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NOTE

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Characterization of ferret Pit1 as a receptor of feline leukemia virus subgroup B

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ABSTRACT. Feline leukemia virus (FeLV) is a retrovirus that causes immune suppression and immunodeficiency, leading to opportunistic infections and leukemia/lymphoma in cats. Today, a variety of domestic mammals are kept in houses, and it is important to evaluate the possibility of interspecies transmission of FeLV. In this study, we assessed the infectivity of FeLV-B in ferrets that belong to Mustelidae. By pseudotype virus infection assay, we revealed that a ferret cell line, Mpf cells, is resistant to FeLV-B infection. The mRNA expression level of the FeLV-B receptor, Pit-1, was approximately half that of cat FEA cells in ferret Mpf cells. There was no significant difference in receptor usage between ferret's and cat's Pit1. These data may indicate the presence of the post-transcriptional modification and/or the restriction factor(s) against the FeLV-B infection in ferrets.

KEYWORDS: feline leukemia virus subgroup B, ferret, interspecies transmission, Pit1, restriction factor

Feline leukemia virus (FeLV) was first discovered in a cat suffering from lymphoma in 1964 [6]. Domestic cats infected with FeLV have been shown to develop immune suppression and immunodeficiency, and leukemia/lymphoma, leading to death within a few years of infection [4]. Therefore, FeLV infections are one of the most important infectious diseases in small animal veterinary medicine. FeLV belongs to the family *Retroviridae* and is classified into six subgroups, A, B, C, D, E, and T, based on the receptor usages defined by envelope protein [2, 9, 10]. The murine leukemia viral vector pseudotyped with the FeLV-B Env proteins can infect a wide range of hosts, including humans, dogs, foxes, and pigs, in addition to feline cells [13]. However, a mink cell line, Mv-1-Lu, showed scant susceptibility to FeLV-B [13], although minks belong to the order Carnivora as cats (Fig. 1). Minks are phylogenetically close to ferrets, which are famous domestic animals and sometimes are kept in houses with cats. Ferrets are reported to have a high incidence of lymphoma [1]; however, FeLV was not detected in ferrets that developed lymphoma according to a case report [3], and the risk of FeLV infection in ferrets is still unclear. Therefore, it is crucial to verify the susceptibility of ferret cells to FeLV-B *in vitro* for ascertaining potential interspecies transmission. In this study, we characterized the infectivity of the FeLV-B pseudotyped virus in a ferret cell line.

Infectivities of FeLV-B- and vesicular stomatitis virus (VSV)-peseudotype viruses were measured in a ferret cell line, *Mustela putorius furo* (Mpf) (ATCC CRL-1656) and feline embryonic fibroblast (FEA) cells. The Mpf and FEA cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 units/mL penicillin, and 10,000 µg/mL streptomycin (NACALAI TESQUE, Inc., Kyoto, Japan) at 37°C in a humidified atmosphere of 5% CO_2 in the air. We determined the infectivity of FeLV-B using a pseudotype virus of murine leukemia virus bearing FeLV-B Env. For the preparation of pseudotyped viruses, human embryonic kidney 293T cells were seeded in 6-well plates (5×10^5 cells/mL). After 24 hr, 293T cells were co-transfected with 700 ng of pMXs-*nls*LacZ (encoding LacZ with nuclear localization signal), 700 ng of pGag-Pol-IRES-bsr [11], and 700 ng of pFB-FeLV-B (GA) [13], pCAG-VSV-G or phCMV3 empty vector with 2 mL of Opti-MEM and transfected with Avalanche everyday transfection reagent (APRO SCIENCE., Tokushima, Japan). Forty-eight hr after transfection, the culture supernatant was passed through a 0.45 µm filter unit (PALL, Tokyo, Japan) and was stored at −80°C until used. Just before inoculation of the pseudotype viruses, polybrene (hexadimethrine bromide) was added to the inocula to be 8 µg/mL (final concentration). As a result, the FeLV-B pseudotype virus did not infect Mpf, although FEA cells were infected with the virus (Fig. 2), suggesting that the viral entry of FeLV-B is impaired in Mpf cells.

FeLV-B utilizes Pit1 as an infection receptor [7, 14]. To clarify whether the infection of FeLV-B depends on the expression levels of Pit1, we quantified the mRNA expression of Pit1 in Mpf and FEA cells by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). We designed primer sets of

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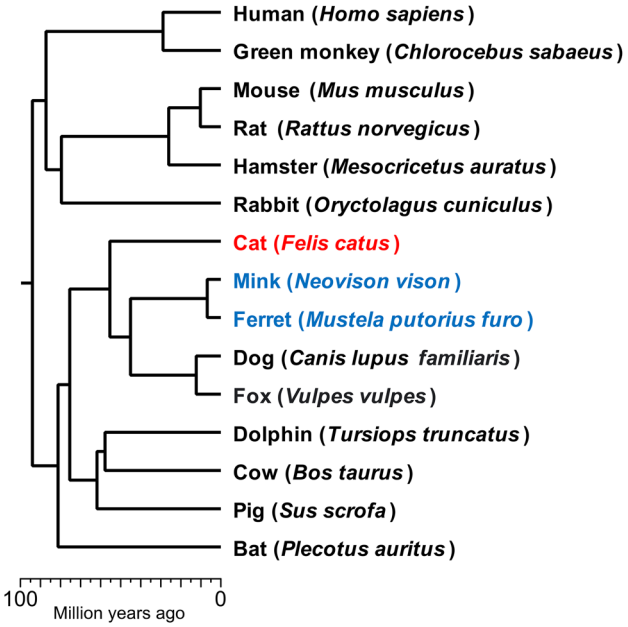

Fig. 1. A phylogenetic tree of mammals based on TimeTree [8]. *Mustelidae* diverged from the feline lineage about 55 million years ago. Mink and ferrets diverged relatively recently, about 5–10 million years ago, and are closely related.

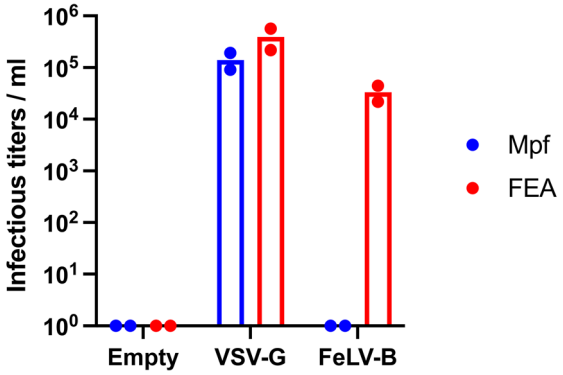

Fig. 2. Infectious titers of the FeLV-B-pseudotyped virus in Mpf and feline embryonic fibroblast (FEA) cells. On the day before infection, Mpf cells (1×10^5 cells) and FEA (4×10^5 cells) were seeded in a 96-well plate. Cells were inoculated with 80 µL/well step diluted pseudotyped virus. Experiments were repeated independently.

5′-TGGTATATGTCTGGAATTTTATTCTTCCTTGTTC-3′ (forward) in the 4th exon and 5′-AAGGTGCTCCAGTATACATGATGGA-3′ (reverse) in the 5th exon of Pit1. These primer sets are designed for consensus sequences to cats and ferrets Pit1. The RT-qPCR reactions were performed using the Power SYBR Green RNA-to-C1-Step Kit (Thermo Fisher Scientific). The reverse transcription (48°C for 30 min) and denature (95°C for 10 min) were followed by the denature (95°C for 10 sec) and extension (60°C for 1 min) with 40 cycles. PCR was carried out using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The RT-qPCR results revealed that the mRNA expression level of Pit1 in Mpf cells was approximately 0.5-fold in FEA cells (Fig. 3). This difference may contribute the reduction of susceptibility of Mpf cells to FeLV-B; however, it does not explain the complete or high resistance of Mpf cells to FeLV-B (Fig. 2).

Next, we performed gene-cloning of ferret and cat Pit1 to evaluate the receptor usages of the FeLV-B-pseudotype virus in *Mus dunni* tail fibroblast (MDTF) cells transiently expressing Pit1 of each species. For cloning of Pit1, cDNA was synthesized from the total RNA of Mpf and FEA cells using Verso cDNA synthesis kit (Thermo fisher scientific). The coding regions of ferret Pit1 (2,049 bp) and cat Pit1 (2,043 bp) were amplified by PCR using KOD One PCR Master mix (TOYOBO, Osaka, Japan). The PCR primer sets were 5′-CGAGCTTCGGGCCACCATGGGCATCTACCG-3′ (forward) and 5′-ACATCGTGATGGTGAGACTGTGAGGATGACATATATTTGAG-3′ (reverse) for ferret Pit1, and 5′-CGAGCTCAAGCTTC GCCACCATGGGCATTTACCGGGGGCAA-3′ (forward) and 5′-ACACATCGTGATGGGTGAGAGGATGACATATTTGAAGACTGC-3′ (reverse) for cat Pit1. Amplicons were cloned into EcoRI and BamHI sites of phCMV3 vector (Genlantis, San Diego, CA, USA) using NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs Inc., Ipswich, MA, USA). The inserted Pit1 sequence was confirmed by Sanger sequencing (Fasmac Co., Ltd., Tokyo, Japan) to be identical to the reference genome of the ferret (XM_004770998.3) and cat (XM_045053320.1), respectively. The plasmids expressing ferret and cat Pit1 are designated as phCMV3-Ferret-Pit1 and phCMV3-Cat-Pit1, respectively. For determining infectious titers, MDTF cells were seeded in 10 cm dishes (1×10^6 cells), and phCMV3-Ferret-Pit1 or phCMV3-Cat-Pit1 were transfected with Avalanche everyday transfection reagent (APRO SCIENCE). Six hr after transfection, MDTF cells were seeded in 96-well plates (1×10^5 cells/mL). Then, 24 hr after transfection, the pseudotyped virus was inoculated into target cells. Forty-eight hr after infection, cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and LacZ-positive foci were counted. We found that MDTF cells expressing ferret Pit1 were susceptible to FeLV-B infection. However, the infectious titers in ferret Pit1-expressing cells were slightly lower than cat Pit1-expressing cells (Fig. 4).

We revealed that ferret Mpf cells were resistant to FeLV-B infection as well as mink Mv-1-Lu cells. However, the efficient infectivity of the FeLV-B-pseudotype virus to MDTF cells expressing ferret Pit1 suggests that ferret-Pit1 can be utilized as a functional infection receptor of FeLV-B (Fig. 4). We consider that combination of the lower expression level of Pit1 in Mpf cells and the slightly low efficiency of viral entry via ferret-Pit1 cannot fully explain the resistance to FeLV-B in Mpf cells. Instead, the translation efficiency of Pit1, post-translational modification of Pit1, or restriction factors in Mpf cells may contribute to the resistance to FeLV-B. The efficiency of Pit1 translation and post-translational modifications (e.g., glycosylation) need to be verified by immunoblotting; however, as far as we know, antibodies that detect the common epitope of Pit1 between cats and ferrets are not commercially available, making it hard

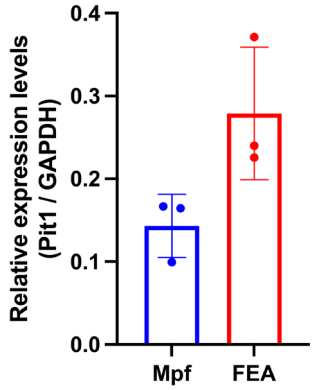

Fig. 3. Relative expression of Pit1 in Mpf and feline embryonic fibroblast (FEA) cells determined by RT-qPCR. GAPDH expression levels normalized relative expression levels of Pit1. Experiments were repeated three times independently. The qPCR product was confirmed identical to the target sequence by Sanger sequencing.

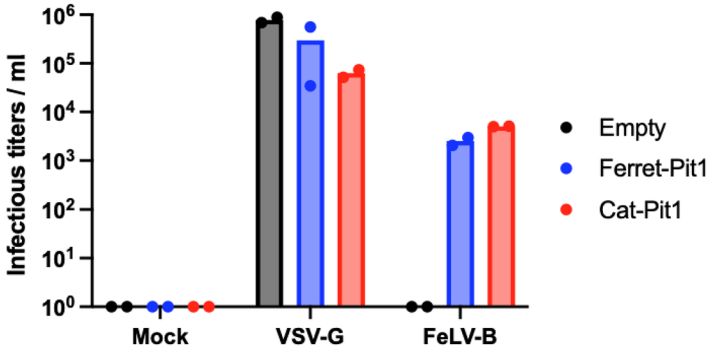

Fig. 4. The feline leukemia virus-B-pseudotyped virus infectious titers of ferret and cat Pit1. Experiments were repeated independently.

to verify the possibility. Endogenous retrovirus-derived restriction factors against retroviral envelope proteins have been identified in various animal species [12]. One example is Refrex-1 against FeLV-D infection in domestic cats [5]. Therefore, we performed a BLAT sequence search against the ferret genome (JIRA1106) using the surface unit of the FeLV-B (strain Gardner-Arnstein) used in this study as a query on the UCSC genome browser. As a result, some significant sequence hits were found, and they may be candidate restriction factors for Pit1-mediated infection of ferrets. Further studies are needed to reveal the mechanism of resistance of FeLV-B infection in Mpf cells.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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