virions is observed.[35,36] However, high copy numbers of episomal PV DNA and the expression of late viral genes are seen in PV-induced cutaneous SCCs in rabbits and in beta PV-induced cutaneous SCCs in people with epidermodysplasia verruciformis.[37-39] Thus, the finding of L1 transcripts does not exclude a role of FcaPV-2 in cancer development but it does suggest that if FcaPV-2 does cause cancer, it probably does so by a different mechanism than that of the high-risk HPVs.

In conclusion, the results of the present study demonstrated the presence of FcaPV-2 E6/E7 gene expression within a proportion of feline cutaneous SCCs. In addition, the presence of E6/E7 gene expression was associated with increased p16 immunostaining, high FcaPV-2 DNA loads, and expression of the FcaPV-2 L1 gene within the cancers. This is the first time that FcaPV-2 E6/E7 gene expression has been detected and the first time that FcaPV-2 DNA has been quantified in cutaneous SCCs. The results support the hypothesis that FcaPV-2 influences the development or progression of a subset of feline cutaneous SCCs.

4.5 References

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Chapter 5

FcaPV-2 Gene Expression in FFPE SCCs

5.1 Introduction

A key finding from the study described in the previous chapter was the detection of *Felis catus* papillomavirus type 2 (FcaPV-2) E6 and E7 gene expression in a proportion of feline cutaneous squamous cell carcinomas (SCCs). Furthermore, FcaPV-2 E6/E7 gene expression was significantly associated with high viral DNA loads and the presence of positive p16 immunostaining. This confirmed that the FcaPV-2 DNA detected in a subset of feline cutaneous SCCs was transcriptionally active and not merely an incidental detection of a latent infection. The detection of E6 and E7 mRNA also supported the involvement of FcaPV-2 in cancer development as deregulation of human papillomavirus (HPV) E6 and E7 gene expression is a key feature in high-risk HPV-induced cancer in people.[1] In order to expand on these findings, and address some limitations of the previous study, a follow-up study was conducted.

The major limitation of the previous study was the small sample size. This was due to the requirement to collect the SCCs as they were being surgically removed from cats in an effort to prevent RNA degradation. The detection of high levels of both viral and reference gene RNA in the previous study was testament to the success of this method. However the results were limited to reporting the presence of high, low, or absent viral gene expression as only very large differences in the relative quantity of FcaPV-2 mRNA were meaningful given the small number of SCCs. It would have been particularly interesting to compare the relative quantity of viral gene expression between SCCs and Bowenoid in situ carcinomas (BISCs) or feline viral plaques (FVPs), which are known to be caused by FcaPV-2 and sometimes progress to invasive cancer. However, only one BISC and no FVPs were included in the previous study and the low incidence of both lesions in the cat population means it is not practical to collect more in this manner. A further limitation of the previous study was that the assay to detect FcaPV-2 late viral gene expression was not optimised to a standard that would have allowed reliable relative quantification of late viral gene expression.

Therefore a follow-up study was conducted, using the previously developed FcaPV-2 E6/E7 assay, to quantify FcaPV-2 gene expression in a large series of formalin-fixed paraffin-embedded

(FFPE) samples including SCCs, premalignant lesions, and normal skin. Using formalin-fixed samples enabled a much larger number of samples to be evaluated, leading to a more reliable assessment of the proportion of SCCs that contained FcaPV-2 gene expression, and also allowing comparison of the relative quantity of gene expression between the different groups of samples. FFPE samples generally contain poor quality RNA but given the high levels of gene expression found in the previously described un-fixed samples, it was hoped that some FcaPV-2 mRNA would still be detectable in the FFPE samples and this indeed proved to be the case. The FcaPV-2 L1 assay was also redesigned to allow relative quantification of late viral gene expression.

5.2 Methods

5.2.1 Sample collection

Formalin fixed paraffin embedded (FFPE) samples of feline cutaneous SCCs were sourced from the local New Zealand Veterinary Pathology diagnostic laboratory archives (NZVP Ltd, Palmerston North, New Zealand). The location of the SCC was recorded from the submission form and the locations were subdivided into: densely haired locations, including the neck, paw, trunk and face; and sparsely haired locations including the eyelids, pinna and nasal planum. Haematoxylin and eosin stained sections from all samples were examined to confirm the diagnosis. Also included were ten FFPE samples of premalignant lesions, including viral plaques, BISCs, and lesions described as late viral plaque/ early BISC, that had been collected and found to contain FcaPV-2 DNA and p16 immunostaining in previous studies. Samples of normal skin were collected from 10 cats that had been euthanized for unrelated reasons; these were fixed in 10% neutral buffered formalin and paraffin embedded.

5.2.2 RNA extraction and cDNA synthesis

Three 10 µm sections were cut from each FFPE sample for RNA extraction, and a further three for DNA extraction. Microtome blades were changed between samples. In cases where > 20% of the section was normal tissue surrounding the SCC, the sections were placed onto slides but left unstained and unfixed. These were then viewed under a dissecting microscope and the neoplastic cells scraped off using a clean scalpel into 1.5 mL microtubes for RNA extraction. This process ensured that all RNA samples contained at least 80% neoplastic cells. Total RNA was extracted from the samples using the Nucleospin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Nucleic acid concentrations were measured with a Qubit 2.0 fluorometer, using Qubit dsDNA High Sensitivity, and Qubit RNA Broad Range assay kits (Life Technologies, Carlsbad, CA, USA). To remove any residual DNA an additional post-extraction DNAse digestion was performed using Ambion Turbo DNA-free DNAse according to the manufacturer's protocol (Life Technologies). Complementary DNA synthesis was performed with the Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) using 0.6 µg total RNA, and both random hexamer and oligo-dT primers

according to the manufacturer's recommendations. For reference gene validation, the cDNA concentration was measured with the Qubit 2.0 fluorometer (Life Technologies) and diluted with DNAse-free, RNAse-free water to a final concentration of 5 ng/µL.

5.2.3 Real-time PCR assays for FcaPV-2 gene expression

The assay for FcaPV-2 E6 and E7 mRNAs was performed as described in chapter 4. This assay amplifies a 111 bp fragment from the E7 ORF that is expected to be in mRNA transcripts coding for both E6 and E7 proteins but not in transcripts coding for other viral proteins. To detect transcripts from the late viral genes, forward (5'CCGATAAGGTGCCTCCCAAA) and reverse (5'ACGGAAATTGGTCGAGGTCC) primers were designed to amplify a 105 bp fragment from the L1 ORF. Based on transcriptomes from human and rabbit PVs this fragment was expected to be present in transcripts coding for both L1 and L2 proteins but none of the transcripts from the early genes.[2,3] Real-time PCR reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD), 0.8 µM of each primer, and 1 µL of template cDNA. The cycling conditions consisted of an initial 5 minute denaturation step at 95°C, followed by 40 cycles of denaturation (95°C for 5 seconds), annealing (60°C for 20 seconds), and elongation (72°C for 15 seconds). The cycling was followed by a melting step from 55°C to 95°C. The efficiency of the L1 assay, calculated from standard curves derived from PCR product, was 93.48%, $r^2 = 0.996$, which was adequate for relative quantification. The assay demonstrated adequate analytical sensitivity as it was able to detect the equivalent of 1000 copies of FcaPV-2 L1 cDNA. All reactions were run in duplicate. RNA from every sample was included as a minus-RT control for the E7 assay to ensure there was no residual FcaPV-2 DNA. Positive and no-template controls were also included on every plate. FcaPV-2 gene expression for each sample was reported as a normalised relative quantity (NRQ), which was the level of FcaPV-2 gene expression relative to the average of all positive samples, normalised to reference gene expression.

5.2.4 Reference genes

Although a reference gene validation study had been done on the RNA-later fixed samples in chapter 4, the effects of formalin fixation were expected to alter the amplification of the different reference gene transcripts to varying degrees. Therefore, another reference gene

validation study was done to assess reference gene stability on a subset of 8 FFPE SCC samples and 8 FFPE normal skin samples. The same nine reference genes as in chapter 4 were assessed, namely: Beta actin (ACTB), Tyrosine 3-monooxygenase/ 5 tryptophan 5-monooxygenase activation protein zeta (YWHAZ), Beta glucuronidase (GUSB), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ribosomal protein S7 (RPS7), Ribosomal protein L17 (RPL17), Ribosomal protein S19 (RPS19), Abelson proto-oncogene 2 non-receptor tyrosine kinase (ABL2), and Beta-2 microglobulin (B2M). Primer sequences, concentrations and reaction conditions were the same as described in chapter 4. Extra RNA was extracted from the selected samples and 10 ng of cDNA used as the template. All reactions were performed in triplicate and Cq values were compared using two statistical algorithms: GeNorm, a pairwise variation model included in the qbase+ software (Biogazelle, Zwijnaarde, Belgium); and NormFinder, an ANOVA-based model of reference gene stability.[4,5]

Normfinder identified ABL2, RPL17, ACTB and GAPDH as the most suitable reference genes. These genes had the lowest relative inter-group variation (between the normal skin and the SCC groups), seen by their location close to zero on the y-axis, and there was minimal intra-group variation shown by the short vertical line (Fig. 5.1). GeNorm identified ABL2, ACTB, RPL17 and RPS7 as the best candidates for reference gene normalisation (Fig. 5.2). Both algorithms identified ABL2 and ACTB as potential candidates, so they were chosen for use again in this study.

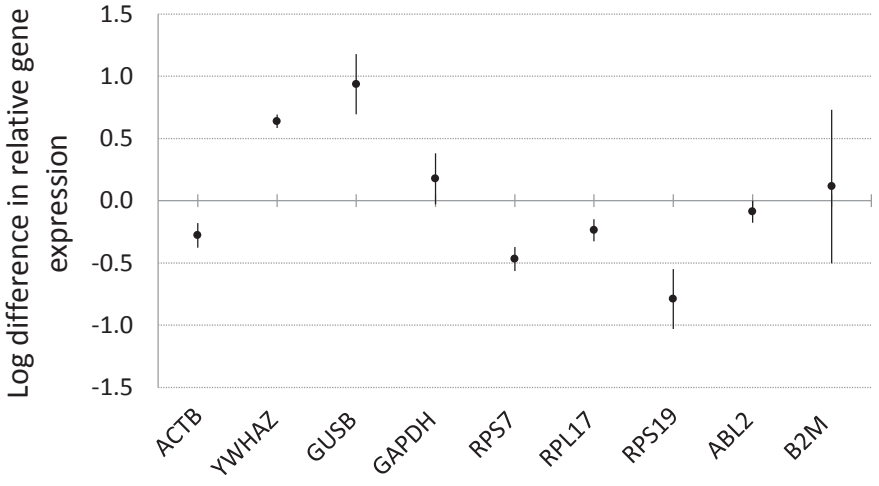

Figure 5.1. Reference gene stability in NormFinder. Point: relative inter-group variation. Line: intra-group variation.

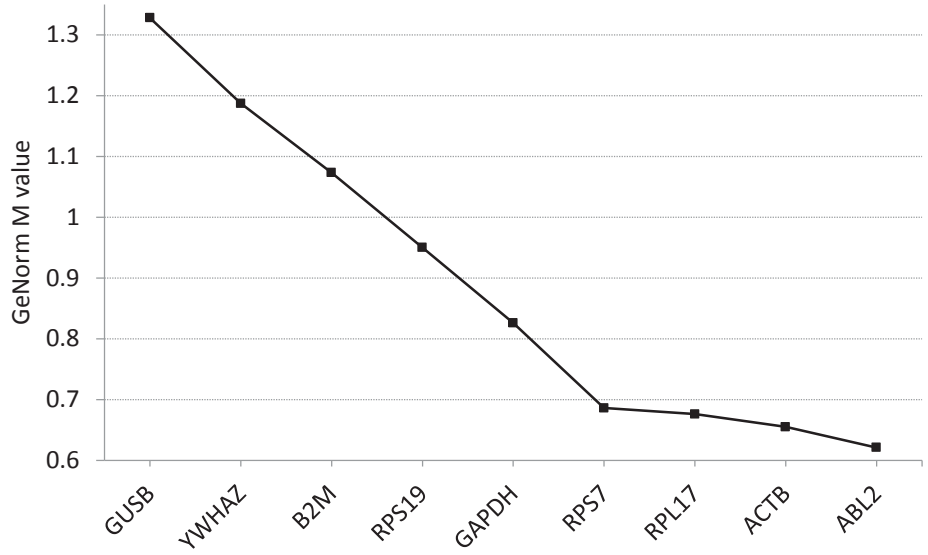

Figure 5.2. Candidate reference genes ranked according to their GeNorm M values (lower values indicate higher stability).

Real time PCR assays for ABL2 and ACTB were performed in duplicate for all samples, as described in chapter 4, with 1 µL of the same template cDNA used for the FcaPV-2 assays. RNA from every sample was included as a minus-RT control for ACTB to ensure there was no residual genomic DNA. Positive and no-template controls were also included on each plate. The geometric average Cq values from ABL2 and ACTB reference genes were used to normalise the relative quantities of FcaPV-2 E6/E7 and L1/L2 gene expression. The use of two reference genes allowed reference gene stability in the RNA samples to be assessed. This identified three outliers: SCC samples which had ABL2 expression greater than three-fold above the average of all the samples. Relative quantification of viral gene expression in these samples would have been unreliable and so they were discarded. The reference gene stability of the remaining samples was good with coefficient of variation (CV) and M values of 0.331 and 0.931 respectively. M values are a measure of gene stability based on the pairwise variation model.[4] For heterogeneous sample panels such as this, acceptable CV and M values are lower than 0.5 and 1 respectively.[6]

5.2.5 Quantification of FcaPV-2 DNA

DNA extraction and absolute quantification of FcaPV-2 DNA copy number was performed as reported in chapter 2, with the additional step that the FFPE scrolls were rehydrated in a graded alcohol series prior to DNA extraction. Real-time PCR absolute quantification was based on standard curves generated with the recombinant plasmids as templates. FcaPV-2 copy number was normalised to copies of *F. catus* 28S ribosomal DNA. This was more appropriate than virus copies per cell as many of the virus-containing neoplastic cells may have been polyploid.

5.2.6 Immunohistochemistry for p16

Immunohistochemistry for p16 was performed at BioMed Central Medical Testing Laboratory (BioMed Central, Palmerston North, NZ), using a mouse antihuman p16 monoclonal antibody (BD Biosciences, San Jose, CA) as has been previously reported.[7] Positivity was defined as greater than 75% of cells exhibiting strong nuclear and cytoplasmic immunostaining for p16.[8]

5.2.7 Statistical analysis

Real-time PCR data was analysed using EcoStudy software (Illumina Inc, San Diego, CA). Plates that showed a Cq variation of < 0.5 for the positive control were considered valid. Gene-expression, DNA copy number and immunohistochemistry results were analysed with SPSS statistics 22 (IBM, Armonk, NY). Results were analysed both as categorical data (for example the presence or absence of FcaPV-2 DNA) using Fisher's exact tests, and as log transformed continuous data using linear regression and ANOVA.

5.3 Results

5.3.1 Sample selection and quality

A total of 60 FFPE cutaneous SCCs, 10 premalignant lesions and 10 samples of normal skin were included in the study. The diagnosis of SCC was confirmed in all cases by examination of haematoxylin and eosin stained sections. Histologically, the premalignant lesions appeared as multifocal areas of well-demarcated epidermal hyperplasia, which involved the follicular infundibula, and had variable degrees of dysplasia including loss of nuclear polarity and disruption of normal epidermal stratification, consistent with BISC.[9] Several of the samples also contained histological features of PV infection including abnormal keratinocytes within the stratum granulosum with swollen blue-grey fibrillar cytoplasm, large vacuoles and vesicular or shrunken nuclei; these samples were diagnosed as FVPs or early BISCs. *F. catus* RNA reference genes were amplified from 60 of 67 initial SCC samples, all of the premalignant lesions and all of the normal skin samples. Four SCC samples without detectable reference gene RNA were discarded, as were three SCCs which had abnormal reference gene amplification. The most common locations for the SCCs were the pinna and nasal planum, although the location was unknown for 26 of 60 SCCs (Table 5.1).

SCC location	***n***	**E6/E7 gene expression**
Sparsely haired skin		
pinna	12	1/12 (8%)
eyelid	7	1/7 (14%)
nasal planum	9	3/9 (33%)
combined		**5/28 (18%)**[a]
Densely haired skin		
face	2	2/2 (100%)
trunk	3	2/3 (67%)
neck	1	0/1 (0%)
combined		**4/6 (67%)**[a]
Unknown	26	11/26 (46%)
Total	**60**	**20/60 (33%)**

Table 5.1. FcaPV-2 E6/E7 gene expression by SCC location. [a] FcaPV-2 E6/E7 gene expression was more common in SCCs from densely haired skin compared to sparsely haired skin ($p < 0.05$).

5.3.2 FcaPV-2 gene expression

FcaPV-2 gene expression was detected in 21 of 60 SCCs (35%), all 10 premalignant lesions (100%), but none of the normal skin samples. The proportion of positive samples was significantly greater in the SCC group than in the normal skin group ($p < 0.05$). FcaPV-2 E6/E7 gene expression was detected in 20 of 60 SCCs (33%), all 10 premalignant lesions and none of the normal skin samples, while late viral gene (L1/L2) expression was detected in 20 of 60 (33%) SCCs, all 10 premalignant lesions and none of the normal skin samples. One SCC had only FcaPV-2 E6/E7 expression and another had only L1/L2 gene expression. Therefore, 19 of 60 SCCs had expression of both FcaPV-2 E6/E7 and L1/L2 genes and there was a strong correlation between expression of the two genes ($r = 0.866$, $p < 0.001$). The relative quantity of both E6/E7 and late viral gene expression in the SCCs that had detectable viral gene expression was no different to the relative quantity of E6/E7 and late viral gene expression in the premalignant lesions (Fig. 5.3; E6/E7 $p = 0.39$, L1/L2 $p = 0.17$). For the 34 SCCs for which the location was known, FcaPV-2 E6/E7 gene expression was present significantly more frequently in SCCs from densely haired skin (4 of 6) than in SCCs from sparsely haired skin (5 of 28, $p < 0.05$, Table 5.1). Details of the individual SCC samples are shown in Table 5.2 and details of the premalignant and normal samples in Table 5.3.

Location	p16	FcaPV-2 DNA	E6/E7 NRQ	L1/L2 NRQ	Location	p16	FcaPV-2 DNA	E6/E7 NRQ	L1/L2 NRQ
eyelid	−	0	−	−	nasal planum	−	26	−	−
unknown	−	0	−	−	nasal planum	+	1472	2.49	4.69
unknown	+	0	−	−	pinna	−	3	−	−
unknown	+	0	−	−	unknown	−	0.1	−	−
nasal planum	−	55	−	−	unknown	+	0	−	−
unknown	+	46236	4.76	5.50	pinna	−	NA	−	−
unknown	−	2	−	−	nasal planum	−	0.2	−	0.01
unknown	+	0	−	−	unknown	+	0	−	−
unknown	−	NA	−	−	eyelid	+	NA	−	−
unknown	−	2	−	−	unknown	−	NA	−	−
unknown	+	41424	1.95	2.42	pinna	−	16	−	−
unknown	−	NA	−	−	nasal planum	+	11297	9.36	4.97
unknown	+	400	0.89	0.17	unknown	+	4	0.02	−
unknown	+	2307	1.71	1.36	unknown	−	1968	0.27	0.49
pinna	−	0.2	−	−	pinna	−	0	−	−
eyelid	+	0.4	−	−	pinna	−	NA	−	−
pinna	−	0.8	−	−	nasal planum	+	25	0.004	0.02
pinna	−	0	−	−	pinna	−	NA	−	−
flank	+	15731	1.90	0.21	unknown	+	73367	10.9	2.84
nasal planum	+	435	−	−	nasal planum	−	5	−	−
pinna	+	0	−	−	unknown	+	0	−	−
unknown	+	1393	5.94	26.1	unknown	+	584	0.02	0.07
pinna	+	32893	1.38	0.44	eyelid	−	0	−	−
eyelid	−	4	−	−	unknown	−	0	−	−
eyelid	+	1643	0.77	1.61	unknown	−	19662	0.02	0.07
neck	+	2	−	−	unknown	+	48673	0.79	0.42
pinna	−	1	−	−	eyelid	−	0	−	−
nasal planum	+	0	−	−	unknown	+	NA	−	−
face	+	7750	1.68	1.79	interscapular	−	0	−	−
face	+	273935	3.70	3.94	lateral thorax	+	77836	6.11	1.94

Table 5.2. Details of individual FFPE SCCs. FcaPV-2 DNA is reported as copies of FcaPV-2 DNA per copy of *F. catus* 28Sr DNA. Gene expression is reported as normalised relative quantity (NRQ). E6/E7: FcaPV-2 transcripts potentially coding for E6 or E7 genes. L1/L2: FcaPV-2 transcripts potentially coding for late viral genes. +: positive result. −: negative result. NA: no amplification of reference gene DNA.

Sample	Location	p16	FcaPV-2 DNA	E6/E7 NRQ	L1/L2 NRQ
premalignant	face	+	20143	7.88	10.12
premalignant	neck	+	246968	2.19	1.02
premalignant	dorsal paw	+	87648	3.12	7.25
premalignant	nasal planum	+	517	0.70	0.83
premalignant	unknown	+	671	NA[a]	NA[a]
premalignant	face	+	2693	0.66	0.68
premalignant	unknown	+	927	1.05	1.35
premalignant	unknown	+	13404	0.77	1.47
premalignant	head	+	2015	3.17	6.63
premalignant	dorsum	+	94540	NA[a]	NA[a]
normal skin	dorsum	−	0	−	−
normal skin	dorsum	−	0	−	−
normal skin	dorsum	−	0.7	−	−
normal skin	dorsum	−	NA	−	−
normal skin	dorsum	−	0	−	−
normal skin	dorsum	−	0	−	−
normal skin	dorsum	−	0	−	−
normal skin	dorsum	−	NA	−	−
normal skin	dorsum	−	0.6	−	−
normal skin	dorsum	−	18	−	−

Table 5.3. Details of individual samples: premalignant skin lesions and normal skin samples. FcaPV-2 DNA is reported as copies of FcaPV-2 DNA per copy of *F. catus* 28Sr DNA. Gene expression is reported as normalised relative quantity (NRQ). E6/E7: FcaPV-2 transcripts potentially coding for E6 or E7 proteins. L1/L2: FcaPV-2 transcripts potentially coding for late viral proteins. +: positive result. −: negative result. NA: no amplification of reference gene DNA. [a] These two premalignant lesions had amplifiable transcripts of both E6/E7 and L1/L2 but only amplified one reference gene (ACTB) and so the normalised relative quantity could not be determined.

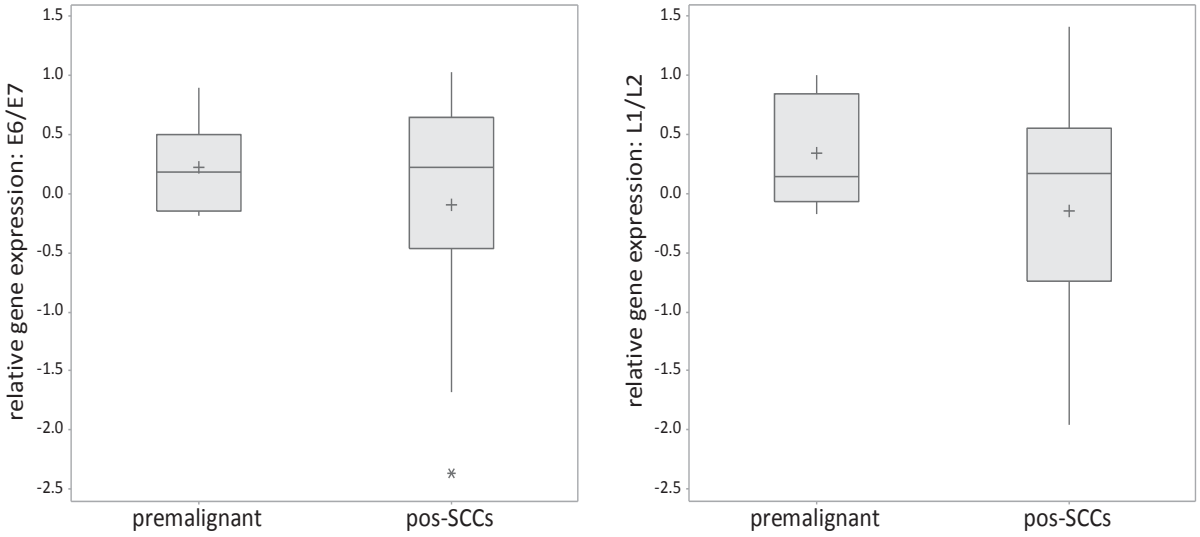

Figure 5.3. Log normalised relative quantity of FcaPV-2 E6/E7 and L1/L2 gene expression in premalignant lesions and SCCs that had detectible FcaPV-2 gene expression (pos-SCCs). The box represents the first to third quartiles with the median indicated by the horizontal line. The vertical lines indicate the minimum and maximum values. ⁎: outlier. +: group mean.

5.3.3 FcaPV-2 viral load

The presence of amplifiable DNA was confirmed in 52 of 60 SCCs, all 10 premalignant lesions and 8 of 10 normal skin samples. Samples from which no reference gene DNA was amplified were excluded from further analysis. FcaPV-2 DNA was amplified from 36 of 52 (69%) SCCs, all 10 (100%) premalignant lesions, and 3 of 8 (38%) normal skin samples. While there was no significant difference in the proportion of positive samples between the SCC group and the other groups, a large range in the quantity of FcaPV-2 DNA was found, from 0.1 to 273,934 copies of FcaPV-2 DNA per copy of reference gene DNA.

Based on the quantity of FcaPV-2 DNA present, two distinct subgroups were apparent in the SCC group. The SCCs without FcaPV-2 E6/E7 gene expression had low FcaPV-2 DNA copy numbers (mean of 17 copies of FcaPV-2 DNA per copy of reference gene DNA), whereas those with FcaPV-2 E6/E7 gene expression had high copy numbers (mean of 32,930 copies of FcaPV-2 DNA per copy of reference gene DNA). When separated into E6/E7-positive and E6/E7-negative groups (Fig. 5.4), the mean copy number in the positive SCCs was similar to that of the premalignant lesions (46,731 copies per copy of reference gene DNA) and both were significantly greater than

the normal skin samples (mean of 2.4 copies per copy of reference gene DNA) and the E6/E7-negative SCCs (mean of 17 copies per copy of reference gene DNA; p < 0.001). Correspondingly, the quantity of FcaPV-2 DNA was significantly correlated to the relative quantity of FcaPV-2 E6/E7 gene expression (r = 0.63, $p < 0.001$) and L1/L2 gene expression (r = 0.57, $p < 0.01$).

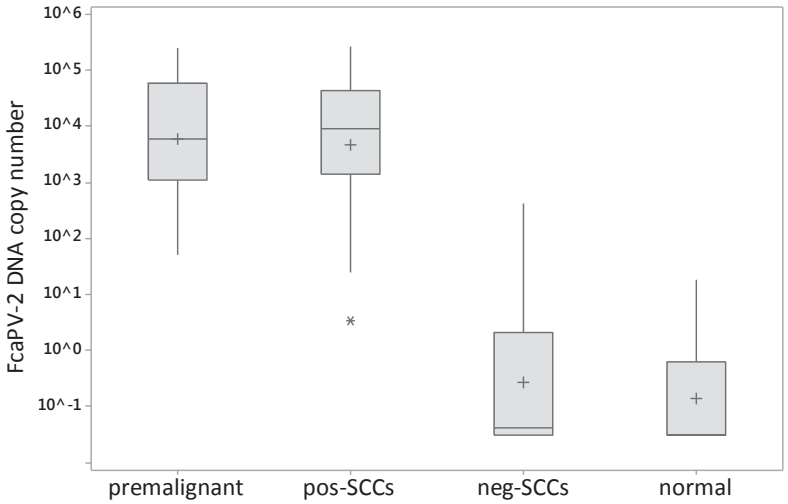

Figure 5.4. Log transformed viral load (copies of FcaPV-2 DNA per copy of reference gene DNA) in FcaPV-2 E6/E7-positive and -negative SCCs, premalignant lesions and normal skin SCCs. pos-SCCs: SCCs that had detectible FcaPV-2 E6/E7 gene expression. neg-SCCs: SCCs that did not have FcaPV-2 E6/E7 gene expression. premalignant: premalignant skin lesions (FVPs and BISCs), all of which had FcaPV-2 E6/E7 gene expression. normal: normal skin samples, none of which had FcaPV-2 E6/E7 gene expression. The box represents the first to third quartiles with the median indicated by the horizontal line. The vertical lines indicate the minimum and maximum values. ⁎: outlier. +: group mean.

5.3.4 P16 immunohistochemistry

Positive p16 immunostaining was seen in 31 of 60 (52%) SCCs, all 10 (100%) premalignant lesions and none of 10 normal skin samples (Fig. 5.5). There was a significant association between FcaPV-2 E6/E7 gene expression and p16 immunostaining in the SCCs: 18 of 20 (90%) E6/E7-positive SCCs were p16 positive, compared to just 13 of 40 (33%) E6/E7-negative SCCs ($p < 0.05$). One of the 13 p16-positive E6/E7-negative SCCs had a large amount of FcaPV-2 DNA, but 8 of remaining 12 had no detectable FcaPV-2 DNA and 2 of 12 had less than 3 copies of FcaPV-2 DNA per copy of reference gene DNA. The two remaining p16-positive E6/E7-negative SCCs had no amplifiable reference gene DNA, despite having amplifiable reference gene cDNA, so FcaPV-2 DNA copy number could not be assessed.

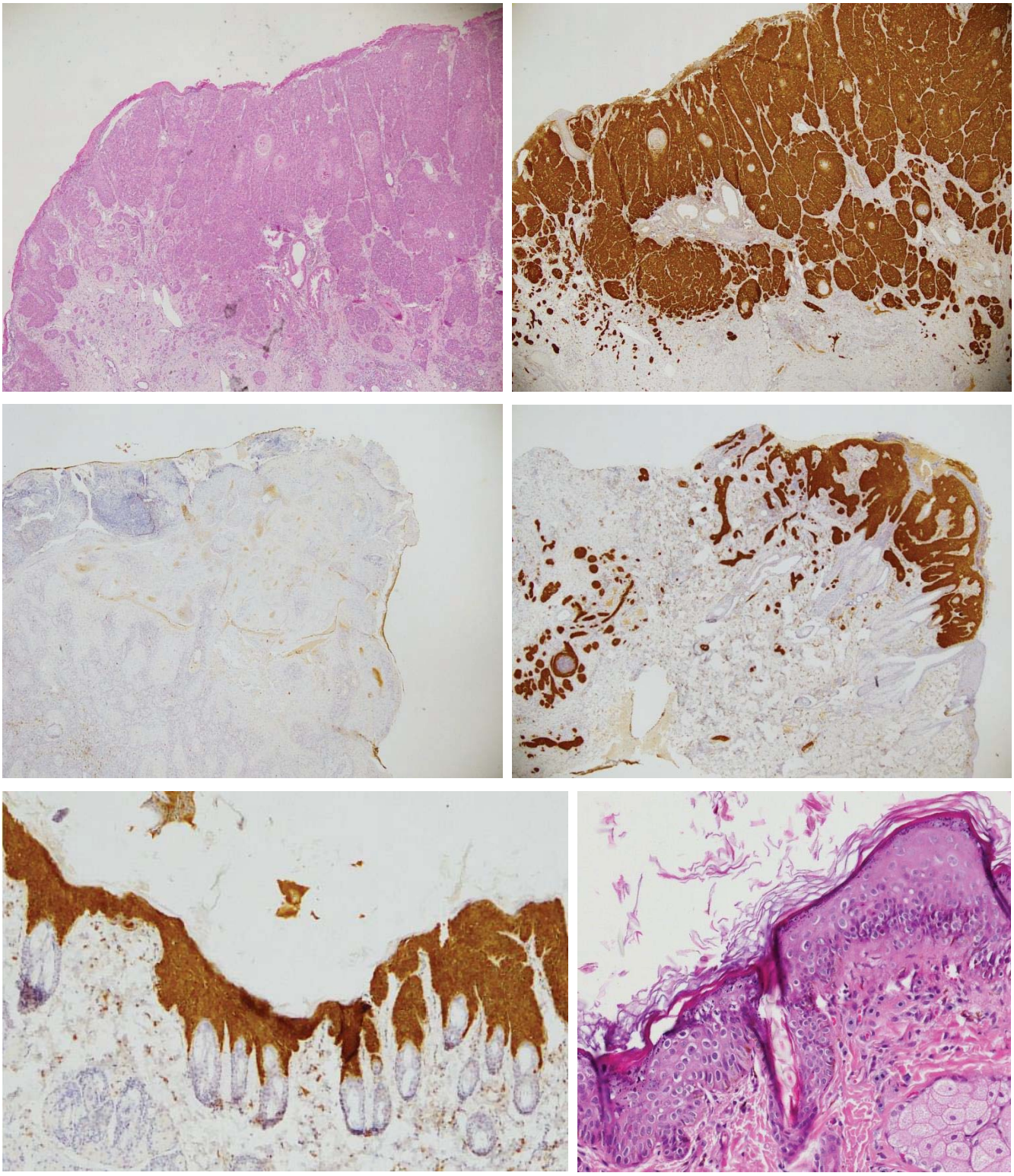

Figure 5.5. p16 immunohistochemistry and haemotoxylin and eosin (H&E) stained sections. Top row: a pinna SCC that contained FcaPV-2 E6/E7 gene expression and was p16 positive, H&E (left) and the corresponding IHC (right), 20x magnification. Middle row: examples of nasal planum SCCs that were p16 negative (left) and p16 positive (right), 20x magnification. Bottom row: premalignant lesions showing the IHC (left) with follicular involvement, 20x magnification, and the same lesion at 200x magnification (right) with abnormal cells in the upper epidermal layers containing swollen blue-grey cytoplasm, large vacuoles and a vesicular or shrunken nucleus consistent with PV cytopathic changes.

5.4 Discussion

FcaPV-2 gene expression was found in 21 of the 60 SCCs in this study, and transcription of the potentially oncogenic E6/E7 genes was present in 20 of 60 (33%) SCCs. This is the first time that FcaPV-2 gene expression has been detected in a large series of FFPE feline cutaneous SCCs and this finding is consistent with the findings of the previous chapter which suggested that a proportion of SCCs harbour an active infection with FcaPV-2 that could be contributing to cancer development, rather than merely an incidental latent infection.

Interestingly, a higher proportion of SCCs had FcaPV-2 E6/E7 gene expression in this study (33%) than in the previous study (12.5%). The findings of this study are more consistent with what has been reported previously; a review by Munday (2014) suggested that approximately 30% of SCCs from UV-exposed skin and 75% of SCCs from UV-protected skin are caused by PVs.[10] The lower proportion in the first study is likely to be due to a predominance of SCCs from the pinna and nasal planum. This may be because SCCs from these locations have a very typical clinical appearance allowing veterinarians to identify the cases prior to surgery, which was needed for the study. In comparison, the SCCs described in this chapter were from a local diagnostic laboratory. It is possible that SCCs from UV-protected areas are over-represented in this study, as these have a less typical appearance so may be more likely to be submitted for histopathology. Therefore, the true proportion of SCCs that have FcaPV-2 E6/E7 gene expression is probably between 12.5% and 33%.

This study was also the first time that copy numbers of FcaPV-2 DNA have been quantified in a large series of FFPE feline cutaneous SCCs. While 69% of the SCCs had amplifiable FcaPV-2 DNA, there was a large range in the copy number of FcaPV-2 DNA detected, with many SCCs having less than 2 copies of FcaPV-2 DNA per cell whereas others had over 10^5 copies per cell. There was a significant relationship between the viral copy number and PV gene expression, both in this and the previous study, and the results of this study in particular showed that the SCCs could be subdivided into two groups based on the quantity and transcriptional activity of the FcaPV-2 infection. The first group of SCCs was characterised by the presence of FcaPV-2 E6/E7 gene expression and high copy numbers of FcaPV-2 DNA. Furthermore, the relative quantity of FcaPV-2 E6/E7 gene expression and the absolute quantity of FcaPV-2 DNA in these SCCs was similar to that in the PV-induced premalignant lesions. As these premalignant lesions are generally considered to be caused by FcaPV-2 infection, the detection of similar levels of FcaPV-2 E6/E7

gene expression and genome copy numbers adds evidence that FcaPV-2 could likewise influence the development of SCCs. While it cannot be confirmed that FcaPV-2 caused the SCCs in this study, the detection of E6/E7 gene expression within the lesions strongly suggests that the presence of the PV in these lesions influenced the growth and replication of the neoplastic cells. In the second group, which accounted for around two-thirds of the SCCs in this study, there was no detectible FcaPV-2 E6/E7 gene expression and little to no FcaPV-2 DNA. The lack of PV gene expression and low copy numbers of FcaPV-2 DNA in this group was similar to what was observed in the normal skin samples. The presence of FcaPV-2 DNA in some of the SCCs in this second group, and in the normal skin samples, appears likely to represent a latent infection which did not significantly influence the growth of the host cells.

The two subgroups of SCCs also had a different distribution of sites on the body. The first group were more common on densely haired skin; 67% of the SCCs from these sites had FcaPV-2 E6/E7 gene transcription. In contrast, the second group were more common on sparsely haired skin such as the eyelids, nasal planum and pinna; only 18% of the SCCs from these sites had FcaPV-2 E6/E7 gene transcription. It has been suggested previously that there may be two different aetiologies of feline cutaneous SCCs, with those occurring on sparsely haired skin caused by UV-induced DNA damage, and those occurring on densely haired skin developing from PV-induced premalignant lesions.[7] The different locations of the two subsets of SCCs found in this study supports this hypothesis and the continued expression of the PV E6/E7 genes in the SCCs also provides evidence that the PV may contribute to the progression from premalignant to malignant lesions. Unfortunately, other than tumour location, no further patient information or follow-up was available in this study, although previously it has been shown that cats with PV-associated SCCs are of a similar age, and tend to have a longer survival time, than cats with non-PV-associated SCCs.[7, 11]

Positive p16 immunostaining was present in around half of the SCCs in this study, including 18 of 20 (90%) FcaPV-2 E6/E7-positive SCCs. In human PV-induced cancers there is increased production of the PV E6 and E7 proteins which interfere with numerous cell-cycle regulatory proteins and induce chromosomal instability leading to cancer development.[1] The PV E7 protein also induces expression of p16, and depletion of p16 in E7-expressing cervical cancer cell-lines results in a marked reduction in cell viability suggesting that p16 is also an essential part of cancer development.[12] Therefore, the significant association between FcaPV-2 E6/E7 expression and positive p16 immunostaining in the feline cutaneous SCCs suggests that FcaPV-2 E6/E7 gene products are similarly manipulating the cell-cycle and the virus is contributing to cancer

development. The two p16 negative SCCs that had FcaPV-2 E6/E7 gene expression showed focal p16 immunostaining that did not reach the threshold for positivity. The lack of p16 immunostaining in parts of these cancers may be because the cells developed additional mutations which allowed them to proliferate in the absence of p16.[12]

Surprisingly, positive p16 immunostaining was also found in 33% of the SCCs that did not have detectable FcaPV-2 E6/E7 gene expression in this study. This is similar to the results reported in chapter 4 where 21% of PV-negative SCCs had increased p16 immunostaining. This result has several possible explanations. Firstly, it is possible that more than one feline PV type may be capable of causing increased p16. Evidence supporting this is the previous detection of *Felis catus* papillomavirus type 3 DNA in 4 of 10 p16-positive, FcaPV-2-negative SCCs.[11] In this study, the primers were specific for transcripts from FcaPV-2 and so the presence of other PV types cannot be excluded. Alternatively, increased p16 in these SCCs may be due to genetic or epigenetic changes which are unrelated to PV infection. Thirdly, FcaPV-2 E6/E7 gene expression may have been present but below the detection limit of the assay. This seems unlikely as 8 of the 13 E6/E7-negative p16-postive SCCs also had no detectable FcaPV-2 DNA, which is strongly correlated to FcaPV-2 gene expression, and the FcaPV-2 DNA assay developed in chapter 2 was able to detect the equivalent of 2 copies of FcaPV-2 DNA. Therefore, the frequent detection of increased p16 in SCCs with FcaPV-2 E6/E7 gene expression is consistent with the virus playing a role in cancer development. However positive p16 immunostaining cannot be used as a biomarker for the presence of a transcriptionally active FcaPV-2 infection. Although beyond the scope of this thesis, the p16-positive, PV-negative SCCs warrant further investigation including sequencing of p16 and related genes and investigation of the samples with a range of PV consensus primers to detect additional PV types.

The mechanism by which PVs are thought to cause cancer in people typically involves integration of the PV DNA into the human genome.[13] This disrupts the normal PV lifecycle, causing an increase in the steady-state levels of the PV E6 and E7 mRNAs and reduced expression of the late viral genes.[14,15] Consequently there is no production of capsid proteins and PV-induced cancers are considered abortive infections.[16] Therefore, human PV-induced cancers contain relatively low copy numbers of PV genomic DNA. In a study of FFPE cervical carcinomas, between 1 and 630 copies of high-risk HPV DNA per cell were reported.[17] In contrast, the consistent expression of FcaPV-2 late viral genes and high copy numbers of FcaPV-2 DNA found in the SCCs in this study was not consistent with PV integration but is more consistent with a

predominance of episomal PV DNA. This supports similar findings from chapter 4 and may suggest that FcaPV-2 is successfully replicating in feline cutaneous SCCs.[18] This would be unusual as PV-induced cancers do not generally support PV replication, although it is possible as PV replication has been reported in cutaneous SCCs from people with the hereditary condition epidermodysplasia verruciformis, which confers a defective immune response to cutaneous PVs.[19]

The possibility that FcaPV-2 may be replicating in cutaneous SCCs brings into question the significance of the viral gene expression found. One possibility may be that the PV is not involved in cancer development but is simply a secondary infection because the SCC provides a suitable environment for FcaPV-2 replication, possibly due to reactivation of a pre-existing latent infection. However this seems unlikely as, in people, high-risk HPV infection is cleared within 24 months and reactivation of latent infections has not yet been conclusively demonstrated.[20-22] Also, if FcaPV-2 were simply replicating in the SCCs due a permissive environment, it would follow that other skin tumours would also harbour active FcaPV-2 infections. Previous studies have demonstrated that this is not the case. Several studies have investigated various inflammatory and neoplastic skin samples, including trichoblastomas which are epidermal skin tumours closely related to SCCs, and have found little to no PV DNA.[7,23-25]

In conclusion, this is the first time that FcaPV-2 gene expression and viral load have been evaluated in a large series of FFPE feline cutaneous SCCs. The results revealed that around a third of the SCCs contained large quantities of transcriptionally active FcaPV-2. In these SCCs, the FcaPV-2 DNA is not simply present as an incidental, latent infection. Rather, these findings suggest that a subset of feline cutaneous SCCs may develop from FcaPV-2-induced premalignant lesions, and also supports the hypothesis that FcaPV-2 E6/E7 gene expression may influence the development, malignant transformation, and continued growth of these SCCs. Therefore, the results of this study support a role of FcaPV-2 in the development of a proportion of feline cutaneous SCCs.

5.5 References

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Chapter 6

The Physical State of FcaPV-2 DNA in SCCs

6.1 Introduction

Felis catus papillomavirus type 2 (FcaPV-2) gene expression was detected in around a third of the feline cutaneous squamous cell carcinomas (SCCs) in the previous chapter, and the level of FcaPV-2 E6/E7 gene expression was similar in the SCCs and the premalignant lesions. This suggests that a proportion of feline cutaneous SCCs may develop from FcaPV-2-induced premalignant lesions and that continued viral gene expression may be necessary for growth and maintenance of these cancers. Alternatively, it is possible that the virus may be a secondary infection because the cancers provide a suitable environment for viral replication. While the association between FcaPV-2 gene expression and the host cyclin dependant kinase inhibitor $p16^{INK4A}$, and the infrequent detection of FcaPV-2 DNA in other epidermal tumours, suggests this is unlikely, further evidence is nevertheless required to prove that FcaPV-2 gene expression is contributing to cancer development.

In high-risk HPV-induced cervical cancer in women, a key event in cancer development is deregulation of HPV E6 and E7 gene expression resulting in expression of these genes in the basal epithelial cells which do not normally support E6/E7 gene expression.[1] A common mechanism of deregulation of HPV gene expression is integration of the PV DNA into the host genome which can result in constitutive expression of highly stable E6/E7 mRNA transcripts.[2] High levels of HPV E6 and E7 proteins in the basal cells are thought to cause chromosomal instability leading to the acquisition of further genetic mutations and the development of invasive cancer.[3] The importance of viral integration in cancer progression is illustrated by the high proportion of cervical cancers that contain integrated high-risk HPV DNA: 100% of HPV-18-induced cancers and around 70% of HPV-16-induced cancers.[4,5] Therefore, the aim of the study presented in this chapter was to analyse the physical state of FcaPV-2 DNA in feline cutaneous SCCs to determine whether the FcaPV-2 DNA is integrated in these cancers. Although the results of previous chapters have suggested that FcaPV-2 exists episomally (separate from the *F. catus* genome) in feline cutaneous SCCs, this does not preclude the presence of integrated FcaPV-2 DNA as PV-induced cancers can contain both integrated and episomal PV DNA. The detection of

integration of the FcaPV-2 genome within the host cell DNA would support a causative role of FcaPV-2 in cancer development.

A range of different techniques have been reported to determine the physical state of the HPV DNA in PV-induced cancers in people. In the early studies, restriction enzyme analysis and southern blotting were used; integrated HPV DNA co-migrated with the bulk of high molecular weight cellular DNA when treated with a single-cut restriction enzyme whereas episomal HPV DNA was seen as a distinct 8 kbp band.[6] An alternative PCR based approach was suggested by Das *et al*. (1992), comparing the amplification of the HPV-16 E6 and E2 genes. Because integration commonly disrupts the E2 gene, the E2:E6 ratio was close to 1.0 when the HPV DNA was exclusively episomal but < 1.0 with integrated HPV DNA.[7] Another PCR-based approach involved detection of integrated HPV DNA by amplifying viral-cellular sequences between viral primers and repeated elements in the host genome such as restriction enzyme binding sites.[8] Amplification of viral-celluar mRNA has also been used to detect transcripts from integrated PV DNA and has the additional benefit of demonstrating functionally active integrated HPV DNA.

In this chapter, two different PCR based approaches were used to investigate the physical state of FcaPV-2 DNA in the RNAlater-fixed feline cutaneous SCCs that were collected for the study reported in chapter 4. The first approach was a long-range PCR aimed at amplifying a sequence between a primer specific for FcaPV-2 DNA and a primer complementary to a repeated element of the cat genome. The second approach was aimed at detecting viral-host mRNA transcripts.

6.2 Experiment 1 Methods

The goal of experiment 1 was to amplify viral-cellular DNA sequences using long-range PCR with one primer specific for FcaPV-2 DNA and another primer complementary to a repeated element of the cat genome.

6.2.1 Identification of repeat elements in the cat genome

The RepeatMasker program was used to screen the cat genome for repeated elements that might provide a target for a host primer.[9] The results showed that 43.7% of the cat genome is comprised of repeated elements and low complexity DNA sequences (Fig. 6.1). The most common type of repeated element was long interspersed nuclear elements (LINEs), accounting for 21.6% of the cat genome. This was followed by short interspersed nuclear elements (SINEs) and long terminal repeats (LTRs), accounting for 11.4% and 5.2% of the cat genome respectively. All three groups (LINEs, SINEs and LTRs) belong to the wider group of repeated elements called retrotransposons. Retrotransposons are sequences of DNA that jump from one location in the genome to another via an RNA intermediate, and are thought to play a role in regulating gene expression.[10]

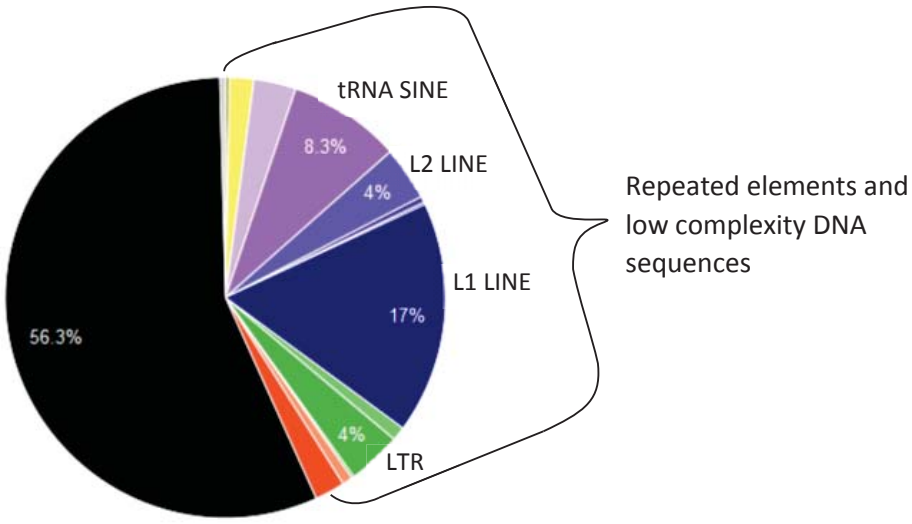

Figure 6.1. Proportion of the cat (*F. catus*) genome that is comprised of repeated elements and low complexity DNA sequences.

Inspection of RepeatMasker analysis from 1000 kbp regions of each *F. catus* chromosome identified SINEC_Fc2, as a potential target for primer development. SINEC_Fc2 belongs to a group of SINEs where the intermediate RNA is tRNA (tRNA SINEs), and these sequences make up approximately 8.3% of the cat genome (Fig. 6.1). The frequency of the SINEC_Fc2 sequence, which occurred at approximately 10 kbp intervals throughout the inspected genome regions, made it a good target because regardless of the location of integrated FcaPV-2 DNA it would likely be within 10 kbp of a SINEC_Fc2 sequence. This was well within the 30 kbp range of the long-range DNA polymerase enzymes. Additionally, SINEC_Fc2 is unique to carnivores so was unlikely to be present in the samples as a contaminant. A forward primer (5'ATCAGACTCTTGATTTCAGCTCA) and inverse primer (5'ACTCGACTTTAGTTCTCAGACTA) were designed to bind to SINEC_Fc2 sequences that were orientated in either direction.

6.2.2 Samples and DNA extraction

Six of the SCC samples described in chapter 4 were used in this experiment. These samples had been collected within 30 minutes of surgical removal and fixed in RNAlater solution so they were expected to contain high-quality DNA. Details of the previous analysis of these samples is shown in Table 6.1. Two SCC samples (SCCs 1 and 2) that had amplifiable FcaPV-2 DNA and E6/E7 gene expression were included, as it was thought that these sample may possibly contain integrated FcaPV-2 DNA. A Bowenoid in situ carcinoma (BISC) was also included; as these lesions have been shown to support viral replication the FcaPV-2 DNA was expected to be episomal in this sample.[11] Three other SCCs were included that had little to no FcaPV-2 DNA and no viral gene expression. These served as FcaPV-2 negative, integration-negative controls.

DNA was extracted from the six samples using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany) according the manufacturers recommendations. This magnetic-bead based protocol is designed to isolate large (> 150 kbp) fragments of DNA. The quantity of extracted DNA was good, ranging from 54.8- 139.4 ng/µL, on Qubit 2.0 fluorometer with a Qubit dsDNA Broad Range assay kit (Life Technologies, Carlsbad, CA, USA).

Sample	Description	FcaPV-2 load [a]	Increased p16	E6/E7 mRNA	Relative quantity [b]	L1 mRNA
BISC	temporal skin BISC	10248	+	+	122.0	+
SCC 1	nasal planum SCC	1131	+	+	27.6	+
SCC 2	nasal planum SCC	464	+	+	57.7	+
SCC 7	left pinna SCC	–	–	–	–	–
SCC 8	left pinna SCC	0.02	–	–	–	–
SCC 9	nasal planum SCC	0.01	+	–	–	–

Table 6.1. FcaPV-2 DNA load, p16 immunostaining and FcaPV-2 gene expression of selected SCCs from chapter 4. [a] Copies of FcaPV-2 DNA per copy of reference gene DNA [b] Relative quantity of E6/E7 expression normalised to reference gene expression

6.2.3 Long range PCR

The long range PCR was performed using GoTaq Long PCR Master Mix (Promega, Madison, WI, USA), a SINEC_Fc2 primer, the E7SF primer designed in chapter 2, and the high molecular weight DNA extracted from the six samples above. E7SF was chosen as the viral primer because it binds to the E7 open reading frame (ORF) which remains intact in transcriptionally active integrated HPV DNA, whereas downstream ORFs are often disrupted.[12] The E7SF primer was paired with either the SINEC_Fc2 forward or inverse primer. The reaction mix included a final concentration of 1 µM of each primer and 2 µL of template DNA in a total volume of 20 µL. Control reactions were performed by omitting either the DNA template or the E7SF primer. The cycling conditions consisted of an initial 2 minute denaturation step at 94°C followed by 35 cycles of denaturation (94°C for 20 seconds), annealing (55°C for 20 seconds) and elongation (72°C for 20 minutes, increasing by 10 seconds per cycle after 15 cycles), with a final extension step (72°C for 10 minutes). PCR products were separated by gel electrophoresis in 0.8% agarose and viewed under a UV-transilluminator.

6.2.4 Next generation sequencing

An 11 kbp gel-purified PCR product was sent to New Zealand Genomics Limited for next generation sequencing (NZGL, Dunedin, New Zealand). A library of DNA fragments was produced from the amplicon using a Nextera XT DNA Library Preparation Kit V1 (Illumina, San Diego, CA). Overlapping reads from the Illumina MiSeq run of this library (paired end sequencing, 1.7 million reads, read length 250 bp) were assembled into 889 continuous sequences (contigs) using three programs: VelvetOptimiser, Velvet, and SPAdes.[13,14] These were annotated using a BLASTn search against the National Center for Biotechnology Information (NCBI) nucleotide database.[15] The majority of the contigs were annotated as having strongest homology to scaffolds from the cat genome assembly, however one contig (contig 1) had 99% identity to the previously published sequence of FcaPV-2 (EU796884). A different assembly approach was also used, starting with the PAUDA method to BLAST short reads against the NCBI database and then utilising the MEGAN program to identify reads consistent with papillomaviridae origin.[16,17] These reads were independently assembled using the Velvet program which resulted in a single assembled contig with 99% identity to FcaPV-2 (contig 2).

6.3 Experiment 1 Results and Interpretation

6.3.1 Long-range PCR

The long range PCR using the viral E7SF primer and the host inverse SINE_Fc2 primer yielded an approximately 11 kbp band from three samples: SCC 1, SCC 2 and SCC 8 (Fig. 6.2). This band was thought to be consistent with an integrated FcaPV-2 sequence because it was too large to be episomal FcaPV-2 DNA (7899 bp) and similar sized bands were not apparent in the BISC or control samples. In comparison, many samples produced 8-10 kbp bands which probably represented SINE- SINE sequences as they were also present in the minus E7SF primer controls.

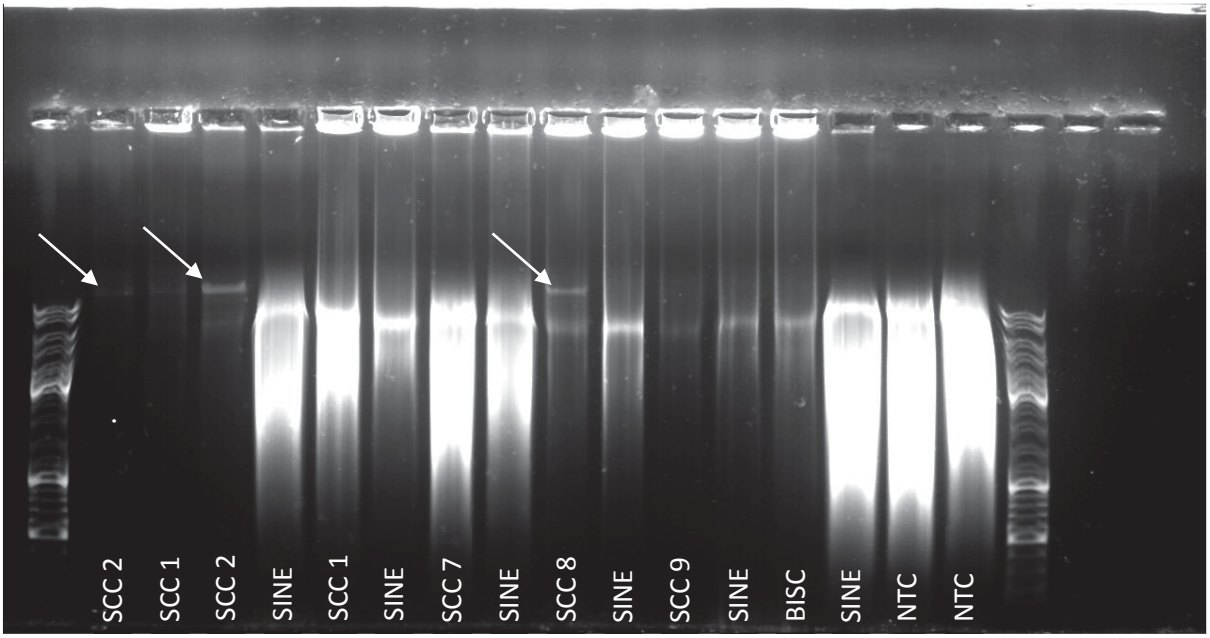

Figure 6.2. Long-range PCR products after gel electrophoresis. SCCs 1, 2 and 8 had a discrete band at 11 kbp (arrows) whereas the other samples had DNA smearing and bands at 8-10 kbp. SINE: long range PCRs where the viral E7SF primer had been omitted. NTC: long range PCRs where the DNA template had been omitted.

A recurring problem in the gel electrophoresis was retention of the PCR product in the wells and/or smearing in the lanes. Numerous troubleshooting attempts, such as reducing the concentration of template, using new reaction components, and reducing the amount of product loaded into the wells, were unsuccessful. However one such attempt did yield a more intense 11 kbp band from SCC 1 (Fig. 6.3A). This band was excised from the agarose gel and

purified using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's recommendations. The purified amplicon (Fig. 6.3B) was submitted for next generation sequencing (NGS). Further attempts to optimise the long-range PCR reaction were unsuccessful and although the 11 kbp band could be reproduced for SCC 2 it remained faint and was not suitable for sequencing. The 11 kbp band could not be reproduced from SCC 8.

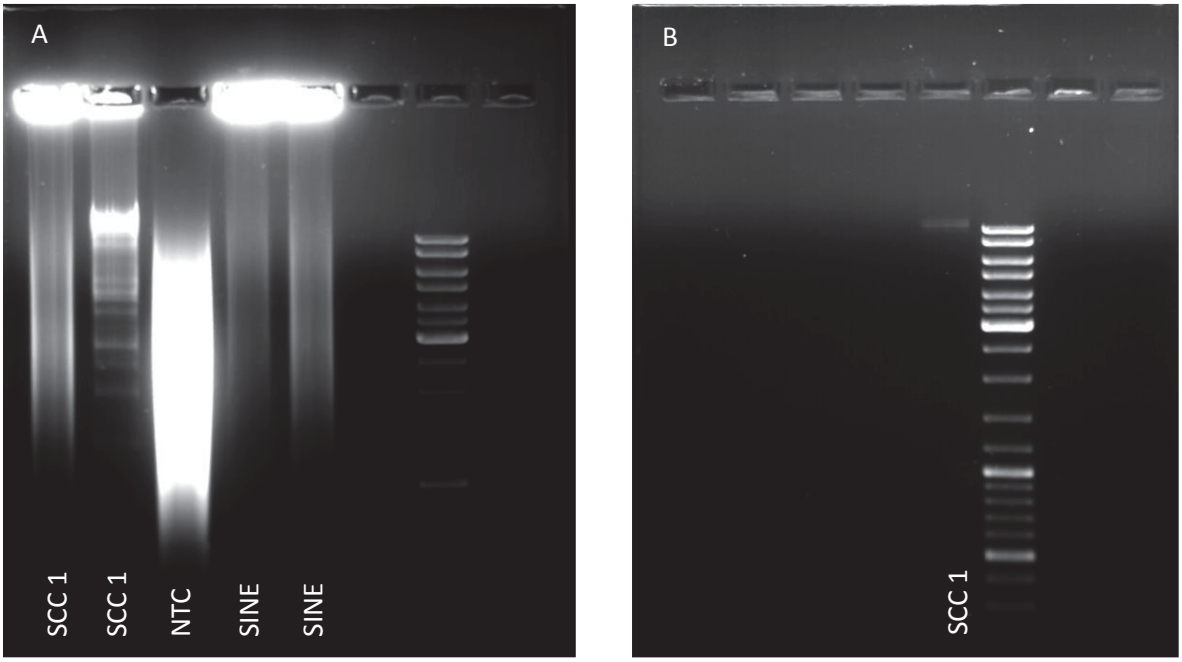

Figure 6.3. The intense 11 kbp band for SCC 1. (A); this band was excised from the gel and purified resulting in a single amplicon (B), which was submitted for NGS.

6.3.2 Next generation sequencing

The two contigs assembled from the NGS of the 11 kbp amplicon had 99% identity to the previously published sequence of FcaPV-2 (EU796884), and are shown alongside this sequence in Fig. 6.4. Contig 1 was 8025 bp in length and differed from the existing FcaPV-2 sequence due to duplication of part of the long control region (LCR) and the E7 ORF, as well as a 26 bp novel nucleotide sequence in the L2 ORF. Contig 2 was 8424 bp long and also contained the novel 26 bp sequence. In addition, contig 2 was missing much of the LCR, although other parts of the LCR, and the E6/E7 ORFs, were duplicated in the reverse orientation.

Single nucleotide polymorphisms (SNPs) existed between the two contigs and the previously published sequence for FcaPV-2 (EU796884). There were nine SNPs in the L2 ORF, five in E1, two in E1 and L1, three in the long control region, one in the non-coding region at the end of the viral genome, and none in the E6 or E7 ORFs (Table 6.2). The majority of these SNPs did not alter the amino acid sequence of the putative protein sequence suggested by Lange *et al*. (2009). Exceptions to this included a single amino acid (AA) substitution from glutamine to arginine at position 128 of the E1 protein, and three AA substitutions at the end of the L2 protein: tryptophan to leucine at AA position 369, serine to alanine at AA position 463, and arginine to lysine at AA position 491. The serine to alanine substitution at AA position 463 was the only substitution which resulted in a change in the affinity of the amino acid for water molecules, in this case from a hydrophilic to a hydrophobic amino acid. Therefore, it is plausible that this substitution may have changed the tertiary structure of L2. Additionally a 50 bp sequence was missing from the L2 ORF and had been replaced with the novel 26 bp sequence. Interestingly, the new sequence from the contigs translated almost exactly to the putative protein sequence described by Lange *et al*. whereas the previously published sequence requires two frame shifts to fit the suggested protein sequence. This finding is difficult to interpret but may suggest an error in the original published DNA sequence of L2.

Neither contig contained sequences of feline genomic DNA therefore it was not possible to conclusively determine whether the sequences resulted from integrated or episomal FcaPV-2 DNA. The duplication of some ORFs may be indicative of viral integration but was more likely to be artefactual due to the method of amplification. This latter option also explains the poor quality of the initial gel electrophoresis results as suboptimal PCR conditions, including high concentrations of primers and low annealing temperatures, were required to amplify non-target episomal DNA. Further evidence for the presence of episomal FcaPV-2 DNA was the finding of intact E2 and downstream ORFs as these are often disrupted in integrated PV DNA.[12]

PV ORF	Nucleotide position (on contig)	Nucleotide change	Amino acid change
LCR	75	G→A	NA
	264	G→C	NA
	278	C→T	NA
E1	267	A→T	none
	383	A→G	Glutamine→Arginine
	489	C→T	none
	1326	C→T	none
	1359	T→A	none
E2	868	A→C	none
	1566	T→C	none
L1	601	C→T	none
	1324	G→A	none
L2	81	T→C	none
	152	C deletion	frame shift
	193	novel sequence[a]	frame shift
	690	A→C	none
	720	C→A	none
	726	T→A	none
	1038	G→A	none
	1106	G→T	Tryptophan→Leucine
	1357	T→G	Serine→Alanine
	1472	G→A	Arginine→Lysine

Table 6.2. Single nucleotide polymorphisms between the two contigs and the previously published sequence for FcaPV-2 (EU796884). [a] The predicted amino acid sequence from the novel sequence in the L2 ORF was identical to the previously published L2 amino acid sequence, see text for more detail. NA: the long control region (LCR) is non-coding.

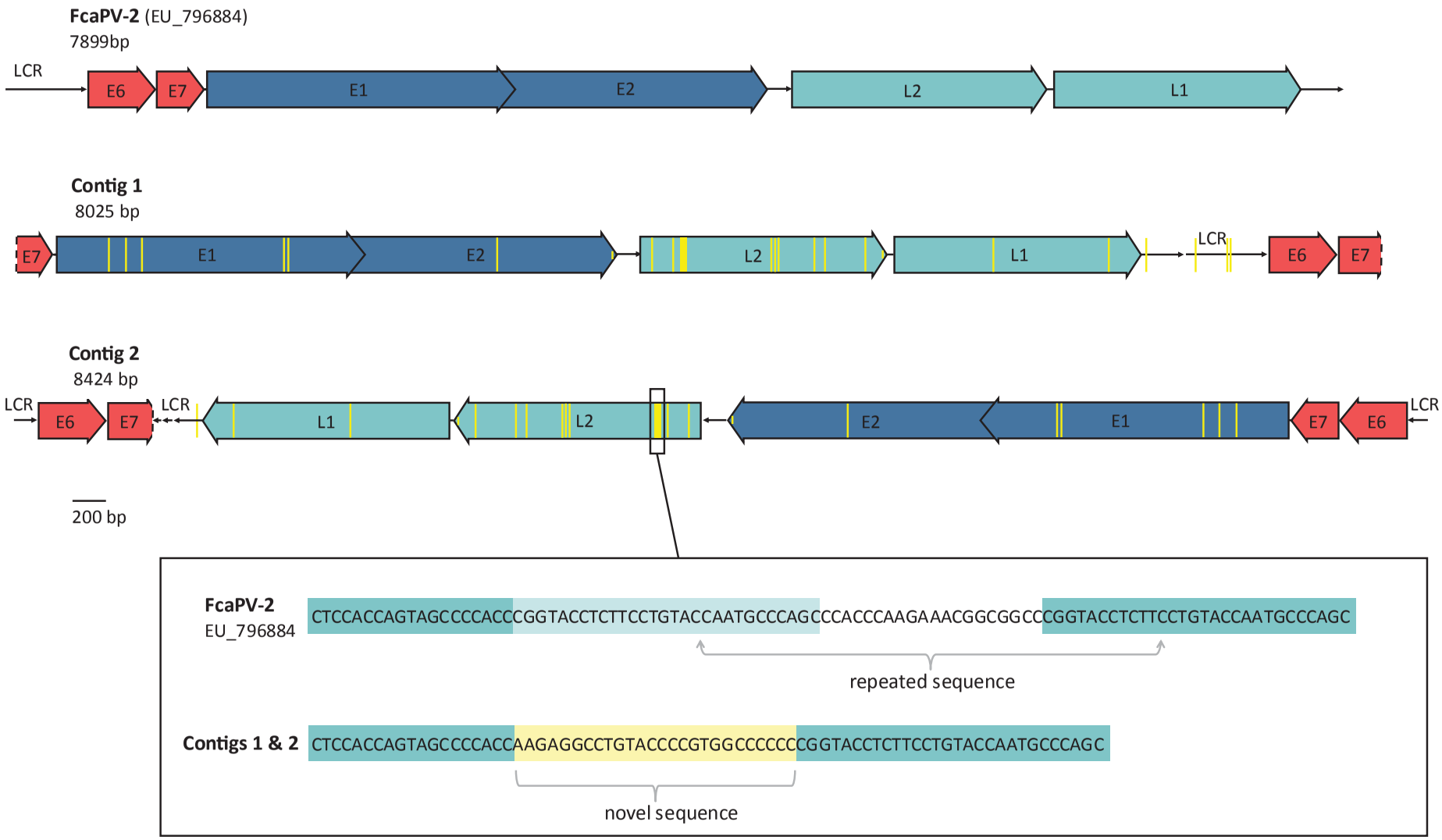

Figure 6.4. Manually annotated contigs from NGS relative to the published genome sequence for FcaPV-2 (EU_796884). Block arrows with E1, E2, E6, E7, L1 and L2 indicate FcaPV-2 ORFs, intervening non-coding sequences are indicated by line arrows, LCR: long control region (non-coding). Yellow lines indicate a single SNP whereas the magnified yellow block is a novel sequence in the contig L2 ORF.

6.4 Experiment 2 Methods

The goal of experiment 2 was to use a nested end-point PCR to detect viral-cellular fusion transcripts in the reverse transcribed mRNA from the feline cutaneous SCC samples. Detection of different sized transcripts in SCCs 1 and 2 compared to the BISC and other samples would suggest these were viral-cellular fusion transcripts from integrated FcaPV-2 DNA.

6.4.1 Sample selection

Selected SCC and control samples that were used in the study reported in chapter 4 were used again in this experiment, with the previous results for these samples shown in Table 6.3. Six SCCs were chosen including SCCs 1 and 2 which were also used in experiment 1 and were thought to possibly contain integrated FcaPV-2 DNA on the basis of having high levels of FcaPV-2 E6/E7 gene expression. The BISC from experiment 1 was also included as a control because it was expected to contain episomal FcaPV-2 DNA. Four SCCs that did not contain E6/E7 mRNA were used as negative controls (SCCs 3-6); these had between 0 and 49 copies of FcaPV-2 DNA and had not been used in experiment 1. Normal skin without any FcaPV-2 DNA was included as an additional negative control. Three formalin fixed paraffin embedded (FFPE) SCCs from chapter 5 were also included. These SCCs contained high levels of FcaPV-2 E6/E7 gene expression, but due to formalin fixation the cDNA quality was expected to be low so it was unknown whether these samples would yield meaningful results. The previously made cDNA from all of the samples was used as template for the nested PCR in this experiment. The cDNA had been reverse transcribed from extracted RNA using oligo-dT and random hexamer primers.

Sample	Description	FcaPV-2 load [a]	Increased p16	E6/E7 mRNA	Relative quantity [b]	L1 mRNA
BISC	temporal skin BISC	10248	+	+	122.0	+
SCC 1	nasal planum SCC	1131	+	+	27.6	+
SCC 2	nasal planum SCC	464	+	+	57.7	+
SCC 3	nasal planum SCC	–	–	–	–	–
SCC 4	nasal planum SCC	49.0	+	–	–	–
SCC 5	right pinna SCC	0.10	+	–	–	–
SCC 6	right pinna SCC	0.05	–	–	–	–
FFPE 1	unknown FFPE SCC	73367	+	+	NA	+
FFPE 2	face FFPE SCC	273935	+	+	NA	+
FFPE 3	unknown FFPE SCC	1393	+	+	NA	+
NORM	control normal skin	–	–	–	–	–

Table 6.3. FcaPV-2 DNA load, p16 immunostaining and FcaPV-2 gene expression in SCCs and controls. [a] Copies of FcaPV-2 DNA per copy of reference gene DNA. [b] Relative quantity of E6/E7 expression normalised to reference gene expression. NA: Because these FFPE SCCs were analysed in a different study, the relative quantity of FcaPV-2 gene expression was not comparable to that of the SCCs fixed in RNAlater.

6.4.2 Nested PCR

A forward primer (5'CACGGTCACGGGGCTTTGCT) was designed to bind at the start of the E6 ORF. The primary PCR used this forward primer and an oligo-dT reverse primer that binds to the poly-A tail of the cDNA transcripts. The PCR was performed using TopTaq Master Mix (Qiagen, Hilden, Germany), a final concentration of 0.2 µM of the forward primer, 0.25 µM of the oligo-dT primer, and 4 µL of template DNA in a total volume of 50 µL. The cycling conditions consisted of an initial 3 minute denaturation step at 94°C followed by 30 cycles of denaturation (94°C for 40 seconds), annealing (60°C for 30 seconds) and elongation (72°C for 4 minutes), with a final extension step (72°C for 7 minutes). PCR products from the first reaction then became the template for the second nested PCR reaction. The nested PCR was performed using TopTaq Master Mix (Qiagen), a final concentration of 0.2 µM of the E7SF primer, which binds at the start of the E7 ORF, 0.25 µM of the oligo-dT primer, and 4 µL of template DNA in a total volume of 50 µL. The cycling conditions were the same as for the primary PCR except that the annealing temperature was raised to 67°C. PCR products from the nested reaction were separated by gel electrophoresis (1.2% agarose) and viewed under a UV-transilluminator.

6.5 Experiment 2 Results and Interpretation

The gel electrophoresis images of the nested PCR products are shown in Fig. 6.5. The BISC and the six SCCs from chapter 4 all had a distinct band at 400 bp. The BISC and SCCs 1 and 2 also had a faint band at 800 bp. Other faint bands were seen in several samples at 200 bp and 300 bp. A faint 400 bp band and a 300 bp band were seen in one of the FFPE SCCs from chapter 5 however no bands were present in the other two FFPE SCCs confirming suspicions that the cDNA quality in these samples was poor. No bands were seen in the normal skin negative control.

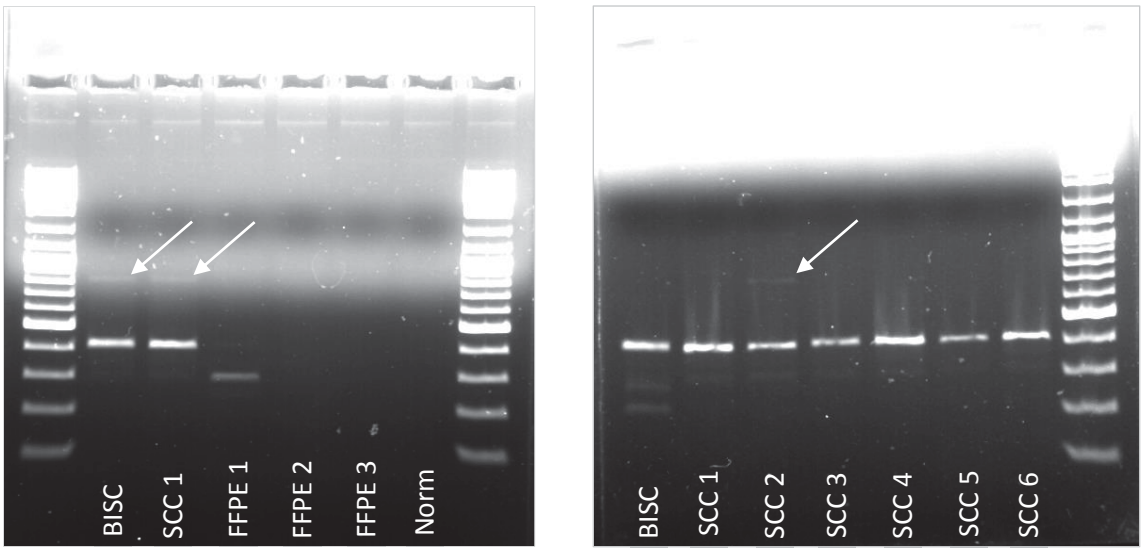

Figure 6.5. Gel electrophoresis of FcaPV-2 reverse transcribed mRNA transcripts. The 800 bp band (arrows) may represent episomal FcaPV-2 transcripts. The intense 400 bp band was thought to be a host transcript due to non-specific binding of the oligodT primer to A-rich regions.

Attempts to sequence the 400 bp band were unsuccessful however it is unlikely that this band represented mRNA transcripts from either episomal or integrated FcaPV-2. Firstly, the band was present in SCCs 3, 5, and 6, which had little to no FcaPV-2 DNA and did not show FcaPV-2 gene expression. Additionally, the band was too small to be an episomal FcaPV-2 transcript. Although the splicing patterns of FcaPV-2 are unknown, the major transcript detected in the HPV-16 or -18 amplification of PV oncogene transcripts (APOT) assay includes part of the E7 and E1 ORFs plus the E4 ORF (E7-E1'E4).[18,19] The size of the HPV-16 E7-E1'E4 transcript is approximately 1050 bp and the HPV-18 transcript is approximately 1000 bp. Even when using a very conservative estimate, the minimum length for a similar FcaPV-2 transcript would be over 500 bp.[20,21] A

possible explanation for the 400 bp band is aberrant binding of the oligodT primer to an A-rich sequence of a host transcript. In contrast, the 800 bp band was more consistent with an episomal transcript, in terms of size and presence in the BISC and the two SCCs with FcaPV-2 E6/E7 gene expression. However only very small quantities of DNA were extracted from these bands and attempts to sequence this were unsuccessful. No distinct bands were present in any of the SCCs that were not also present in the BISC therefore there was no indication of mRNA transcripts from integrated FcaPV-2.

6.6 Discussion

Integration of high-risk HPV DNA into the host genome is a frequent occurrence in human PV-induced cancer and is thought to cause deregulated PV E6/E7 expression, which results in chromosomal instability leading to the development of invasive cancer.[1] The aim of this chapter was to determine the physical state of the FcaPV-2 DNA in feline cutaneous SCCs. Two different PCR-based approaches were used: the first aimed to amplify a sequence of viral-host DNA between integrated FcaPV-2 DNA and a repeated element in the cat genome, whereas the second method aimed to detect viral-host fusion mRNA transcripts from integrated FcaPV-2 DNA. Unfortunately neither method provided conclusive results although the sequences detected were more consistent with the presence of episomal rather than integrated FcaPV-2 DNA. The inconclusive results reflect the generality that it is easier to prove the existence of something compared to its absence.

The aim of experiment 1 was to identify viral-cellular DNA sequences by using long-range PCR with primers complementary for a PV sequence and for a repeated element in the cat genome. This method was adapted from Thorland *et al*. (2000) although a host tRNA SINE (SINEC_Fc2) was used rather than restriction enzyme binding sites.[8] As template, high molecular weight DNA was extracted from SCCs that had been collected in a previous study, reported in chapter 4, including two SCC that contained FcaPV-2 E6/E7 mRNA. These samples were used instead of the formalin-fixed samples from chapter 5 as formalin fixation degrades nucleic acids and long intact sequences were needed to identify viral-cellular sequences. Although the intial amplification of an 11 kbp band from three of the SCCs seemed promising, next generation sequencing of the band revealed the entire FcaPV-2 genome, with duplication of the E6 and E7 ORFs and deletion of small parts of the LCR. Although it is possible that these changes were due to integration of the PV DNA, integration often results in the loss of large parts of the viral genome so the presence of the entire genome means it is more likely that these changes were artefactual. Therefore, it appeared that the nested PCR reaction had amplified episomal FcaPV-2 DNA between two viral primers rather than a viral-cellular sequence between a viral and a host primer.

While further optimisation of the PCR assay used in this study could potentially reduce the amplification of non-target episomal FcaPV-2 DNA, the lack of a positive control for integrated FcaPV-2 DNA meant that all potential products would need to be sequenced which was not

possible due to financial constraints in this study. A similar method has been reported recently in which DNA was extracted from cervical cancer biopsies, fragmented, and then enriched for HPV-16 sequences by PCR prior to next generation sequencing.[5] This differed from method used in experiment 1 because the PCR with PV-specific primers was done after, instead of before, DNA fragmentation which made the method more sensitive and less prone to amplification artefacts. Another potential method that could also be used, and is also based on amplification of PV DNA, is to compare the relative quantity of PV E2 and E6 amplicons.[7] Because integration commonly disrupts the E2 gene, the E2:E6 ratio is close to 1.0 when the PV DNA is episomal but < 1.0 when the PV DNA is integrated into the host genome. The advantage of this method is that amplification of episomal PV DNA does not interfere with amplification of integrated PV DNA. In the present study, there were only two SCCs which were hypothesized to contain integrated PV DNA. Therefore, the sample size would have been too small to detect a difference in the E2:E6 ratio between SCCs with integrated versus episomal PV DNA. However, with a larger number of samples, determining the E2:E6 ratio could potentially be a better approach to determine the physical state of FcaPV-2 within SCCs than trying to directly amplify viral-cellular DNA sequences.

The aim of experiment 2 was to detect viral-host fusion mRNA transcripts from integrated FcaPV-2 DNA. The method was based on the previously published amplification of PV oncogene transcripts (APOT) assay which detects transcripts from integrated high-risk HPV DNA.[22] The APOT assay is a nested PCR on reverse transcribed RNA using two PV forward primers and a poly A-adaptor linked reverse primer. The major transcript from episomal HPV DNA contains part of the E7 ORF and the start of the E1 ORF spliced onto the E4 ORF, resulting in a 1050 bp amplicon. In comparison, the transcripts from integrated HPV DNA have part of the E7 and E1 ORFs spliced onto cellular sequences resulting in transcripts that are either greater or less than 1050 bp.[19,22] A limitation of applying this assay to the feline samples was the lack of knowledge regarding the splicing patterns of FcaPV-2 mRNA transcripts and this made the results more difficult to interpret. One 800 bp band was present in the BISC and the two SCCs that were hypothesized to contain integrated FcaPV-2 DNA. The FcaPV-2 mRNA present in the BISC was expected to have come from episomal DNA as these lesions suport PV genome replication.[11] Therefore, the presence of the 800 bp band in the BISC suggested it was a transcript from episomal FcaPV-2 DNA, although this was not confirmed as the gel-extracted band failed to sequence properly due to an inadequate concentration of DNA. While none of the bands were consistent with transcripts from integrated PV DNA, the presence of such transcripts cannot be ruled out because the assay did not perform well. While the APOT assay is designed to only amplify viral mRNA, host transcripts were also amplified presumably due to non-specific binding of the oligo-

dT primer to A-rich sequences in host transcripts. This was probably due to the lack of an adaptor on the oligo-dT primer. The use of such an adaptor means that the first APOT PCR reaction can use a reverse primer that is complementary to the adaptor, rather than the oligo-dTs, which prevents non-specific binding. Unfortunately the use of an adaptor-linked primer was not possible in this study because it requires the RNA to be reverse transcribed using the same adaptor-linked oligo-dT primer. This study used cDNA that had already been made with a standard oligo-dT primer in the study reported in chapter 4. There was no tissue left to extract more RNA from these samples, and the samples from chapter 5 had been formalin fixed so were not suitable to detect long viral-host fusion transcripts. To improve the experiment, new SCCs would need to be collected, as was done in the study reported in chapter 4, and the nested PCR repeated with an adaptor-linked oligo-dT primer. However, while this could confirm the presence of mRNA transcripts from episomal FcaPV-2 DNA, it may still be difficult to exclude the presence of transcripts from integrated FcaPV-2 DNA. This again relates to the lack of a positive control for transcriptionally active integrated FcaPV-2, as it is difficult to tell if the lack of transcripts from integrated PV DNA is real or due to failure of the assay. However, if the presence of integrated PV DNA could be excluded by a non-PCR based method, such as restriction enzyme analysis with southern blotting, this would negate the need to exclude the presence of mRNA transcripts from integrated FcaPV-2 DNA.[6]

Overall, neither of the methods used in this chapter provided conclusive results although the sequences detected were more consistent with the presence of episomal rather than integrated FcaPV-2 DNA. In the first experiment the entire FcaPV-2 genome was amplified from a feline nasal planum SCC. Because integration often disrupts the PV genome, amplification of the complete genome suggests that the FcaPV-2 DNA was episomal. In the second experiment, amplification of mRNA transcripts from several feline cutaneous SCCs resulted in an 800 bp band in the BISC and SCC samples but not in any of the control samples. As the BISC was expected to contain only episomal FcaPV-2 DNA, the 800 bp band was consistent with an mRNA transcript from episomal FcaPV-2 DNA.

The results from this chapter support the findings of chapters 4 and 5, which also suggested that episomal FcaPV-2 DNA was present in feline cutaneous SCCs. In chapters 4 and 5, the SCCs that contained FcaPV-2 mRNA also contained large quantities of FcaPV-2 DNA, ranging from several hundred to over 100,000 copies of FcaPV-2 DNA per cell. This was much higher than copy numbers of PV DNA that have been found in HPV-induced cancers where integration of the PV DNA disrupts normal viral gene expression and prevents viral replication. HPV copy numbers in

HPV-18-induced cervical cancers, which contain exclusively integrated HPV DNA, range from one to 111 copies per cell.[23] Further evidence for the presence of episomal FcaPV-2 DNA in chapters 4 and 5 was the consistent detection of FcaPV-2 L1/L2 transcripts. These late PV genes are not usually expressed in HPV-induced cancers.[24] This is because these ORFs are downstream of the E2 ORF which is usually disrupted in transcriptionally active sites of integrated PV DNA.[12] If the E2 ORF is intact, it is thought that the E2 protein inhibits transcription from that site of integrated PV DNA.[25] Therefore, the presence of late viral gene expression in feline cutaneous SCCs suggests that the FcaPV-2 DNA is episomal.

Given the evidence from this and previous chapters, there are two likely possibilities for feline cutaneous SCCs: either the FcaPV-2 DNA is present in both integrated and episomal forms or it is entirely episomal. However, even if integrated FcaPV-2 DNA was present it may not be contributing to E6/E7 gene expression, as it has been suggested that E2 protein from intact episomal PV DNA can inhibit transcription of integrated PV DNA, although this has not yet been confirmed to occur *in vivo*.[25,26] It is also plausible that the FcaPV-2 DNA may be entirely episomal in feline cutaneous SCCs. Although integrated high-risk HPV DNA is present in most PV-induced cancers in people, there is some contention as to whether integration occurs early or late in cancer development and it has been suggested that a degree of PV E6/E7 deregulation exists prior to integration.[22] This means that integration of PV DNA is not the only mechanism of PV E6/E7 deregulation in these cancers and this is supported by the presence of exclusively episomal HPV-16 DNA in 23- 37% of HPV-16-induced cervical cancers.[4,5] Additionally, spontaneously occurring PV-induced cancers in other species, such as rabbits and mice, contain episomal PV DNA.[27,28] The mechanism of cancer development in the absence of integrated PV DNA is not well understood. A recent study attempting to re-create the early events in HPV-16-induced cancer induced both low and high grade cancer phenotypes in a keratinocyte cell line transfected with episomal HPV-16 DNA.[29] Interestingly, the high grade lesions had elevated E6/E7 protein levels and reduced cell-cell contact inhibition despite the absence of integrated PV DNA. This suggests that the cell cycle or differentiation status of the cell at the time of virus entry and/or episomal copy number may determine the cells ability to regulate PV E6/E7 expression and thus be important for cancer development.[29] Because PV gene expression is tightly regulated to epithelial cell differentiation, it has also been suggested that certain populations of epithelial stem cells, such as the junctional cells of the cervix, may be susceptible to infection but unable to regulate PV gene expression.[30] It is interesting to note that many of the feline cutaneous SCCs that contained FcaPV-2 E6 and E7 mRNA occurred on the nasal planum where epithelial cells transition from skin to mucosa and so a susceptible cell population

may also exist in this transition zone. To further speculate, if a susceptible cell population did exist in this location, infection of cells at a specific stage of replication or differentiation may lead to unregulated FcaPV-2 E6/E7 expression and the development of cancer without the FcaPV-2 DNA becoming integrated. The host immune response may also play a role as episomal PV DNA is usually detected and cleared by a cell-mediated immune response. In the studies reported in chapters 2 and 3, we detected consistently high levels of FcaPV-2 DNA on several adult cats who were believed to be shedding the virus, suggesting that the immune response against FcaPV-2 may be poor in some cats.

Another finding from the first experiment in this chapter was the presence of several single nucleotide polymorphisms between the amplified FcaPV-2 sequence and the FcaPV-2 sequence published by Lange *et al.* (2009), as well as a novel 26 bp sequence in the L2 ORF.[31] The majority of these changes are likely to be insignificant. However, the three amino acid substitutions in the carboxy terminus of the L2 protein could have a functional significance as they are located in the region that complexes with the PV L1 protein.

In summary, although the results from this chapter were not conclusive, they were more consistent with the presence of episomal FcaPV-2 DNA in feline cutaneous SCC and there was no evidence for integration of FcaPV-2 DNA. At present, the most plausible hypothesis is that the E6/E7 mRNA detected in feline cutaneous SCCs in chapters 4 and 5 came from episomal FcaPV-2 DNA.

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Chapter 7

General Discussion

This thesis was inspired by the work performed at Massey University between 2008 and 2013 that showed an association between the virus *Felis catus* papillomavirus type 2 (FcaPV-2) and feline cutaneous squamous cell carcinomas (SCCs), a common and aggressive skin cancer of domestic cats. At the beginning of this project, little was known about the epidemiology of the virus in cat populations, other than the virus could be detected in skin swabs from around half of adult cats.[1] Given the success of prophylactic HPV vaccines at preventing infection and cancer development in women, the possibility that a proportion of feline cutaneous SCCs could be prevented by a similar vaccine against FcaPV-2 warranted investigation.[2] To investigate this, two questions needed to be answered. Firstly, the timing of FcaPV-2 infection in cats needed to be established in order to determine whether there was an opportunity to vaccinate kittens before they became infected with the virus. This was done by developing a real-time PCR assay and measuring the quantity of FcaPV-2 DNA present in skin swabs and hair plucks from a series of kittens, from local cat breeders and a research colony. The second question that needed to be answered was whether FcaPV-2 was actually involved in the development of the SCCs, or whether it was just an incidental finding in the cancers. A FcaPV-2 vaccine would only be effective if the virus was involved in the development of cancer, so it was important to rule out the possibility that the FcaPV-2 DNA found in the cancers was from a latent infection that was not contributing to cancer development. This was done by investigating the quantity and transcriptional activity of the FcaPV-2 DNA present in feline cutaneous SCCs and these studies also provided insights into the mechanism by which FcaPV-2 may contribute to cancer development.

This chapter will start by summarising the findings presented in chapters 2 and 3, which addressed the timing of FcaPV-2 infection in cat populations, and then discussing how these fit in with what is already known about the epidemiology of PV infection in people and in other animals. This will be followed by a summary of the findings presented in chapters 4, 5 and 6, which addressed the role of FcaPV-2 in feline cutaneous SCCs. To put these latter findings into context, the cumulative evidence for a role of FcaPV-2 in cancer development will be discussed and compared to the key evidence that demonstrated a causative association between high-risk

HPV and cervical cancer in women. Finally, the potential options to reduce the incidence of FcaPV-2-associated disease will be briefly outlined.

7.1 The timing of FcaPV-2 infection in cats

Two studies were conducted to investigate the timing of FcaPV-2 infection in both privately owned and colony cats, and these were presented in chapters 2 and 3. In the first study a robust and highly repeatable assay was developed to quantify FcaPV-2 DNA in feline skin swabs and hair plucks. This assay was then used to measure the FcaPV-2 DNA load in serial samples from eleven pure-bred queens and their kittens that belonged to five local cat breeders. FcaPV-2 DNA was found on the skin of all eleven clinically normal adult cats and 20 of 22 (91%) of the two day old kittens. Therefore, asymptomatic FcaPV-2 infection appears to be common in cats. Additionally, it appears that most kittens are exposed to FcaPV-2 in the first few days of life and the primary source of exposure is likely to be direct contact with other cats in the household, particularly their queen. Another interesting finding from this study was that all of the cats from a single breeder (i.e. all of the in-contact cats within a household) had similar FcaPV-2 DNA loads, whereas there was a large variation in the quantity of FcaPV-2 DNA detected on cats from different households. For instance, the FcaPV-2 DNA load seldom rose above 10 copies per swab in two of the households whereas it was rarely less than 10,000 copies per swab in two other households. The implications of this finding were that some of the adult cats were potentially shedding large quantities of the virus and the FcaPV-2 DNA found on the kittens may have been shed from other cats in the household rather than the kittens themselves. Therefore, the first study demonstrated the ubiquitous nature and early exposure of kittens to FcaPV-2, however it was not conclusive regarding the timing of infection.

To further investigate the timing of FcaPV-2 infection and to determine the significance of the FcaPV-2 DNA detected on young kittens, a follow up study was done on serial swab samples collected from queens and their kittens at a research colony. This study had several advantages: firstly, the queens and kittens were isolated from other cats removing an unknown variable, and secondly, the kittens could be followed for a longer period of time which included the time after they had been weaned from the queen. Kittens were also cross-fostered from queens with high FcaPV-2 DNA loads to queens with low FcaPV-2 DNA loads and *visa versa* to determine the effect of different litters on the viral DNA load. Finally, 35 additional swabs and three post-mortem tissue samples were also investigated for FcaPV-2 mRNA. The results of this study strongly

suggested that several adult cats were shedding large quantities of virus and that this was being detected on the kittens. However, some kittens did become infected over the course of the study as evidenced by the detection of FcaPV-2 mRNA in three swab samples and one tissue sample from 1-8 day old kittens. From these two studies it can be concluded that most cats probably become infected with FcaPV-2 early in life from close contact with other cats, particularly their queens. In some cats this infection either persists, or they are subsequently re-infected with FcaPV-2, which accounts for the frequent detection of large quantities of FcaPV-2 in clinically normal adult cats. These results suggest that there is little opportunity for prophylactic vaccination against FcaPV-2.

7.2 Comparison of the natural history of FcaPV-2 infection with other species

The results from the first two studies of this thesis show that FcaPV-2 shares features with many PV types, particularly the beta HPVs, but overall the natural history of infection with FcaPV-2 differs from any of the current PV models. This is not surprising as FcaPV-2 is, at present, the only recognised member of the *Dyothetapapillomavirus* genus.

The natural history of infection with FcaPV-2 is most similar to that of the beta HPVs that infect people. Beta HPV DNA has been found on newborn babies within an hour of birth and the source of this exposure was thought to be close contact with family members.[3,4] This is consistent with what was found for FcaPV-2. Over 50 beta HPV types have been identified in people and the prevalence varies greatly for the different types: DNA from the most common beta HPV types was present in hair plucks from over 50% of people, while the least common types were present in less than 1%.[5,6] The overall prevalence of FcaPV-2 DNA was 100% in both studies presented in this thesis but this included multiple types of samples and multiple time points. The cross-sectional prevalence of FcaPV-2 in hair plucks from 28 day old kittens was 65%. In addition, studies in people have used consensus primers to detect multiple different PV types and the sensitivity of this assay is not as good as the assay used here to detect FcaPV-2 DNA. Therefore, the prevalence of FcaPV-2 in cats is probably similar to that of the most common beta HPVs in people. The quantity of FcaPV-2 DNA detected in hair pluck samples from cats was also similar to the quantity of beta PV DNA found in plucked eyebrow hairs from people.[7] Other similarities between the beta HPVs and FcaPV-2 include the frequent presence of asymptomatic infections and the frequent detection of PV DNA in adults.[6] However, beta HPVs have only been conclusively associated with cutaneous SCCs in immunocompromised people, whereas FcaPV-2 has been associated with cutaneous SCCs in immunocompetent cats.[8-10] The inability of the beta HPVs to cause lesions in immunocompetent people has been hypothesised to be due to the lack of an E5 gene.[11] Interestingly, an E5 gene has not been identified in FcaPV-2 despite its ability to cause lesions in immunocompetent cats. It may be that FcaPV-2 has developed an alternative method to bypass the hypothesized EVER1/EVER2/zinc mediated inhibition of the transcription factors necessary for cell proliferation in suprabasal skin layers that results in lesion development.

Like FcaPV-2, several other animal PVs also cause skin tumours in immunocompetent hosts. The most well-studied are *Mastomys natalensis* papillomavirus type 1 (MnPV-1), that infects African multimammate mice, and the cottontail rabbit papillomavirus, *Sylvilagus floridanus* papillomavirus type 1 (SfPV-1). FcaPV-2 shares some similarities with MnPV-1. In both instances the most likely mode of transmission appears to be close skin contact between mothers and their offspring, and there was no evidence of a protective effect from maternal antibodies.[12] Interestingly, both beta HPVs and MnPV-1 have been localised to hair follicles and it has been suggested that the virus may gain entry to basal stem cells through the hair follicle rather than micro-wounds.[13] However it is possible that the virus uses both routes of entry. In support of this view, Nafz *et al*. (2007) noted that male multimammate mice, which often fought, had a higher incidence of PV-induced tumours than female mice, which rarely fought.[12] Important differences between MnPV-1 and FcaPV-2 include the incidence and type of skin tumours. MnPV-1 frequently induces exophytic warts (papillomas) and keratoacanthomas, which are benign skin tumours, whereas FcaPV-2 rarely induces viral plaques, bowenoid *in-situ* carcinomas (BISCs), and may also cause invasive skin cancer. Some similarities also exist between FcaPV-2 and SfPV-1. SfPV-1-induced warts frequently occur on the face of cotton tail rabbits and, as described in chapter 5, many of the FcaPV-2-associated SCCs occurred on the face and nasal planum. Possible reasons for this include transmission by biting insects, exposure to UV light, or a particularly susceptible population of cells in these areas. An important difference between the two PVs is that while a high proportion of SfPV-1 induced warts spontaneously regress, PV-associated lesions on cats seldom regress.[12,14]

The alpha genus of PVs includes the high-risk HPV types that cause cancer at mucosal sites and the cutaneous HPVs that cause skin warts. The natural history of both mucosal and cutaneous alpha HPVs is very different to that of FcaPV-2, particularly in terms of the host's age at the time of initial infection, and subsequent clearance of the virus. Infection with cutaneous alpha HPVs is uncommon in infants, peaks in school aged-children, and is rare in adults.[15] Infection with the high-risk mucosal HPVs occurs soon after the onset of sexual activity.[16] Infections with both cutaneous and mucosal alpha HPVs typically resolve within 1-2 years and reinfection or reactivation of latent infection is uncommon.[17,18] Consequently, the prevalence of high-risk HPV DNA is low in adults 35 years and older.[19] In contrast, FcaPV-2 infection is acquired early in life and there is a high prevalence of FcaPV-2 DNA in adult cats. These differences have significant implications. Firstly, the early onset of infection with FcaPV-2 leaves little opportunity for prophylactic vaccination, whereas the late onset of infection with the high-risk mucosal HPVs in people allows plenty of opportunity for prophylactic vaccination to prevent both infection and

the development of cancer. Secondly, the frequent detection of FcaPV-2 DNA on adult cats compared to very low prevalence of alpha PVs on adult humans may reflect differences in the immune response generated against the PVs. Infection with alpha HPVs usually generates a humoral immune response that protects against reinfection.[20] In contrast, the immune response to FcaPV-2 appears to be ineffective at either clearing infection or preventing reinfection. This has implications for the possibility of vaccinating regardless of past infection, which is discussed in the later section on reducing the incidence of FcaPV-2 associated disease.

The reason why FcaPV-2 infection is acquired soon after birth but cutaneous alpha HPV infection is not acquired until later in childhood is not known. It may be related to different routes of infection, for instance FcaPV-2 may access basal cells predominantly via hair follicles whereas cutaneous alpha HPVs are thought to access basal cells through micro-wounds. The prevalence of infection in adults may also be important as infection requires a minimum infectious dose. So even if human infants do acquire cutaneous micro-wounds, they may not be exposed to sufficiently large quantities of alpha HPVs to become infected, as most adults would have cleared previous infections. In comparison, many adult cats appear to shed large quantities of FcaPV-2, so kittens are exposed to infectious doses of FcaPV-2 early in life and infection probably occurs rapidly via hair follicles or micro-wounds. Alternatively maternal antibodies may protect human infants from infection with alpha-HPVs but not kittens from FcaPV-2, possibly due to a greater strength of the humoral immune response to the alpha HPV types.

The results of the studies reported in this thesis suggest that FcaPV-2 is unique because infection is acquired early in life and is common in clinically normal adult cats, yet the virus also appears to cause invasive cancer in immunocompetent hosts. The frequent detection of large quantities of FcaPV-2 DNA on adult cats, and the lack of any evidence of clearance, suggests that the virus is highly host-adapted and usually well-tolerated. The sequence of events that lead from a normally innocuous FcaPV-2 infection to the development of invasive cancer, without immunosuppression, are currently unknown and were the focus of the second part of this thesis.

7.3 FcaPV-2 gene expression in SCCs

The second aim of the research presented in this thesis was to investigate the role of FcaPV-2 in the development of feline skin cancer. Prior to the start of this project, there was a known association between FcaPV-2 and feline cutaneous SCCs although it was not known whether this association was causal. The previous finding of reduced levels of host retinoblastoma protein (pRb) and increased levels of host cyclin dependent kinase inhibitor $p16^{CDK2A}$ (p16) in the SCCs that contained FcaPV-2 DNA suggested that FcaPV-2 may cause cancer by a similar mechanism to the high-risk alpha HPVs.[21] However, FcaPV-2 DNA was also detected in a large proportion of swab samples from normal skin and a number of SCCs that contained FcaPV-2 DNA did not contain increased p16 immunostaining.[1,22] Therefore, it remained possible that FcaPV-2 was present in the cancers without contributing to cancer development. A key event in high-risk HPV-induced cancer is deregulation of expression of the HPV E6 and E7 genes, such that these genes are expressed in the basal cell layer. Production of p16, which is a tumour suppressor protein, increases in response to oncogenic stress caused by the PV but is ineffective because the PV E7 protein bypasses its actions by binding and degrading pRb.[23,24] [23] Therefore, it was hypothesized that the detection of FcaPV-2 E6/E7 mRNA would provide the missing link between the detection of FcaPV-2 DNA and the increased p16 immunostaining and provide additional evidence that FcaPV-2 was contributing to cancer development rather than just being a latent infection.

The aim of the research presented in chapter 4 was to detect FcaPV-2 E6/E7 gene expression in feline cutaneous SCCs. Although previous studies had used formalin-fixed paraffin-embedded (FFPE) tumour samples, RNA preservation in these samples is usually poor, so tumour samples were collected from cats as they were undergoing surgical removal and fixed in RNAlater solution (Life Technologies, Carlsbad, CA, USA) to preserve the quality of the RNA. Using these high-quality samples, a method was developed to quantify FcaPV-2 early and late viral gene expression. To accurately measure relative gene expression it was necessary to normalise the quantities against stably expressed reference genes. Nine reference gene qPCR assays were developed and a reference gene validation study identified two stably expressed reference genes which were then used in the study. Analysis of the 17 test samples found high levels of E6/E7 gene expression in one BISC and two SCCs and these cancers also had increased p16 immunostaining, high copy numbers of FcaPV-2 DNA, and expression of the FcaPV-2 late viral gene. One SCC had increased p16 immunostaining and a high copy number of FcaPV-2 DNA but

no E6/E7 mRNA. Two SCCs had increased p16 immunostaining but little to no FcaPV-2 DNA and no E6/E7 mRNA. The remaining 11 SCCs and the normal skin controls did not demonstrate increased p16 and had little to no E6/E7 mRNA, although low copy numbers of FcaPV-2 DNA were detected in 7 of 11 SCCs and six of 8 normal skin controls. This study demonstrated, for the first time, the presence of FcaPV-2 gene expression in feline cutaneous SCCs, and the results supported a role of FcaPV-2 in the development of 2 of the 16 SCCs included.

A limitation of the study reported in chapter 4 was the small sample size which made it difficult to compare the relative quantities of FcaPV-2 gene expression between groups of samples, particularly between the SCCs and premalignant lesions which are thought to be caused by FcaPV-2. Therefore, another study was conducted using a similar methodology to investigate a larger number of FFPE samples that were collected from the archives of a local diagnostic laboratory. This study was reported in chapter 5. FcaPV-2 gene expression was found in 21 of 60 (35%) SCCs, 10 of 10 (100%) premalignant lesions (including BISCs and viral plaques), and none of 10 normal skin samples. The results showed two distinct subsets of SCCs. The majority of the SCCs had low copy numbers of FcaPV-2 DNA (mean of 17 copies per copy of reference gene DNA) and no FcaPV-2 gene expression, suggesting the virus was an incidental finding. In contrast, around a third of the SCCs had detectable FcaPV-2 E6/E7 gene expression and very high copy numbers of FcaPV-2 DNA, with a mean of 32,930 copies per copy of reference gene DNA. There was also a significant association between FcaPV-2 E6/E7 expression and positive p16 immunostaining in these SCCs which suggests that the FcaPV-2 E6/E7 proteins were manipulating the cell-cycle in a similar manner to the high-risk HPV E6 and E7 proteins. Furthermore the relative quantity of E6/E7 mRNA transcripts and the viral copy number in these SCCs was similar to that found in the PV-induced premalignant lesions. Taken together, these results strongly suggest that FcaPV-2 played a role in the development of a third of the feline cutaneous SCCs in this study. Additionally, the mechanism of cancer development appeared to be similar to that of the high-risk HPVs.

Given the similarities between high-risk HPV-induced cancer and FcaPV-2-associated SCCs, the aim of the research presented in chapter 6 was to detect integration of the FcaPV-2 genome into the host genome; another important event in high-risk HPV-induced cancer. No integrated FcaPV-2 DNA was detected in the feline cancers but non-target sequences were amplified in both assays, so it was not possible to determine whether the lack of integrated FcaPV-2 was due to a true absence of integrated FcaPV-2 DNA or a failure of the method. However, sequences consistent with episomal FcaPV-2 DNA were detected by both assays, which confirmed the

findings in chapters 4 and 5 where the detection of large quantities of FcaPV-2 DNA and late viral gene transcripts in SCCs suggested the presence of episomal FcaPV-2 DNA. This was not consistent with the expected mechanism of cancer development as most HPV-induced cancers contain integrated HPV DNA.[25] However, despite the frequent occurrence of integration events in high-risk HPV-induced cancer it is not a necessary event for cancer development, as a small proportion of human cancers contain exclusively episomal HPV DNA.[26] In addition, PV-induced tumours in rabbits and African multimammate mice contain predominantly episomal PV DNA.[27,28] It is currently unknown how gene expression from episomal PV DNA can become deregulated and contribute to cancer development but it appears likely that this mechanism may be important for FcaPV-2.

The key findings from chapters 4-6 were the detection of FcaPV-2 E6/E7 gene expression in around a third of feline cutaneous SCCs, the significant association between FcaPV-2 gene expression and increased p16 immunostaining, and the similar quantity of FcaPV-2 gene expression between a subset of SCCs and the premalignant lesions. These findings suggest that FcaPV-2 causes a subset of feline cutaneous SCCs, and some of the oncogenic mechanisms may be similar to those of the high-risk HPVs. In addition, low quantities of FcaPV-2 DNA were found in some SCCs without detectible FcaPV-2 gene expression, and in these cancers, the FcaPV-2 is probably an incidental, latent infection. The large quantities of FcaPV-2 DNA and the presence of late viral gene expression in almost all of the feline cutaneous SCCs suggested there was episomal FcaPV-2 DNA. This was surprising and may indicate that FcaPV-2 causes cancer by a mechanism which does not require integration of the viral DNA into the host genome. Such a mechanism is already thought to exist in a proportion of human cancers and some animal cancers but is poorly understood.

7.4 Cumulative evidence for a role of FcaPV-2 in feline skin cancer

While the evidence so far suggests that FcaPV-2 may be involved in the development of a proportion of feline cutaneous SCCs, more evidence is required to definitely prove that FcaPV-2 causes these cancers. By comparison, the high-risk HPVs have been proven to cause almost all cervical cancer in women.[29] Therefore, to put the evidence for a role of FcaPV-2 in cancer development into context, it will be compared to the information that was required to demonstrate a causal association between high-risk HPVs and cervical cancer in women.

7.4.1 Transitional lesions between PV-induced premalignant lesions and cancer

The initial interest in the involvement of HPV in cervical cancer stemmed from the observation of viral cytopathic changes in dysplastic cervical lesions, some of which progressed to invasive cancer.[30] A very similar series of events occurred in the early stages of investigation into PV infection of domestic cats. Miller *et al.* (1992) first suggested a PV aetiology for BISCs based on their clinical and histologic similarities to FVPs.[31] The following year, in a case report of 12 cats with BISC, Baer *et al*. (1993) noted that several cats also had areas of invasive SCC that appeared to have arisen within the BISCs.[32] These transitional lesions between BISCs and SCCs represented the first evidence of a PV-aetiology in feline cutaneous SCCs.

7.4.2 The detection of PV DNA in a high proportion of cancers

Following the observation of transitional lesions between HPV-induced premalignant lesions and cervical cancers, a major advancement in understanding of the role of HPV in cervical cancer came with the discovery of HPV-16 DNA in a high proportion of cervical cancers but only rarely in benign lesions.[33] Other high-risk HPV types were subsequently identified and this was followed by the frequent detection of high-risk HPV DNA in cervical cancers compared to lower rates of detection in other types of cancers and samples of normal cervix.[34-36] Eventually, with improved detection methods, high-risk HPV DNA was found in 99.7% of more than 1000 cervical cancer samples collected from 22 different countries, which was considered strong evidence that the virus was necessary for the development of cervical cancer.[37,38]

FcaPV-2 DNA has also been found in a high proportion of SCCs (51%) compared to other skin samples (9.5%, Table 1.1). Around 200 feline cutaneous SCCs have been investigated for the presence of PV DNA, predominantly from cats in New Zealand but also from cats in North America, England, Belgium and Switzerland.[21,22,39-43] The lower rates of detection of FcaPV-2 compared to high-risk HPV most likely reflects the existence of two different aetiologies for feline cutaneous SCCs. If only the feline SCCs that occur in densely haired skin are considered, then 68% have been reported to contain FcaPV-2 DNA, which is similar to the detection rates of HPV-16 DNA in cervical cancer in women.[38] Therefore, the frequent detection of FcaPV-2 DNA in feline cutaneous SCCs has been well demonstrated and provides strong evidence for an association between the virus and feline skin cancer.

7.4.3 PV infection as a risk factor for the development of cancer

Further evidence for a causative association between high-risk HPV and cervical cancer was provided by epidemiological studies that identified high-risk HPV as the predominant risk factor for cervical cancer and its precursors. For example, in a series of 13 case-control studies the prevalence of HPV DNA in cervical cytology samples was 70 times higher in women with cervical cancer compared to cancer-free women.[29] Importantly, high-risk HPV infection has also been shown to precede the development of cervical cancer and prospective studies have documented persistent HPV infections that progressed to cervical dysplasia and then invasive cancer.[17] Epidemiological evidence has also shown that both HPV infection and cervical cancer have a transmission pattern that is consistent with being sexually transmitted.[16,44]

Such epidemiological evidence is lacking for FcaPV-2 as neither the prevalence nor the risk factors for feline cutaneous SCCs are currently known. While case-control studies investigating the risk factors for cervical cancer in women usually categorise HPV DNA as positive (present) or negative (absent),[29,45] such an approach may not be appropriate for FcaPV-2 as the prevalence of FcaPV-2 DNA is likely to be very high in both case and control cats. Rather, it may be more informative to compare the viral load of FcaPV-2 in cats with and without cutaneous SCCs. In the studies reported in chapters 2 and 3 of this thesis, large quantities of FcaPV-2 DNA were consistently present on some adult cats even when they were isolated for several weeks pre-kittening, which suggested the adult cats were infected with FcaPV-2 and shedding large quantities of virus. It would be very interesting to determine whether the presence of high viral loads in these cats correlated with an increased risk of developing SCC. However, before this

could be assessed a more reliable method is needed to determine the true FcaPV-2 burden of an individual cat. While skin swabs are a quick, non-invasive sampling method that could easily be used on a large number of cats at a routine veterinary appointment, a single skin swab is not a reliable measure of the viral load of an individual cat, as such swabs may also pick up virus-laden squames shed from other cats in the household. The detection of FcaPV-2 mRNA in swabs or biopsy samples could provide more convincing evidence for a true infection but this method has a low sensitivity due to the focal nature of infection. Therefore, an alternate method is needed; a possibility that may warrant further investigation could be the detection of FcaPV-2 mRNA in pooled samples of hair plucks from multiple body sites.

7.4.4 PV-induced immortalisation of cell lines

Equally important as the early epidemiological studies into high-risk HPV were the numerous *in-vitro* experiments demonstrating the oncogenic potential of these HPVs. Although the inability to propagate HPV in cell culture hindered early studies, infection of primary human keratinocytes in culture was eventually achieved by transfecting cells with recombinant plasmids containing the entire HPV genome. Using this procedure, the high-risk HPV types readily immortalised primary human keratinocytes whereas the low-risk HPV types did not.[46] Furthermore, high-risk HPV DNA was present at high copy numbers (1-20 copies per cell) and frequently integrated in HPV-immortalised cell lines compared to cell lines containing low-risk HPVs which had <1-2 copies per cell of exclusively episomal HPV DNA.[46] Studies on cervical cancer specimens confirmed the presence of integrated HPV DNA in a high proportion of cancers and demonstrated that integration was associated with retention of the E6 and E7 open reading frames.[25] The consistent detection of HPV E6 and E7 gene expression in cervical cancer cell lines was further evidence for the importance of these genes in cancer development.[47] This was later confirmed by the finding that E7 gene expression alone or in co-operation with E6 expression was capable of immortalising primary human keratinocytes, and this provided strong evidence for the oncogenic potential of the high-risk HPVs.[48]

The oncogenic potential of FcaPV-2 has not yet been demonstrated *in vitro*. However, based on the frequent detection of E6 and E7 mRNA in feline cutaneous SCCs, it is hypothesized that transfection of a primary feline keratinocyte cell line to constitutively express FcaPV-2 E6 and E7 mRNA would result in immortalisation of the cell line. This would add evidence to the biological plausibility of a causative association between FcaPV-2 and cutaneous SCCs. Currently there are

no major limitations preventing this work and it would be a logical next step in the investigation of the association between FcaPV-2 and cutaneous SCCs. In contrast, it is less clear whether FcaPV-2 plasmids would be integrated in FcaPV-2-immortalised cell lines. The results of the study presented in chapter 6 suggested that FcaPV-2 may not be integrated in feline cutaneous SCCs and it would be more informative to conclusively determine whether integration occurs in the cancers before investigating this in cell lines.

7.4.5 Interactions between PV and host proteins

Another important aspect of the *in vitro* investigations of HPV were the identification of numerous interactions between cellular proteins and the PV E6 and E7 proteins, that occurred with the high-risk HPV types but not with the low risk HPV types.[49,50] These interactions were shown to result in increased cell proliferation, cell immortalisation, and chromosomal instability leading to the acquisition of additional genetic mutations and the development of invasive cancer.[51-53]

Direct interactions between FcaPV-2 proteins and cellular proteins have not yet been demonstrated. However several changes in host proteins have been noted on immunohistochemistry of feline cutaneous SCCs (that contain PV DNA), including reduced immunostaining for pRb, variable immunostaining for p53, and increased immunostaining for p16.[21] FcaPV-2 contains a pRb binding site that is frequently present in the high-risk HPV types and is thought to be important for cellular transformation.[54,55] Therefore, the association between the presence of PV DNA and reduced pRb in the feline cutaneous SCCs was presumed to be due to E7-mediated degradation of pRb. However this needs to be confirmed by the demonstration of FcaPV-2 E7 binding to pRb, and resulting in reduced pRb levels, in cell culture. Similarly, the interactions between FcaPV-2 E6 protein and p53 need to be investigated in cell culture to explain the equivocal findings on immunohistochemistry. The increased p16 immunostaining in feline cutaneous SCC (containing PV DNA) was presumed to occur in response to high levels of the PV E7 protein; this is supported by the results of the study presented in chapter 5 which found a strong association between the presence of increased p16 immunostaining and detectible FcaPV-2 E6/E7 gene expression. The high-risk HPV E7 protein does not directly interact with p16, rather the increased p16 is thought to be a cellular defence mechanism that is triggered by high levels of the high-risk HPV E7 protein.[56] Furthermore, the induction of p16 expression appears to be necessary for the malignant phenotype in human

cervical cancer cell lines.[24] Therefore, it would be interesting to determine whether FcaPV-2-immortalised cell lines have increased levels p16 expression, and whether depletion of p16 (using short hairpin RNAs) reduces cell viability as it does in human cervical carcinoma cell lines.[24] Numerous other biological interactions have been described for the high-risk HPV proteins, including activation of telomerase and binding to PDZ domains of many cell proteins.[57,58] Therefore, a systematic analysis of the interactions between FcaPV-2 and host cellular proteins, possibly using a mass spectrometry-based platform, would be highly beneficial to identify protein interactions both analogous to those of the high-risk HPVs and unique to FcaPV-2. The identification of such interactions would provide further insight into the possible mechanism by which FcaPV-2 is involved in cancer development.[59]

7.4.6 Deregulated PV E6/E7 gene expression

Despite the oncogenic potential of the high-risk HPV E6 and E7 proteins, these proteins are normally expressed in productive lesions, such as condyloma acuminatum (a type of genital wart), without the development of cancer. This is because, in these lesions, high-risk PV E6/E7 gene expression is tightly co-ordinated to the differentiation state of the host cells. Therefore, another important line of evidence for a role of high-risk HPV in cervical cancer was the demonstration that high-risk HPV E6 and E7 gene expression was deregulated in these cancers. The most conclusive evidence for this came from studies of the spatial pattern of PV gene expression and protein levels, in both raft cultures and clinical specimens.[60-62] In low grade lesions E6/E7 mRNA and the corresponding proteins were restricted to the suprabasal layer of cells, whereas in the high grade and cancer samples E6/E7 gene expression and the corresponding proteins were present throughout all layers of the sample (Fig. 1.5 and Fig. 1.6).[60,62]

In cats, FcaPV-2 E6/E7 mRNA has been found in a proportion of cutaneous SCCs (chapters 4 and 5), and the level of E6/E7 gene expression in these SCCs was similar to that in the premalignant lesions. However, this finding does not distinguish between regulated or deregulated PV gene expression as, in people, both productive and neoplastic PV-induced lesions have similar overall levels of PV gene expression. This has been demonstrated in cell culture, where cell lines with integrated high-risk HPV DNA produce large quantities of E6/E7 mRNA from a small number of HPV copies with deregulated gene expression, whereas cell lines with episomal high-risk HPV DNA produce small amounts of E6/E7 mRNA from a large number of episomal copies of the virus

resulting in a similar level of gene expression overall.[47] Therefore, to determine whether FcaPV-2 gene expression is deregulated in feline cutaneous SCCs, the spatial pattern of FcaPV-2 gene and protein expression needs to be investigated in viral plaques, BISCs, and SCCs. The detection of gene expression is done with *in situ* hybridisation for viral mRNAs, whereas the detection of PV proteins is done using immunofluorescent staining using antibodies directed against these proteins. Therefore, this would require the production of antibodies directed against the major FcaPV-2 proteins, which would also need to be isolated and the amino acid sequences confirmed; a potentially difficult and time-consuming process. Alternatively, this process could be made easier if commercially available antibodies directed against the corresponding HPV proteins could be shown to cross-react with the proteins from FcaPV-2, although the likelihood of this occurring is currently unknown.

7.4.7 PV-induced cancer models in transgenic mice

In vivo evidence for a role of HPV in cancer development was difficult to provide, given the high host specificity of HPVs, the low incidence of cancer following infection, and the extended period between infection and cancer development. The most convincing *in vivo* model of high-risk HPV carcinogenesis came from studies of transgenic mice that constitutively expressed HPV-16 E6 and E7 in mucosal basal cells. High rates of cervical cancer occurred in these mice when they were also treated with oestrogen, which demonstrated proof of concept that deregulated HPV gene expression could lead to the development of cancer *in vivo,* as well as confirming the role of oestrogen in HPV-induced cervical cancer.[63] Although this model was a necessary step in the investigation of high-risk HPV-induced cancer, a major limitation was that it did not represent a naturally acquired infection. Now that the proof of concept already exists, the use of transgenic mouse model for FcaPV-2 would simply provide *in vivo* evidence for the oncogenic potential of FcaPV-2 that could also be demonstrated *in vitro.* Therefore, it is possible that the investigation of FcaPV-2 could proceed directly to vaccination trials without the need for a transgenic mouse model.

7.4.8 PV vaccine trials

The epidemiological evidence for a role of HPV in cervical cancer, combined with molecular studies identifying a plausible mechanism, and the development of cancer in HPV transgenic mice, provided sufficient basis for clinical trials of HPV vaccination in women. These trials showed that prophylactic vaccination prevented high-risk HPV infection and provided complete protection against the development of high grade cervical *in situ* cancer (CIN 2+).[2] This provided conclusive evidence that high-risk HPV causes almost all cervical cancer in women.

Therefore, a large, randomised, placebo-controlled vaccine trial represents the gold standard for demonstrating a role of FcaPV-2 in feline cutaneous SCCs. However, the results presented in chapters 2 and 3 of this thesis suggest that there is little opportunity to prophylactically vaccinate kittens against FcaPV-2. An alternative approach may be to vaccinate kittens that are already infected with the hope of reducing the viral load and preventing new infections that may lead to cancer; this is discussed further in the following section. If such an approach can be shown to reduce the viral load on cats, a large, randomized, controlled trial on client owned cats may be able to demonstrate that reducing the FcaPV-2 viral load by vaccination significantly reduces the incidence of feline cutaneous SCCs, and completely protects cats against PV-induced SCCs. This would provide conclusive evidence that FcaPV-2 causes a proportion of feline cutaneous SCCs.

7.5 Reducing the incidence of FcaPV-2 associated disease

In practical terms, the significance of discovering a causal association between FcaPV-2 and a subset of feline cutaneous SCCs relies on the use of this knowledge to either prevent or more effectively treat these cancers. While this thesis did not prove conclusively that FcaPV-2 causes cancer, if this can be proven, reducing the incidence of FcaPV-2 infection could reduce the incidence of cancer development. The remainder of this chapter will discuss the possible vaccination options for prevention of feline cutaneous SCCs. In addition, a brief discussion of potential therapies targeted at PV-induced SCCs is included.

7.5.1 Vaccination

A major finding from this thesis was that most cats are exposed to FcaPV-2 within the first few days of life, which leaves little opportunity for prophylactic vaccination of kittens. The notion that PV vaccination is only effective when administered prophylactically came from early studies on the immune response to vaccination against *Canis familiaris* papillomavirus type 1 (CPV-1) in dogs and *Sylvilagus floridanus* papillomavirus type 1 (SfPV-1) in rabbits. These studies showed that vaccination could prevent PV-induced lesions following experimental challenge but had no effect on existing lesions.[64,65] The vaccine used in these studies, and subsequently developed for use in people, were based on virus-like particles (VLPs), which are empty PV capsids that are created by self-assembly of recombinantly expressed PV L1 and L2 capsid proteins. Although vaccination with VLPs elicits both humoral and cell-mediated responses, it is the antibodies that confer protection, as passive transfer of serum from vaccinated animals protects naïve animals from experimental challenge.[64,66] Subsequently, it has been found that the mechanism of protection at high antibody levels is through occlusion of the heparin sulphate proteoglycan binding sites on the virions which explains why vaccination with VLPs does not prevent progression of disease once the PV has entered the basal cells.[67] The effectiveness of a high-risk HPV VLP vaccine has recently been demonstrated in a large vaccine trial in women, with the vaccine demonstrating a significant protective effect against CIN2/3 lesions when given to women who were both seronegative, and negative for HPV-16/-18 DNA, at baseline.[2] In contrast, no significant protective effect was seen in vaccinated women who had evidence of current or past HPV-16/-18 infection at baseline. The implication of this is that a FcaPV-2 VLP vaccine given to kittens at several weeks of age is unlikely to prevent infection with FcaPV-2

because, by this time, many of the kittens will have already been infected with the virus and the viral genome will be present in the basal cells where it is unreachable by vaccine-induced antibodies.

An alternative way to prophylactically vaccinate kittens against FcaPV-2 may be to vaccinate pregnant queens to provide maternal transfer of protective antibodies to the neonate, followed by vaccination of the kittens around the time the maternal antibodies are waning. In people, maternal antibodies to high-risk HPVs have been detected in newborns following both natural infection and vaccination.[68,69] However it was not determined whether the titre of maternal antibodies in the newborns was sufficient to block PV binding to the basement membrane and therefore prevent HPV infection. In chapters 2 and 3 of this thesis there was no evidence of a protective effect of maternal antibodies against FcaPV-2 in the young kittens. However vaccination generally results in a much stronger humoral response than natural infection and so vaccination of pregnant queens could still potentially protect kittens from infection.[70] However, while this may be possible in some breeding catteries, many feline matings are unplanned so this approach is not practical to prevent cancer in most of the cats in New Zealand.

While most studies suggest that PV vaccines only work in uninfected people,[70] the widely accepted notion that all PV vaccination must be given prophylactically to reduce the incidence of PV-induced cancer was recently challenged by Vinzon *et al.* (2014). In this study, vaccination of multimammate mice (*Mastomys coucha*) already infected with *Mastomys natalensis* papillomavirus type 1 (MnPV-1) resulted in a 10-20 fold reduction in viral load and completely prevented the development of tumours, compared to non-vaccinated mice of which 17.5-28% developed tumours.[71] The authors suggested that this effect was most likely due to neutralizing antibodies blocking reinfection of neighbouring cells in traumatized epithelium. Therefore, it may be that completely preventing PV infection is not always necessary to prevent PV-induced cancer. This finding is not as contradictory to the findings of the HPV vaccine trials in people as it first seems. In the bivalent HPV-16/-18 VLP vaccine trial, a protective effect of vaccination was seen when HPV-16/-18 seropositive but DNA negative women were vaccinated, with a vaccine efficacy of 69%, but the effect was not significant due to the small number of seropositive DNA negative women who developed CIN2.[2] One possible explanation may be that vaccination offers little advantage to women who have already been infected and mounted an immune response because naturally acquired anti-HPV antibodies are generally protective against reinfection.[20] In comparison, the frequent presence of MnPV-1 in adult mice suggests that spread or reinfection with MnPV-1 is common, and naturally acquired antibodies, if present, do not prevent new

infection. Therefore vaccination, which induces a stronger antibody response than natural infection, would be expected to protect against reinfection in both species but the clinical effects are only relevant in mice because reinfection with MnPV-1 appears to be common in mice. In the studies reported in chapters 2 and 3 of this thesis, it was found that most cats were exposed to FcaPV-2 within the first few days of life. Despite this, high levels of FcaPV-2 DNA were consistently found on adult cats suggesting that those cats were either unable to clear FcaPV-2 infection or prevent reinfection, similar to MnPV-1 infected multimammate mice. Therefore, although vaccination of cats with FcaPV-2 VLPs is unlikely to prevent infection with the virus, there is reason to believe that it may reduce the viral load and could potentially prevent FcaPV-2 induced cancer.

Therefore, another potential approach to preventing FcaPV-2-induced cancer may be vaccination of cats with the aim of reducing the viral load rather than completely preventing infection. The validity of this approach would need to be confirmed by first testing whether a vaccine could reduce the FcaPV-2 viral load on cats and then testing whether the reducing the FcaPV-2 viral load would protect cats against the development of FcaPV-2-associated cutaneous SCCs. Testing whether a FcaPV-2 VLP vaccine reduces the viral load on cats should be reasonably simple if done in a closed population of cats. A closed population would be required so that vaccinated cats could be isolated (in groups), from contact with other cats to prevent surface contamination on their skin with virus-laden squames from other non-vaccinated cats, which would otherwise confound the results. Such a study would also provide important information on the safety of a FcaPV-2 VLP vaccine, although it would be expected to be safe given that similar vaccines are safe and well-tolerated in people.[72] In contrast, it would be far more difficult to test whether reducing the cats FcaPV-2 viral load by vaccination would protect against the development of cutaneous SCCs. While the lack of a reliable measure of true FcaPV-2 viral load in this study could be avoided by having previously demonstrated that vaccination significantly reduces the true FcaPV-2 viral load in colony cats, the primary outcome would need to be the development of cutaneous SCCs, which would require many years of follow-up. Additionally, although cutaneous SCCs are a common type of cancer in cats, as with any specific type of cancer the overall prevalence is low. Thus a large number of cats would be needed, probably around 20,000 based on the HPV vaccine clinical trials,[70] although the prevalence of cutaneous SCCs in cats in New Zealand would need to be determined to generate a more accurate estimate. Although this seems like a lot of cats, New Zealand has some of the highest rates of cat ownership in the world; in 2015 there were over 1.1 million pet cats in New Zealand, many of which visit their local veterinarian at yearly intervals.[73] Therefore, while a large scale,

randomised, placebo-controlled clinical trial of a FcaPV-2 VLP vaccine in New Zealand cats is possible, the major limitation would be financial. However, if such a clinical trial was to proceed and demonstrated that reducing the FcaPV-2 viral load by vaccination significantly reduced the incidence of feline cutaneous SCCs, and completely prevented the subset of FcaPV-2-associated SCCs, this would provide conclusive evidence that FcaPV-2 causes a proportion of cutaneous SCCs in cats, as well as showing that vaccination could prevent these cancers.

7.5.2 PV-targeted immunotherapy

Continued expression of the PV E6 and E7 genes is a key feature of HPV-induced cancer and was also found in around a third of feline skin cancers in this thesis.[23] Therefore, it would be expected that the PV E6 and E7 proteins would make excellent targets for tumour immunotherapy, and indeed various immunotherapy approaches have been tried on HPV-induced cancer. Therapeutic vaccines, based on the HPV E6 or E7 complete proteins or peptides (sometimes fused to other immunogenic proteins such as adenylate cyclase (CyaA) from Bordetella pertussis), have generated cytotoxic T cell responses in people and caused tumour regression mice.[74] However, despite the cytotoxic T cell response, so far tumour regression in human patients has been minimal.[75] Viral based vaccines have also shown some promise. A modified vaccinia virus (MVA) expressing HPV E6, E7 and IL-2 caused lesion regression and HPV clearance in 48% of patients presenting with CIN 2/3, which was significantly higher than the expected spontaneous regression rate of 20%.[76] Similar rates of regression were found in a study using a DNA plasmid containing modified HPV E6 and E7 genes that was delivered by intramuscular injection followed by electroporation to increase plasmid entry into the nucleus. Histological regression of CIN2/3 lesions was seen in 49.5% of treated patients compared to 30.6% of the controls.[77] Phase II clinical trials are currently underway for CyaA/E7 proteins and recombinant MVA virus, and have recently been completed the DNA plasmid vaccine.[77-79] However these therapies are directed against dysplastic lesions and, so far, no therapeutic vaccine has demonstrated efficacy against invasive cancer. Therefore, although there is potential for a therapeutic vaccine directed against FcaPV-2 E6 or E7, this would only be expected to help treat the relatively small proportion of cats who present with premalignant lesions and would be unlikely to benefit the majority of cats which present with invasive cancer.

An alternative approach to therapeutic vaccination is T-cell therapy, and a phase II clinical trial is currently underway to assess the response of HPV-induced cancer to this treatment.[80] Briefly, a

patient's own T-cells are genetically modified to express a receptor that is known to bind to HPV-16 E6 peptides presented on a particular allele of the α2 subunit of MHC I molecules. The modified T-cells are multiplied *in vitro* then transfused back into the patient. A preliminary study showed complete regression of widespread cervical cancer metastasis in two out of nine patients treated with HPV E6/E7 T cell therapy.[81] Two of the other patients had strong anti-PV cytotoxic T-cell responses *in vitro* but had only partial or no clinical response.[81] This highlights a common problem in PV-targeted immunotherapy which is the rapid downregulation of anti-PV cytotoxic T cell activity. Improved understanding of the immunosuppressive tumour micro-environment, particularly the role of regulatory T cells and checkpoint inhibitors, will hopefully lead to the development of ways to overcome this local immunosuppression.

Therefore, while PV-targeted immunotherapy theoretically represents an ideal treatment for FcaPV-2-associated SCCs, it is still in the development phase and more immediate solutions are required. With prophylactic vaccination not an option, and maternal vaccination highly impractical, vaccination to prevent reinfection currently represents the most promising therapy to reduce the incidence of FcaPV-2 associated disease in cats. However the feasibility of this approach needs to be verified by experiments demonstrating a reduction in the FcaPV-2 DNA load following vaccination with FcaPV-2 VLPs and further molecular evidence of the oncogenic potential of FcaPV-2 is also needed before a large-scale clinical trial of a vaccine is conducted.

7.6 Final summary

Prior to the start of this project, relatively little was known about FcaPV-2, apart from the virus had been associated with feline cutaneous SCCs, and asymptomatic infection in adult cats appeared to be common. Feline cutaneous SCCs are a common and aggressive type of skin cancer in cats, therefore the possibility of a viral aetiology of these cancers was of interest as it suggested that it may be possible to prevent some of these cancers by vaccination.

The major contributions of the research reported in this thesis were two-fold. Firstly, FcaPV-2 was found to commonly infect young kittens and the likely source of exposure was close contact with adult cats, some of which appeared to be shedding large quantities of the virus. This suggested that vaccination with a FcaPV-2 VLP vaccine would not be effective at preventing FcaPV-2 infection, but could possibly reduce the viral load and may still be effective at reducing the incidence of FcaPV-2-induced skin lesions. The second major contribution from this thesis was the detection of FcaPV-2 gene expression in feline cutaneous SCCs, which separated the SCCs into two distinct subgroups. The first group contained little to no FcaPV-2 DNA and no FcaPV-2 gene expression, thus the FcaPV-2 present in these cancers probably represented a latent infection, and was not the cause of the cancer (which was probably UV-induced DNA damage). The second group, which was around one third of the cancers, contained very high copy numbers of FcaPV-2 DNA and E6/E7 gene expression at a level similar to that seen in FcaPV-2 induced premalignant lesions. The presence of E6/E7 gene expression in the second group was strongly associated with increased p16, which suggested that the virus may be playing a role in cancer development.

However some of the findings reported in this thesis require further investigation, such as the presence of increased p16 immunostaining in a proportion of SCCs that did not contain detectible FcaPV-2 gene expression, and the series of results that suggested that FcaPV-2 may be replicating in feline cutaneous SCCS. These findings were not consistent with the mechanism of high-risk HPV-induced cancer. Therefore, while there is a clear association between FcaPV-2 and feline cutaneous SCCs, and the results from this thesis support a role of FcaPV-2 in cancer development, a greater body of evidence is required to demonstrate that the association is causative. In particular, it needs to be shown that FcaPV-2 E6/E7 gene expression is deregulated in feline cutaneous SCCs compared to benign FcaPV-2 induced lesions. Furthermore, the oncogenic potential of FcaPV-2 needs to be confirmed by demonstrating that FcaPV-2 is capable

of immortalising primary feline keratinocytes. These FcaPV-2-immortalised cell lines could also be used to investigate the interactions between FcaPV-2 proteins and host cellular proteins. Epidemiological evidence that FcaPV-2 is a major risk factor for the development of a proportion of feline cutaneous SCCs is also lacking and this will be difficult to provide given the current lack of a reliable and practical way of determining the true FcaPV-2 load on an individual cat. It may in fact be easier to demonstrate that reducing the FcaPV-2 load by vaccination protects against the development of FcaPV-2-associated SCCs rather than demonstrating that a high FcaPV-2 load is a risk factor for cancer development. Therefore, assuming FcaPV-2 gene expression has been shown to be deregulated in feline cutaneous SCCs, and the oncogenic potential of the virus has been confirmed *in vitro,* the next step would be the development and trial of a FcaPV-2 VLP vaccine in a highly controlled situation in a closed population of cats. If it was conclusively demonstrated that such a vaccine was safe, and significantly reduced the viral load on the treated cats, the next stage would be a randomized, placebo-controlled, clinical trial of a vaccine on a large number of client-owned cats, run through multiple veterinary practices in New Zealand. While such a trial would be a huge undertaking, demonstration of a reduced incidence of cutaneous SCCs (particularly the PV-associated SCCs) in the vaccinated cats would provide conclusive evidence that FcaPV-2 is involved in the development of a subset of feline cutaneous SCCs and could potentially provide basis for the production of a commercial vaccine to prevent disease caused by this virus.

7.7 References

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Appendix A

List of Publications
Statements of Contribution

Publications

Thomson NA, Dunowska M, Munday JS. The use of quantitative PCR to detect *Felis catus* papillomavirus type 2 DNA from a high proportion of queens and their kittens*. Vet Microbiol*. 2015;175:211-217.

Thomson NA, Munday JS, Dittmer KE. Frequent detection of transcriptionally active *Felis catus* papillomavirus type 2 in feline cutaneous squamous cell carcinomas. *J Gen Virol*. 2016;97(5):1189-1197.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Neroli Thomson

Name/Title of Principal Supervisor: Associate Professor John Munday

Name of Published Research Output and full reference:

Thomson NA, Dunowska M, Munday JS. The use of quantitative PCR to detect Felis catus papillomavirus type 2 DNA from a high proportion of queens and their kittens. Veterinary Microbiology. 2015;175:211-217.

In which Chapter is the Published Work: 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and carried out the experiment, and prepared the manuscript. The co-authors provided advice and technical support, and helped with the preparation of the manuscript.

Candidate's Signature — Date: 9/11/16

Principal Supervisor's signature — Date: 9.11.16

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Name/Title of Principal Supervisor: Associate Professor John Munday

Name of Published Research Output and full reference:

Thomson NA, Munday JS, Dittmer KE. Frequent detection of transcriptionally active Felis catus papillomavirus type 2 in feline cutaneous squamous cell carcinomas. The Journal of General Virology. 2016;97(5):1189-1197.

In which Chapter is the Published Work: 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

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