REPORT

Cell cycle S phase markers are expressed in cerebral neuron nuclei of cats infected by the Feline Panleukopenia Virus

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ABSTRACT

The cell cycle-associated neuronal death hypothesis, which has been proposed as a common mechanism for most neurodegenerative diseases, is notably supported by evidencing cell cycle effectors in neurons. However, in naturally occurring nervous system diseases, these markers are not expressed in neuron nuclei but in cytoplasmic compartments. In other respects, the Feline Panleukopenia Virus (FPV) is able to complete its cycle in mature brain neurons in the feline species. As a parvovirus, the FPV is strictly dependent on its host cell reaching the cell cycle S phase to start its multiplication. In this retrospective study on the whole brain of 12 cats with naturally-occurring, FPV-associated cerebellar atrophy, VP2 capsid protein expression was detected by immunostaining not only in some brain neuronal nuclei but also in neuronal cytoplasm in 2 cats, suggesting that viral mRNA translation was still occurring. In these cats, double immunostainings demonstrated the expression of cell cycle S phase markers cyclin A, cdk2 and PCNA in neuronal nuclei. Parvoviruses are able to maintain their host cells in S phase by triggering the DNA damage response. S139 phospho H2A1, a key player in the cell cycle arrest, was detected in some neuronal nuclei, supporting that infected neurons were also blocked into the S phase. PCR studies did not support a co-infection with an adeno or herpes virus. ERK1/2 nuclear accumulation was observed in some neurons suggesting that the ERK signaling pathway might be involved as a mechanism driving these neurons far into the cell cycle.

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Introduction

Feline Panleukopenia virus (FPV) is a small minus sense single stranded DNA virus. Canine parvovirus (CPV) and FPV are close to the rodent parvovirus, minute virus of mice and H1 rat parvovirus, all belonging to the genus *Protoparvovirus*, *Parvovirinae* subfamily of the *Parvoviridae* family.¹ These parvoviruses are small (18–25 nm) non enveloped icosahedral particles. Their 5 kb genome includes no more than 2 genes: the first one, under the control of the P4 promoter, codes for 2 major non-structural proteins, NS1 and NS2, while the second one, under the control of the P38 promoter, codes for 2 major capsid proteins, VP1 and VP2; the different proteins are generated by alternative splicing.² The palindromic DNA extremities are folded up, and the 3' end serves as a primer for viral DNA replication. This process calls for proliferating cell nuclear antigen (PCNA), and depends on the expression of cyclin A and the cyclin dependent kinase (cdk) 2 activity.³ With this very simple organization, these parvoviruses are strictly dependent on host cells reaching the cell cycle S phase for the first step in their life cycle which is the conversion of their single stranded DNA into duplex forms.

FPV develops in various dividing feline cell stores, depending on the age of infection. In young and adult individuals,

lymphoid tissue, bone marrow and intestinal Lieberkühn cells support virus multiplication, while in case of *in utero* fetus infection or neonate infection, the dividing neuroblasts of the cerebellar external granular layer will be hit.^{4,5} Should the infection occur during the last 3 weeks of pregnancy or the 3 first weeks of life, various degrees of granulo-prival cerebellar hypoplasia will be observed. During this period, Purkinje cells, although immature, are obviously postmitotic.⁶ However, in 1976 Csiza et al. reported the presence of viral antigen in Purkinje cells of kittens experimentally infected at birth.⁴ This paradoxical observation has been repeatedly reported in naturally occurring cases.^{5,7,8} Moreover, CPV was detected by immunohistochemistry in cerebral neurons of 18/100 cats submitted for necropsy,⁹ and more recently, FPV protein expression was evidenced in cerebral neurons of 4/23 cats that presented enteritis.¹⁰ In the latter study, the thalamus was the most affected area, and the cerebellum was spared. In the present study, we retrospectively investigated the whole brain of cats with FPV-associated, PCR-confirmed cerebellar hypoplasia. In addition to FPV capsid protein expression in cerebellar Purkinje cells of most infected cats, we found FPV antigen expression in various parts of the brain in 4/12 subjects. Our study also showed that parvoviral DNA replication conditions were found in neurons

in FPV infected cats: cell cycle S phase markers (cyclin A, cdk 2, and PCNA) were detected by immunohistochemistry while G1 phase markers (cyclin D1 and cdk4) could not be found. The expression of a key player of the cell cycle arrest, S 139 phosphorylated H2A1 histone (γ H2AX),^{11,12} was observed in some neuronal nuclei, suggesting a long lasting stay in S phase of some neurons. The repeated demonstration of external signal related kinase (ERK 1/2) expression in some neuron nuclei of affected kittens suggests that the ERK signaling pathway is activated to lead to cell division.¹³

Over the last 2 decades, neuronal cell cycle re-entry, as a possible common bottle neck for most nervous system degenerative processes, has been subjected to intensive researches and controversies.^{14–23} To our knowledge, this study is the first one demonstrating the existence of neuronal nuclei expressing S phase markers in the frame of a spontaneously occurring infectious disease and represents an example of inflammatory/ infectious link to the neuronal cell cycle reentry phenomenon.

Results

Histopathology

The histopathological features of the cerebellum from cats #1–3 were already reported as well as those of cats #4–7.^{5,7} They were characterized by a cerebellar atrophy involving all folia, reduction of the granular and molecular layers, and rarefaction of the Purkinje cells. The severity of these changes varied from one subject to the other, probably as a result of the developmental stage, viral load, and immunological competence at the time of infection. In the most affected cats, Purkinje cell heterotopia and marked changes in their dendritic tree shape were obvious. The new affected cats included in the present study (Cats #8–12) showed similar changes. In the oldest cat (#3, 4-year-old), despite a relatively modest cerebellar atrophy, very few Purkinje cells remained, as evidenced by calbindin immunostaining, suggesting that affected neurons eventually died. In addition, in area showing FPV VP2 expression (Fig. 1a), some vacuolization, satellitosis, gliosis and microgliosis were found at various places and occasional perivascular mononuclear cell cuffing was observed in the thalamus of cat #10 (Fig. 1b,c). Foci of leptomeningeal mononuclear cell infiltration were previously described at the cerebellar level in cats #2 and 3,⁸ indicating that inflammation may be present at some stages of this infection.

Detection of DNA segments of NS and VP genes of FPV

Various DNA segments of the NS and/or VP genes were amplified from the cerebellar tissue of cats #1–7, confirming the presence of FPV infection.^{5,7} For Cats #5, 7, 9–12, the same primers as those used in a previous study,¹⁰ which reported FPV proteins in thalamic and brain stem neurons, were used to amplify a 357 bp product coding for the C-terminus of the FPV NS1 protein. For cat #12, no product was obtained and a different pair of primers was used to amplify a shorter, 206 bp product of the NS1 gene (Fig. 2). Both products included the aa #582 codon. Indeed, in this previous study, an L to S substitution in the NS1 C-terminus was found, that might explain the unusual cellular tropism. However, sequencing the amplified products from cats #5, 7, 9–12 (which includes the cats with extracerebellar neuronal infection) did not show this mutation in any of them. Consequently, this mutation does not explain the propensity of FPV to complete its cycle in extracerebellar neurons.

Absence of concurrent viral infection

Gamma herpes viruses and some adenoviruses are able to drive quiescent cells into the cell cycle.^{24–27} Nested PCRs using either pan herpes (215–235 bp products from the viral DNA polymerase) or pan adeno primers (324 bp product from the viral DNA polymerase gene) were conducted on cats 7, 10–12 (Fig. 2). With the adenovirus PCR, a weak band was retrieved from cat #10 close to the expected size. With the herpes PCR, a weak band close to the expected size was observed in cat #12. However, the sequence of gel-extracted, purified DNA from these bands corresponded in both cases to irrelevant feline DNA. These data make unlikely a co-infection with viruses of these 2 families.

FPV VP2 capsid protein expression in FPV infected cats

In the cerebellum of 10 cats among 12 (except cats #3 and 4, one- and 4-year-old, respectively), FPV VP2 capsid protein was expressed in the nucleus of some Purkinje cells. The cerebellum of cat #10 was particular because up to 100 Purkinje cells with positive nuclei were observed on a single sagittal section, and because viral proteins were also detected in the cytoplasm of rare Purkinje cell and in rare glial cells. In 4 cats (cats #7, 10, 11 and 12), FPV VP2 capsid protein expression was also observed in nuclei of neurons and of micro- and macroglial cells in other brain areas. A few FPV VP2 positive neurons were found in the

Figure 1. Thalamus of cat #10. (A) Three neuronal nuclei show a FPV protein immunoreactivity, which is also present in the cytoplasm of one of them; anti-CPV immunostaining, light hematoxylin staining. (B) Perivascular cuffing, perivascular and perineuronal edema; hematoxylin and eosin staining. (C) Neuronal satellitosis, neuropil vacuoles, microgliosis and astrogliosis; hematoxylin and eosin staining. (D) Nuclear accumulation of ERK 1/2 in a neuron nucleus; anti-ERK1 immunostaining, light hematoxylin staining. Bar = 30 μ m in a, 100 μ m in b, 50 μ m in c and d.

Figure 2. PCRs for a 357 bp product from the coding region of the C-terminus of the NS1 protein (NS1, cats 7, 10–11) or a 206 bp product coding for the same part (NS1, cat 12); nested PCR using degenerated primers for the detection of a 324 bp products from the viral DNA polymerase of adenoviruses (Adeno); nested PCR using degenerated primers for the detection of a 215–235 bp product from the viral DNA polymerase gene of herpes viruses (Herpes). Results for the 4 cats with extracerebellar neuronal FPV expression.

cortex of cat #11; they were more numerous in the cortex and thalamus of cat #7; FPV-positive neurons were especially numerous in the thalamus of cat #10 but were also present in the cortex, hippocampal formation, ventral tegmental area, and pontine gray matter; in cat #12, FPV-positive neurons were the most numerous in the ventral tegmental area and were also present in the cortex, hippocampus, pontine and vestibular nuclei. In cats #10 and 12, numerous neurons also expressed FPV VP2 proteins in their cytoplasm, indicating that FPV mRNA translation was still active in neurons of these cats (Fig. 1a). Notably, no positive cell was observed in the neuroblast-containing subventricular and subgranular areas.

Immunodetection of cell cycle markers in FPV infected cats

Immunostaining for PCNA, cyclin A, cyclin D1, cdk4, cdk2, and γ H2AX were performed on sections from cats #7, 10–12, selecting sections adjacent to those where FPV VP2 proteins were evidenced. Neuronal and non-neuronal nuclei were marked with antibodies to cycle S phase markers PCNA, cyclin A and cdk2, while no positive neuron nuclei were found using antibodies to G1 phase markers cyclin D1 and cdk4. Double immunostainings using anti microtubule associated protein 2 (MAP2) antibodies were performed in conjunction with either anti-PCNA, cyclin A, cdk4 or γ H2AX antibodies, and confirmed that these proteins were indeed expressed in neuronal nuclei (Fig. 3). In brain regions where FPV mRNA translation

was active in the cytoplasm of neurons, neurons exhibiting cell cycle S phase markers (PCNA, cyclin A and cdk4) were similarly found. In addition, a key player of cell cycle arrest (γ H2AX) was also expressed in some neurons.

Detection of ERK 1/2 nuclear expression

In a previous study involving 10 cases of FPV-linked cerebellar atrophy, we observed that ERK 1/2, involved in a signaling cascade that may lead to cell division, was strongly expressed in rare Purkinje cell nuclei in one case.⁸ In the present study, ERK 1/2 nuclear expression could also be observed in rare neurons of cat #10 and 11 (Fig. 1d).

Validation of antibodies ability to recognize feline proteins

The anti-canine parvovirus antibody recognizes the FPV VP2 protein as demonstrated previously through immunoprecipitation.⁶ Both anti-PCNA antibodies have been validated for use in the feline species in previous studies, notably through the observation of a single band at the expected molecular weight on western blot (WB), and the anti ERK1 antibody recognizes the feline ERK1 and 2 based on the same method.⁸ Data bank (PubMed protein) analysis indicated that the amino acid sequences of feline proteins shows 100% identity with sequences from corresponding proteins from other species used to generate the following antibodies: aa 70–120 of mouse Cyclin

Figure 3. Thalamus of cat 10, double immunostainings; (A) MAP2 (green), PCNA (red); (B) MAP2 (red), Cdk2 (green); (C) MAP2 (red), cyclin A (green); (D) MAP2 (green), γ H2AX (red). Neuronal perikaryon and dendrites express MAP2 while some neuronal nuclei express PCNA, Cdk2, cyclin A and γ H2AX (arrows). Bar = 25 μ m.

Figure 4. Specificity in the feline species of the antibodies used. Western blot using proteins from HEK293T cells (A, positive control) and Crandel Feline kidney cells (B, CrFK). Anti-cyclinA, anti-Cdk2, anti Cdk4 antibodies label a single band at the expected molecular weight.

D1 (AAO13813.1) and feline Cyclin D1 (XP 011285404); aa 132–143 of the human histone H2AX (NP 001073248.1) and feline histone H2AX (XP 003992480.1). For cyclin A, cdk2 and cdk4, the feline proteins show 89%, 99%, and 95% identity with the corresponding proteins from other species used to generate rabbit polyclonal antibodies. These antibodies detected by WB after denaturing SDS-PAGE a single band at the expected molecular weight of cyclin A, cdk2 and cdk4 (54, 33 and 34 kDa, respectively) in Crandell Feline kidney cell lysates (Fig. 4). Moreover, these antibodies showed a coherent immunostaining on tissue sections of a feline mammary adenocarcinoma (notably strong nuclear expression in some tumor cells (Fig. 5). The anti-calbindin antibody has been repeatedly used in cats.^{5,6,8} The anti-MAP2 antibodies gave probing results with an immunostaining limited to neuronal perikaryon and processes.

Discussion

Since about 2 decades, a hypothesis linking degeneration of neurons to their failure to keep cell cycle reentry in check has emerged.^{14–20} Immature neurons die quickly under these

conditions while fully mature neurons might be weakened and succumb to a second hit.¹⁹ This view is supported by evidence of expression of cell cycle-related proteins in neurons. However, in the frame of naturally-occurring diseases, these cell cycle markers are most often expressed in cytoplasmic compartments and not in the nucleus. Consequently, they may fulfill different functions than their known contribution to the cell cycle progress.^{21,22} Polyploidy of some neurons has also been viewed as supporting the cell cycle-related neuronal death hypothesis, but physiologic neuronal polyploidy has been described.²³ In the present study, we demonstrated that S phase markers cyclin A, cdk2 and PCNA were expressed in the nuclei of neurons in brain areas where FPV proteins were produced in the cytoplasm and accumulated in the nuclei of neurons. These observations support the hypothesis that these neurons reached the S phase of the cell cycle. To our knowledge, observation of cell cycle S phase markers in neuron nuclei has not been reported before in the frame of a naturally-occurring disease. A study using 2 anti-PCNA antibodies and a Ki-67 antibody for immunolabelling of brains from 50 necropsied cats reported somewhat contradictory results, but nevertheless, positive neuron nuclei were observed in some cats.²⁸ The presence of FPV proteins or viral DNA sequences was however not investigated in the latter study. Thus our results indicate that FPV infection is an entity linking an inflammatory / infectious process to the neuronal cell cycle reentry phenomenon.

Neuronal expression of G1 phase markers cyclin D1 and cdk4 could not be detected in neuron nuclei. Parvoviruses are able to maintain their host cells in S phase by triggering the DNA damage response.¹¹ In this way they hijack the host cell replication machinery for their own profit for longer period of time. DNA damage sensor proteins recruit several transducer kinases into the nucleus where they phosphorylate different targets: among them phosphorylation of H2A1 histone on S 139 seems to be the main event leading to cell cycle arrest.¹² S 139 phosphorylated H2A1 histone was expressed in some neuronal nuclei, suggesting that they were indeed blocked in cell cycle S phase. This situation may explain that S phase markers were observed while G1 phase markers were not.

FPV protein accumulation in Purkinje cells of perinatally-infected kittens has long been described,^{4,5,7,8} implicating a cell cycle reentry of these Purkinje cells.² At these perinatal

Figure 5. Immunohistochemical validation in the feline species of the antibodies used. Immunostainings on a feline mammary adenocarcinoma. (a and h) representative examples of negative controls (no primary antibody); (B) anti-cyclin D, (C) anti-cyclin A, (D) anti-PCNA, (E) anti-Cdk4, (F) anti-Cdk2, (G) anti-H2A1. Some tumor cell nuclei are immunostained. Bar = 100 μm.

development stages, Purkinje cells are obviously postmitotic although they have not yet reached morphological maturity.⁶ Immaturity of Purkinje cells and rarefaction of their input cells (granule cells) might be considered as a cause of cell cycle reentry. Indeed, the reverse situation, lack of target cells (Purkinje cells), caused cell cycle dysregulation and loss of immature granule cells in the developing cerebellum of the *staggerer* mutant mouse.¹⁴ However, FPV protein expression in our cases was far from being restricted to immature neurons in the cerebellum. We observed FPV proteins expression in extracerebellar neurons in cats of 10 weeks (this study, an age when even the cerebellar cortex is adult-like) and FPV expression in extracerebellar neurons has been described in individuals up to 4.5 y.¹⁰ Consequently, neuron immaturity and lack of trophic factors from the connected neurons (cerebellar granule cells) are not likely to be the major cause of their cell cycle reentry after FPV infection.

In a previous study, a mutation in the C terminus of the NS1 protein (L to S at position 582) was found in the 4/23 cats showing FPV protein expression in extracerebellar neurons.¹⁰ This mutation was not present in the tested cats of the present series. It is thus unlikely that this mutation plays any role in the particular neuronal tropism of the FPV. The parvoviruses are extremely resistant in the environment, and as such they are highly contagious. In the previous series, the enteritis cases were collected over a relatively short period and a relatively restricted area. An outbreak with the KP769859 strain would better explain the repeated observation of the mutation in this previous study.¹⁰

Expression of the SV40 T-antigen in developing Purkinje cells drove these neurons into cell cycle-associated death.²⁹ Viral proteins like the SV40 T-antigen that inactivate tumor suppressors of the retinoblastoma family (pocket proteins), free the E2F family transcription factors and might be able to launch neuronal cell cycle reentry.²⁴ The E1A protein from adenoviruses also displays this property.^{26,27} A potential co-infection with an adenovirus was investigated by PCR analysis using cerebellum-extracted DNA from the 4 cats with extracerebellar FPV protein expression, but no evidence of an adenoviral co-infection was found. Herpes viruses interfere with the E2F transcription factors through different mechanisms, either by retinoblastoma protein phosphorylation and E2F dependent genes expression, or by the production of viral retinoblastoma-inactivating proteins.²⁵ A PCR analysis using pan herpes primers did not yield positive results, and a co-infection with a herpes virus is unlikely as a cause of cell cycle reentry. Papilloma viruses might also bind pocket proteins through their E7 protein and induce their degradation, freeing the E2F family transcription factors.²⁴ However, papilloma viruses specifically infect mucosal or cutaneous epithelia and were not considered here.

We repeatedly observed ERK 1 / 2 accumulation in neuronal nuclei in FPV infected cats, suggesting that, the ERK

signaling pathway is the most plausible pathway responsible for driving neurons into the cell cycle S phase during FPV infection. The upstream, earlier, probably short-lived interplay of kinases and phosphatases in this cascade should be analyzed to this aim but cannot be easily investigated using our necropsy material.

As a result of the promising oncotropic and oncolytic properties of parvoviruses,³⁰ the H-1 parvovirus has reached the I/IIa clinical trial phase for glioblastoma treatment in human.³¹ The safety studies did not disclose specific risk.³² However, our results also raises questions about the use of parvoviruses as anti-cancer weapon in their natural target species.

In summary, in this retrospective study on 12 cats exhibiting FPV-related cerebellar hypoplasia, nuclear FPV proteins expression was found in extracerebellar neurons in 4 cats and cytoplasmic expression, supporting ongoing viral mRNA translation, was observed in 2 cats. In the same brain areas, nuclear expression of PCNA, cyclin A and cdk2 was demonstrated in some neurons of these 2 latter cases, indicating that neurons entered into the cell cycle S phase. This observation in a naturally occurring inflammatory / infectious entity supports the cell cycle-associated neuronal death hypothesis and its significance as a mechanism of neurodegeneration. Future studies will need to investigate how the FPV and / or neuroinflammation drive postmitotic, mature neurons into the cell cycle S phase.

Material and methods

Animals

Twelve privately owned cats, males and females, aged from 6 to 208 weeks (mean: 32; median 12 weeks, Table 1) with cerebellar ataxia were euthanized at the owner request and necropsy was granted. According to the local regulations, diagnostic procedures conducted on dead animal or animal tissues do not require approval by an ethical committee. The encephalon was harvested immediately after death and immersed in 4% buffered formalin within 30 minutes. After 4 days, it was rinsed in 0.01 M phosphate buffer added with 9 g/L NaCl (PBS). Each brain was cut along the sagittal plane; the left half was cut in 10 transverse segments along fixed landmarks, and the right half was cut in rostral and caudal halves. These 12 pieces were routinely dehydrated and paraffin embedded. The left half was used to generate transverse sections, while the right half was used to gather sagittal and parasagittal sections. Sections from an 8-week-old kitten and an adult cat, euthanized for reasons unrelated to a brain problem, were available as normal controls. Sections from a surgically removed feline mammary adenocarcinoma and from the intestine of a cat that died from an ELISA-confirmed, FPV-associated enteritis were also used as positive controls.

Table 1. Age of the cats enrolled in the study.

Cats												
Cat #	1	2	3	4	5*	6*	7	8**	9**	10	11	12
Age	24 w	12 w	1 y	4 y	13 w	13 w	12 w	10 w	10 w	10 w	6 w	7 w

Note. w: weeks; y: years; *and **: from a same litter.

Histology and immunohistochemistry

The following primary antibodies were used with different dilutions for light (L) or fluorescence (F) microscopy, without or with amplification (A), and, for some antibodies, after pretreatment with citrate buffer (C) or TRIS EDTA buffer (T); dilutions for western blot (WB) were also different: mouse anti canine parvovirus (Santa Cruz sc-57961, L 1/50); rabbit anti calbindin (Swant, L 1/5000); rabbit anti MAP2 (Abcam ab32454, L 1/500, F 1/100 A, C or T); mouse anti MAP2 (Sigma Aldrich, clone HM-2, M4403, L 1/500, F 1/100 A, C); mouse anti PCNA (Abcam clone PC10, Ab29, L 1/1000, F 1/100, C); rabbit anti PCNA (Santa Cruz, sc-7907, L 1/500); rabbit anti cyclin D1 (Bios bs-0623, L 1/2000, C); rabbit anti cyclin A (Santa Cruz, sc-751, L 1/100, F 1/100 A, C; WB 1/1000); rabbit anti cdk2 (Spring Bioscience, clone SP80, L 1/100, F 1/50 A, C; WB 1/1000); rabbit anti cdk4 (Novus Biologicals, NBP1-31308, L 1/100, T; WB 1/100); mouse anti phosphohistone H2A.X (Merck Millipore, clone JBW301, L 1/400, F 1/50, T); rabbit anti ERK1 (Santa Cruz, sc93, L 1/100).

Sections were deparaffinised and rehydrated. After three rinses in water, they were routinely colored with Mayer hematoxylin and eosin. For the immunostainings, whenever needed, a pre-treatment was conducted (C or T buffer, maintained close to the boiling point during 15 minutes); normal sheep serum 1/20 in PBS was used for the blocking stages; primary antibodies were left overnight in a humid chamber at 4°C; either goat anti rabbit, 1/160, or goat anti-mouse, 1/160 (323-0005-024 and 223-0005-024, respectively, Jackson ImmunoResearch) were used as secondary antibodies. Binding was visualized using the peroxidase anti-peroxidase method, with diaminobenzidine as the chromogen; sections were counterstained with hematoxylin. A negative control (no primary antibody) was included in all runs. Sections from normal cats were also used as negative controls. For the double immunostainings, amplification was used for one primary antibody using either biotinylated goat anti rabbit (A-11012 Invitrogen) followed by Alexa 488 (green)-bound streptavidin (S11223, Invitrogen) or biotinylated horse anti mouse antibodies (BA-2001, Vector) followed by Alexa 594 (red)-bound streptavidin (S11227, Invitrogen), while the second primary antibody was visualized using either Alexa 488 (green)-bound goat anti rabbit (A-11008, Invitrogen) or Alexa 594 (red)-bound goat anti mouse antibodies (A-11005, ThermoFisher), all reagents diluted at 1/100.

PCR

DNA extraction

DNA was extracted from the paraffin-embedded samples as previously described.⁵ Briefly, thick sections were rehydrated, digested with proteinase K, and total DNA was extracted using phenol-chloroform-isoamyl alcohol and phenol-chloroform successively; DNA was precipitated using 100% propanolol and rinsed with 75% ethanol.

DNA was extracted from the gels using Gel and PCR Clean Up kit (Macherey-Nägel).

NS1 PCR

Primer forward (F): CAACCAATAAGAGACAGAATGTTGA; primer reverse (R): CCCCACTTTACTAACACACC or F:

TCAACCATGGCAAACCTATACACA; R: GTACTCCACG GTTCCAGTGC. For 20 µL of mixture: DNA:2 µL; primers (1 µM) 2 µL each; Hot Star master mix: 10 µL; H₂O: 4 µL. Start: 94°C 5 minutes; 45 cycles: 94°C 30 seconds, 46°C 1 minutes, 72°C 1 minute; elongation: 72°C 7 minutes. Two % gel agarose migration; tampon dye, O' GeneRuler 1 kb Plus DNA ladder (both from Thermo Fischer Scientific); Midori Green Advanced DNA Stain (NIPPON Genetics). Positive control: Nobivac DHP vaccine (Intervet/MSD); negative control: no DNA. DNA extracted from the cerebellum of a normal cat was also used as a negative control.

Pan-adenovirus nested PCR

According to Wellehan et al.³³

Primers outer F (OF): TNMGNGGNGGNMGNTGY TAYCC; outer R (OR): GTDGCRAANSHNCCRTABARNGM RTT; inner F(IF): GTNTWYGAYATHTGYGGHATGTAYGC; inner R (IR): CCANCCBCDRTTTRTGNARNGTRA, where B = G, T, or C; D = A, G or T; H = A, T, or C; M = A or C; N = A, C, G or T; R = A or G; S = G or C; Y = C or T. Same cycling, migration and revelation as above. Positive control: Nobivac DHP vaccine (Intervet/MSD); negative control: no DNA.

Pan-herpes nested PCR

According to Ehlers et al. and to Chmielewicz et al.^{34,35}

Primers: OF1: GAY TTYGCIAGY YTITAYCC; OF2: TCC TGG ACA AGC AGC ARIYSGCIM TIAA; OR: GTC TTG CTC ACC AGITCIACICCY TT; IF: TGT AAC TCG GTG TAY GGITTYACIGGIGT; IR: CAC AGA GTC CGT RTCICC IAT. Where Y = C or T, and I stands for deoxyinosine. PCR 1, for 50 µL mixture: DNA 1 µL, Master Mix 25 µL, OF1 5 µL, OF2 5 µL, OR 5 µL, H₂O 9 µL. PCR2, for 50 µL mixture: PCR1 product 5 µL, Master Mix 25 µL, IF 5 µL, IR 5 µL, H₂O 10 µL. Same cycling, migration and revelation as above. Positive control: purified MuHV-4 suspension; negative control: no DNA.

Sequencing

Sanger sequencing was conducted by GATC Biotech (Konstanz, Germany).

Western blots

Pellets of cultured CrFK cells were used as a source of feline proteins,⁸ while pellet of HEK 293T cells were used as human protein controls.³⁶ Thawed pellets were lysed in a modified RIPA buffer composed of 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Sodium deoxycholate, 5 mM EDTA, Complete protease inhibitor (Roche, 11 873 580 001), 1 mM EGTA, phosphatase inhibitor cocktail (SIGMA, P5726) and 1 mM PMSF. Cell lysates were sonicated on ice for 30 s and rocked at 4°C for 30 min, followed by a centrifugation at 20,000 g for 20 min at 4°C. The supernatant was mixed with 5x Laemmli sample buffer and heated at 100°C for 10 min. 100 µg of protein was applied to 10% Tris-glycine gels. After transfer, PVDF membranes (Bio-Rad, 162-0177) were blocked with 10% skimmed milk for 1 h and immersed in the antibody solutions for 2 h. Membranes were incubated with goat anti-rabbit-HRP antibody (1/2500, Cell signaling 7074S) and then with Super-Signal West Pico or Femto substrate (Pierce, 34077 or 34095,

respectively). The chemiluminescent signal was detected by a DARQ-7 CCD cooled camera (Vilber-Lourmat) in a SOLO 4S WL system.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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