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Systematic Identification of Endogenous Retroviral Protein-Coding Genes Expressed in Canine Oral Malignant Melanoma

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Endogenous retroviruses (ERVs) are remnants of ancestral retroviruses that infected host germ cells in the past. Most ERVs are thought to be non-functional elements, but some ERVs retain open reading frames (ORFs) capable of expressing proteins. The proteins encoded by ERV-ORFs have potential roles in oncogenesis; however, studies on mammals other than humans and mice are limited. Here, we identified ERV-derived genes expressed in canine oral malignant melanoma (OMM). We identified 11 ERV-derived genes in our OMM samples. Differential expression gene analysis revealed that four ERV-derived genes (*PEG10*, *LOC102155597*, and two newly identified genes) were upregulated in OMM compared to healthy tissues. *PEG10* is a conserved long terminal repeat (LTR)-type retrotransposon-derived gene among mammals and is involved in human cancers. *LOC102155597* is a retroviral *env* gene conserved in Carnivora. This Env protein harbors an immunosuppressive domain, implying the potential adverse effects on the immune system. While the production of viral particles from ERVs has been reported in human and mouse melanoma, we found no ERV-derived genes having the potential to produce viral particles. These results provide insights into the different and conserved features of ERV-derived genes in mammalian melanoma.

Keywords: dog, endogenous retrovirus, oral malignant melanoma, protein-coding gene, RNA-Seq

INTRODUCTION

Endogenous retroviruses (ERVs) are remnants of ancient retroviral infections to germ cells and have accumulated in all mammalian genomes (1). Retroviruses have three structural and enzymatic genes: *gag* gene encoding the major structural protein; *pol* gene encoding a polyprotein consisting of multiple proteins including protease, reverse transcriptase, RNase H, and integrase; *env* gene encoding the envelope protein. In addition, some retroviruses encode regulatory and/or accessory genes that control viral gene expression and/or suppress host defense mechanisms. Most ERVs have lost their open reading frames (ORFs) due to the accumulation of mutations. However, some genes

derived from ERVs are expressed as proteins in host cells when the ORFs are still intact because they are young ERVs, or when the ORFs are domesticated as *de novo* host genes (1).

In human melanoma, aberrant expression of ERVs has been reported to be associated with oncogenesis (2). Human ERV-K (HERV-K) is actively expressed in human melanoma, and their protein expression, as well as particle formation, have been reported (3, 4). ERV3-1, an *env* gene of HERV-R, is also reported to be expressed in colorectal cancers (5) and acute myelogenous leukemia (6). In mice, active expression of an ERV called MelARV has been reported in B16/BL6 melanoma cells (7, 8). Knockdown of MelARV reduces the aggressiveness of B16 melanoma in transplanted mice (9). They proposed a functional model in which Env protein of MelARV regulates regulatory T cells (9). Thus, aberrantly expressed ERV genes may be a promising therapeutic target for melanoma. However, the association between melanoma and ERVs in mammals other than humans and mice remains unresolved.

Long terminal repeat (LTR)-type retrotransposons that mainly correspond to ERVs comprise 8 and 10% of the human and mouse genomes, respectively (10, 11). The overall proportion of ERVs as well as their lineages varies greatly among mammalian species (12). In the dog genome, for example, a proportion of genomic regions corresponding to ERVs is relatively low at 4.85% (13). According to a comprehensive study identifying ERV-derived ORFs (ERV-ORFs) in the genome, the number of canine ERV-ORFs is one-third of those in humans (14). Therefore, the expressed ERV-derived genes in canine melanoma are supposed to be fewer than in human and mouse melanoma; however, comprehensive characterization of ERVs expressed in canine melanoma has not been performed.

Canine oral malignant melanoma (OMM) is a highly metastatic tumor in dogs, spreading to the lung, lymph nodes, and liver, and shows a grave prognosis (15). Surgical resection and radiation are first-line treatments for OMM cases with no metastasis; however, there are no effective systemic treatments after metastasis (15). Canine cancers, including OMM, have been proposed as research models for human cancer studies (16, 17). On the contrary, the applications of the cancer therapies developed in humans to dogs are also underway, such as the administration of immune checkpoint inhibitors (18, 19). These studies have shown the effectiveness of strategies targeting genes conserved in humans and dogs. Though most ERVs are not conserved in humans and dogs, considering the active expression of ERVs in human and mouse melanoma, it is plausible that ERVs, including canine-specific ones, are expressed in canine OMM. Since cancer-specific ERVs may not be detectable in normal tissues, *de novo* transcripts in OMM samples, in addition to the reference transcripts, are required for the comprehensive gene expression analysis. Here, we made a catalog of transcripts retaining the ERV-ORFs that are expressed in canine OMM samples and conducted differential gene expression analyses. Since ERVs are involved in oncogenesis in humans and mice, these canine ERVs have the potential to be new therapeutic targets and markers. This study also will provide a model for studying ERVs' common roles in melanoma across mammalian species.

TABLE 1 | Tumor location, breed, age, and gender.

Case	Age	Gender	Number of reads	Mapping rate (%)
1	Unknown	Unknown	76,852,209	93.94
2	13Y9M	Castrated male	83,562,483	94.29
3	Unknown	Unknown	81,698,415	93.14
4	8Y3M	Spayed female	64,190,554	92.68
5	16Y	Castrated male	82,291,328	92.87
6	Unknown	Unknown	83,826,245	94.46
7	14Y	Spayed female	74,349,680	93.93
8	9Y7M	Castrated male	79,606,779	94.20

Reads/Mapping rate.

MATERIALS AND METHODS

RNA Sequencing

Canine OMM samples ($n = 8$) were obtained following surgical resection as treatment at Animal Medical Center, Yamaguchi University (**Table 1**). The bleed of all dogs used in this study was Dachshund. The patient's owners were informed before sample collection. Total RNA was isolated from fresh OMM from eight dogs using RNA Premium Kit (FastGene #FG-81050). We collected mRNA by NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). Strand-specific libraries were generated by NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420) and indexed by NEBNext Multiplex Oligos for Illumina (NEB #E7335). All libraries were sequenced on Illumina NextSeq 500. We also obtained transcriptome sequencing data of canine OMM and oral healthy tissues (8 and 3 samples, respectively) from NCBI sequence read archives (https://www.ncbi.nlm.nih.gov/sra) (20) to confirm our RNA-seq analyses. Accession numbers and read mapping results were summarized in **Supplementary Table 1**.

Transcript Assembly

All paired-end FASTQ files were trimmed, and their sequence qualities were checked using Trim Galore (version 0.6.4) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Then, the filtered short reads were aligned to the canine reference genome (un-masked canFam3 genome from UCSC genome browser: https://hgdownload.soe.ucsc.edu/goldenPath/canFam3/bigZips/) using HISAT2 (version 2.1.0) (21). For each RNA-seq sample, we conducted transcript assemblies using StringTie2 (version 2.1.1) (21) with "–rf" options to specify strandedness. Resultant GTF files obtained from the eight canine OMM samples and the canine RefSeq GTF file (canFam3, downloaded from UCSC genome browser on June 15, 2021) were merged using StringTie2 with "–merge" option to

generate a merged GTF file. The "-m 500 -f 0.05" options were combined to improve the specificity of the transcript assembly. Each melanoma transcript identical to a RefSeq transcript was allocated to the RefSeq nomenclature. The resultant transcript assemblies are available in **Supplementary File 1** (a compressed GTF file).

TABLE 2 | Curated 11 ERV-derived genes, TPMs, and overlapped ERV-ORFs.

RefSeq name	AverageTPM[a]	ERV-ORF (gEVE ID)	Amino acid length[b] (ATG-starting)	Overlapping ratio[c]	HMM profile
NA (NMG-1)	1.28	Cfam31.chr1.17538.18311.-	258 (222)	1	POL
		Cfam31.chr1.18315.18689.-	125 (3)	1	RNaseH
		Cfam31.chr1.18799.19509.-	237 (212)	1	GAG
		Cfam31.chr1.19475.19843.-	123 (109)	0.37	GAG
ASPRV1	2.11	Cfam31.chr10.68586978.68587886.-	303 (263)	1	AP
LOC102155597	9.56	Cfam31.chr12.71944605.71944928.-	108 (0)	0.37	POL
		Cfam31.chr12.71944903.71945781.-	293 (224)	1	POL
		Cfam31.chr12.71945669.71946616.-	316 (271)	1	POL
		Cfam31.chr12.71946828.71947217.-	130 (130)	1	RT
		Cfam31.chr12.71947221.71947559.-	113 (70)	1	AP
		Cfam31.chr12.71947774.71948322.-	183 (141)	1	PRO
		Cfam31.chr12.71948376.71948984.-	203 (170)	1	GAG
		Cfam31.chr12.71948785.71949063.-	93 (75)	1	GAG
		Cfam31.chr12.71949067.71950053.-	329 (320)	1	GAG
LOC111098614/LOC102154912	14.94	Cfam31.chr13.45478479.45479102.+	208 (168)	0.21	INT
		Cfam31.chr13.45479000.45479401.+	134 (27)	0.49	POL
		Cfam31.chr13.45480576.45480908.+	111 (87)	0.85	ENV
PEG10	11.11	Cfam31.chr14.20127798.20128877.+	360 (305)	1	GAG
		Cfam31.chr14.20128379.20129443.+	355 (163)	1	AP
NA (NMG-2)	2.01	Cfam31.chr2.10127277.10127657.-	127 (108)	0.36	GAG
LOC111093532	18.55	Cfam31.chr31.38015118.38015360.-	81 (68)	1	ENV
		Cfam31.chr31.38015533.38016231.-	233 (29)	1	ENV
		Cfam31.chr31.38016117.38016590.-	158 (34)	1	ENV
		Cfam31.chr31.38016809.38017414.-	202 (188)	0.52	INT
		Cfam31.chr31.38017418.38018188.-	257 (150)	0.12	POL
LOC111094052	1.65	Cfam31.chr35.24271063.24272484.+	474 (447)	1	ENV
LOC111094054	7.17	Cfam31.chr35.24996009.24996902.-	298 (194)	0.80	GAG
		Cfam31.chr35.24996945.24997460.-	172 (71)	0.28	GAG
		Cfam31.chr35.24991408.24991809.-	134 (31)	1	ENV
		Cfam31.chr35.24991822.24992172.-	117 (107)	1	ENV
		Cfam31.chr35.24992111.24992359.-	83 (21)	1	ENV
		Cfam31.chr35.24992564.24992989.-	142 (0)	1	ENV
		Cfam31.chr35.24993046.24993933.-	296 (187)	1	POL
		Cfam31.chr35.24995423.24995950.-	176 (104)	0.72	POL
NA (NMG-3)	5.12	Cfam31.chr8.4429849.4430145.+	99 (68)	0.10	INT
NA (NMG-4)	3.56	Cfam31.chrX.107190737.107191321.+	195 (110)	0.30	GAG
		Cfam31.chrX.107193576.107193956.+	127 (7)	1	RNaseH
		Cfam31.chrX.107194162.107194650.+	163 (129)	1	INT
		Cfam31.chrX.107194524.107194814.+	97 (73)	1	INT
		Cfam31.chrX.107195119.107195367.+	83 (9)	1	ENV

TPM, Transcripts per kilobase million; HMM profile, significant retroviral motif profiles obtained by hidden Markov models (HMM); NMG, newly identified melanoma gene; AP, aspartic protease; INT, integrase.
[a] *TPM values are mean values of eight canine oral malignant melanoma samples.*
[b] *Including non-ATG starting.*
[c] *Ratios of ERV-ORFs overlapping with transcripts.*

Detection of ERV-Derived Genes

A BED file of canine ERV-ORFs was downloaded from gEVE (version 1.1) (http://geve.med.u-tokai.ac.jp/) (14). In gEVE, *pol* genes that were thought to be derived from long interspersed nuclear elements (LINEs) were included (14). To remove LINE-derived sequences, ERV-ORFs which were annotated as "LINE" by RepeatMasker and/or "YP_073558.1" or "NP_048132.1" by BLASTP against the NCBI Viral Genome Database were removed. Transcripts in the merged GTF file overlapped with ERV-ORFs were identified using BEDtools intersect with "-s" option to consider strandedness (22). Forty-three ERV-derived genes detected at this step were listed in **Supplementary Table 2**. All ERV-derived genes were manually checked using Integrative Genomics Viewer (version 2.4.9) (23). Transcripts per kilobase million (TPM) was calculated using StringTie2 with "-e –rf" options using the merged GTF file. Eleven ERV-derived genes with the mean TPM > 1 were listed including *Canis-env2* (**Table 2**). Differentially expressed genes were identified and visualized with an MA plot and a volcano plot using DESeq2 (version 1.26.0) (24) in R (version 3.6.1).

RT-PCR

Total RNA was extracted from cultured OMM cells from four animals (**Supplementary Table 3**), which were maintained in Dulbecco's modified Eagle medium (Sigma-Aldrich #D5796) supplemented with 10% heat-inactivated fetal bovine serum (Gibco #10270), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Nacalai tesuque # 09367-34). To synthesize the cDNA, RNA was reverse transcribed using Verso cDNA Synthesis kit (Thermo Fisher Scientific #AB1453B). For negative controls, samples without reverse transcriptase were prepared. PCR was performed using KOD One (TOYOBO #KMM-101). Primer pairs used for NMG-1 and NMG-4 were 5′-ACCCGCTGACTATGACTCAGGAAC-3′ (forward) and 5′-AACAGTCTTTGCCTCTGCTGTCAG-3′ (reverse), and 5′-AGGCACTCCTCCACGCCACTACTAG-3′ (forward) and 5′-TCAAGTCTGTGCTTATGTAGGGACCAG-3′ (reverse), respectively. For loading control, GAPDH was amplified with a primer pair, 5′-AAGGTCGGAGTGAACGGATTTGG-3′ (forward) and 5′-CGGTTGCTGTAGCCAAATTCATTG-3′ (reverse). After preheating at 94°C for 2 min, thermal cycling reaction (98°C for 10 s and 68°C for 10 s) was repeated 35 times (for NMG-1 and NMG-4) or 30 times (for GAPDH). The sequences of the amplicons were checked by the Sanger sequencing (Fasmac Co., Ltd.).

Evolutionally Analyses of *Canis-Env2*

To collect *Canis-env2* orthologs in Carnivora genomes, coding sequences of *Canis-env2* in the dog was searched using Blat (25) in the following represented species in the UCSC genome browser (https://genome.ucsc.edu): Southern Sea otter, enhLutNer1 (Jun. 2019); Ferret, musFur1 (Apr. 2011); Hawaiian monk seal, neoSch1 (Jun. 2017); panda, ailMel1 (Dec. 2009); and Cat, felCat9 (Nov. 2017). The hits with the highest similarity scores were confirmed to be located between *BTN2A1* and *BTN1A1*. To conduct the following evolutionary analyses of *Canis-env2*, we used MEGA-X (26). We first aligned nucleotide sequences of *Canis-env2* genes by MUSCLE program with "Align codons" option (27), and pairwise *p*-distance values of amino acids, which are proportions of amino acid sites at which the two sequences to be compared are different, were calculated. The numbers of non-synonymous substitutions (*dN*) and synonymous substitutions (*dS*) per site were estimated by Nei-Gojobori method with Jukes-Canter correlation (28).

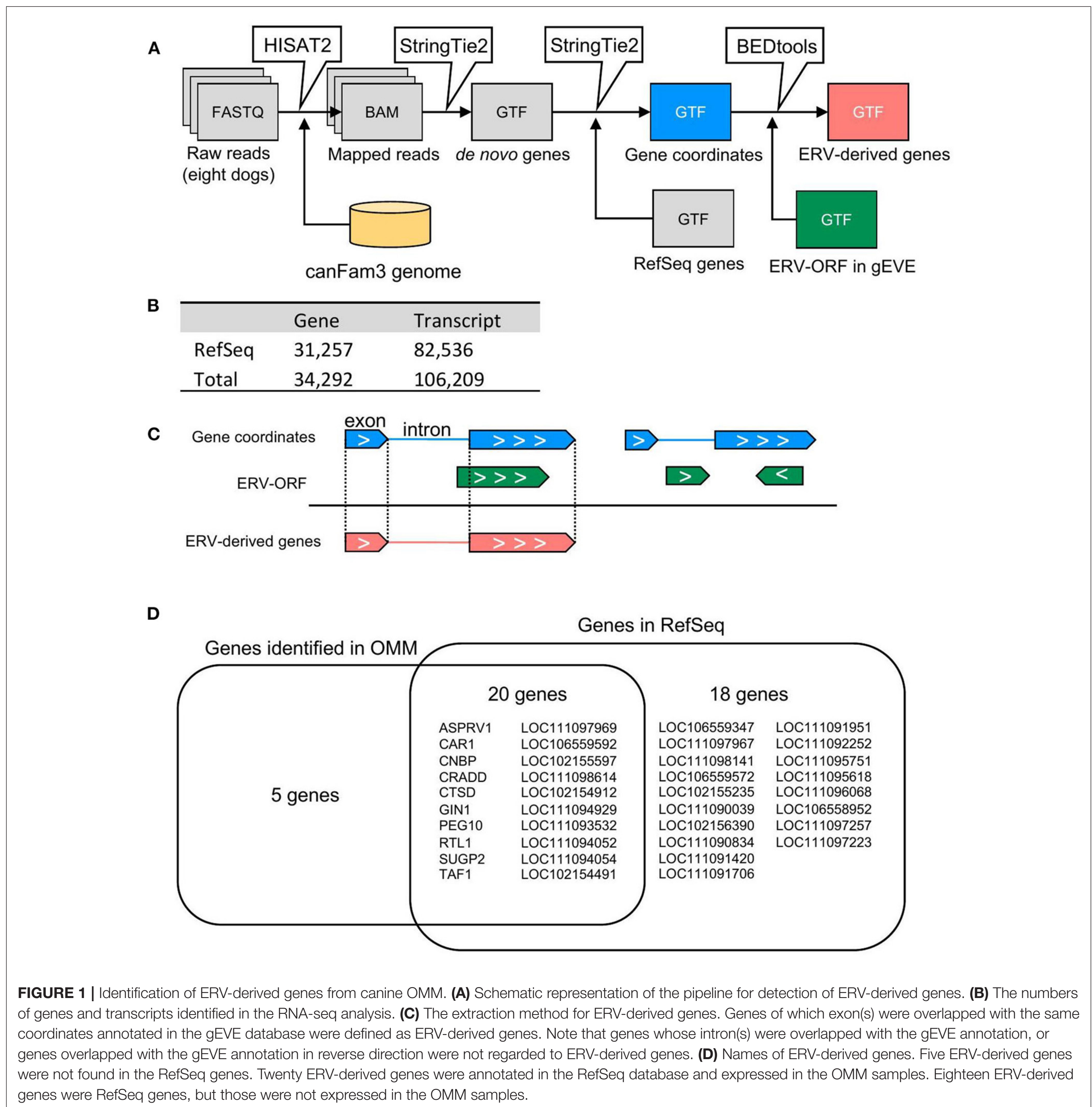
RESULTS

Identification of Transcriptionally Active ERV-Derived Genes in Canine OMM

We conducted RNA-seq analyses using canine oral malignant melanoma (OMM) cases ($n = 8$). To identify ERV-derived genes from these sequence data, we constructed a pipeline to identify ERV-ORFs expressed in canine melanoma (**Figure 1A**). First, we constructed *de novo* transcript assemblies with reference to the RefSeq gene coordinates using StringTie2. In this procedure, transcripts expressed from the overlapped locus were given a single gene name. In total, we obtained 106,209 transcripts from the 8 OMM RNA-seq samples, and these transcripts were organized into 34,292 genes, of which 3,035 genes were not reported in the reference gene annotation (**Figure 1B**). Next, we extracted genes whose transcripts were overlapped with ERV-ORFs in the gEVE database (http://geve.med.u-tokai.ac.jp/) (14) (**Figure 1C**). As a result, we obtained 43 genes, of which five genes were newly identified (**Figure 1D**, **Supplementary Table 2**).

Characterization of ERV-Derived Genes

Next, we examined the structures of these 43 genes overlapped with ERV-ORFs. In nine genes, ERV-ORFs were identical to full or partial coding sequences of host genes: *ASPRV1*, *CAR1*, *CNBP*, *CTSD*, *GIN1*, *PEG10*, *RTL1*, *SUGP2*, and *TAF1* (**Figure 2**). In these genes, four genes—*ASPRV1, CAR1, PEG10,* and *RTL1* — were known to originate from ERVs or LTR-type retrotransposons and mammalian or Carnivora-specific genes. *ASPRV1* codes an aspartic protease conserved in mammals and has roles in skin maintenance (29). *PEG10* and *RTL1* are *gag-pol* genes derived from Ty3/Gypsy retrotransposon and were involved in placental development (30, 31). *CAR1*, also known as *syncytin-Car1*, is a retroviral *env* gene conserved in Carnivora (32). Syncytin-Car1 protein is involved in the cell fusion of syncytiotrophoblast during placentation (32). We used these four definite retroviral genes for further analysis. The remaining five genes—*CNBP*, *CTSD*, *GIN1*, *SUGP2*, and *TAF1*—consist of several exons, and one exon identified as containing ERV-ORFs (**Figure 2A**). Orthologs of these exons were found in at least chickens, mice, and humans and may be derived from ERVs before the divergence of birds and mammals. This possibility has been particularly suggested for *GIN1*, where its integrase domain shows a high similarity to the GIN element, an animal-specific DNA transposon that encodes a Gypsy/Ty3 retrotransposon-like integrase (33). On



	Gene	Transcript
RefSeq	31,257	82,536
Total	34,292	106,209

FIGURE 1 | Identification of ERV-derived genes from canine OMM. **(A)** Schematic representation of the pipeline for detection of ERV-derived genes. **(B)** The numbers of genes and transcripts identified in the RNA-seq analysis. **(C)** The extraction method for ERV-derived genes. Genes of which exon(s) were overlapped with the same coordinates annotated in the gEVE database were defined as ERV-derived genes. Note that genes whose intron(s) were overlapped with the gEVE annotation, or genes overlapped with the gEVE annotation in reverse direction were not regarded to ERV-derived genes. **(D)** Names of ERV-derived genes. Five ERV-derived genes were not found in the RefSeq genes. Twenty ERV-derived genes were annotated in the RefSeq database and expressed in the OMM samples. Eighteen ERV-derived genes were RefSeq genes, but those were not expressed in the OMM samples.

the other hand, to the best of our knowledge, no evidence has been reported to indicate that *CNBP*, *CTSD*, *SUGP2*, and *TAF1* were derived from transposons. It is also plausible that these genes independently acquired protein motifs similar to ERVs. Since it is beyond the focus of this study to make conclusions, we did not regard these five genes as ERV-derived genes in this study. Two splicing variants of *CRADD* were overlapped with two ERV-ORFs, although they were in the 5′ UTR. Therefore, *CRADD* was also removed for further analyses. Others were uncharacterized genes in the RefSeq database (i.e., genes whose names start with *LOC*), including non-coding genes or newly identified non-RefSeq genes. To identify sufficiently expressing genes, we collected genes that were expressed in the eight canine OMM samples with an average TPM > 1. Finally, we identified 11 ERV-derived genes, including the newly identified melanoma genes 1 to 4 (*NMG-1* to *4*), which were

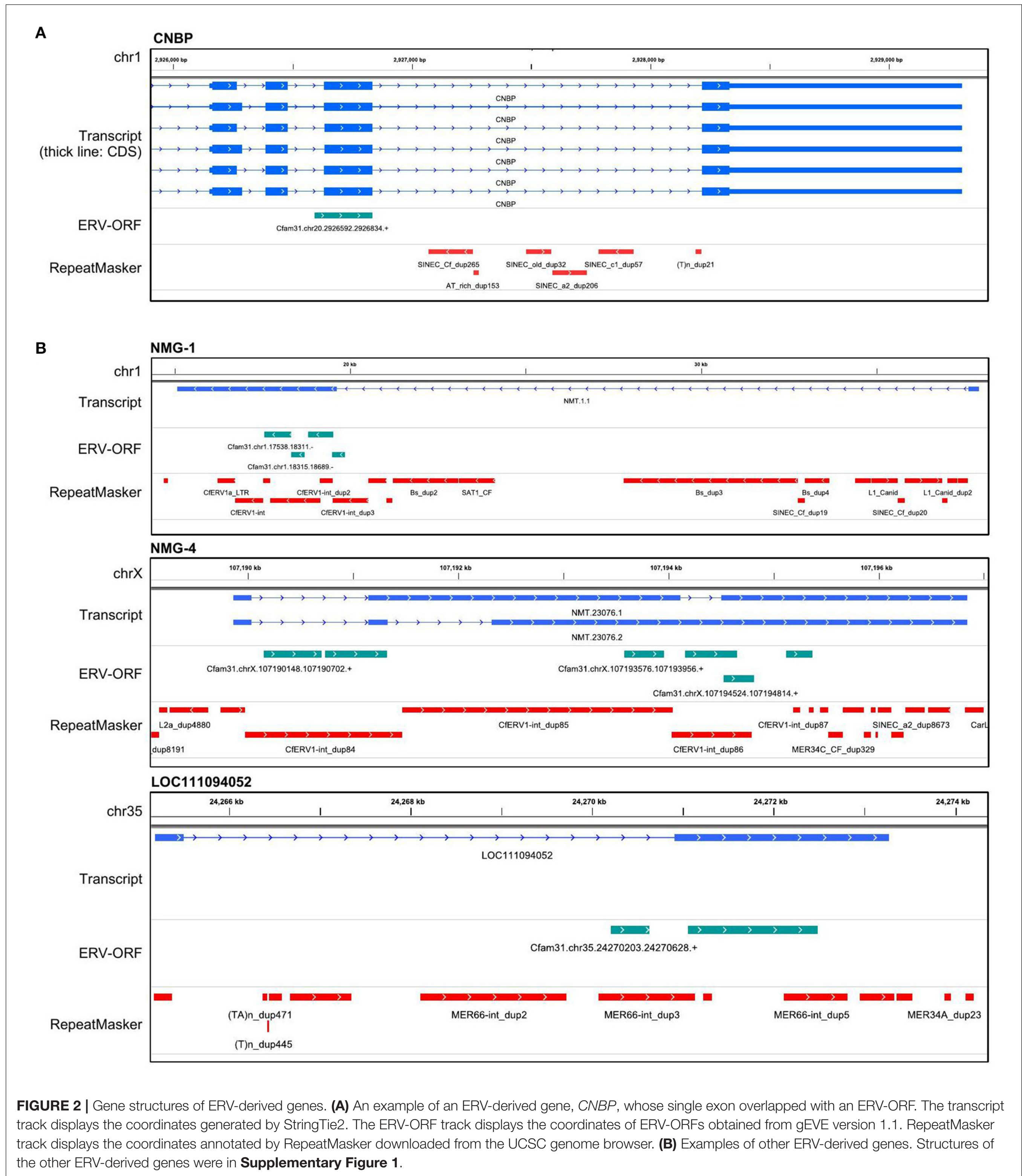


FIGURE 2 | Gene structures of ERV-derived genes. **(A)** An example of an ERV-derived gene, *CNBP*, whose single exon overlapped with an ERV-ORF. The transcript track displays the coordinates generated by StringTie2. The ERV-ORF track displays the coordinates of ERV-ORFs obtained from gEVE version 1.1. RepeatMasker track displays the coordinates annotated by RepeatMasker downloaded from the UCSC genome browser. **(B)** Examples of other ERV-derived genes. Structures of the other ERV-derived genes were in **Supplementary Figure 1**.

expressed in canine oral melanoma (**Table 2**). Since *NMG-1* to *4* overlapped with RepeatMasker track of an ERV group, CfERV1-int (**Figure 2B**; **Supplementary Figure 1**), these genes are presumed to be derived from ERVs.

Identification of Upregulated ERV-Derived Genes in Canine OMM

Next, we identified the ERV-derived genes that showed higher expression levels in the OMM samples than those in healthy

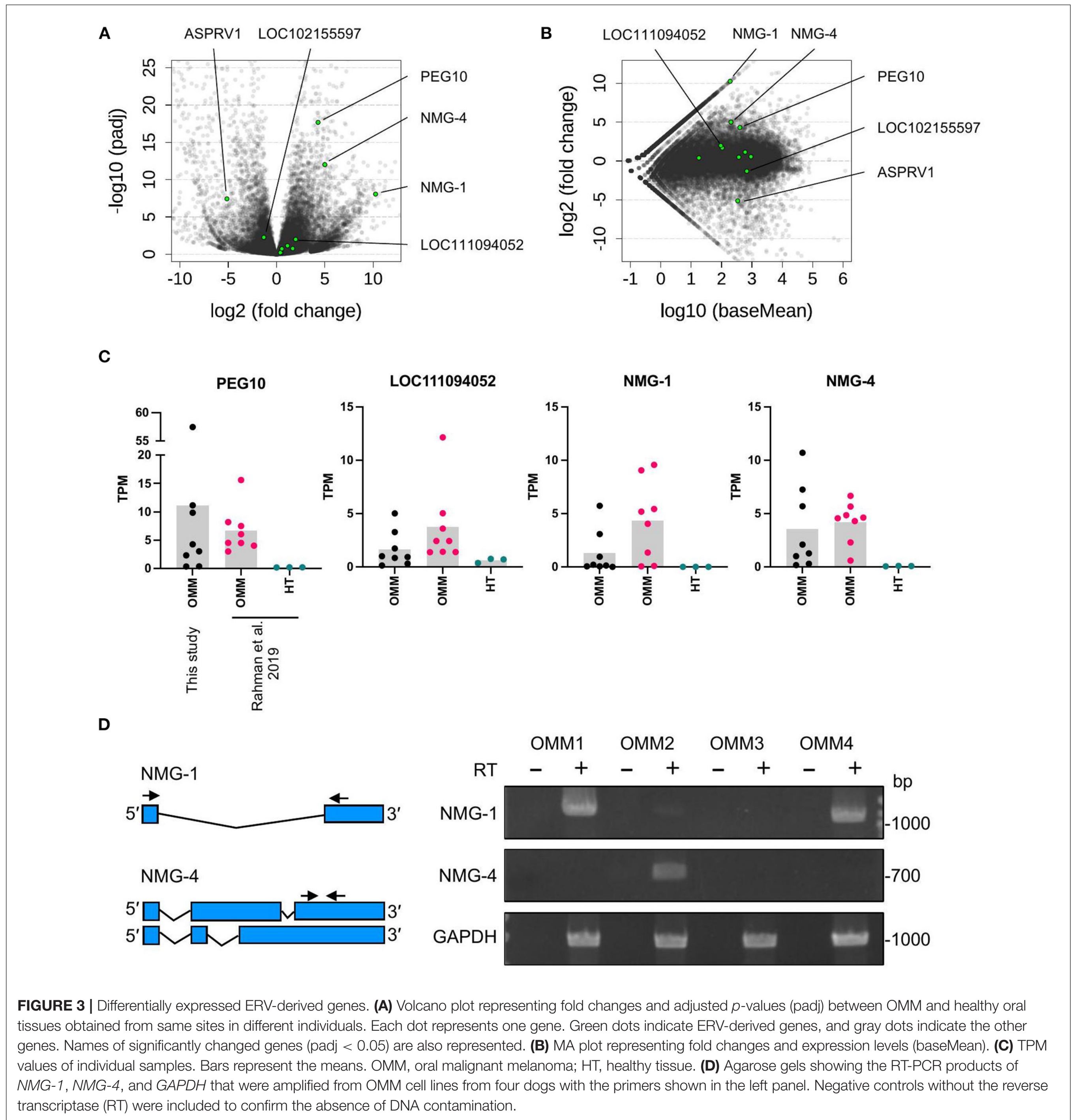


FIGURE 3 | Differentially expressed ERV-derived genes. **(A)** Volcano plot representing fold changes and adjusted p-values (padj) between OMM and healthy oral tissues obtained from same sites in different individuals. Each dot represents one gene. Green dots indicate ERV-derived genes, and gray dots indicate the other genes. Names of significantly changed genes (padj < 0.05) are also represented. **(B)** MA plot representing fold changes and expression levels (baseMean). **(C)** TPM values of individual samples. Bars represent the means. OMM, oral malignant melanoma; HT, healthy tissue. **(D)** Agarose gels showing the RT-PCR products of *NMG-1*, *NMG-4*, and *GAPDH* that were amplified from OMM cell lines from four dogs with the primers shown in the left panel. Negative controls without the reverse transcriptase (RT) were included to confirm the absence of DNA contamination.

tissues. Although we did not conduct the transcriptome sequencing of control samples, we utilized publicly available RNA-seq data of eight canine OMM samples and three canine oral healthy samples, all of which were obtained from the SRA database (20) (**Supplementary Table 1**). Then, we found that four ERV-derived genes were significantly upregulated (*PEG10, NMG-1*, *NMG-4*, and *LOC102155597*), and two ERV-derived genes were down-regulated (*ASPRV1* and *LOC10215559*) in OMM (adjusted $p < 0.05$) (**Figures 3A,B**, **Supplementary Table 4**). TPMs of these four upregulated genes varied among melanoma samples, but their expression levels were low in all healthy tissues (**Figure 3C**). *PEG10* was reported as an important gene in several species of human cancers, such as hepatocellular carcinoma (34), breast cancer (35), lung cancer (36), and neuroendocrine prostate cancer (37). *NMG-1* and *NMG-4* are newly identified genes, and their expressions were

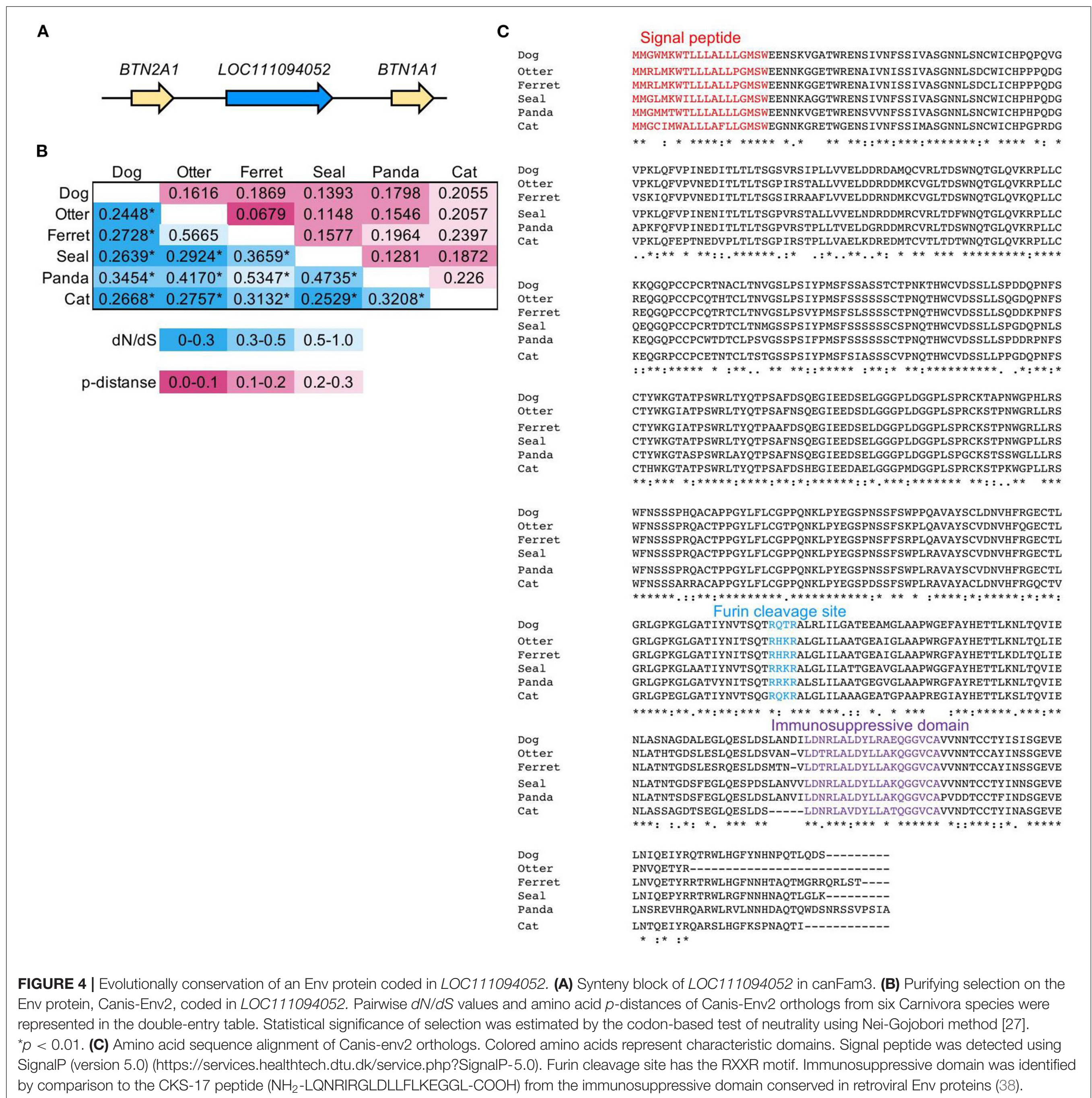


FIGURE 4 | Evolutionally conservation of an Env protein coded in *LOC111094052.* **(A)** Synteny block of *LOC111094052* in canFam3. **(B)** Purifying selection on the Env protein, Canis-Env2, coded in *LOC111094052.* Pairwise *dN/dS* values and amino acid *p*-distances of Canis-Env2 orthologs from six Carnivora species were represented in the double-entry table. Statistical significance of selection was estimated by the codon-based test of neutrality using Nei-Gojobori method [27]. $^*p < 0.01$. **(C)** Amino acid sequence alignment of Canis-env2 orthologs. Colored amino acids represent characteristic domains. Signal peptide was detected using SignalP (version 5.0) (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). Furin cleavage site has the RXXR motif. Immunosuppressive domain was identified by comparison to the CKS-17 peptide (NH_2-LQNRIRGLDLLFLKEGGL-COOH) from the immunosuppressive domain conserved in retroviral Env proteins (38).

experimentally verified by RT-PCR (**Figure 3D**). *NMG-1* gene has a single transcript isoform that is composed of two exons, and its second exon was overlapped with four ERV-ORFs annotated by gEVE database (**Figure 2B**). *NMG-4* gene has two transcripts, and both are composed of three exons. *LOC102155597* was overlapped with an Env-like ERV-ORF of 447 amino acid length (**Figure 2B**). This *env* gene was previously designated as *Canis-env2* (32). We found that *Canis-env2* homologs located between *BTN2A1* and *BTN1A1* among six Carnivora species available in UCSC genome browser, and they were presumed to be orthologs (**Figure 4A**). We found that the coding sequences of *Canis-env2* orthologs were under purifying selection, suggesting that the proteins are functionally important (**Figure 4B**). This Canis-Env2 protein retains the signal peptide, the furin cleavage site, and the immunosuppressive domain, but not the transmembrane (TM) domain (**Figure 4C**). Together, we successfully identified newly identified or uncharacterized retroviral coding genes expressed in canine OMM.

DISCUSSION

In this study, we identified and characterized ERV-derived genes that are expressed in canine OMM samples (**Figures 1**, **2**). Among them, four ERV-derived genes are upregulated in melanoma compared to healthy tissues (**Figure 3**). Though ERV-derived viral particles have been found in melanoma in humans and mice (3, 7), we could not find such ERV-derived genes that could potentially produce viral particles. Since this study is based on the canine reference genome (canFam3), we could not detect newly generated genes in OMM genomes. Therefore, the relationship between polymorphisms and ERVs in the genome of canine OMM remains to be investigated. Even with these limitations, evolutionarily conserved as well as expressed ERV-derived genes were newly identified in canine OMM in this study (**Figure 4**).

PEG10 is upregulated in several human cancers (39). Comprehensive expression analysis revealed that PEG10 is upregulated, especially in hepatocellular carcinoma and breast carcinoma (39). In hepatocellular carcinoma, PEG10 is involved in the TGF-β1-triggered epithelial-mesenchymal transmission, progressing metastasis (34). PEG10 interacts with SIAH1 and suppresses apoptosis of hepatocellular carcinoma (40). Though a correlation between *PEG10* and human melanoma has not been reported yet, considering the upregulation of *PEG10* in canine OMM, this gene may be involved in tumor development in various mammalian species.

NMG-1 and *NMG-4* are two ERV-derived genes that were newly identified in this study. Both retained fragmented ORFs and overlapped with CfERV1-int, a canine-specific ERV family. Therefore, these two genes are relatively new ERVs and may be under being disrupted in the canine genome by mutation. Since their expressions were suppressed to very low levels in healthy samples, they may become a valuable marker for OMM. Future studies on the correlation of the expression levels with malignancy are needed.

LOC111094052 is an Env-coding gene, conveniently named *Canis-env2* in the previous study (32). *Canis-env2* orthologs were identified in all mammals belonging to the four major lineages of the order Carnivora (**Figure 4A**). Pairwise dN/dS values among orthologs were <1 (**Figure 4B**), and their amino acid sequences were conserved (**Figure 4C**), suggesting the functional importance of the protein. Therefore, *Canis-env2* has been under co-option as a host gene in the Carnivora. While the function of Canis-Env2 protein is still unknown, the structure of Canis-Env2 is very similar to a primate-specific Env protein, HEMO, which lost the TM domain by protease cleavage (41). In addition, *env-panMars*, an *env* gene conserved in marsupials, also lost the TM domain due to a stop codon before the TM domain. HEMO, Env-panMars, and Canis-Env2 retain immunosuppressive domains and may be involved in cancer immunity. Tumors expressing Env can avoid immune rejection when transplanted into mice (42). MCA205 tumor cells expressing ERV-FRD1 Env (Syncytin-2) and ERV3 Env suppress the immune rejection in mice depending on the immunosuppressive domains. Syncytin-2 and ERV3 are expressed in the placenta, and their immunosuppressive activities are thought to be conserved for fetal-maternal immunosuppression (43). Thus, aberrant expression of Canis-Env2 may have a role in suppressing immune systems against OMM.

Our current study has some limitations: the small number of samples as well as the lack of well-designed control groups. These are partially due to the inevitable circumstances that our sample collection was dependent on the hospital surgeries for domesticated dogs. Furthermore, the expression of the transcripts and proteins identified in our RNA-seq analysis were not immunohistologically validated. However, our analysis clearly indicated that four ERV-derived genes (*PEG10*, *LOC111094052,* and two fragmented-ORF genes) was expressed in canine OMM. These results indicate that even in dogs with relatively low ERV copy numbers, ERV-derived genes similar to those of humans and mice are expressed.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Experiment Ethics Committee of Yamaguchi University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KK and TMiy designed the study. AS, MY, and TMiz performed experiments. KK performed the computational analyses, with supervision by SN. KK, SN, TMiz, and TMiy prepared the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2021.785678/full#supplementary-material

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Conflict of Interest: MY was employed by the company Anicom Specialty Medical Institute Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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