Frequent detection of transcriptionally active *Felis catus* papillomavirus 2 in feline cutaneous squamous cell carcinomas

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*Felis catus* papillomavirus 2 (FcaPV-2) causes premalignant skin lesions in cats and has also been found in a proportion of cutaneous squamous cell carcinomas (SCCs) – a common and potentially fatal cancer of cats. Whilst this could suggest a role of the virus in cancer development, FcaPV-2 has also been detected in skin swabs of normal cats, making it difficult to discern whether the papillomavirus is causing the cancer or merely an ‘innocent bystander’.

To distinguish between these two possibilities, real-time PCR was used to determine the viral copy number and the transcriptional activity of FcaPV-2 infections present in 70 formalin-fixed paraffin-embedded skin lesions including 10 papillomavirus-induced premalignant lesions and 60 SCCs. FcaPV-2 gene expression was found in 21 of 60 (35 %) SCCs, all 10 premalignant lesions 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, 20 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. The relative quantity of E6/E7 gene expression and the viral copy number in this group were similar to those found in the papillomavirus-induced premalignant lesions, suggesting that FcaPV-2 may play a role in the development of a subset of feline cutaneous SCCs.

**INTRODUCTION**

Papillomaviruses are small, non-enveloped DNA viruses that infect skin and mucosal surfaces, and are thought to cause ~6 % of all human cancers (Thun et al., 2010). A key event in papillomavirus-induced cancer is deregulation of the papillomavirus oncogenes E6 and E7, which are normally expressed in the suprabasal layers of the epidermis and 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 (Flores et al., 2000). Overexpression of the papillomavirus E6/E7 genes in basal cells leads to abnormal cell proliferation and the acquisition of additional mutations, which can progress to invasive cancer (Isaacson Wechsler et al., 2012). This process is an uncommon and aberrant sequela of papillomavirus infection, and these lesions do not support viral replication (Middleton et al., 2003).

*Felis catus* papillomavirus 2 (FcaPV-2) is a member of the genus (*Dyothetapapillomavirus*) that infects the skin of domestic cats. FcaPV-2 is thought to cause premalignant skin lesions including viral plaques and Bowenoid *in situ* carcinomas, as these lesions frequently contain FcaPV-2 DNA and cytopathic changes consistent with papillomavirus replication (Munday & Peters-Kennedy, 2010; Munday et al., 2007). Additionally, FcaPV-2 DNA has been found in skin swabs from normal cats, suggesting that subclinical infections may be common (Munday & Witham, 2010).

In addition to the premalignant lesions, some evidence suggests that FcaPV-2 may also cause cutaneous SCCs, which are the most common malignant skin cancer of cats (Miller et al., 1991). Early evidence of this was the observation of transitional lesions between papillomavirus-induced premalignant lesions and SCCs (Baer & Helton, 1993). Subsequently FcaPV-2 DNA was detected in ~50 % of all feline cutaneous SCCs, in particular 76 % of those that occur in densely haired skin (Munday et al., 2011b). In comparison, FcaPV-2 DNA was found in <10 % of non-SCC skin lesions (Munday et al., 2011a). Additionally, the SCCs that harbour FcaPV-2 DNA frequently have increased levels of the host cyclin-dependent kinase inhibitor p16\(^{INK4A}\) (p16) (Munday et al., 2011b). Increased p16 is frequently seen in human papillomavirus (HPV)-induced SCCs and this increase is thought to be an innate tumour suppressor response that is bypassed by papillomavirus E7 protein-induced degradation of the retinoblastoma protein (Smeets et al., 2007; McLaughlin-Drubin et al., 2013). Interestingly, increased
p16 in both feline cutaneous SCCs and human oropharyngeal SCCs has been associated with a more favourable prognosis compared with similar p16-negative SCCs (Smeets et al., 2007; Munday et al., 2013).

 Whilst current evidence suggests a role of FcaPV-2 in the development of some SCCs, the possibility that the FcaPV-2 DNA detectable in the SCCs is simply an incidental silent infection cannot be excluded. If the FcaPV-2 were a silent infection, low viral copy numbers and minimal gene expression would be expected, as has been described in other species (Maglennon et al., 2011). Alternatively, if FcaPV-2 was involved in the development of the cancer, it would be expected to be present in high copy numbers and actively transcribing genes (Wilczynski et al., 1998). The presence of FcaPV-2 E6 and E7 gene expression is especially informative as the products from these genes could influence cell proliferation and neoplastic transformation.

 Therefore, the aim of this study was to determine FcaPV-2 copy number and gene transcription in a series of feline cutaneous SCCs. By doing this it should be possible to distinguish between an incidental silent infection with FcaPV-2 and an active infection that could be contributing to cancer development. In addition, viral copy number and gene expression were compared between SCCs, papillomavirus-induced premalignant lesions and normal skin. If the viral copy numbers and gene expression levels in the SCCs were similar to those in normal skin, this would suggest that the FcaPV-2 was merely an incidental finding. However, if the viral copy numbers and the relative quantity of viral gene expression in the SCCs were similar to those observed in the papillomavirus-induced premalignant skin lesions, this would provide additional evidence that FcaPV-2 is involved in the development and progression of feline cutaneous SCCs. The presence of p16 protein within the SCCs was also investigated to determine whether increased p16 correlated with E6/E7 gene expression. To the best of our knowledge, this is the first time that FcaPV-2 gene transcription and viral load has been investigated in a large number of feline cutaneous SCCs.

### RESULTS

#### Sample selection and quality

In total, 60 formalin-fixed paraffin-embedded (FFPE) cutaneous SCCs, 10 premalignant lesions and 10 samples of normal skin were included in the study. *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 1).

<table>
<thead>
<tr>
<th>SCC location</th>
<th>E6/E7 gene expression [n/N (%)]</th>
</tr>
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<tbody>
<tr>
<td>Sparsely haired skin</td>
<td></td>
</tr>
<tr>
<td>Pinna</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Eyelid</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td>Nasal planum</td>
<td>3/9 (33)</td>
</tr>
<tr>
<td>Combined</td>
<td>5/28 (18)</td>
</tr>
<tr>
<td>Densely haired skin</td>
<td></td>
</tr>
<tr>
<td>Face</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Trunk</td>
<td>2/3 (67)</td>
</tr>
<tr>
<td>Neck</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>4/6 (67)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11/26 (46)</td>
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#### FcaPV-2 gene expression

FcaPV-2 gene expression was detected in 21 of 60 SCCs (35 %) and 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 %), whilst one SCC with amplifiable FcaPV-2 mRNA had only a small amount of L1/L2 gene expression. The relative quantity of E6/E7 gene expression in the SCCs that did have detectable E6/E7 gene expression was no different to the relative quantity of E6/E7 gene expression in the premalignant lesions (Fig. 1(a); *P*=0.39). Of 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 (four of six) than in SCCs from sparsely haired skin (five of 28; *P*<0.05). Details of the individual SCC samples are shown in Table 2, and details of the premalignant and normal samples are shown in Table 3.

Expression of the FcaPV-2 late viral genes (L1/L2) was detected in 20 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 the expression of the two genes (*r*=0.866, *P*<0.05). There was no difference in the mean relative quantity of late viral gene expression between the SCCs that had late viral gene expression and the premalignant lesions (Fig. 1(b); *P*=0.17).

#### FcaPV-2 viral load

The presence of amplifiable DNA was confirmed in 52 of 60 SCCs, all 10 premalignant lesions and eight 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 three of eight (38 %) normal skin samples. Whilst 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 very 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. 2), 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 < 0.05). Correspondingly, the quantity of FcaPV-2 DNA was significantly correlated with the relative quantity of FcaPV-2 E6/E7 gene expression (r = 0.63) and L1/L2 gene expression (r = 0.57).

**DISCUSSION**

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

This study was also the first time that copy number of FcaPV-2 DNA was quantified in a large series of feline cutaneous SCCs. Whilst 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 fewer than two copies of FcaPV-2 DNA per cell whereas others had > 10^5 copies per cell. There was a significant relationship between the viral copy number and gene expression, and thus the results of this study 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 characterized by having FcaPV-2 E6/E7 gene expression and high copy numbers of FcaPV-2 DNA. It is interesting to note that the relative quantity of FcaPV-2 E6/E7 gene expression and the absolute quantity of FcaPV-2 DNA in these SCCs were similar to those in the papillomavirus-induced premalignant lesions.

**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. 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 with 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 eight of the remaining 12 had no detectable FcaPV-2 DNA and two of 12 had fewer than three copies of FcaPV-2 DNA per copy of reference gene DNA. The two remaining p16-positive, E6/E7-negative SCCs had no amplifiable DNA so FcaPV-2 DNA copy number could not be assessed.

Fig. 1. Log-normalized relative quantity of gene expression of FcaPV-2 E6/E7 (a) and L1/L2 (b) viral genes in premalignant lesions and SCCs. Box-and-whisker plots; +, group mean; *, outlier.
normal skin samples, appears likely to represent a normal inci- incidental infection with this virus in which the infection is silent and does not significantly influence the growth of the host cells.

The two subgroups of SCCs also showed a different distribution of sites on the body. The first group was more common on densely haired skin; 67% of the SCCs from these sites contained FcaPV-2 E6/E7 gene transcription. In contrast, the second group was more common on sparsely haired skin; such as the eyelids, nasal planum and pinna; only 18% of the SCCs from these sites contained FcaPV-2 E6/E7 gene transcription. It was suggested previously that there may be two different aetiologies of feline cutaneous SCCs, with those occurring on sparsely haired skin caused by UV-induced genetic damage and those occurring on densely haired skin developing from papillomavirus-induced premalignant lesions (Munday et al., 2011a). The different locations of 

Table 2. Details of individual SCCs

E6/E7, FcaPV-2 transcripts potentially coding for E6 or E7 proteins; L1/L2, FcaPV-2 transcripts potentially coding for capsid proteins; +, positive result, −, negative result; NA, no amplification of reference gene DNA.
the two subsets of SCCs found in this study support this hypothesis and the continued expression of the papilloma-
virus E6/E7 genes in the SCCs also provides evidence that the papillomavirus may contribute to the progression from premalignant to malignant lesions.

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 HPV-induced cancers there is increased production of the papillomavirus E6 and E7 proteins which interfere with numerous cell cycle regulatory proteins and induce chromosomal instability, thus contributing to cancer development (Pett & Coleman, 2007; Isaacson Wechsler et al., 2012). The papillomavirus E7 protein also induces expression of p16; 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 (McLaughlin-Drubin et al., 2013). Therefore, the significant association between FcaPV-2 E6/E7 expression and positive p16 immunostaining in the feline cutaneous SCCs may suggest 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 showed FcaPV-2 E6/E7 gene expression had focal p16 immunostaining that did not reach the threshold for positivity. The lack of p16 immunostaining in parts of these cancers may have been due to the cells developing additional mutations which allowed them to proliferate in the absence of p16 (McLaughlin-Drubin et al., 2013). Surprisingly, positive p16 immunostaining was also found in 33 % of the SCCs that did not contain detectable FcaPV-2 E6/E7 gene expression. This result has several possible explanations. (1) It is possible that more than one feline papillomavirus type may be capable of causing increased p16. Evidence supporting this is the previous detection of Felis catus papillomavirus 3 DNA exclusively in four of 32 p16-positive SCCs (Munday et al., 2013). In the present study, the primers only amplified transcripts from FcaPV-2 and so the presence of other papillomavirus types cannot be excluded. (2) Increased p16 in these SCCs may be due to genetic or epigenetic changes which are unrelated to papillomavirus infection. Although p16 is frequently downregulated in non-HPV-induced cancers in people, some distinct subsets, including triple-negative breast cancers, treatment-resistant prostate carcinomas and non-small cell lung cancers, can show high-level p16 expression (Brambilla et al., 1999; Lee et al., 1999; Subhawong et al. 2009). (3) FcaPV-2 E6/E7 gene expression may have been present in the SCCs, but not detectable by the PCR primers used in the present study. (4) Other factors, such as the presence of an oncogenic human papillomavirus type, may have contributed to the increased p16 expression. (5) There may be a third papillomavirus type capable of causing increased p16. Evidence supporting this is the previous detection of Felis catus papillomavirus 3 DNA exclusively in four of 32 p16-positive SCCs (Munday et al., 2013).
but below the detection limit of the assay. This seems unlikely as eight of the 13 E6/E7-negative p16-positive SCCs also had no detectable FcaPV-2 DNA and the FcaPV-2 DNA assay used in this study has previously been shown to be able to detect the equivalent of two copies of FcaPV-2 DNA (Thomson et al., 2015). 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.

The mechanism by which papillomaviruses are thought to cause cancer in humans typically involves integration of the papillomavirus DNA into the human genome (Klaes et al., 1999). This disrupts the normal papillomavirus life cycle, causing an increase in the steady-state levels of the papillomavirus E6 and E7 mRNAs, and reduced expression of the late viral genes (Jeon & Lambert, 1995; Schmitt et al., 2011). Consequently, there is no production of capsid proteins and papillomavirus-induced cancers are considered abortive infections (Middleton et al., 2003). Therefore, HPV-induced cancers contain relatively low papillomavirus copy numbers: a study of FFPE cervical carcinomas found that around a third of the SCCs contained 10 copies of high-risk HPV DNA per cell (Kalantari et al., 2009). 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 papillomavirus integration, but is more consistent with a predominance of episomal papillomavirus DNA (Stoler et al., 1992). It is interesting to note that similar copy numbers of papillomavirus DNA have been reported in the upper epithelial layers of oral papillomas in rabbits, suggesting that FcaPV-2 may be successfully replicating in feline cutaneous SCCs (Maglennon et al., 2011). This would be unusual as papillomavirus-induced cancers do not generally support papillomavirus replication, although it is possible as papillomavirus replication has been reported in cutaneous SCCs from people with the hereditary condition epidermodysplasia verruciformis, which confers a defective immune response to cutaneous papillomavirus (Orth, 1986).

In conclusion, to the best of our knowledge, this is the first time that FcaPV-2 gene expression and viral load have been evaluated in a large series of 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, silent infection. Rather, these findings suggest that a subset of feline cutaneous SCCs may develop from FcaPV-2-induced premalignant lesions, and also support 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.

METHODS

Sample collection. FFPE samples of feline cutaneous SCCs were sourced from the local New Zealand Veterinary Pathology diagnostic laboratory archives. 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 eosin-stained sections from all samples were examined by one author (N. T.) to confirm the diagnosis. Ten FFPE samples of premalignant lesions that had been collected and found to contain FcaPV-2 DNA and p16 immunostaining in previous studies were also included. 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 (Gross et al., 2005). Two of the samples contained histological features of papillomavirus infection, including abnormal keratinocytes within the stratum granulosum that contained swollen blue–grey fibrillar cytoplasm, large vacuoles and a vesicular or shrunken nucleus. Samples of normal skin were collected from 10 cats that had been euthanized for unrelated reasons; these were fixed in formalin and paraffin embedded.

RNA extraction and cDNA synthesis. Three 10μm sections were cut from each FFPE sample for RNA extraction and a further three samples 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 a Nucleospin totalRNA FFPE XS kit (Macherey-Nagel) according to the manufacturer’s recommendations. Nucleic acid concentrations were measured with a Qubit 2.0 fluorometer (Life Technologies). To remove any residual genomic DNA, an additional post-extraction DNase reaction was performed using Ambion Turbo DNA-free DNase according to the manufacturer’s protocol (Life Technologies). Complementary DNA synthesis was performed with the Roche Transcriptor first-strand cDNA synthesis kit (Roche Applied Science) 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 a Qubit 2.0 fluorometer and diluted with DNase-free, RNase-free water to a final concentration of 5 ng µl⁻¹.

**Real-time PCR assays for viral mRNAs.** The assay for FcaPV-2 E6 and E7 mRNAs was performed as described elsewhere (Thomson et al., 2015) (unpublished). This assay amplifies a 111 bp fragment from the E7 ORF that is expected to be in 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'-CGGATAAGGTGCTCCTCCAAA-3') and reverse (5'-ACGGAAATT-GTTCAGGTCC-3') primers were designed to amplify a 105 bp fragment from the L1 ORF. Based on transcriptomes from HPV and rabbit papillomavirus this fragment was expected to be present in transcripts coding for both L1 and L2 proteins, and none of the transcripts from the early genes (Zeltner et al., 1994; Johansson & Schwartz, 2013). Real-time PCRs were performed using AccuMelt HRM SuperMix (Quanta Biosciences), 0.8 µM both primers and 1 µl template cDNA. The cycling conditions consisted of an initial 5 min denaturation step at 95 °C, followed by 40 cycles of denaturation (95 °C for 5 s), annealing (60 °C for 20 s) and elongation (72 °C for 15 s). The cycling was followed by a melting step from 55 to 95 °C. The efficiency of the L1 assay was calculated from standard curves derived from PCR product: E=93.48 %, r²=0.996. All reactions were run in duplicate. RNA from every sample was included as a no-reverse-transcriptase (−RT) control for the E7 assay to ensure there was no contamination with FcaPV-2 DNA. Positive and no-template controls were also included on each plate.

**Reference genes.** A reference gene validation study (Figs 3 and 4) was performed to assess the stability of nine reference genes on a subset of samples: eight FFPE SCC samples and eight FFPE normal skin samples, which were selected because ample tissue was available. The reference genes assessed were: β-actin (ACTB), tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein ζ (YWHAZ), β-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 β2-microglobulin (B2M). Primer sequences, concentrations and reaction conditions are described elsewhere (Thomson, N. A., Munday, J. S. & Dittmer, K. E., submitted for publication). Extra RNA was extracted from the selected samples and 10 ng cDNA used as the template. All reactions were performed in triplicate and Ct values were compared using two statistical algorithms: geNorm, a pairwise variation model included in qbase+ software (Biogazelle), and NormFinder, an ANOVA-based model of reference gene stability (Vandesompele et al., 2002; Andersen et al., 2004).

NormFinder identified ABL2, RPL17, ACTB and GAPDH as the most suitable reference genes. These genes had the lowest 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. 3). geNorm identified ABL2, ACTB, RPL17 and RPS7 as the best candidates for reference gene normalization (Fig. 4). As both algorithms identified ABL2 and ACTB as potential candidates, these reference genes were chosen for use in this study.

Real-time PCR assays for ABL2 and ACTB were performed in duplicate for all samples, with 1 µl of the same template cDNA used for the FcaPV-2 E6/E7 and L1/L2 gene assays. RNA from every sample was included as a −RT control for ACTB to ensure there was no contamination with genomic DNA. Positive and no-template controls were also included on each plate. The geometric mean Cc values from ABL2 and ACTB reference genes were used to normalize the relative quantities of FcaPV-2 E6/E7 and L1/L2 gene expression. The use of two reference genes allowed reference gene stability to be assessed. This identified three outliers: SCC samples which had ABL2 expression greater than threefold above the mean 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 and M values of 0.331 and 0.931, respectively. M values are a measure of gene stability based on the pairwise variation model (Vandesompele et al., 2002). For heterogeneous sample panels such as this, acceptable coefficient of variation and M values are <0.5 and 1, respectively (Hellemans et al., 2007).

**Quantification of FcaPV-2 DNA.** DNA extraction and absolute quantification of FcaPV-2 DNA copy number were performed as previously reported, with the additional step that the FFPE scrolls were rehydrated in a graded alcohol series prior to DNA extraction (Thomson et al., 2015). Real-time PCR absolute quantification was based on recombinant plasmid generated standard curves (Thomson et al., 2015). FcaPV-2 copy number was normalized to copies of F. catus 28S rDNA. This was more appropriate than virus copies per cell as many of the virus-containing neoplastic cells may have been polyploid.

**Fig. 4.** Candidate reference genes ranked according to their geNorm M values (lower values indicate higher stability).
Immunohistochemistry for p16. Immunohistochemistry to detect p16 was performed as previously reported using a mouse anti-human p16 mAb (BD Biosciences) (Munday et al., 2011a). Positivity was defined as >75% of cells exhibiting strong nuclear and cytoplasmic immunostaining for p16 (Lewis et al., 2012).

Statistical analysis. Real-time PCR data were analysed using EcoStudy software (Illumina). Plates that showed a C₅ variation of <0.5 for the positive control were considered valid. Gene expression, DNA copy number and immunohistochemistry results were analysed with SPSS Statistics version 22 (IBM). Results were expressed as mean ± standard deviation. Differences were considered significant at P<0.05.

REFERENCES


