Studies on the Pathogenesis of Feline Morbillivirus in Kidneys of Infected Cats

（ネコモルビリウイルス感染ネコ腎臓における病原性に関する研究）

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2. ABBREVIATIONS

B	B-cells	B lymphocytes
C	cDNA	complementary DNA
	CD4	Cluster of differentiation 4
	CD20	Cluster of differentiation 20
	CDV	Canine distemper virus
	CeMV	Cetacean morbillivirus (CeMV)
	CKD	Chronic kidney disease
	CPE	Cytopathic effect
	CRF	Chronic renal failure
	CRFK	Crandell-Rees Feline Kidney Cell
D	dA	Deoxyadenosine
	DAB	3,3'-diaminobenzidine
	DAPI	4',6-diamidino-2-phenylindole
	$DAPI^+$	DAPI positive cell
	DNA	Deoxyribonucleic acid
E	*E.coli*	*Escherichia coli*
	ELISA	Enzyme-linked immunosorbent assay
F	F	Fusion protein
	FeLV	Feline leukemia virus
	FeMV	Feline morbillivirus
	$FeMV^+$	FeMV positive cell
	FeMV-GT2	Feline morbillivirus genotype 2
	FFPE	Formalin fixed paraffin-embedded
	FGF2	Fibroblast growth factor 2

	FITC	Fluorescein isothiocyanate
	FIV	Feline immunodeficiency virus
	FIP	Feline infectious peritonitis virus
	FmoPV	Previously known abbreviation for feline morbillivirus
G	GST	Glutathione S-transferase
H	H	Hemagglutinin protein
	HIV	Human immunodeficiency virus
	H_2O_2	Hydrogen peroxide
	HRP	Horseradish peroxidase
	H&E	Hematoxylin and eosin staining
	H+L	Heavy chain and light chain of antibody
I	IFA	Immunofluorescence assay
	Ig	Immunoglobulin
	IgG	Immunoglobulin G
	IHC	Immunohistochemistry
	IRIS	International Renal Interest Society
K	Kb	Kilo base
L	L protein	Large protein
	LB medium	Lysogeny medium
	LVOT	Left ventricular outflow tract obstruction
	RVSD	Right ventricular septal detect
M	M	Matrix protein
	MeV	Measles virus
	MERV-CoV	Middle east respiratory syndrome coronavirus
N	N	Nucleocapsid protein

	NA	Not determined
	N/A	Not available
	NS	Not significance difference
O	ORF	Open reading frame
P	P	Phosphoprotein
	PBS	Phosphate-buffered saline
	PBS-T	Phosphate-buffered saline containing 0.02% Tween 20
	PCR	Polymerase chain reaction
	PDV	Phocine morbillivirus
	pMD20-T	TA cloning vector plasmid
	pGEX4T-1	Expression vector plasmid
	pH	Potential hydrogen
	PRRV	Peste-Des-Petits ruminant virus
	p-value	probability value
R	RNA	Ribonucleic acid
	RPV	Rinderpest virus
	RT	Room temperature
	RT-PCR	Reverse-transcription polymerase chain reaction
S	SDS	Sodium dodecyl sulfate
	SDS-PAGE	SDS-polyacrylamide gel electrophoresis
	SDMA	Serum symmetric dimethylarginine
	SEM	Standard error of the mean
	SPF	Specific-pathogen-free
	SLAM (CD150)	Signaling lymphocytic activation molecule (Cluster of differentiation 150)

	Smad7	Mothers Against Decapentaplegic homolog 7
T	T-cells	T lymphocytes
	TdT	Terminal deoxynucleotidyl transferase
	TIN	Tubulointerstitial nephritis
	Tris-HCl	Tris (hydroxymethyl) aminomethane (THAM) hydrochloride
	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
	$TUNEL^+$	TUNEL positive cell
	$TUNEL^+FeMV^+$	TUNEL and FeMV positive cells
U	UPC ratio	Urine protein creatinine ratio
V	V	V protein
W	WB	Western blot analysis

3. UNIT ABBREVIATIONS

D	dL	deciliter
	˚C	Degree Celsius
H	h	Hour
L	L	Liter
M	µL	Microliter
	µm	Micrometer
	µM	Micromolar
	µg	Microgram
	mg	Milligram
	mmHg	Millimeters of mercury
	min	Minute
	M	Molar
P	%	Percent
S	sec	Second
X	x g	Times gravity of the relative centrifugal force (RCF)

4. GENERAL INTRODUCTION

1 General introduction of feline morbillivirus (FeMV)

1.1 Background information and taxonomical classification

Feline morbillivirus (FeMV, previously known as FmoPV) is a member of the Genus *Morbillivirus* in the Family *Paramyxoviridae* of the Subfamily *Paramyxovirinae* in the order *Mononegavirales* classified according to the International Committee on the Taxonomy of Viruses in 2016 (1,2). Morbilliviruses are highly contagious viruses, which cause respiratory, nervous, and gastrointestinal diseases with high morbidity and mortality rates (3,4). Genus *Morbillivirus* includes important viruses; for example, measles virus (MeV) in human (4), rinderpest (RPV) (5) and peste-des-petits-ruminant virus (PRRV) (6) in ruminants, canine distemper (CDV) in dogs (7), cetacean morbillivirus (CeMV) in marine mammals (8), and phocine morbillivirus (PDV) in seals (9).

It has been known that FeMV is widely spread over many regions in the world and draws attention to concern about this transmitted disease in domestic cats since the first discovery of FeMV in Hong Kong and some parts of mainland China in 2012 (10). The apparent reason, which brings the FeMV to be one of the interesting topics for conducting the morbillivirus research, is the unusual viral pathogenicity characteristic among other morbilliviruses regarding the association of FeMV with tubulointerstitial nephritis (TIN) based on the case-control study described in the original paper (10).

Whereas pathogenicity of other morbilliviruses connects to the clinical signs of neurological, respiratory, and gastrointestinal related to infectious diseases, FeMV is the only one of morbilliviruses with the uniqueness of its pathogenicity related to urological diseases. According to its pathogenicity, it has been concerned that whether FeMV belonged to the Genus *Morbillivirus* or not. However, many studies have already confirmed that FeMV is one of the members of the Genus *Morbillivirus* (10-13).

Furthermore, the new genotype of FeMV was isolated from urine samples of cats with urinary tract diseases in Germany, and it was tentatively named as feline morbillivirus genotype 2 (FeMV-GT2) (14). Besides, the molecular and biological of FeMV-GT2 is quite distinctive for further study according to the ability to infect renal and pulmonary epithelial cells, primary nervous cells, and immune cells, particularly $CD4^+$ T cells, $CD20^+$ B cells, and monocytes (14).

1.2 Epidemiology

The first FeMV detection in 2012 reported the prevalence of FeMV in cats captured from various locations of Hong Kong and mainland China over two years from March 2009 to February 2011. Fifty-six out of 457 cats (12.3%; fifty-three urine, four rectal swabs, and one blood sample, with two cats in this study positive in both urine and rectal swabs) were FeMV positive by reverse-transcription polymerase chain reaction (RT-PCR) (10). Since then, FeMV has been globally detected in various countries on different continents such as Japan, the USA, Turkey, Brazil, Thailand, Italy, UK, Germany, and Malaysia (13,15,16,11,17-20) (Fig. 1). To investigate the prevalence of FeMV infection in domestic cats, RT-PCR has been performed with primers designed from the FeMV nucleotides of the L gene to detect the viral RNA from urine samples (10,24,13). The prevalence of FeMV infection detected from urine samples, and the seroprevalence of FeMV viral antibodies from the serum of domestic cats is summarized in Table 1 and Table 2.

By comparison, the prevalence of FeMV infection in male cats is higher than female cats according to the behavior corresponded to the male gender, which increases the opportunity to contact the virus such as territorial fighting, aggressiveness, and marking behaviors (20). Moreover, FeMV co-infect with other cat viruses such as feline infectious peritonitis virus (FIP), feline immunodeficiency virus (FIV), and feline leukemia virus (FeLV) (21,17) or is possible to be incidentally found with other diseases such as adenocarcinoma (21).

1.3 Biological characteristics

The structure of FeMV is similar to the other morbilliviruses. It is an enveloped negative-sense single-stranded RNA virus, which consists of six non-overlapping genes in the order N-P/V/C-M-F-H-L encoded for eight structural and non-structural protein including nucleocapsid protein (N), phosphoprotein (P), V protein (V), C protein (C), matrix protein (M), fusion protein (F), hemagglutinin (H), and large protein (L) (Fig. 2). In addition, the two accessory proteins (C and V) expressed from the P open reading frame contribute to innate immune response interference and viral infectivity. The size of the linear pattern genome is about 16 kb in length, and the viral genome is enclosed in an enveloped nucleocapsid of helical symmetry (10).

The viral tropism to various kinds of cell lines derived from cats has been clarified. FeMV infects in epithelial, fibroblastic, lymphoid, and glia cells from cats; however, Vero cells originated from a kidney of African green monkey is only one of the cell lines derived from other species susceptible to FeMV infection (22). Therefore, the possibility of transmission of FeMV from cats to humans might be limited.

It is reported that the virus successfully isolated from clinical samples using Crandell-Rees Feline Kidney Cell (CRFK) and FeMV induces cytopathic effects (CPEs) in the form of cell rounding, cell detachment, cell lysis, and also cellular fusion (syncytial formation) (10,12). The structure of virion revealed by electron microscopy that FeMV virions are relatively large and highly variable in size (130-380 nm in diameter) (10).

The study of stability and infectivity of FeMV on various environmental temperatures demonstrated that the infectivity of FeMV gradually decreases when the virus stays at 37°C, but the infectivity potential still remains up to 12 days (23). Also, heat-treatment above 70°C is a reliable method for inactivating FeMV to reduce the risk of FeMV infection and viral contamination (23).

Based on the biological characteristic of FeMV, there is still no specific method for disease prevention, control, and treatment for FeMV infection. For disease prevention and control, there is still no commercial vaccine available; therefore, the sanitation is essential for disease prevention and control in pets or shelter cats. Symptomatic and supportive treatment should be considered as a therapeutic method for controlling fluid and electrolyte imbalances according to viral infection and preventing secondary bacterial infections.

2. Detection of FeMV

Nowadays, there is no any commercial diagnostic tool available for FeMV detection for veterinary practitioners yet. However, virology and pathogenicity of FeMV are still not clear. Definitive evidence is needed for proving the connection between FeMV and feline urological diseases, which requires the combination of molecular biological and immunological assays for FeMV detection based on genetic, serological, and histological detection for FeMV detection.

2.1 Genetic detection

The most rapid and accurate method for FeMV diagnosis is the nucleic acid detection of viral RNA by using either conventional RT-PCR or real-time RT-PCR (24,10). The advantage of this method is high specificity and sensitivity for FeMV detection, and is applicable for handling many samples. The real-time RT-PCR can be used to detect FeMV from urine, blood, and kidney samples (13,10). The primers are designed based on the consensus of the L gene of FeMV to detect the RNA fragments (10,24,13). The total viral RNA is extracted and reverse transcribed to be complementary DNA (cDNA). Then PCR is performed for DNA amplification to detect the virus as a conventional or real-time method. The prevalence of FeMV is quite variable according to the geographical area (25). Although RT-PCR is a high sensitivity assay, RT-PCR might be possible to include a false-positive result

in the prevalence of FeMV study. Therefore, the other assays are necessary for confirming FeMV diagnosis.

2.2 Serological detection

As mentioned above, the other assays are required for confirming the prevalence of FeMV infection studied by genetic detection; serological diagnosis is the alternative method for confirming the study of FeMV epidemiology, particularly seroprevalence of FeMV investigation. Besides, combining the urine RT-PCR and serology results can be used for determining the phase of FeMV infection in domestic cats (25,26), as summarized in Table 3.

Although the gold standard for serological detection has not been successfully developed yet, many investigators expressed and purified either FeMV P or N protein for conducting seroprevalence study such as enzyme-linked immunosorbent assay (ELISA) (27), immunofluorescence assay (IFA) (12,26), and western blot analysis (WB) (10).

2.3 Histological detection

The immunohistological assays have been used for diagnosis and disease surveillance of FeMV by detecting either viral P protein or N protein as IFA and immunohistochemistry (IHC) from formalin-fixed paraffin-embedded (FFPE) kidney tissues (26,28,17). Although the specificity of the immunohistological assays is high, the sensitivity is lower than genetic methods. To determine the FeMV prevalence based on the immunohistological assays, the results should be confirmed more than one immunoassay because it is possible that sometimes the viral protein can be not detected according to the difference in the sensitivity of the assays. The FeMV viral protein can be found in the renal epithelial tubules, transitional epithelium, hepatocytes, and immune cells such as lymphoid cells, plasma cells, and mononuclear cells (26,28,10,17).

In the case-control study of the original article, FeMV infection is significantly associated with tubulointerstitial nephritis with the histological details as the aggregation of

inflammatory cells in the interstitium with tubular degeneration and necrosis and the lesions are found together with the viral N protein antigen positive in the renal tubular cells and mononuclear cells (10). Moreover, the presence of FeMV infection has been described together with microscopic findings such as severe granular, vacuolar degeneration of epithelial cells along with mononuclear infiltration (26,17), but there is no statistical evidence to support those lesions with FeMV infection.

Some chronic kidney disease lesions are significantly associated with FeMV infection such as tubular atrophy, luminal expansion, urinary casts, interstitial inflammation, and renal fibrosis; however, glomerulosclerosis is only the glomerular lesion associated with FeMV infection (28). These findings are essential information for a veterinary pathologist to consider those lesions together with IHC or IFA for FeMV diagnosis from a biopsy or necropsy samples of cats showing clinical signs regarding urological diseases.

3. Pathogenicity of FeMV

3.1 Reported evidences

The first report described by *Woo et al.* (10) suggested that the pathogenicity of FeMV is significantly connected to the incidence of tubulointerstitial nephritis (TIN) in cats based on the case-control study and they found in microscopic details that there was inflammatory cell infiltration in the interstitial area corresponded with tubular degeneration and necrosis lesions found together with FeMV N protein-positive cells in renal tubular cell and macrophages from 12 cats with FeMV infection ($p<0.05$). In addition, they also reported that FeMV causes typical CPEs such as cell rounding, detachment, lysis, and syncytia formation in CRFK cells (10). The report confirmed the pathogenicity of FeMV with feline urinary tract disease.

The first report in Japan provided the information between the connection of FeMV infection and nephritis. The FeMV RNA is detected from FFPE kidney tissues of cats with

nephritis, and the positive rate is 40% (4/10) by using a nested-set primer designed from the FeMV L genes (13).

Based on the pathogenicity of Genus *Morbillivirus*, acute infection and the clinical symptoms focused mainly on the central nervous system and respiratory system are the main targets of morbillivirus, not the urinary system. However, many articles suggest that FeMV connected to chronic kidney disease (15,29,11). Chronic kidney disease (CKD) or chronic renal failure (CRF) is a common feline disease whose prevalence varies in the middle-aged to old aged populations. CKD usually results from progressive kidney disease with loss of nephrons and severe scarring of kidneys. CKD cats typically lack the ability to concentrate urine according to the severely scarred kidney leading polyuria and polydipsia. The International Renal Interest Society (IRIS) suggested the new modified guideline in 2019 for staging of CKD based on the serum creatinine level and serum symmetric dimethylarginine (SDMA) in cats and sub-stage of CKD based on the urine protein creatinine (UPC) ratio and systolic blood pressure for disease diagnosis, treatment, and prognosis in cats (Table 4) (30,31).

The first report of FeMV in the USA convinced the connection between FeMV and CKD with the ability to persistently infect the urinary tract rather than the central nervous system as the other morbilliviruses (29). At the beginning of the study, the first strain of feline morbillivirus in the USA was detected from a healthy 4-year-old male domestic shorthair cats in October 2013. The followed-up of the FeMV detection was performed from urine from 2013 to 2015 and found the cat was chronically infected and persistently shedded the virus in the urine detected by RT-PCR (29).

The other supporting evidence which confirmed that FeMV is persistently shaded in urine was described in Italy (15). FeMV was identified in a 15-year-old domestic shorthair neutered stray male cat referred to an animal hospital according to a car hitting accident. Based on the renal ultrasonography and urinalysis, the cat was diagnosed as chronic inflammatory

renal disease. According to RT-PCR results, they found that the cats still shedded the virus in its urine for up to 14 days following the first detection (15). However, they did not confirm whether the virus was continuously or intermittently shedded with viral infectivity in their study. The study of feline paramyxovirus in Germany in 2015 did find any FeMV infection in cats without clinical signs of uropathy; hence, the study strongly highlighted the association between FeMV infection and CKD (11). Nevertheless, there are no specific gross lesions specific to FeMV infection and CKD from necropsy, but the details of microscopic pathology found in the necropsy of cats such as TIN, mononuclear infiltration confirmed the association of FeMV infection with CKD, found in the original articles (17). In addition, FeMV has been found to associate with CKD such as glomerulosclerosis, tubular atrophy, luminal expansion, the presence of urinary casts, renal fibrosis, and inflammatory cell infiltration based on IHC and IFA of FFPE kidney samples (28).

3.2 Contradicting cases

Despite the association between FeMV and CKD, there were still contradicted reports for the connection between FeMV infection and CKD. For instance, McCallum *et al. (16)* performed a cross-sectional, case-control study to find the association between azotemic cat and seroprevalence of FeMV by Western blot and they found that there was no significant difference between azotemic CKD (1/16) and non-azotemia group (4/24; p-value = 0.36). A group of Stransnieri *et al. (21)* also studied the prevalence of FeMV from urines and kidney samples, and they found FeMV in cats without evidence of CKD from 3 out of 108 (one urine sample, two kidney sample) by using RT-PCR.

3.3 Possible mechanisms for pathogenicity

Even though the possible pathogenicity mechanisms have not been determined yet, the Genus *Morbillivirus* is a model for the FeMV pathogenicity and transmission mechanisms. Based on many evidences, it is possible to assume that FeMV might transmit via bodily fluid, contaminated foods, and airborne contact as other morbilliviruses. According to pathological findings based on histopathological examination and *in vitro* study (10), it is strongly suggested that the virus induces renal epithelial injury, causes kidney inflammation, and influences to renal lesions related to CKD, and the virus can persistently infect in the urinary tract and shedded the new viral particles up to 14 days in order to infect to the healthy cats (29) (Fig. 3).

Whereas viral tropism is different from the other morbilliviruses, FeMV corroborates to renal tissues such as renal tubules, transitional epithelium of urinary tract, and immune cells for viral replication and transmission. As same as the other paramyxoviruses, FeMV is possible to use viral attachment proteins, hemagglutinin (H), or glycoprotein bind through receptor such as SLAM (CD150) that is usually expressed on dendritic cells, macrophages, B-and T-cells (32,33) while F protein directly fuses through the host cell membrane (34).

The replication process follows as the virus with a negative RNA genome. The virus uses RNA-dependent RNA polymerase to synthesis the positive-strand RNA. RNA-dependent RNA polymerase binds to the 3`end of the viral genome before starting the transcription process. The viral L protein has an essential role for capping and polyadenylation of the product in the mRNA synthesis, which occurs in the host cytoplasm (35).

Based on the renal tubular injuries found adjacent to the area of FeMV infection in the kidney, the pathogenesis mechanism of FeMV is interesting topic for the study of renal tissue damage through viral induction leading to CKD. The virus-induced apoptosis is one of the possible potential pathogenesis mechanisms to cause renal tissue injury leading to CKD. Commonly, apoptosis is responsible for homeostatic mechanism during development and

aging while inappropriate apoptosis causes abnormality in case of neurodegenerative disease, ischemic damage, autoimmune disorders, and cancers; nevertheless, apoptosis is also responsible as a defense mechanism in the immune reaction according to cellular damage by disease or pathogens (36). The first study about apoptosis was described in 1972 in a classical paper by Kerr, Wyllie, and Currie, who described the morphology of apoptosis by light microscopy as cell shrinkage and pyknosis observed by light microscopy in the early process of apoptosis (37). In routine hematoxylin and eosin (H&E) examination, apoptosis can be seen as single cells, small clusters of cells regularly appeared as a round, or oval marks with dark eosinophilic cytoplasm and dense purple nuclear fragments (36).

To study the virus-induced apoptosis, it is better to use the apoptotic assay, which makes it possible to evaluate both quantitative and qualitative analysis together with viral detection. There are many assays to detect apoptosis, such as observing the cytomorphological alteration, detecting the DNA fragmentation, checking the level of caspases or cleaved substrates, and investigating the membrane alteration visualized either light microscopy or fluorescent microscopy (36). Despite the fact that the evaluation of apoptosis is necessary for virus induction mechanism study, the apoptosis assessment should be carefully done due to the physiological role of controlling program cell death in the kidney. Viruses are possible to induce cell apoptosis indirectly triggering the intrinsic pathway or indirectly induce cytokines to trigger the intrinsic pathway of apoptosis independent of death receptors (38).

The connection between virus and apoptosis of kidney disease has been found in some viruses; for example, human immunodeficiency virus-1 (HIV-1) kills renal tubular epithelial cells by triggering an apoptotic pathway of caspase activation and Fas upregulation related to HIV-associated nephropathy (39). Middle east respiratory syndrome coronavirus (MERS-CoV) causes acute respiratory distress syndrome and multiple organ failure, particularly triggering renal failure via apoptotic induction through upregulation of Smad7 and fibroblast

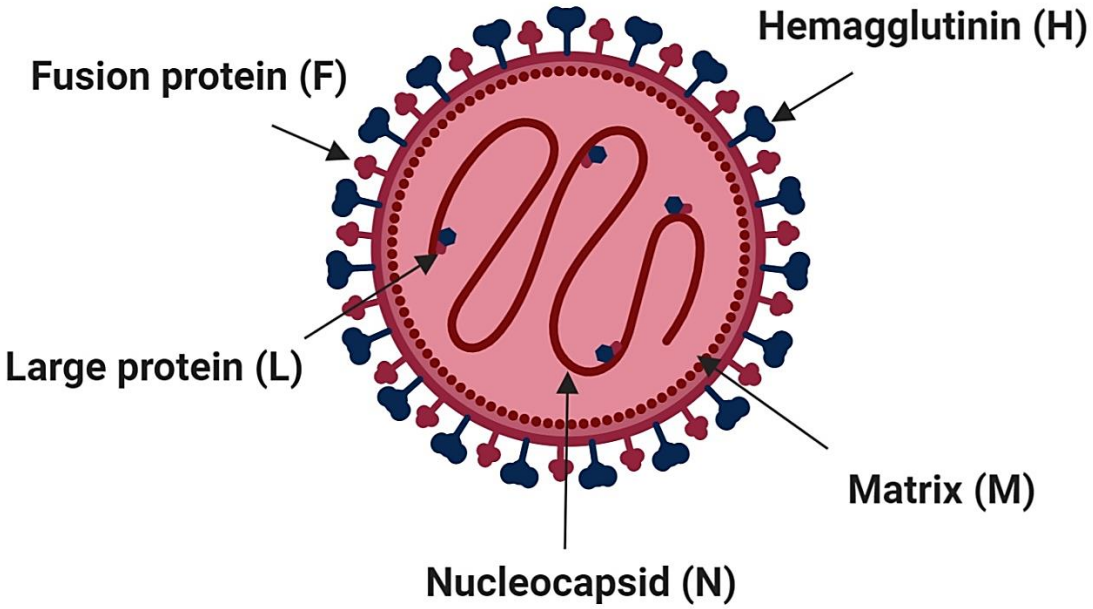
growth factor 2 (FGF2) in kidney cells (40). Therefore, the association of pathogenesis of the novel virus with cellular apoptosis is essential to investigate the virus suspected for kidney disease.

5. The objective of the thesis dissertation

Infectious diseases are particularly common causes of severe morbidity and mortality in cats, especially vial disease. The new emerging morbillivirus, feline morbillivirus (FeMV), is a morbillivirus in domestic cats (*Felis catus*) discovered in Hong Kong in 2012. The original report suggested that the FeMV infection associated with tubulointerstitial nephritis leading to renal failure and possible to be correlated with CKD in cats. Therefore, the objective of this study focuses on the pathological changes and virus pathogenesis mechanism of FeMV infection associated with kidney disease.

Fig. 1. Epidemiology of the geographical map of FeMV infection from 2012 to 2020. Since the first discovery of FeMV in Hong Kong in 2012, FeMV spreads in many regions in the world, including Hong Kong and mainland China (10), Japan (13), Thailand (19), Malaysia (20), Turkey (17), Germany (11), Italy (15), UK (16), the USA (29), and Brazil (18).
The epidemiology of FeMV was plotted by the author based on published articles reporting the first FeMV detection from each country on the geographical map.
(https://commons.wikimedia.org/wiki/File:A_large_blank_world_map_with_oceans_marked_in_blue.svg)

A.



B.

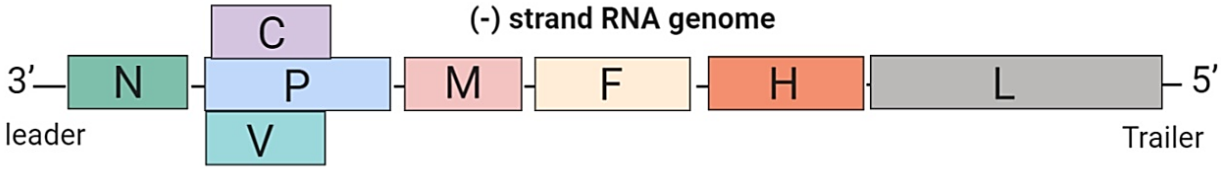


Fig. 2. FeMV virion structure and genome. A. FeMV is an enveloped negative-sense single-stranded RNA virus belonging to Genus *Morbilivirus* in the Family *Paramyxoviridae*. **B.** FeMV genome consists of six non-overlapping arranged genes in the order N-P/V/C-M-F-H-L encoded for eight structural and non-structural proteins including nucleocapsid protein (N), two accessory protein V and C protein (C), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H), and the large protein (L). The size of a linear pattern genome is about 15-16 kilobases (kb) in length (10). Fig.2. was created with Biorender.com under paid subscription, https://biorender.com/.

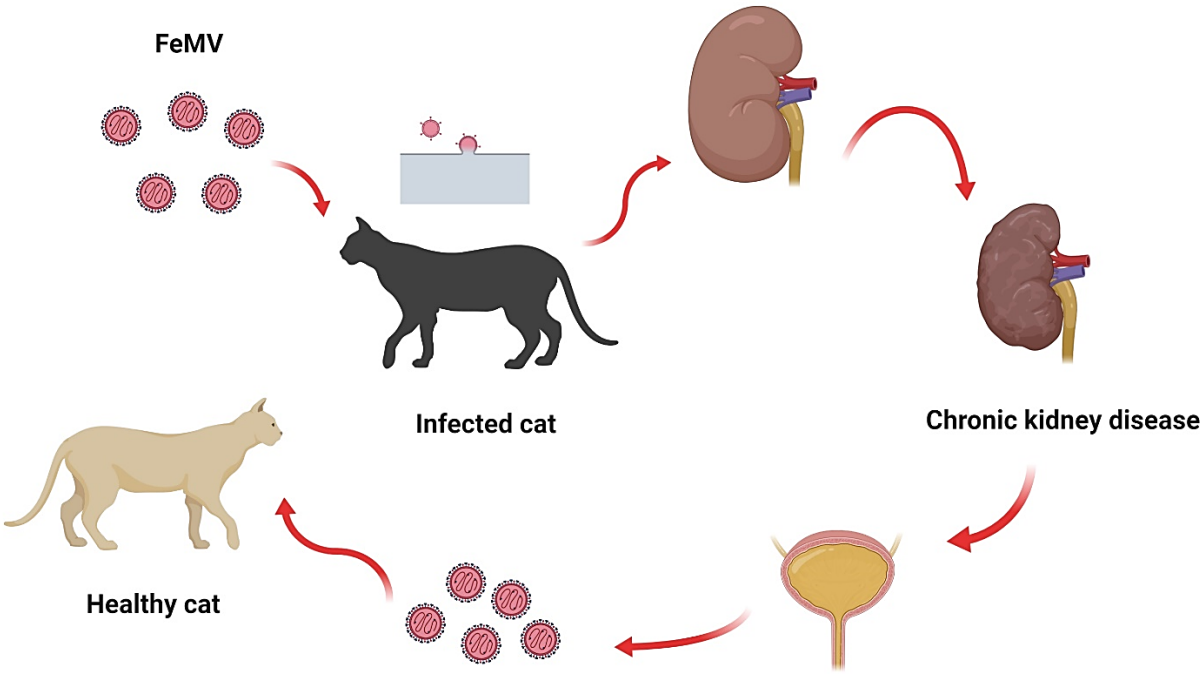


Fig. 3. The possible mechanism of FeMV transmission. FeMV infects the epithelial cells of the urinary tract, including renal tubular cells and transitional epithelial cells leading to chronic infection and potential to be persistently infection and shedding to infect a healthy cat. Fig. 3. was created with Biorender.com under paid subscription, https://biorender.com/.

Table 1. Beatty *et.al* (25) summarized the prevalence of FeMV infection from cat urine by RT-PCR based on published articles

Country	Prevalence of FeMV infection	References
Asia		
Hong Kong	12.4% (53/427), stray cats	(10)
Japan	13% (53/383), stray cats	(13,12,41,24,26)
Malaysia	50.8% (63/124), shelter cats	(20)
Europe		
Italy	10.33% (16/156), stray cats	(42)
	30.6% (22/72), colony cats	
Germany	0% (0/86), healthy control cats	(11)
	4.2% (5/120), diseased cats	
Turkey	4.4% (3/68), unhealthy cat urine samples	(17)
UK	6.3% (1/16), azotemic cats	(16)
	16.7% (4/24), non-azotemic cats	
Americas		
USA	3% (3/10), CKD cats	(29)
	7% (7/10), non-CKD cats	

Table 2. Beatty *et.al (25)* summarized the seroprevalence of anti-FeMV antibodies from published articles.

Country	**Seropositivity in RT-PCR positive cats**	**Seropositivity in RT-PCR-negative cats**	**Overall seroprevalence**	**References**
Hong Kong	96.4% (54/56)	19.5% (78/401)	28.9% (132/457)	(10)
Japan	63.6% (14/22)	9.0% (7/78)	21.0% (21/100)	(26)
Italy	75% (18/24)	42.1% (16/38)	54.8% (34/62)	(42)
UK	100% (5/5)	42.6% (12/26)	66.7% (46/69)	(16)

Table 3. Interpretation of serology results with RT-PCR

RT-PCR	**Serology results**	**Interpretation**
Negative	Seronegative	Never been exposed to FeMV
Negative	Seropositive	Exposed to FeMV but not shedding the virus
Positive	Seronegative	Either acute phase of infection or in the early period of seroconversion
Positive	Seropositive	Either early phase of infection or still in the phage of fully developed seroconversion Period of viral elimination or persistently chronic infection

Table 4. Modified guideline for staging feline chronic kidney disease in 2019 from International Renal Interest Society (IRIS) (31)

	Stage 1 No azotemia (Normal creatinine)	**Stage 2** Mild azotemia (Normal or mildly elevated creatinine)	**Stage 3** Moderate azotemia	**Stage 4** Severe azotemia
Creatinine (mg/dL)	<1.6	1.6-2.8	2.9-5.0	>5.0
Serum symmetric dimethylarginine (SDMA) (µg/dL)	<18	18-25	26-38	>38
Urine protein creatinine (UPC) ratio	Nonproteinuric<0.2, Borderline proteinuric, 0.2-0.4 Proteinuric >0.4 (*Substage based on proteinuria)*			
Systolic blood pressure (mm Hg)	Normotensive <140, Prehypertensive 140-159 Hypertensive 160-179, Severe hypertensive *(Substage based on blood pressure)*			

6. Chapter 1

Association of feline morbillivirus infection with pathological changes in cat kidney tissues

1-1. Introduction

Feline morbillivirus (FeMV) is an enveloped negative-sense single-stranded RNA virus. It was originally discovered in Hong Kong in 2012 (10) and has been detected across different regions of the world since then (18,42,43,13,44,15,16,26,41,12,29,11,45,17). Although officially classified as *Morbillivirus* (2), FeMV has a genome sequence relatively distant from the other members of the genus and has some distinctive biological characteristics, such as infection of kidney tissues and lack of known severe acute clinical signs upon infection (13,26,29,10). The biology of FeMV, including its pathogenicity, is still not well understood partly due to this uniqueness. A case-control study in the original report suggested an association of FeMV infection with tubulointerstitial nephritis (TIN) using histopathological samples (10) and some studies have been conducted concerning the links between FeMV infection and chronic kidney disease (CKD) in cats.

FeMV was detected in 40 % of the fixed kidney tissues (4 out of 10) from cats with nephritis (13), in urine samples from the cats with lower urinary tract diseases (5 in 120 cats), but not from the ones without clinical signs (86 cats) (11). Inflammatory lesions were detected in the kidney tissues of a higher percentage of cats in the FeMV-positive group (90.0 % (26/29)) than in cats in the -negative group (62.0 % (44/71)) (26). In contrast, the studies in Brazil (FeMV-positive: 12 in 52 cats) (18) and Turkey (FeMV-positive: 3 in 110 cats) (17) did not find clear correlation between detection of FeMV and kidney diseases in cats, based on clinical information, and both clinical and histological information, respectively. In the UK, correlation between FeMV infection and azotemic CKDs was not found (FeMV positive in the azotemic and non-azotemic groups, 1/16 and 4/24 cats, respectively) by serum creatinine level and urinary specific gravity (16).

Therefore, based on the studies to date, standard clinical records or test results of the cat patients may not be enough, and histological examinations may also be required to find the important effects of FeMV infection, including pre-clinical changes of the kidney tissues which lead to CKDs in FeMV-infected cats. For this purpose, immunohistological assays were performed to detect the FeMV antigens in kidney tissues from cats and analyzed the relationship between FeMV infection and pathological changes in the tissues.

1-2. Materials and Methods

Reagents and chemicals

All reagents and chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) unless otherwise noted.

Cat kidney samples

Kidney tissues were collected from 38 cats of mixed breeds and genders, which were admitted to clinics for various health conditions (information of some donors is stated in Table 1-1). Though ages and genders of all the donors were not provided, the average age and male/female ratio were 12.1 years $\pm$ 1.18 (Mean $\pm$ SE) and 50 % (11/11), respectively, for the ones whose records were provided. This study was reviewed and approved by Ethics Committee for Animal Study and Research at the Faculty of Agriculture, Tokyo University of Agriculture and Technology (Approval Number 0016015).

All kidney samples were fixed in the 10% neutral buffered formalin for at least 24-48 h at room temperature. The whole tissues should be covered with fixative with the volume of fixative more than 5-10 times of tissue volume. The samples were trimmed into appropriate size and shape in the midsagittal plane and placed in embedding cassettes. Then, the samples were processed as formalin fixed paraffin embedded (FFPE) kidney tissues for pathological evaluation and detection of FeMV antigens. The samples were placed in reagents as follows, 70% ethanol in two times for 1 h, 80% ethanol for 1 h, 95% ethanol for 1 h, 100% ethanol in three times for 1.5 h, xylene for three times in 1.5 h, and paraffin wax at 58-60°C in two times for 2 h and embedded into paraffin blocks for sectioning process.

Staining and microscopic evaluation of cat kidney tissues

The FFPE kidney samples were sectioned at 3 μm with a microtome. Paraffin ribbons were placed in a water bath at 42°C and mounted onto slides, Micro slide glass (Matsunami, Osaka, Japan). The sections were airdried for overnight or incubated into 45-50°C at least for

3 h before the deparaffinization process. The sections were deparaffinized in 3 changes of xylene for 5 min in each time, rehydrated in 3 changes of 100% ethanol for 5 min in each time, and washed with distilled water 3 times for 5 min in each time.

The sections were stained with hematoxylin and eosin (H&E). In brief, the sections were stained with hematoxylin for 7 min and washed by distilled water for 5 min to remove excess dye. Counter-stained was performed with eosin for 5 min, then washed with 100% methanol, and xylene before mounting with a mounting reagent, Bioleit (Oken Shoji Co., LTD, Tokyo, Japan) covered by a coverslip, Neo micro cover glass (Matsunami, Osaka, Japan) for histopathological evaluation.

For evaluation of the HE sections, the pathological scoring criteria were modified from the criteria to evaluate the kidney section of human lupus nephritis (46). The severity of histopathological changes was scored in categories grouped by kidney structures as follows: Glomerular variables; cell proliferation, the thickness of capillaries, glomerulosclerosis, and mesangial area expansion. Renal tubular variables; tubular necrosis, tubular atrophy, luminal expansion, and urinary casts. Interstitial variables; interstitial inflammation, and interstitial fibrosis. All variables were graded from 0 to 3 based on the percentages of observed tissue areas that included lesions: 0, no lesion; 1, less than or equal to 25 %; 2, more than 25 % and less or equal to 50 %; 3, > 50%. To estimate the impact of FeMV on each kidney structure, total scores of glomerular variables, tubular scores, and interstitial scores were calculated.

Antibodies

Recombinant FeMV P protein was expressed and purified as reported previously (27). To clone the FeMV P gene, the total RNA was isolated from a FeMV-positive urine sample by TRIzol LS reagent (Invitrogen, Tokyo, Japan) and reverse transcribed using a ReverTra Ace^{R} qPCR RT Kit (Toyobo, Japan). The open reading frame (ORF) of P gene was amplified by PCR using KOD Plus Neo (Toyobo, Tokyo, Japan) and specific primers as follows,

forward primer, 5'-ATGTCGACTCATGTCCTCTCACCAAATCC-3'; reverse primer, 5'-ACGCGGCCGCTAGTTCTTATTCTTTATCAATATCATGACC-3'-) which were designed base on FeMV 76 stain sequences.

Deoxyadenosine (dA) was added to the 3' ends using the 10x A-attachment mix (Toyobo, Tokyo, Japan), and then the P gene ORF was ligated into a TA cloning vector plasmid, pMD20-T (Takara, Tokyo, Japan). The plasmid was digested at SlI and NotI sites to excise the ORF before inserting into the expression vector pGEX4T-1 (GE Healthcare, Tokyo, Japan), which was digested at the same restriction sites. The pGEX4T-1 containing a glutathione-S-transferase with P gene as a glutathione S-transferase (GST)-fused protein were transformed to *Escherichia coli* BL21 (DE3) (Cosmo Bio, Tokyo, Japan) and initial cultured in LB medium at 37° for 3 h before Isopropyl-β-d(-)-thiogalactopyranoside (Wako, Tokyo, Japan) induction at 0.5 mM and further cultured for 4 h.

The supernatant was ultra-sonicated (Handy Sonic UR-20P, Tomy Seiko Co., LTD, Tokyo, Japan) and centrifugated for 10 min at 16,000 xg at 4°C. The recombinant FeMV P-GST fusion protein was affinity-purified from the soluble fraction with glutathione-conjugated sepharose 4B (GE Healthcare, Tokyo, Japan) and eluted with a buffer containing 10 mM reduced glutathione in 50mM Tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Tris-HCl) pH8.0 and then was removed by the Amicon® Ultra 0.5 mL10 K, Ultracel®-10 K membrane (Merck Millipore, Germany). For GST purification, the P protein was removed from the GST-P bound to the glutathione Sepharose beads by digesting at the upstream thrombin site with 1 unit of thrombin per mL at room temperature overnight. The remaining GST on the beads was eluted with 10mM reduced glutathione in 50 mM Tris-HCL, pH8.0 and also removed the reduced glutathione with the Amicon device. The P-protein was analyzed by 10% SDS-poly acrylamide gel electrophoresis (SDS-PAGE), and the concentration was measured using a Qubit fluorometer (Thermo Scientific, USA).

To produce a specific antibody against FeMV P proteins, a specific-pathogen-free (SPF) Japanese white rabbit was immunized with 200 μg of the purified FeMV P-GST protein three times during the 56-day immunization period, and whole blood was collected for the serum. All this process was produced as a commercial service (Sigma-Aldrich®, Tokyo, Japan). A specific antibody against FeMV N protein was produced and provided by Dr. Morikawa from the Department of Veterinary Science, National Institute of Infectious disease, Tokyo, Japan. Production of the antibody was described elsewhere (26). The antibody has no cross-reaction with other viral antigens.

Immunohistochemistry and immunofluorescence for detection of FeMV antigens

For immunohistochemistry (IHC), the FFPE kidney tissues were sectioned at 6 μm and placed on the adhesive glass slide, Platinum pro micro slide (Matsunami, Osaka, Japan), deparaffinized, and rehydrated for staining preparation as same as a preparation method for H&E staining. Then the sections were autoclaved at 121°C for 20 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. After the sections were cooled down, the endogenous tissue peroxidase was performed using 0.3% H_2O_2 in absolute methanol to inactivate the endogenous tissue reaction for 30 min. For blocking non-specific reactions, the sections were incubated with 10% normal goat serum in phosphate-buffered saline (PBS) at room temperature for 30 min. After the non-specific blocking, the sections were incubated with 1:200 dilutions of the primary antibodies for overnight at 4°C in a moist chamber. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-labeled polymer conjugated with the secondary antibody (Envision solution, Dako Envision®+System-HRP Labelled Polymer Anti-Rabbit, Tokyo, Japan) at room temperature for 30 min. The signal was developed in 0.05% 3, 3'-diaminobenzidine tetrahydrochloride/H_2O_2 (Wako, Tokyo, Japan). After each step of the process, the sections were washed with PBS for 3 times in 5 min of each time. All sections were counterstained with hematoxylin, mounted with a mounting reagent, Bioleit

(Oken Shoji Co., LTD, Tokyo, Japan), and covered by a coverslip, Neo micro cover glass (Matsunami, Osaka, Japan). All images were recorded by a photo-micrographic software cell Sens Standard 1.9 (Olympus DP26 model camera, Tokyo, Japan).

For immunofluorescence (IFA), all FFPE kidney tissues were sectioned at 3 μm. After the same deparaffinization and rehydration process as the immunohistochemistry staining, the section slides were enzymatically treated with 0.05% trypsin for 30 min at 37°C for antigen retrieval. This antigen retrieval method resulted in better signal/background ratio than the autoclaving method in the case of IFA. The sections were washed with PBS, blocked with 10% normal goat serum in PBS for 1 h at room temperature and incubated with rabbit pre-immune serum or the antibody against FeMV P protein diluted to 1:400 with PBS for 1 h at room temperature. The tissues were then washed with PBS and incubated with 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (Invitrogen, Tokyo, Japan) for 1 h at room temperature. For detection of feline IgG, the sections were treated as above until the blocking, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-cat IgG (H+L) antibody (Rockland Immunochemicals, Tokyo, Japan) diluted to 1:200 with PBS. For the washing process with PBS, the sections were washed for 3 times in 5 min of each time. All stained slides were mounted with ProLong™ Glass Antifade Mountant (Invitrogen, Tokyo, Japan), covered by a coverslip, Neo micro cover glass (Matsunami, Osaka, Japan). The IFA sections were examined with a fluorescence microscope, and the IFA images were recorded by a digital camera system (FSX 100 Olympus, FSX-BSW Ver 02.02, Tokyo, Japan).

Statistical analysis

For the statistical analysis, FeMV test results were determined by taking the most stringent criteria with the obtained results and samples were regarded positive only when all the three immunological examination (two IHC results with antibodies against the FeMV P and N protein, and IFA with antibody against the FeMV P protein) were positive. To evaluate the

statistical significance in the differences of the pathology scores between FeMV-positive and -negative kidney tissues, the scores in the two groups were analyzed with two-sided Mann Whitney U test using a publicly available tool (http://astatsa.com/WilcoxonTest/).

1-3. Results

Microscopic evaluation of pathological changes in cat kidney tissues

To find the morphological abnormalities in the kidney tissues used in this study, HE-stained sections of the cat kidney tissues were firstly evaluated. Demonstrations of the tissue injuries in the kidney samples were observed microscopically and grouped into the three kidney tissue areas: glomeruli, renal tubules and interstitial areas (categories for each area are shown in Fig. 1-5). Such morphological changes include; cell proliferation (Fig. 1-1, A), thickness of capillaries (Fig. 1-1, B), glomerulosclerosis (Fig. 1-1, C) and mesangial area expansion (Fig. 1-2, A) for the glomerular tissues; tubular necrosis (Fig. 1-2, B), tubular atrophy (Fig. 1-1, D), luminal expansion (Fig. 1-1, E) and urinary casts (Fig. 1-1, F) for the renal tubules; and inflammatory cell infiltration (Fig.1-1, G) and fibrosis (Fig. 1-1, H) for the interstitial areas. Also, the severity of the tissue injuries was scored based on the frequencies of the morphological changes above in each tissue (Fig. 1-1, table 1-2 for all the scores).

Immunological detection of FeMV antigens and immune complex in cat kidney tissues

To study association between FeMV infection and cat kidney tissue injuries, the immunological detection of the FeMV antigens was performed in the kidney tissues from 38 individual cats using FeMV-specific antibodies. IHC was conducted with antibodies against FeMV P or N protein (Fig. 1-3). FeMV P protein was observed in epithelial cells of proximal, distal, and collecting duct of renal tubules in transitional epithelial cells (Fig. 1-3, A and B, and Table 1-3, D, negative example). Some positive cells were observed together with inflammatory cells infiltrating into the interstitial areas (Fig. 1-3, B, arrows). IHC was also performed with antibody against the FeMV N protein and the protein was mostly localized on the renal tubular cells, similar to the FeMV P protein localization in the tissues (Fig. 1-3, C, Table 1-3). With both antibodies, positive signals were observed in the cells of renal tubules and transitional epithelium, but not in the cells of glomeruli or interstitial areas (Table 1-3).

The FeMV antigens were also detected in the renal tubular cells in all the tissue samples in which the FeMV antigens were detected in the transitional epithelial cells (Table 1-3). Among the 38 total tissues, 20 (52.6 %) were positive for the P protein and 16 (42.1 %) were positive for N proteins, while 14 (36.8 %) tissues were positive for the both proteins (Table 1-3).

Since eight tissues had signals for only one of the two antigens (21.1%, sample numbers 10, 11, 12, 15, 16, 18, 23, and 30), IFA was additionally performed with the anti-P protein antibody to confirm the IHC results. In the IFA, signals were detected in 14 samples (36.8%), with the tubular localizations similar to the ones with the IHC (Fig. 1-3E, and F for a negative sample). I could determine IFA results for three kidney samples, due to strong backgrounds on the samples (Table 1-3, indicated as "ND"). Based on the one IFA and the two IHC results, I diagnosed the kidney sample FeMV antigen positive only when all these three tests were positive (Table 1-3, FeMV antigen diagnosis).

To study association between presence of immune complex formation and FeMV infection in the cat kidney tissues, I performed IFA with anti-cat IgG (H+L) antibody conjugated to FITC. I observed feline IgG-localizations along glomerular tuft of five tissues in total (Table 1-3 and Fig. 1-3G), among which two were also positive for FeMV (Table 1-3, columns for "FeMV antigen diagnosis" and "Feline IgG localization"). In some tissues feline IgG were observed in some interstitial and glomerular spaces (Fig. 1-3G, Fig. 1-2F). Colocalization of the FeMV antigen and feline IgG was not commonly but only observed in limited occasions in interstitial cells in these tissues (Fig. 1-2F).

Statistical evaluation of the correlation between the FeMV antigens and pathological changes in the cat kidney tissues

In order to study the association between FeMV infection and kidney tissue injuries, I analyzed the score differences between the FeMV-positive and -negative groups for each pathological category (Fig. 1-5A-J). The differences between the two groups were especially significant in the three pathological categories of the tubular tissues ($p < 0.0005$, tubular atrophy, luminal expansion, urinary casts, Fig. 1-5F-H) and the two categories of the interstitial areas ($p=0.0058$ for inflammatory cell infiltration and 0.0008 for fibrosis, Fig. 1-5I-J). Difference between the FeMV-positive and -negative groups was also significant in glomerulosclerosis ($p=0.0013$) and thickness of capillaries ($p=0.0390$) of the glomerular tissues, although the latter value was close to the threshold (Fig.1-5B-C). Based on these results, the presence of the FeMV antigens in cat kidney tissues was significantly associated with some pathological changes, particularly those in the tubular and the interstitial areas in the tissues.

1-4. Discussion

In this study, detection of the FeMV antigens was significantly associated with some morphological abnormalities in cat kidney tissues, particularly those in renal tubular and interstitial areas. Correlation between FeMV infection and kidney diseases has not yet been clear to date. However, our results are in accordance with lines of evidence which have been suggestive of association between FeMV infection and conditions related to chronic kidney diseases (CKD) (13,26,10,11). On the other hand, in a study in UK, they did not find significant difference in FeMV positive rates between azotemic CKD (6.0 %, 1/16) and nonazotemic (17 %, 4/24) cat groups when they studied association between detection of FeMV RNA and clinically diagnosed azotemic CKD (16). Although the reason for this discrepancy is not clear at this point, it is possible to point out some potential causes as explained in their report (16). First, TIN and CKD can be developed even after resolution of the viral infection in cats when the viral RNA is no more detected in their urine samples. Also, they could have missed some subclinical azotemic and nonazotemic CKD since they relied on relatively insensitive indirect markers (serum creatinin concentration and urinary specific gravity) to detect azotemic CKD rather than more sensitive means, such as measurement of glomerular filtration rates (16). Finally, they might have missed some FeMV RNA due to sub-optimal primers used in their detection method, since the copy numbers of FeMV RNA can be low in the majority of urine samples from FeMV-posibive cats, in which RT-PCR detection of the viral RNA is difficult for some FeMV strains due to sequence variations (24). Regardless of sensitivity of the diagnostic methods for detection of CKD or FeMV RNA used in the study, they could not detect the histological abnormalities in kidney tissues, since they needed to rely on clinical diagnostic methods for live cat patients, which explains at least some differences observed in their study.

The FeMV antigens were mainly detected in tubular epithelial cells in cat kidney tissues in this study, and such localizations of FeMV were consistent with the analysis results of the severity scores for the pathological abnormalities in which the differences between the FeMV-positive and negative groups were particularly significant in the tubular and interstitial areas (Fig. 1-5E-J). On the other hand, less morphological changes were affected by presence of the FeMV antigens in the glomerular areas (Fig. 1-5A-D), agreeing to the fact that no FeMV antigens were localized in glomerular areas (Table 1-3). Despite the lack of FeMV antigen localization, severity scores of glomerulosclerosis and thickness of capillaries were significantly affected by detection of the FeMV antigens, though the significance was near to the threshold in the latter category (Fig. 1-5B and C). Glomerulosclerosis is known to occur in association with interstitial inflammation and tubular atrophy in cats with CKD (30), both of which were significantly observed more often in FeMV-positive tissues, which explains the result of our study. It is also possible that thickness of capillaries occurred due to the injuries in the other areas, or secondary injuries such as glomerulosclerosis above.

The FeMV antigens were also detected in transitional epithelial cells (Fig. 1-3A, and Table 1-3). However, observation of pathological changes in these cells was difficult, since tissues were damaged in many samples around that area, and the numbers of the samples with the FeMV antigens in these cells were smaller than those with the signals in tubules (Table 1-3). Further study will be required to find the effect of FeMV in such cells, including studying more samples with the FeMV localizations in the transitional cells.

In this study, I detected the feline IgG along capillaries of glomerular tufts in some kidney tissues (Fig. 1-2 F and Fig.1-3G, Table 1-3). However, I did not find significant association of the FeMV antigens with the feline IgG (Table 1-3 and Fig. 1-2F), therefore, formation of immune complex in glomerular tissues or interstitial spaces may be secondary to

the other injuries associated with FeMV. Further study is needed to find the role of immune complex in FeMV-associated kidney injury.

Although this study suggests correlations between the FeMV infection and the pathological changes in cat kidney tissues, it is still possible that the observed correlations might be results of bystander effects and, thus, FeMV may more frequently infect cats with pre-existing kidney diseases. Any potential cause-effect relationship needs to be examined further with experimental infections of cats with FeMV or studies with continuous clinical records of FeMV-infected cats. However, studies suggest that the irreversible lesions, associated with FeMV antigens detection in this study, such as interstitial inflammation, fibrosis, and glomerulosclerosis, significantly relate to the later stages of CKDs (47). In humans, infections of viruses such as polyomavirus and herpesviruses are known to induce interstitial and tubular pathological changes in infected kidney tissues (48). In this regard, it is possible that FeMV-infected cats may have higher chance of developing CKDs later in their lives, particularly at old ages.

In the IHC results in this study, some samples became positive for only one of the FeMV antigens (N or P antigen) (Table 1-3). Such results might be due to variation of amino acid sequences of the viral proteins among tissues or due to the difference in sensitivity of IFA performed by these antibodies. Since it was difficult to additionally perform a genetic test due to RNA degradation in the tissues, I performed IFA as an additional assay since non-specific signal can be lower in IFA in some tissues. However, I cannot use RT-PCR data from FFPE tissues together with IHC and IFA in this study according to formalin crosslink from the samples.

Based on the all assays to detect the FeMV antigens, I diagnosed tissue samples positive for the FeMV antigens when all the three results were positive, in order to minimize the chance of false positive results. Although it is difficult to know which assay is the most reliable for the

FeMV antigens since the assay results were affected by conditions of the tissue samples, I determined the final results of detection of the FeMV antigens as stringently as possible, so that the following statistical analysis would be more reliable.

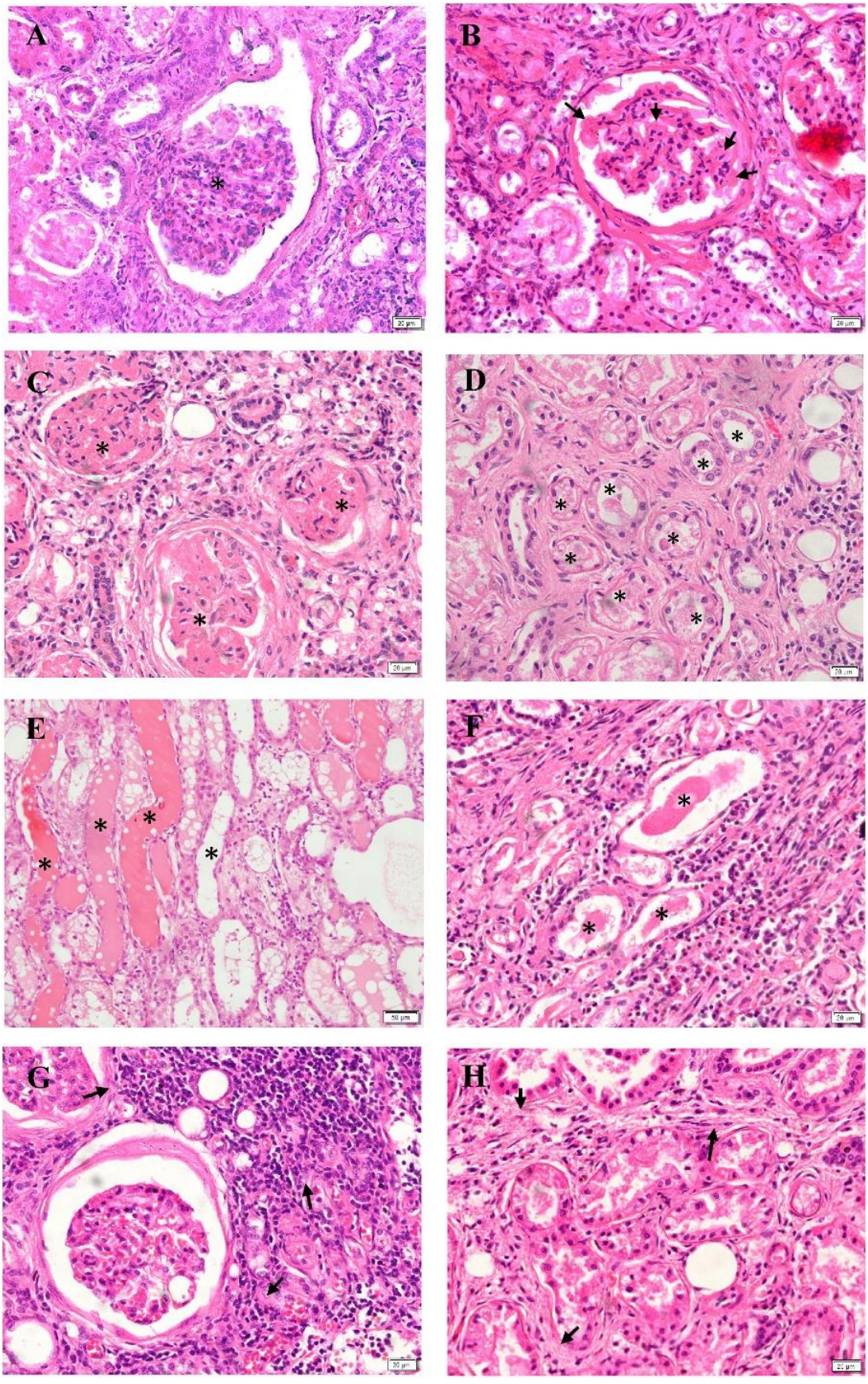

A
B
C
D
E
F
G
H

Fig. 1-1. Representative images for specific pathological changes observed in cat kidney tissues. A, glomerular cell proliferation; B, the thickness of capillaries; C, glomerulosclerosis; D, tubular atrophy; E, tubular luminal expansion; F, urinary casts; G, interstitial cell infiltration; H, fibrosis. Arrows and asterisks indicate typical affected areas. All images are in 40× magnification and scale bars are 20 μm except E, which is in 20× magnification and has the scale bar of 50 μm.

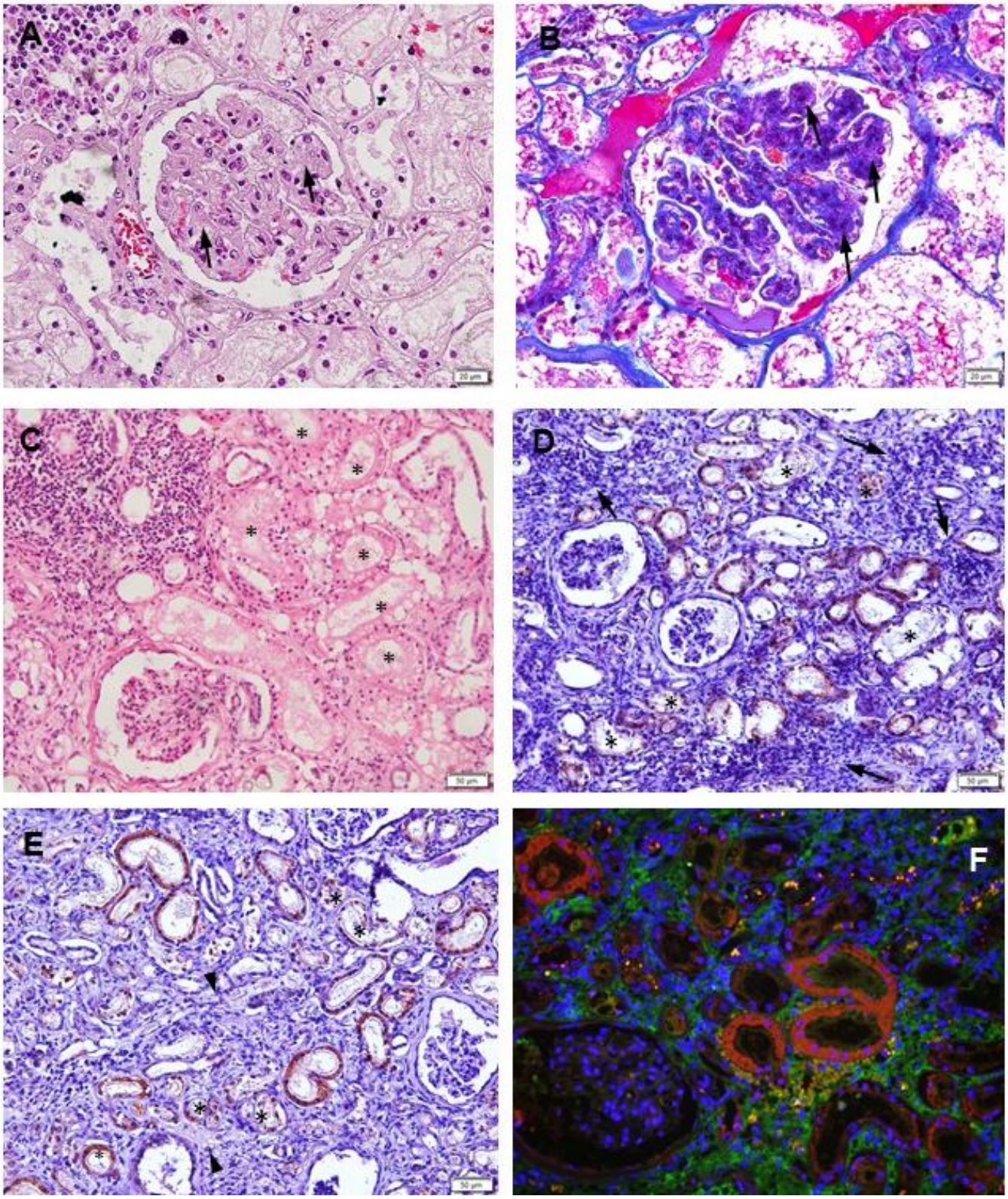

A
B
C
D
E
F

Fig. 1-2. Representative images for pathological changes observed in cat kidney tissues, A and B, mesangial area expansion (arrows) (40x) H&E (A), Masson trichrome staining (B); C, tubular necrosis (20x). Arrows and asterisks indicate affected areas. D and E, significant pathological lesion found with FeMV immuno-staining (anti-P antibody) (20x). Asterisks, arrows, and arrow heads indicate tubular atrophy, inflammatory cell infiltration, and fibrosis, respectively. F, dual-fluorescent staining of a cat kidney tissue with anti-FeMV P protein antibody (red) and anti-feline IgG (green) together with DAPI (blue).

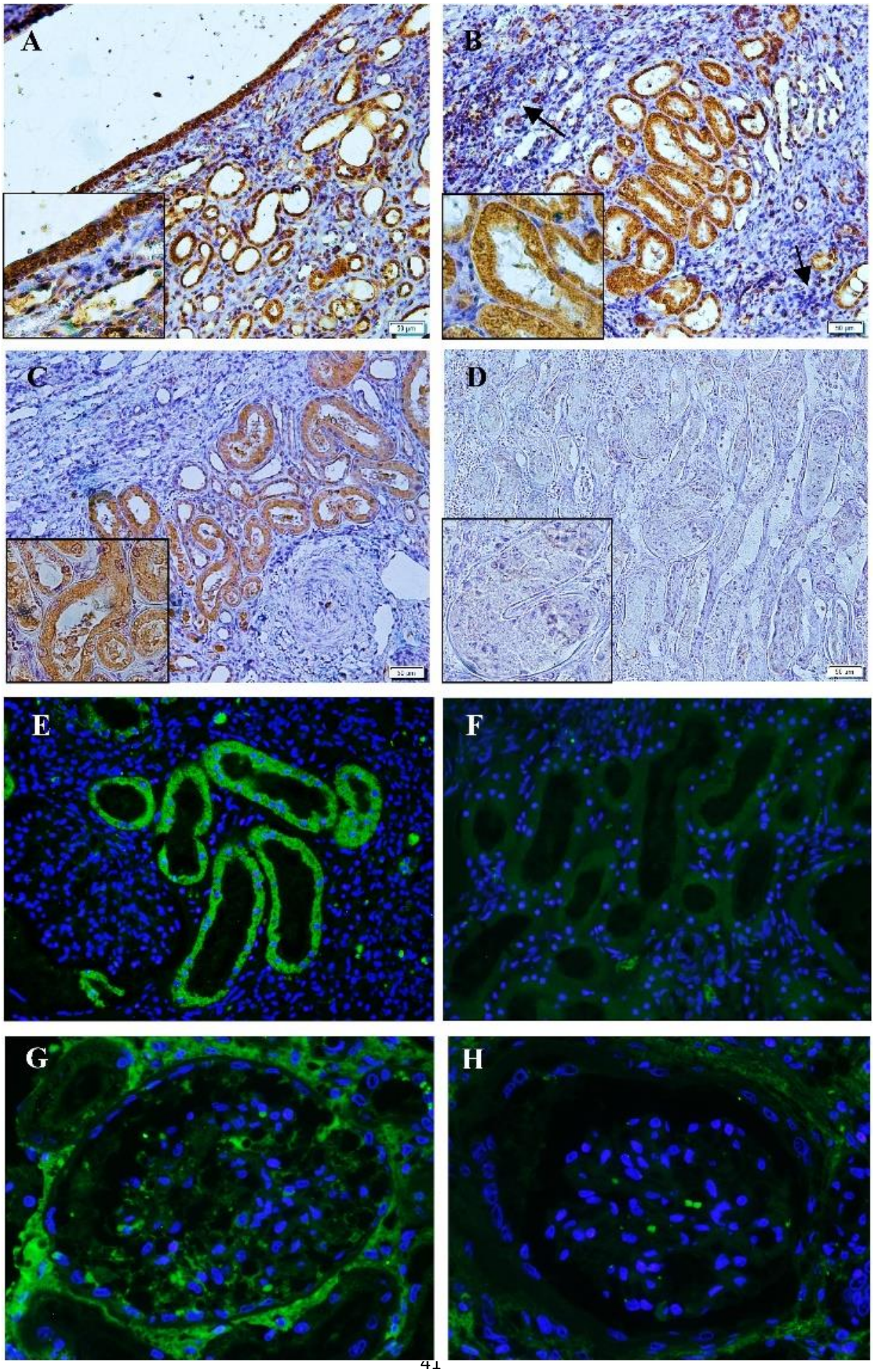

A
B
C
D
E
F
G
H

Fig. 1-3. Representative images for IHC and IFA staining of FFPE cat kidney tissues with antibodies against the FeMV antigens. A–D, IHC images; A, transitional epithelial cells; B and D, renal tubular cells, stained with antibody against the FeMV P protein (D is a sample without the signal for comparison). C, renal tubular cells stained with antibody against the FeMV N protein. Arrows indicate proliferating inflammatory cells. All images in 20× magnification the scale bars are 50 μm. E–H, IFA images; E and F, FeMV P protein-positive and -negative renal tubular cells, respectively (20× magnification). The small figures in A-D represent the magnification of each image. Arrows indicate proliferating inflammatory cells. G and H, feline IgG-positive and -negative glomerular cells (40x original magnification). IFA images (E–H) were taken at the same imaging conditions (exposure time, gain and contrast) for fair comparison.

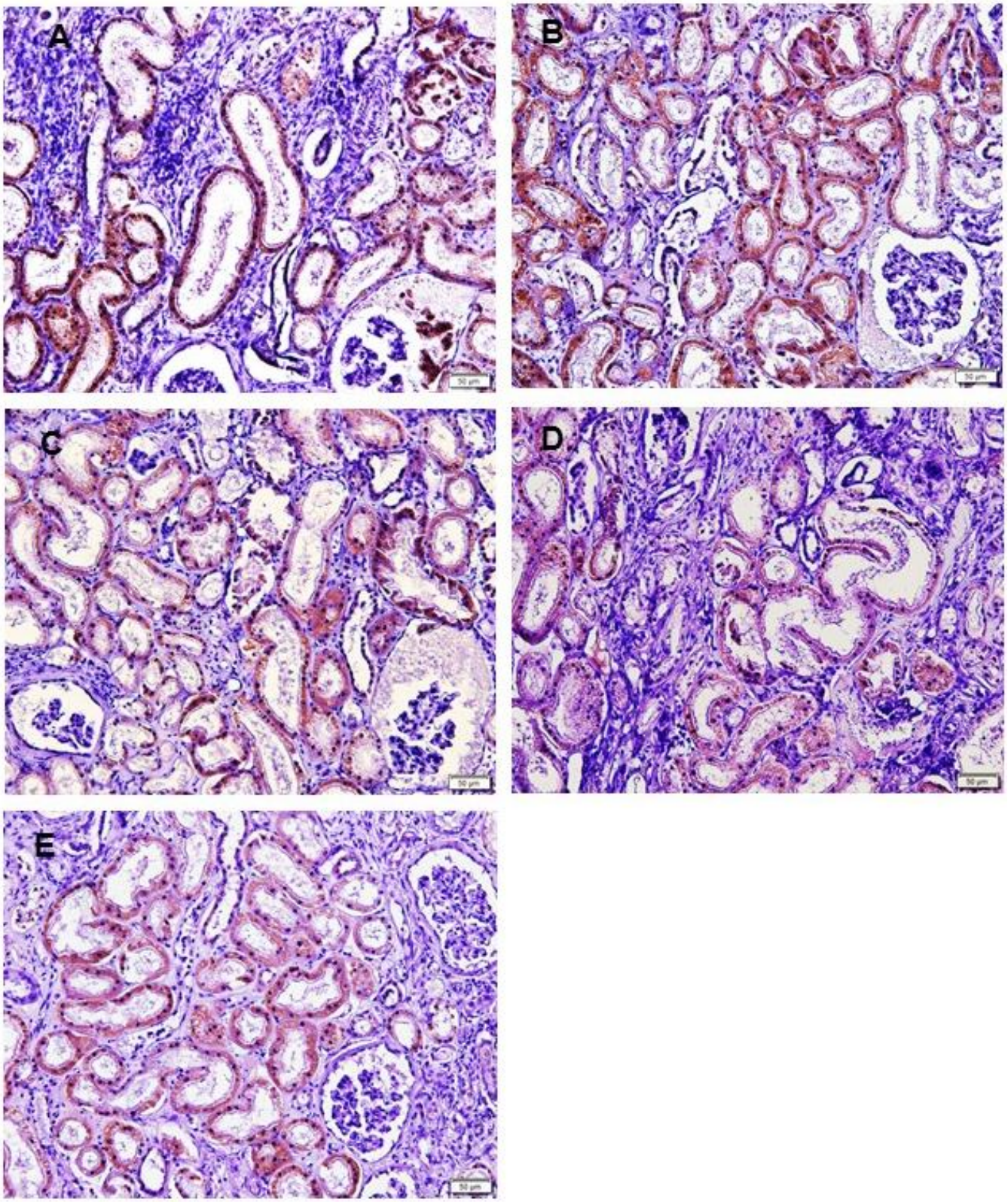

A
B
C
D
E

Fig. 1-4. IHC of serial sections from a cat kidney tissue with anti-FeMV P protein antibody. Tissue slices were sectioned at 6 μM. Ten sections were made and every other slide were stained in the same manner as IHC in Fig. 2-2.

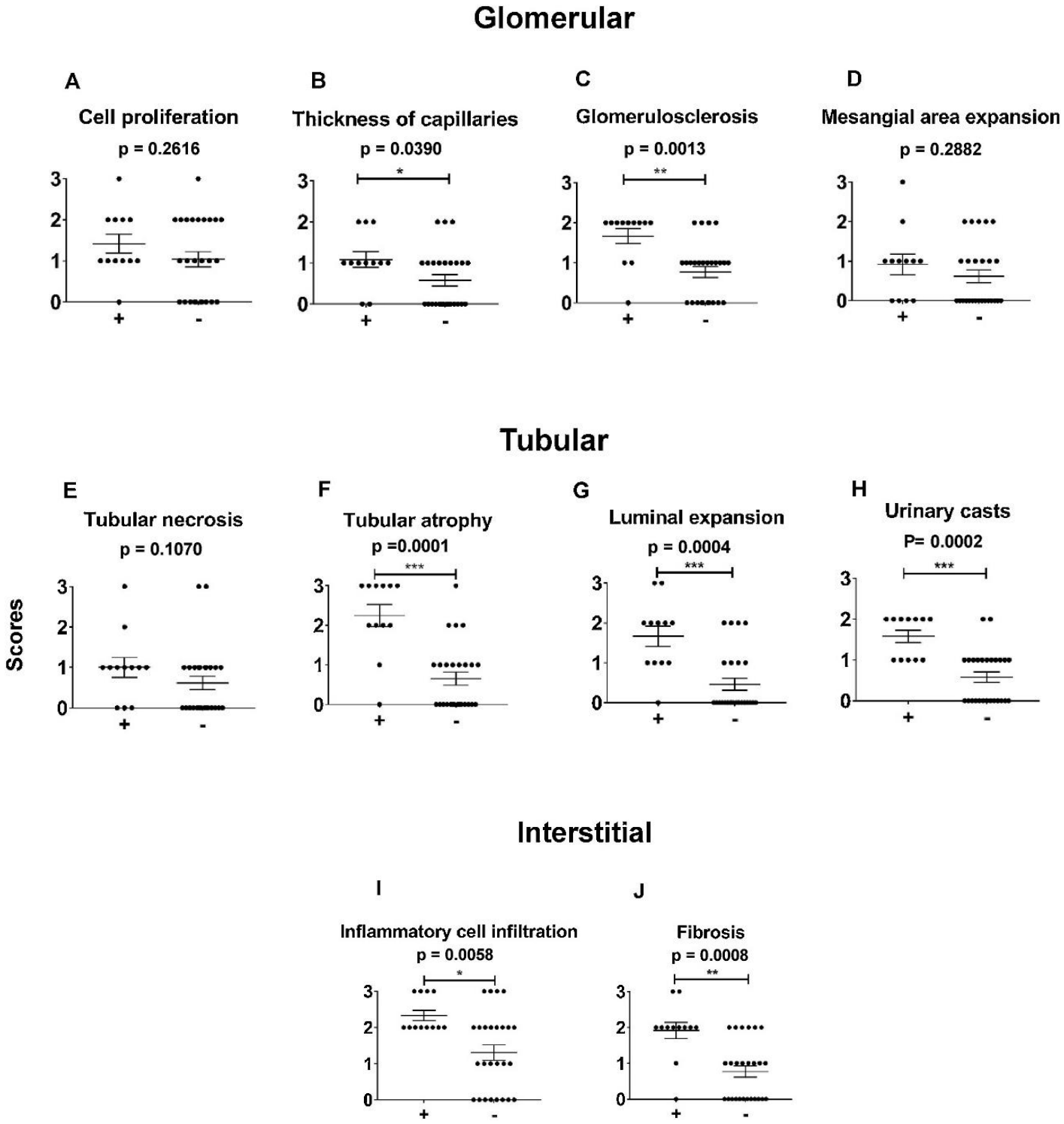

Glomerular
A
Cell proliferation
p = 0.2616
B
Thickness of capillaries
p = 0.0390
C
Glomerulosclerosis
p = 0.0013
D
Mesangial area expansion
p = 0.2882
Tubular
E
Tubular necrosis
p = 0.1070
F
Tubular atrophy
p =0.0001
G
Luminal expansion
p = 0.0004
H
Urinary casts
P= 0.0002
Scores
Interstitial
I
Inflammatory cell infiltration
p = 0.0058
J
Fibrosis
p = 0.0008

Fig. 1-5. The correlation between FeMV antigens and pathological changes in the cat kidney tissues. Scores of detailed pathological changes are plotted for groups with (+) or without (-) the FeMV antigens. Significant differences are marked with * ($p < 0.05$), ** ($p < 0.005$), or *** ($p < 0.0005$).

Table 1-1. Clinical information of the cat kidney sample donors

Sample number	Age (Years)	Breed	Gender	Clinical signs
1		-	-	-
2	8	Japanese breed	Male	Urothelial carcinoma
3	12	Persian	Neutered male	Nephrosclerosis
4	13	Japanese breed	Neutered male	Renal failure, renal mass, hepatic tumor
5	2	Mixed breed	Female	Thromboembolism by cardiomyopathy
6	10	-	Male	Gastroesophageal hiatal hernia, dilated cardiomyopathy
7	18	Japanese breed	Male	Restrictive cardiomyopathy
8	8	Mixed breed	-	-
9	16	Mixed breed	Spayed female	Chronic renal failure
10	10	Mixed breed	Spayed female	Restrictive cardiomyopathy, dilated cardiomyopathy, arterial thrombosis
11	16	Mixed breed	Neutered male	Thromboembolism
12	16	Mixed breed	Spayed female	Filariasis
13	0.08	Mixed breed	Male	Petechiae over the left lung lobe
14	16	Mixed breed	-	-
15	15	Mixed breed	-	-
16		Mixed breed	Neutered male	Atrial thrombosis
17	17	Mixed breed	Neutered male	-
18	6	-	Female	Mitral valve dysplasia
19	20	-	Neutered male	Ventricular aneurysm
20	6	-	-	Sudden death during anesthesia

Sample number	Age (Years)	Breed	Gender	Clinical signs
21	9	Calico	Spayed female	Wolff Parkinson White syndrome
22	17	American shorthair	Spayed female	Left Ventricular Outflow Tract Obstruction (LVOT), stenosis, and right ventricular septal defect (RVSD)
23	14	-	Female	Renal failure, pulmonary emphysema
24	17	Himalayan	Male	Renal hypertension
25		-	-	-
26		-	-	-
27		-	-	-
28		-	-	-
29		-	-	-
30		-	-	-
31		-	-	-
32		-	-	-
33		-	-	-
34		-	-	-
35		-	-	-
36		-	-	-
37		-	-	-
38		-	-	-

-: no information

Table 1-2. Severity scores for pathological changes

Pathological changes										
	Glomeruli				Tubules				Interstitial areas	
Sample number	Cell proliferation	Thickness of capillaries	Glomerulosclerosis	Mesangial expansion	Tubular necrosis	Tubular atrophy	Luminal expansion	Urinary casts	Inflammatory infiltration	Fibrosis
1	2	1	2	1	0	1	1	2	2	1
2	0	0	1	0	0	1	2	2	2	0
3	1	1	2	0	0	3	2	2	2	3
4	1	0	2	0	1	3	1	2	2	2
5	0	2	2	1	3	0	0	2	3	0
6	1	1	0	1	1	0	0	0	1	0
7	2	1	2	1	2	2	1	2	3	2
8	2	1	1	0	1	1	0	1	2	2
9	1	2	2	1	1	3	2	2	2	2
10	1	1	1	1	1	1	0	1	3	2
11	2	1	1	2	0	1	1	1	2	1
12	2	2	2	2	0	1	1	1	3	1
13	1	0	0	0	0	0	0	0	1	0
14	3	1	0	2	1	3	2	1	2	2
15	2	1	1	2	1	2	1	1	2	2
16	0	1	0	0	1	0	0	1	2	1
17	2	2	2	3	1	2	2	1	3	2
18	3	2	1	2	1	0	0	1	2	1
19	0	1	2	1	1	3	2	1	3	2
20	2	0	1	1	0	0	0	0	0	0
21	2	0	1	0	1	1	0	0	1	1
22	2	1	1	1	1	2	1	1	2	2
23	2	2	1	2	1	2	2	1	3	2
24	1	0	1	0	0	2	3	1	2	2
25	1	1	2	0	1	3	2	2	3	3
26	1	0	1	0	3	0	0	0	1	1
27	1	1	2	1	1	3	3	1	2	2
28	0	0	2	0	0	2	2	2	2	2
29	2	1	1	1	0	0	0	0	1	0
30	2	1	1	1	3	1	0	1	2	1
31	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
37	1	0	2	0	1	1	1	1	1	1
38	1	0	0	0	0	0	0	0	0	0

Table 1-3. Immunological detection of FeMV antigens on the cat kidney tissues

Sample number	IHC (anti-P)		IHC (anti-N)		IFA (anti-P)	FeMV antigen diagnosis	Feline IgG localization
	Tubules	Transitional epithelium	Tubules	Transitional epithelium			
1	+	–	+	+	+	+	–
2	–	–	–	–	–	–	–
3	+	–	+	–	+	+	–
4	+	–	+	–	+	+	–
5	+	–	+	+	+	+	+
6	–	–	–	–	–	–	–
7	+	+	+	+	+	+	–
8	–	–	–	–	+	–	–
9	+	–	+	–	+	+	–
10	+	–	–	–	–	–	–
11	–	–	+	–	–	–	–
12	+	+	–	–	–	–	+
13	–	–	–	–	–	–	–
14	+	+	+	–	+	+	–
15	+	–	–	–	–	–	–
16	–	–	+	–	–	–	–
17	+	–	+	–	+	+	–
18	+	–	–	–	–	–	–
19	+	–	+	–	–	–	–
20	–	–	–	–	–	–	+
21	–	–	–	–	–	–	–
22	+	–	+	–	+	+	–
23	+	–	–	–	–	–	–
24	+	–	+	–	+	+	–
25	+	–	+	–	+	+	+
26	–	–	–	–	–	–	–
27	+	+	+	+	+	+	–
28	+	–	+	+	–	–	–

Sample number	IHC (anti-P)		IHC (anti-N)		IFA (anti-P)	FeMV antigen diagnosis	Feline IgG localization
	Tubules	Transitional epithelium	Tubules	Transitional epithelium			
29	–	–	–	–	–	–	+
30	+	–	–	–	–	–	–
31	–	–	–	–	+	–	–
32	–	–	–	–	–	–	–
33	–	–	–	–	–	–	–
34	–	–	–	–	ND	–	–
35	–	–	–	–	ND	–	–
36	–	–	–	–	–	–	–
37	–	–	–	–	–	–	–
38	–	–	–	–	ND	–	–
IFA positive signal	52.6% (20/38)	10.5% (4/38)	42.1% (16/38)	13.2% (5/38)	36.8% (14/38)	31.6% (12/38)	13.2% (5/38)

ND: not determined.

Orange highlighted: samples which were positive for FeMV antigen with feline IgG localization

Grey highlighted: samples which had signals for only one of the two antigens

7. Chapter 2

Induction of renal tubular apoptosis by feline morbillivirus

2-1. Introduction

Morbilliviruses are enveloped negative-sense single-stranded RNA viruses in Family *Paramyxoviridae*, which causes highly contagious infectious diseases in human in case of measles virus (MV) and animal diseases with highly morbidity and mortality rates including, canine distemper virus (CDV), rinderpest virus (RV), peste des petits ruminant virus (PRRV) etc. (1,49). In recent years, the new cat virus classified as feline morbillivirus (FeMV, previously known as FmoPV) (2) was initially discovered in Hong Kong (10). Since the first discovery in 2012, the new cat virus has drawn the attention of researchers to focus on the morbillivirus in cats highlighted as the first member of Morbilliviruses linked with the urinary disease in cats. Until now, FeMV has been reported from different countries including Japan, the USA, Turkey, Brazil, Thailand, Italy, UK, Germany, and Malaysia (18,42,13,24,44,15,16,50,26,12,29,11,45,17,14,28). Besides, FeMV-GT2, the new genotype of FeMV was recently isolated from the urine of cats with urinary tract disease in Germany; this virus showed the viral tropism to primary feline cells from kidney, lung, and immune cells as wells to organotypic slice cultures from feline nervous tissues (14).

In the first report of FeMV, a case-control study suggested that FeMV infection relevant to the incidence of tubulointerstitial nephritis (TIN), which involves primary injury in renal tubules and could be the leading cause of renal failure in cats (10). Even though the association between FeMV infection and kidney diseases has been reported from many articles (13,10,28,15,29,11), some controversy evidence contradicted the connection between FeMV and kidney disease (16,21). As far as more clear evidence regarding the FeMV has been concerned, I demonstrated that FeMV associated to renal tubular and interstitial lesions rather than glomerular lesions, particularly lesions specific to chronic kidney disease (CKD) such as tubular atrophy, urinary cast, interstitial inflammation, and renal fibrosis based on a statistical analysis between a histopathological score of the kidney disease and incidence of FeMV

examined by immunohistochemistry (IHC) and immunofluorescence (IFA) assays in the previous report (28). Although the pathological changes have been found associated with the localization of FeMV antigen at the microscopic level, the viral pathogenesis mechanism behind the renal injury and inflammation according to FeMV infection has not been investigated.

Apoptosis, or programmed cell death mechanism, which is an essential process for cellular injury responses to viral infection, is associated with morbillivirus infection. For instance, CDV associated with apoptosis in the central nervous system and lymphoid system leading to lymphoid depletion and immunosuppression *in vivo* (51-54) and induce apoptosis in Vero cells (55) and HeLa cells (56) *in vitro*. MV induces cellular apoptosis and necrosis through interaction between viral nucleoprotein and host cell receptors in MV infected cells *in vitro* (57) and also promotes human peripheral blood mononuclear cell apoptosis via interaction of hemagglutinin with the cellular receptor to induce caspase-dependent pathway (58). In ruminant, PPRV (59) and RPV (60) induce peripheral blood mononuclear apoptosis, particularly lymphocytes in goats and cattle.

To study whether apoptosis is the potential viral pathogenesis mechanism induced by FeMV, I performed a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which is the apoptotic assay together with immunofluorescence double-labeling assay with FeMV P-protein antibody to find whether there is a correlation between the numbers of apoptotic cells and FeMV-infected cells.

2-2. Materials and Methods

Reagents and chemicals

All chemical reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and used as received unless otherwise stated.

Cat kidney samples and tissue fixation

Thirty-eight kidney samples were collected from mixed breeds and gender cats with various disease conditions, which were admitted from animal clinics. The samples I used in this experiment were the same sample from the experiment in Chapter I. Urine and other samples were not provided in this experiment. All experimental protocol used in this study were reviewed and approved by the Ethics Committee for Animal Study and Research at the Faculty of Agriculture, Tokyo University of Agriculture and Technology (Approval Number 0016015).

All kidney samples were prepared as formalin-fixed paraffin-embedded tissues (FFPE). In brief, the kidneys were fixed in the 10% neutral buffered formalin and the fixative was covered the whole tissues. The samples were cut into in the midsagittal plane and placed in embedding cassettes. Then, the samples were processed as FFPE kidney tissues. The samples were incubated in reagents as follows, two times of 70% ethanol for 1 h, 80% ethanol for 1 h, 95% ethanol for 1 h, three times of 100% ethanol for 1.5 h, three times of xylene for 1.5 h, and two times of paraffin wax at 58-60°C for 2 h and embedded into paraffin blocks for sectioning process.

The FFPE kidney samples were sectioned at 6 μm with microtome and place paraffin ribbon in a water bath at 42°C and mounted onto adhesive slides, Platinum Pro micro slide glass (Matsunami, Osaka, Japan). The sections were airdried overnight or incubate into 45-50°C at least for 3 h before further process. Deparaffinization was performed by incubating the section with xylene for 5 min in three times and rehydrated by incubating with 100% ethanol

for 5 min in three times and washed with PBS for 5 min in three times before immunostaining process.

Antibody for FeMV P antigen detection

The antibody for FeMV P antigen detection in this experiment was produced with the same method from the antibody I used in Chapter I, as described previously by expressing and purifying the recombinant FeMV P protein (27). Briefly, the total RNA was isolated from FeMV positive samples, reversed transcribed and amplified by PCR as described in detail in Chapter I. The P gene was first ligated in to a TA cloning vector plasmid, pMD20-T (Takara, Tokyo, Japan) and digested at SalI and NotI before inserting the the P gene into pGEX4T-1 (GE Healthcare, Tokyo, Japan) and were transformed into *Escherichia coli* BL21 (DE3) (Cosmo Bio, Tokyo, Japan) for gene expression as a glutathione S-transferase (GST)-fused protein. The recombinant FeMV P-GST fusion protein was used for producing a specific antibody against FeMV P protein by a commercial service, as described in detail in Chapter I (Sigma-Aldrich®, Tokyo, Japan).

***In situ* apoptosis assay Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and immunofluorescence double-labeling assay with FeMV antibody**

After sectioned, FFPE kidney tissues were deparaffinized and rehydrated as a routine staining procedure. The antigen retrieval process was performed by incubating the sections with 0.5% Triton X for 10 min at a room temperature. In each step of the staining procedure, the sections were washed with phosphate buffered saline containing 0.02% Tween 20 (PBS-T) for 5 min three times whenever the incubating reagents were changed. For non-specific blocking, the sections were incubated with 10% goat serum for 1 h before primary antibody incubation.

The FeMV P protein antibody (1:400 dilution) was used as a primary antibody for incubating the sections for 1 h at room temperature in a moist chamber. After washed with PBS,

the sections were incubated with labeling reaction mixture from Takara In Situ Apoptosis Detection Kit (Takara, Tokyo, Japan) consisted of TdT enzyme, labeling safe buffer and Alexa Fluor 546Fab α anti-rabbit IgG (H+L) antibody (1:1000 dilution) as a secondary antibody (Invitrogen, Tokyo, Japan) for 1 h at room temperature. The sections were washed with PBS-T and incubated with Anti-fluorescein isothiocyanate (FITC) horseradish peroxidase (HRP) conjugate provided from the reagent kit for 30 min at room temperature. The counterstained was performed by incubating the sections with DAPI (1:1000 dilution) (DOJINDO, Tokyo, Japan) for nuclear staining for 5 min.

All double-stained slides were mounted with ProLong™ Glass Antifade Mountant (Invitrogen, Tokyo, Japan), covered with a coverslip, Neo mico cover glass (Matsunami, Osaka, Japan), and observed by a fluorescence microscope with a digital camera system (FSX 100 Olympus, FSX-BSW Ver 02.02, Tokyo, Japan). Recording pictures were taken at 20x magnification in 10 different fields of each sample for further analysis. The number of TUNEL positive cells and colocalization of TUNEL positive cells with FeMV infected cells was counted manually from the digital recorded image. The total number of nucleated cells stained by DAPI was calculated automatically from the digital recorded image. All manual and automatic cell counting were performed on the ImageJ program version 1.52a (National Institutes of Health, Maryland, USA).

Statistical analysis

Quantitative data were presented as Mean ± SEM values. The normality of data was performed by the Kolmogorov-Smirnov normality test, and homogeneity of variance was conducted by Levene's test before analyzing the data for a parametric statistic (the two-sided t-test, $p<0.05$ was taken as statistically significant) for the statistical analysis using the IBM SPSS PASW® Statistics Version 18.0.0 (Mahidol University license, New York, USA).

2-3. Results

Immunofluorescence of FeMV antigens

In the IFA assay of 38 kidney tissues using anti-FeMV P protein, 12 samples (31.58%) were diagnosed FeMV positive, and 26 samples (68.42%) were FeMV negative based on the localization of FeMV P-protein which was present in renal tissues such as proximal tubules, distal tubules, and collecting ducts as described previously (28).

TUNEL and immunofluorescence double-labeling assay with FeMV antibody

To determine the effect of FeMV infection on apoptosis as a potential mechanism for the viral pathogenesis and to find whether FeMV promotes apoptosis in infected tissues or surrounding tissues, a TUNEL assay was performed together with immunofluorescent staining for the FeMV antigen. By using TUNEL and immunofluorescence double-labeling assay with FeMV P protein antibody, a green signal of immunofluorescence indicated the TUNEL positive cells ($TUNEL^+$) (Fig. 2-1A, 2-2A), suggesting these cells were undergoing through the apoptotic process. A red signal of immunofluorescence represented the localization of FeMV P protein antigen ($FeMV^+$ cells) (Fig. 2-1B, 2-2B), and nuclei were stained by DAPI ($DAPI^+$) (Fig. 2-1C, 2-2C) cells which were shown as a blue signal. The colocalization of $TUNEL^+$ was observed together with the localization of the FeMV antigen signal in renal tubules (Fig. 2-1F, 2-2F).

Based on these images, I quantitated the number of $TUNEL^+$ cells, $DAPI^+$ cells, and colocalization of $TUNEL^+$ $FeMV^+$ cells counted by the ImageJ program in FeMV positive and negative tissues. The results were statistically analyzed using the parametric test, independent t-test ($p<0.05$) (Table 2-1.) I found that the total average of nucleated number based on $DAPI^+$ cell in FeMV positive (1045.33 ± 12.00) was higher than FeMV negative group (856.31 ± 43.43) ($p = 0.03$) (Fig. 2-3A). In this study, I found that average numbers of $TUNEL^+$ cells (Fig.

2-3B) were statistically higher in FeMV positive group (85.17 $\pm$ 12.71) compared to FeMV negative group (22.46 $\pm$ 4.11) ($p<0.0001$). To exclude counting of the dead cells, according to necrosis, I normalized the $TUNEL^+$ cells with the number of the $DAPI^+$ cells described as the ratio of $TUNEL^+$ cells and $DAPI^+$ cells. I found that the ratio of $TUNEL^+$ cells per the numbers of $DAPI^+$ cells in FeMV infected group (0.08 $\pm$ 0.01) was statistically higher than FeMV un-infected group (0.03 $\pm$ 0.01) ($p<0.0001$) (Fig. 2-3C).

In this study, the colocalization of the TUNEL signal and FeMV P-protein antigen ($TUNEL^+$ $FeMV^+$; Fig. 2-1F, 2-2F) was also found. The localization of FeMV P protein antigen in the renal tubules (the total number of $TUNEL^+$ $FeMV^+$ was 30.17 $\pm$ 4.45 and the ratio of $TUNEL^+$ $FeMV^+$ /Total $TUNEL^+$ cells was 0.37 $\pm$ 0.05). Based on these results, our results indicated that FeMV induces renal apoptosis in the FeMV infected cells and surrounding tissues as I found the statistical significance of the number of apoptotic cells in FeMV infected group compared to FeMV un-infected group.

2-4. Discussion

In this study, the FeMV antigens localized in the renal epithelial cell structures, including proximal, distal, collecting ducts as described previously (18,26,28,10,17). Even though many studies reported the association between FeMV infection and kidney diseases (13,26,29,28,10), a viral pathogenesis mechanism of FeMV infection-causing lesions associated with CKD has been remained unclear.

According to the renal lesions adjacent with FeMV antigen found connected to CKD (28), virus-induced apoptosis is possible to be a pathogenesis mechanism to cause renal tissue injury leading to CKD. Even though apoptosis is responsible for either homeostatic mechanism or defense mechanism (36), the study of the virus inducing apoptosis should be considered carefully either quantitative or qualitative detection assay such as observing the cytomorphological alteration, detecting the DNA fragmentation, checking the level of caspases or cleaved substrates, and investigating the membrane alteration visualized either light microscopy or fluorescent microscopy (36).

In this study, the total nucleated cells were higher in a group of FeMV positive than a group of FeMV negative. The reason is DAPI or 4′,6-diamidino-2-phenylindole is a fluorescent dye strongly binding to adenine-rich regions in DNA; therefore. DAPI will bind not only the cells, which are the main component of renal structure but only bind to the moveable cells such as inflammatory cells, which was attracted during the viral infection and inflammation. Therefore, I observed that the total number of nucleated cells was higher in the FeMV group than FeMV negative group.

In this study, I determined the number of apoptotic cells using the TUNEL and immunofluorescence double-labeling assay to detect the localization of FeMV P protein antigen quantitated by the ImageJ program. I detected the significantly higher numbers of the apoptotic cells and the ratio of $TUNEL^{+}$ to $DAPI^{+}$ in FeMV-positive than negative tissues,

suggesting that FeMV infection induces renal cell apoptosis in FeMV infected cat kidney tissues as the colocalization of TUNEL signal found together with the localization of FeMV P-protein antigen. Therefore, this result confirms the possibility that apoptosis contributes to pathological changes localized together with FeMV antigen, as described previously (28).

Apoptosis is one of the potential mechanisms in Genus *Morbiliviruses*; for example, CDV, MV, PPRV, and RPV induce apoptosis *in vivo* and *in vivo* of the central nervous system, lymphoid tissues, and peripheral blood mononuclear cells leading to immunosuppression condition (51-56,58,57,59,60). Therefore, the viral mechanism to promote apoptosis can be either the intrinsic pathway or cytokine induction for triggering the apoptotic pathways (38).

Although the connection of Morbillivirus infection and apoptosis remains unclear in kidney tissues, pieces of evidence showed the connection of other viruses in different genus inducing apoptosis in kidney tissues such as human immunodeficiency virus-1 (HIV-1) induced renal tubular epithelial cell apoptosis via caspase activation and Fas upregulation in case of HIV-associated nephropathy (39). Middle East respiratory syndrome coronavirus (MERS-CoV) triggered apoptotic induction and induced renal failure through the upregulation of Smad7 and fibroblast growth factor 2 (FGF2) in kidney cells (40).

In conclusion, FeMV is the only one of morbilliviruses directly targeting the kidney tissues causing CKD in cats and promote apoptosis in kidney tissues in FeMV infected cats. However, the apoptotic mechanism regarding the interaction of viral protein and cellular receptors and apoptotic pathways is required to be identified and has not been investigated in this study. Therefore, this study suggests further research of the apoptotic pathway associated with FeMV infection either *in vitro* or *in vivo* study, which is possible to be related to pathological lesions associated with CKD.

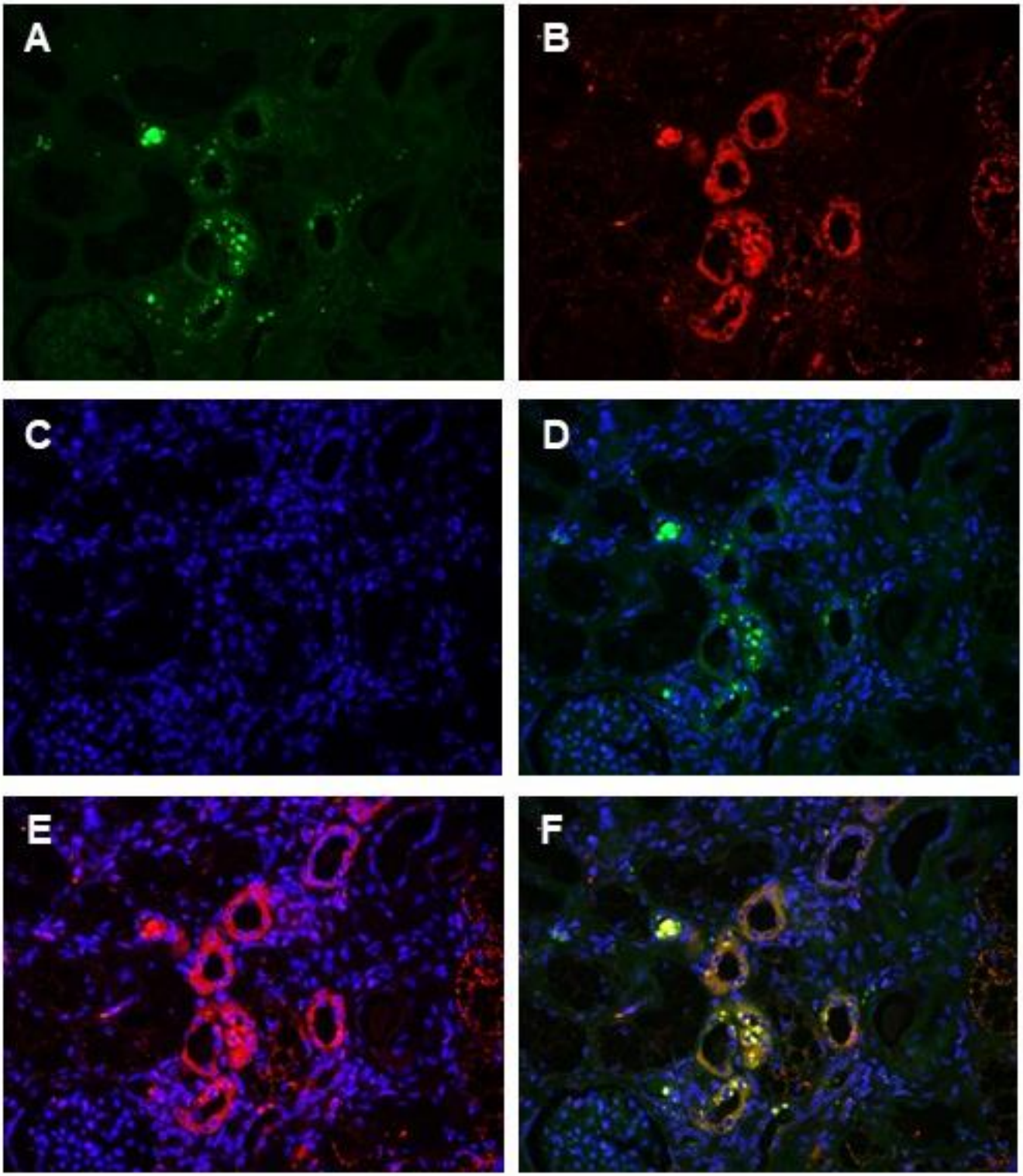

A
B
C
D
E
F

Fig. 2-1. Representative image of FeMV-positive sample with double-labeling with TUNEL assay and IFA with antibody against FeMV P-protein (A) The green signal indicated $TUNEL^+$ cells, suggesting these cells were undergoing the apoptotic process. (**B)** The red signal shows $FeMV^+$ cells of the P protein antigen. **(C)** $DAPI^+$ cells **(D)** Merged image of TUNEL and DAPI signal **(E)** Merged image of FeMV P protein antigen, and DAPI signal (F) Merged image of TUNEL, FeMV signal, and DAPI showed the colocalization of $TUNEL^+$ $FeMV^+$ (yellow signal) with the localization of FeMV P protein antigen. All images were taken at 20x magnification at the same imaging conditions (exposure time, gain and contrast) for fair comparison.

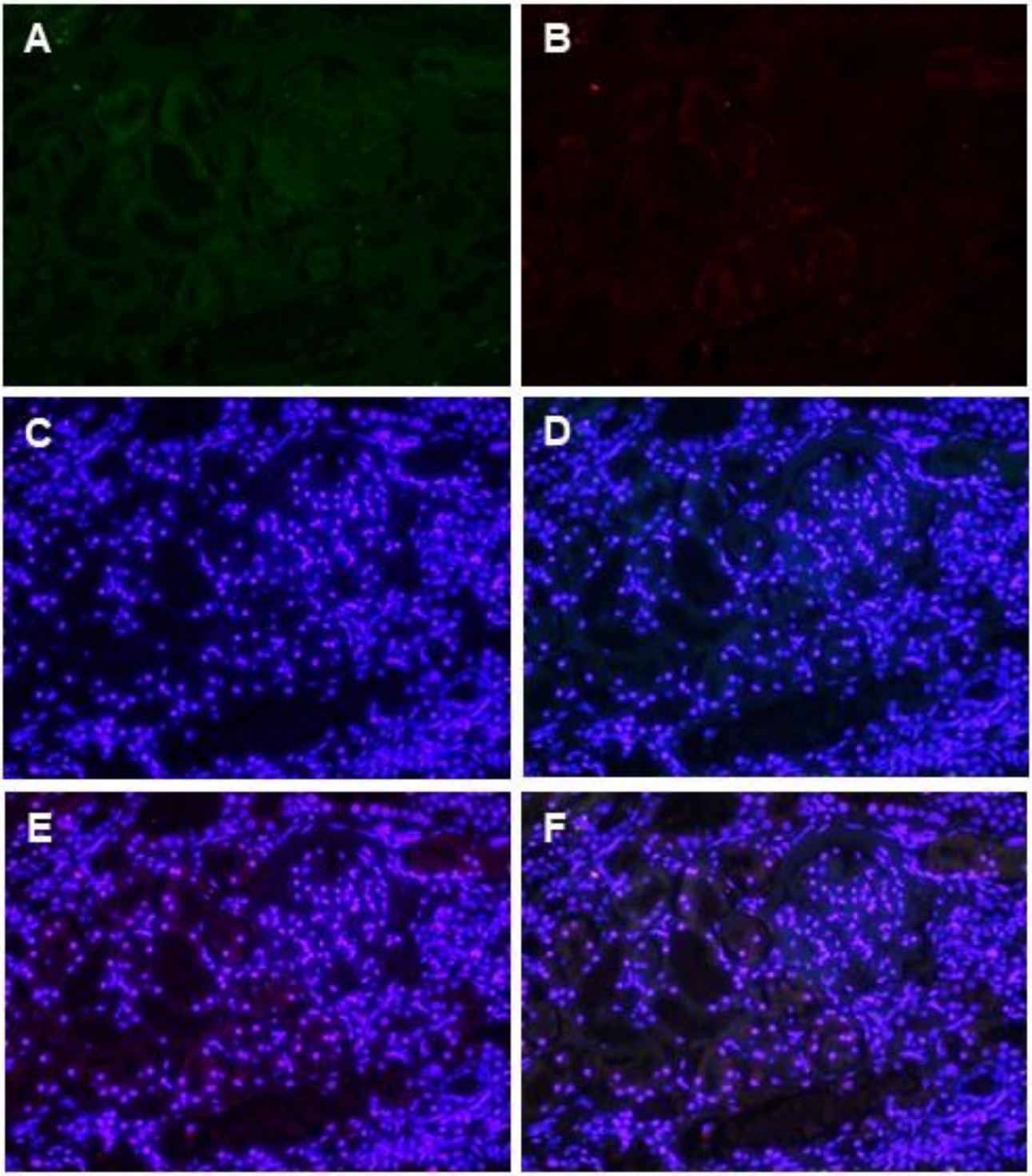

A
B
C
D
E
F

Fig. 2-2. Example image of FeMV-negative sample with double-labeling with TUNEL assay and IFA with antibody against P-protein (A) TUNEL signal (**B)** FeMV signal **(C)** DAPI signal **(D)** Merged image of TUNEL and DAPI signal **(E)** Merged image of FeMV P protein antigen, and DAPI signal **(F)** Merged image of TUNEL, FeMV signal, and DAPI. All images are in 20× magnification. All images were taken at 20x magnification at the same imaging conditions (exposure time, gain and contrast) for fair comparison.

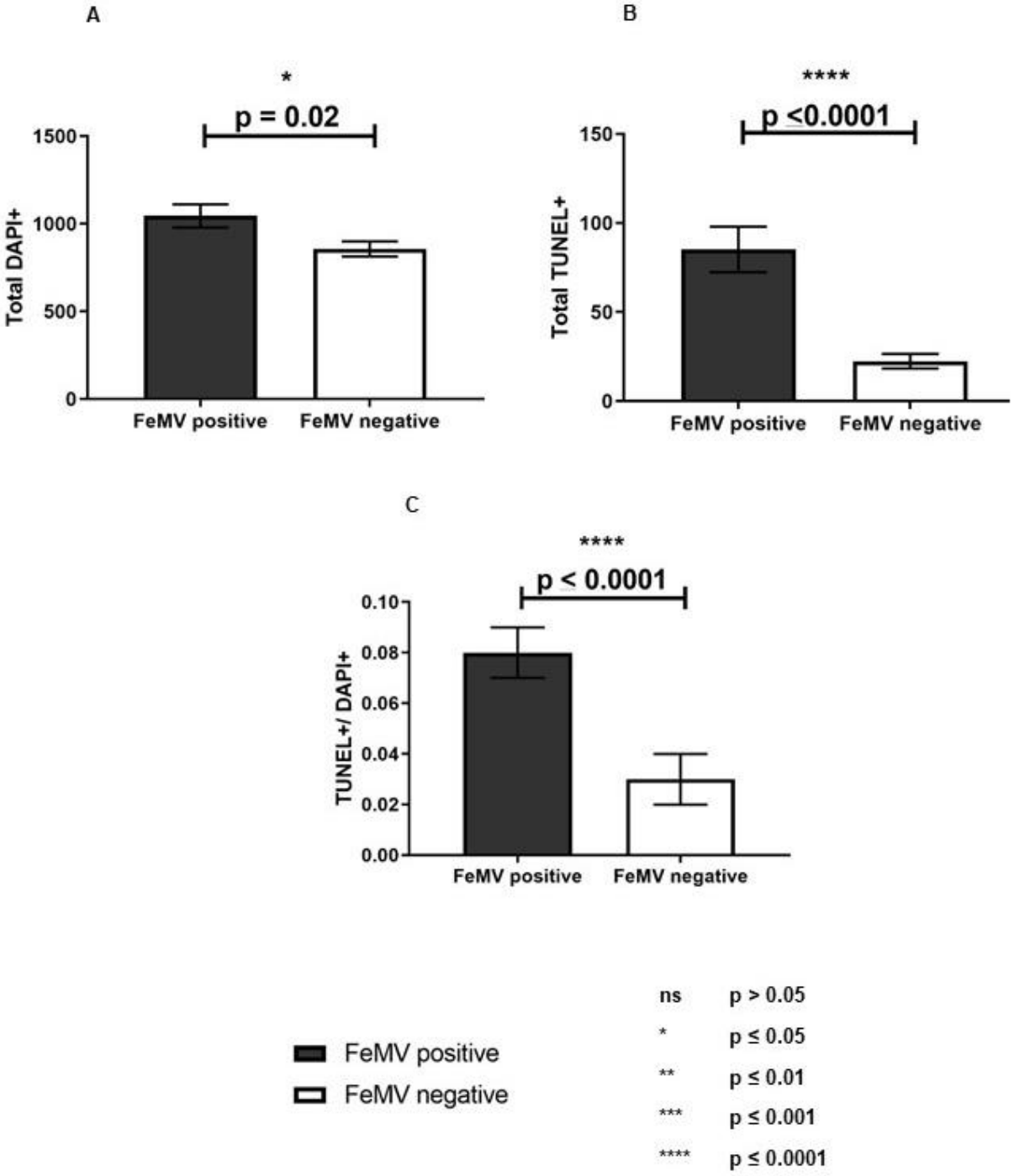

A
*
p = 0.02
1500
1000
500
0
Total DAPI+
FeMV positive
FeMV negative
B
****
p <0.0001
150
100
50
0
Total TUNEL+
FeMV positive
FeMV negative
C
****
p < 0.0001
0.10
0.08
0.06
0.04
0.02
0.00
TUNEL+/ DAPI+
FeMV positive
FeMV negative
FeMV positive
FeMV negative
ns p > 0.05
* p ≤ 0.05
** p ≤ 0.01
*** p ≤ 0.001
**** p ≤ 0.0001

Fig. 2-3. The study of renal tubular cell apoptosis induced by FeMV infection evaluated by TUNEL assays and double-labeling immunofluorescence assay with FeMV-specific P protein antibody counter-stained by DAPI. The number of each parameter was calculated from 20x magnification of 10 different fields of immunofluorescence digital image of each sample by using the ImageJ program. The percentage of FeMV infection assessed by FeMV P-protein Immunofluorescence was as follows, FeMV positive (12/35 samples; 31.58%) and negative (26/38 samples; 68.42%). **(A)** $DAPI^+$ positive cells counted automatically by the ImageJ program **(B)** Total $TUNEL^+$ cells counted manually by ImageJ program **(C)** The ratio of $TUNEL^+/DAPI^+$. The bar-chart represents mean and standard error of each parameter in FeMV P-protein positive and negative groups analyzed by non-parametric t-test ($p<0.05$) Significant differences are marked with ns ($p>0.05$), ** ($p \leq 0.05$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

Table 2-1. TUNEL$^+$, DAPI$^+$, and the colocalization of TUNEL$^+$ FeMV$^+$

Sample number	FeMV diagnosis	TUNEL$^+$ (cells/20x of 10 fields)	DAPI$^+$ (cells/20x of 10 fields)	TUNEL$^+$ /DAPI$^+$	TUNEL$^+$ FeMV$^+$	TUNEL$^+$ FeMV$^+$ /total TUNEL$^+$
1	+	198	1292	0.15	54	0.27
2	+	85	1372	0.06	45	0.53
3	+	105	1115	0.09	41	0.39
4	+	52	1034	0.05	14	0.27
5	+	50	580	0.09	13	0.26
6	+	69	1074	0.06	12	0.17
7	+	39	996	0.04	16	0.41
8	+	124	818	0.15	52	0.42
9	+	86	834	0.10	21	0.24
10	+	88	1147	0.08	35	0.40
11	+	41	949	0.04	34	0.83
12	+	85	1333	0.06	25	0.29
13	-	64	779	0.08	N/A	N/A
14	-	43	895	0.05	N/A	N/A
15	-	32	1167	0.03	N/A	N/A
16	-	55	1015	0.05	N/A	N/A
17	-	22	710	0.03	N/A	N/A
18	-	45	912	0.05	N/A	N/A
19	-	4	909	0.00	N/A	N/A
20	-	71	769	0.09	N/A	N/A
21	-	17	474	0.04	N/A	N/A
22	-	43	974	0.04	N/A	N/A
23	-	13	818	0.02	N/A	N/A

Sample number	FeMV diagnosis	TUNEL$^+$ (cells/20x of 10 fields)	DAPI$^+$ (cells/20x of 10 fields)	TUNEL$^+$ /DAPI$^+$	TUNEL$^+$ FeMV$^+$	TUNEL$^+$ FeMV$^+$ /total TUNEL$^+$
24	-	25	959	0.03	N/A	N/A
25	-	0	1021	0.00	N/A	N/A
26	-	20	1010	0.02	N/A	N/A
27	-	0	268	0.00	N/A	N/A
28	-	35	1120	0.03	N/A	N/A
29	-	0	734	0.00	N/A	N/A
30	-	9	769	0.01	N/A	N/A
31	-	1	770	0.00	N/A	N/A
32	-	21	748	0.03	N/A	N/A
33	-	14	868	0.02	N/A	N/A
34	-	6	677	0.01	N/A	N/A
35	-	8	546	0.01	N/A	N/A
36	-	2	1041	0.00	N/A	N/A
37	-	0	1297	0.00	N/A	N/A
38	-	34	1014	0.03	N/A	N/A

N/A: not available

8. GENERAL CONCLUSIONS

In recent years the new morbillivirus called feline morbillivirus (FeMV, or previously known as FmoPV) was discovered in Hong Kong in 2012. The original paper reported the association between FeMV infection with urological disease, particularly tubulointerstitial nephritis leading to renal failure based on a case-control study in domestic cats. Since 2012, FeMV has been reported from different countries and continents. Despite many reports tried to find out the connection between FeMV and kidney disease, there is some controversy regarding the biology and pathogenesis of this virus.

This thesis mainly focused on the study of the association of FeMV infection with defined pathological changes in cat kidney tissues and investigated a possible potential viral pathogenesis mechanism related to pathological lesions found adjacent with FeMV protein antigen in cat kidney tissues. Therefore, the summaries of the main findings of this thesis are as follows,

In Chapter 1, the study is focused on microscopic pathological changes found in the kidney of FeMV infected cats. This study revealed the significant pathological lesions found together with FeMV antigen investigated by immunohistochemistry and immunofluorescence for detecting the presence of FeMV viral antigen, especially both viral P-protein and viral N-protein.

In this study, I found that FeMV antigen either viral P and N proteins localized in renal epithelial tissues, including proximal renal tubules, distal renal tubules, collecting ducts, and transitional epithelium. Based on the statistical analysis between the score of pathological lesions and both immunohistological assays, the localization of FeMV antigen significantly found together with tubular lesions such as tubular atrophy, luminal expansion, and urinary cats. In the interstitial area, I found that inflammatory cell infiltration and renal fibrosis are significantly found with FeMV antigen. In comparison, glomerulosclerosis is the only lesion

found considerably in the glomerular area. Therefore, the results indicate a significant association between FeMV infection and tubular and interstitial lesion as the original reported described. Thus, this study provided insights pathological lesions regarding FeMV infection, suggesting further research regarding the viral pathogenesis mechanisms related to those pathological lesions, which I found significance difference between FeMV infected group and un-infected group statistically.

In Chapter 2, I studied potential mechanisms for the viral pathogenesis using TUNEL assay to find whether there was a correlation between the numbers of apoptotic cells and FeMV-infected cells. To find the effect of FeMV infection on the induction of apoptosis, I performed TUNEL assay together with immunofluorescent staining for the FeMV antigen. I found significantly more numbers of the cells with apoptosis in FeMV-positive tissues than FeMV-negative tissues, suggesting that FeMV infection induces apoptosis in infected cat kidney tissues and contributes to pathological changes associated with FeMV.

In conclusion, pathological lesions, including inflammatory cell infiltration, glomerulosclerosis, tubular atrophy, and fibrosis, were significantly associated with the presence of the localization FeMV antigen in renal epithelium. Those lesions were commonly associated with CKD, suggesting the strong association of FeMV infection with such diseases. I found more apoptotic cells in the kidney tissues infected with FeMV than FeMV negative tissues, suggesting induction of apoptosis as a potential pathogenic mechanism of FeMV, which is possible to be connected with those pathological lesions in the CKD.

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10. References

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