SUMMARY: Project IZS LT 03/15 RC "Feline Papillomavirus and feline skin neoplasms: new diagnostic approaches for an emerging oncogenic virus"

Objectives

- To evaluate the presence of viral DNA and the expression of FcaPV-2 potential oncogenes in cutaneous and mucosal neoplasms in domestic and wild felines and in exotic felids.

• To develop biomolecular techniques for the diagnosis of papillomavirus (PV) and their use for the diagnosis of PV in squamous cell carcinoma of wild and exotic felines.
• To clarify the role of the virus under study in cutaneous and mucosal neoplasms in cats
• To phylogenetically correlate the identified strains with those previously isolated, described in literature

Methods

- Anatomo-pathological examination: collection of neoplastic cutaneous and mucosal lesions from animals received for necroscopic examination and from live animals, possibly anesthetized.
- Histological examination by staining with Hematoxylin-Eosin on 10% formalin fixed samples and included in paraffin.
- Immunohistochemical examination for evidence of the viral antigen and of the host cell p16 protein on paraffin-embedded samples.
- Virological exams: biomolecular techniques for highlighting DNA, RNA, structural genes, and transcription and expression genes; negative staining electron microscopy (nsEM) techniques to highlight PV particles by morphology and size.

The RCA PCR, RT-PCR and PCR consensus protocols for the molecular diagnosis of feline papillomavirus have been studied through detailed research in literature and then developed for use. Also performed: Sanger method for sequencing; quantitative real-time PCR for comparison of viral loads in neoplastic and non-neoplastic oral lesions; in situ hybridization to highlight FcaPV-2 genomic DNA.

Results

On the 132 biopsies of skin and mucous membranes of cats, collected for histological examination during the project period, 48 neoplasms compatible with papillomavirus infection were diagnosed including 44 squamous cell carcinomas, 2 oral papillomas and 2 actinic keratoses.
Overall, in the period January 2013 - April 2019, 85 samples of lesions compatible with papillomavirus infection were collected and selected at the IZSLT[1] (37 from the archive and 48 collected during the project period). Of these, 81 were squamous carcinomas, two were oral papillomas and two actinic keratoses; 27 involved the mucous membranes, in particular that of the oral cavity (25 samples).
On all the samples of skin and mucous membranes, the presence of lesions indicative of papillomavirus infection was evaluated histologically, in particular the presence of Coilocytes, ie epithelial cells modified by the action of the virus, characteristics of this infection. These lesions were detected in a sample of squamous cell carcinoma taken from the palate and in the two cases of papillomas located on the tongue.
All the 85 samples selected were subjected to virological investigations as foreseen by the project.
PCR RCA allows to amplify circular DNA viral genomes using random primers with low specificity, a particular TaqPolymerase and, in the case of feline Papillomaviruses, three different restriction enzymes: EcoRI, BamHI, HindIII. The RCA-PCR followed by the enzymatic restriction analyzes confirmed the presence of the bands of about 7.9Kbp in all the 19 samples examined, confirming the presence of the Feline Papillomavirus genome, without however indicating the type. These results confirmed both the diagnosis of squamous cell carcinoma performed by histological examination and the presence of FcaPV verified by molecular tests (selected samples as positive or to both specific PCR JMP and PCR E2 protocols, or to at least one panPV PCR protocol).

[1] Istituto Zooprofilattico del Lazio e della Toscana "M.Aleandri"

Both pan-PV CODHEOP PCR have been developed for the detection of Papillomaviruses in skin lesions of a wide range of mammals and in our work, have also been tested on skin lesions found in reptiles.
The products of the six samples tested positive in panPV PCR were confirmed with the JMP and E2 PCR specific for FcaPV-2 and sequenced as FcaPV-2 with 100% sequence identity and 99% query-cover with the sequence A.N. EU796884.1, Feline Papillomavirus type 2 isolates Main Coon 2007. Of the 34 samples sent by CEROVEC, 10 were positive, of which five confirmed with PCR JMP and E2. The PCR JMP and E2 amplifiers showed 99% sequence identity and 91% query-cover with the sequence NC_038520.1, Feline papillomavirus type 2 isolate Main Coon 2007.
The PCR molecular protocols JMP and PCR E2 have been developed considering that the most widespread FcaPV is type 2 and are characterized by using primers that recognize highly conserved sequences within the FcaPV-2 genome. The two protocols are applicable to phylogenetic analyzes. Of the 88 samples examined, 27 were positive in PCR JMP and 28 in PCR E2. Of the 34 samples sent by the CEROVEC[2], 7 were positive in PCR JMP and 12 positive in PCR E2. Five samples coincided with those positive in PCR panPV. The sequencing result of two amplifiers was 99% sequence identity and 100% query cover with the sequence A.N. NC_038520.1, Feline papillomavirus type 2 isolate Main Coon 2007.
For 23 samples among those positive in PCR, it was possible to proceed with the examination in nsEM. In four lesions with auricular, back skin, lingual mucosa and periocular skin site, papillomavirus particles were observed. Three lesions were cutaneous SCCs and one papilloma on tongue.
The immunohistochemical examination was performed on 25 oral lesions (23 samples of squamous cell carcinoma and two papillomas), which had tested positive for biomelecular examinations. Positivity was detected in the three samples (one squamous carcinoma of the hard palate and two lingual papillomas) that histologically presented coilocytes. One of the two oral papillomas was also positive in nsEM.
In order to evaluate the presence of FcaPV-2 in mucosal lesions, 32 samples of FOSCC (feline oral SCC, located on the head and neck), two of SCC of the nasal cavities and an oral papilloma were selected from the archives of the IZSLT and of CEROVEC.
First, the presence of the specific amplicons related to FcaPV-2 DNA was demonstrated in 10/32 FOSCC (31%), in the two nasal SCC samples and in the papilloma.
Molecular methods have been accompanied by in situ hybridization (ISH). Of the 10 samples of FOSCC positive for viral DNA, n.9 were available for this technique. These were processed for ISH using a probe specifically for FcaPV-2 gnomic DNA already available in the literature. The results showed a positive reaction in 4/9 (44%) samples, confirming the presence of viral genetic material inside the neoplastic cells.
Specific primers were studied for the development of RT-PCR protocols to evaluate the synthesis of viral particles, through the research of E1, E6 and E7 (potential oncogenes) and of the viral capsid proteins L1 and L2. To verify the expression of the viral genes, the RNA coming from PCR positive samples was subjected to reverse transcription PCR (RT-PCR) methodology, employing the specific primers for L1 capsidic protein transcripts and for the transcripts of regulatory E2 gene and E6E7 oncogenes. The results demonstrated transcriptional activity of the virus in seven samples out of 10 FOSCC positive for DNA.
In order to further evaluate the biological action of FcaPV-2 in tumors, the available samples (11/13 total) for immunohistochemistry (IHC) were subjected to this method using an anti-p16 antibody, a cell cycle protein considered marker of HR-HPVs infection in human oral and cervical SCCs. The results showed that p16 was expressed in 6/9 samples (67%).

Discussion and Conclusions

[2] Centro di Referenza nazionale di Oncologia Veterinaria e Comparata

The immunohistochemical, virological and biomolecular investigations conducted on the samples examined during the project period, showed both the presence of FcaPV-2 in the lesions of SCC of skin and mucous membranes of the cat, and its biologically active role.
The developed PCR protocols were found to be sensitive and specific.
The viral strain shown in 25 SCC lesions and one papilloma lesion was always the same, that is Feline papillomavirus type 2 isolate Main Coon 2007.
During the project period, the role of FcaPV-2 in the oral SCC of the cat was investigated, achieving very interesting results. To evaluate the transcription of the E6, E7 oncogenes and the synthesis of the L1 capsidic FcaPV-2 protein, the specific RT-PCR protocols for the biologically active detection of FcaPV-2 in oral SCC tumors have been developed, since viral type had most frequently found in cutaneous SCCs. FcaPV-2 is also the best characterized in terms of biological activity *in vivo* and *in vitro*, given that the viral genes E6 and E7 show transforming characteristics similar to those of high-risk human PVs (HR-HPVs).

Based on the obtained results, the following can be stated:

- A percentage of FOSCC is associated with FcaPV-2 infection (PCR and ISH positive)
- The infection is active and can be productive (E2 and L1 expression)
- Virus The virus can have transforming activity (viral load, E6E7 expression, increased p16)
- Cat as an animal model: FOSCC FcaPV-2 (+) compared to human HNSCC HR-HPV (+)