Mouse Mammary Tumor Virus-like Nucleotide Sequences in Canine and Feline Mammary Tumors

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Short Title: MMTV-like nucleotide sequences in dogs and cats
Abstract

Mouse mammary tumor virus (MMTV) has been speculated to be involved in human breast cancer. Companion animals, dogs and cats with intimate human contacts, may contribute to the transmission of MMTV between mouse and man.

The aim of this study was to detect MMTV-like nucleotide sequences in canine and feline mammary tumors by nested PCR. Results showed that the presence of MMTV-like env and LTR sequences in canine malignant mammary tumors was 3.49% (3/86) and 18.60% (16/86), respectively. For feline malignant mammary tumors, both env and LTR sequences were found to be 22.22% (2/9).

Nevertheless, the MMTV-like LTR and env sequences were also detected in normal mammary glands of dogs and cats. Comparing the MMTV-like DNA sequences of our findings with those of NIH 3T3 (MMTV-positive murine cell line) and human breast cancer, the sequence similarities ranged from 94% to 98%.

Phylogenetic analysis revealed that intermixing among sequences identified from tissues of different hosts, i.e. mouse, dog, cat, and human, indicated the MMTV-like DNA existing in these hosts. Moreover, as the env transcript was detected in one of the 19 MMTV-positive samples by reverse transcription-PCR. Taken together, our study provided evidence on the existence and expression of MMTV-like sequences in neoplastic and normal mammary glands of dogs and cats.

Keywords: MMTV-like sequence / dog / cat / mammary gland / mammary tumor
Introduction

Several environmental risk factors have been proposed for human sporadic breast cancer including mouse mammary tumor virus (MMTV). MMTV is an oncogenic retrovirus inducing breast cancer in mice that can be isolated either as endogenous or as exogenous virus (3). It has been indicated the possible links of MMTV to human breast cancer. Previous studies have demonstrated that MMTV-like sequences, which share at least 95% identity with MMTV, are highly expressed in human breast cancer (7, 28-30). Furthermore, viral particles produced in primary cell cultures derived from breast cancer are similar to the MMTV (18).

In addition, it was correlated geographical variations in the incidence of human breast cancer with distribution map of the natural range of certain species of mice, particularly *Mus domesticus*. The area where *M. domesticus* mice are endemic coincides to a large extent with the countries having high prevalence of breast cancer (22). A previous study reported that MMTV-positive samples were found only in the Australian group (high incidences of breast cancer and *M. domesticus*) but not in the Vietnamese group (low prevalence of breast cancer and *M. domesticus*) (10). These results suggest that *M. domesticus* may harbor and transfer a human-tropic strain of MMTV. However, the contribution of MMTV in breast carcinogenesis was not endorsed by recent studies, of which reported no evidence for the existence of MMTV-like sequences in human breast cancer of Germanic (11) and Japanese patients (12). The controversial finding could be
explained as *Mus domesticus* is not widely distributed in both Germany (11) and Japan (12).

Moreover, besides human and mouse, the association of MMTV with breast cancer of other animal species were also investigated; Howard et al., reported that MMTV variant isolated from mouse could productively replicate both in canine and human cells by serial passages (14). Recently, it was found that a significantly increased frequency of dog owners among female patients with breast cancer as compared to an age matched group of the female population (16). Additionally, MMTV-like sequences were present in a much wider range of species than previously known, including rodents, felines, and rhesus macaques (24). Based on these observations, two hypotheses were proposed: first, the MMTV transmitted from mice to humans is considered to be of exogenous origin; second, Szabo *et al.* and Laumbacher *et al.* proposed that cats and dogs may transmit MMTV from mouse to human (16, 23). However, to date the potential role that MMTV-like virus in canine or feline mammary tumorigenesis is not understood yet.

Therefore, the aims of this study were to examine MMTV-like sequences and to investigate whether MMTV infection could be a risk factor of mammary tumors in dogs. To address these questions, detection of MMTV-like sequences and transcript in canine mammary tumors was conducted. The sequence and phylogenetic relation of those PCR products were determined and analyzed. Moreover, the correlation between presence of MMTV-like sequences and clinical and pathological features was performed by statistical analysis.
Materials and Methods

Animals and tissue specimens

Frozen specimens of neoplastic and normal mammary tissues from 145 dogs and 11 cats that had undergone surgery were obtained from 1995 to 2008 in the Veterinary Medical Teaching Hospital, National Chung Hsing University. Immediately after surgery, specimens were frozen in liquid nitrogen and then stored at -80 °C for further use. The number of tissue specimens used in this study including neoplastic and normal mammary glands of dogs and cats were listed in Table 1. A total of 113 neoplastic canine mammary tissues from 108 dogs included three recurrent tumors and two obtained from different sites of the same dogs.

Medical history and thorough clinical examination were taken at the animal patient’s first presentation and follow up information after surgery was conducted. Medical records included breed, age, sex, tumor size, tumor number, tumor location and affected glands, ovariohysterectomy (OHE) status and the reason for OHE, time between tumor identification and surgical removal (observed time), tumor stage by TNM system (20), surgical procedure, tumor type based on the World Health Organization-Armed Forces Institute of Pathology (USA) classification system (19) and overall survival time. Metastasis of lymph node was identified by histologic examination after surgical removal of the affected glands by the techniques described previously (6). The status of distant metastasis was confirmed by radiographs of the thorax. Follow-up examinations including
physical examination and thoracic radiography were performed every two to three
months in the first 6 months to one year. Afterwards, owners were contacted by
telephone interview every 6 months. Questions included, but were not limited to,
activities of daily life, observation of surgical site for recurrence, pet housing
(kept outside or inside of house).

Isolation of Nucleic acid

Approximately 0.1g of frozen neoplastic, or normal (mammary) tissues was
homogenized with TissueLyser II (Qiagne) and genomic DNA was extracted
using a standard phenol–chloroform extraction method. Chromosome DNA of
NIH3T3 cells was extracted using Genomic DNA purification kit (Geneaid).
RNA was extracted from the homogenized tissue using Trizole reagent
(Invitrogen) according to the manufacturer’s instructions. The concentration of
DNA or RNA was determined by FLUOstar OPIMA (BMG LABTECH).

Synthesis of the first strand cDNA

Total RNA (1 µg) and random 8-mer primers (50 µM) were denatured at 65
°C for 5 min and cooled down on ice. To synthesize the first-strand cDNA, the
RNA and primers were mixed in 5x reaction buffers, 0.1 M DDT, 0.5 mM of each
deoxyribonucleotide, 200 U SuperScript III reverse transcriptase (Invitrogen) and 40
U RNase inhibitor. A total of 20 µL of the mixture was initially incubated at 25
°C, then the reaction was held at 65 °C for 60 min and finally it was terminated by
incubation at 70 °C for 10 min.
Detection of MMTV-like env and LTR sequences by nested PCR

DNA integrity was assessed by amplifying a 292-base paired (bp) sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. DNA of NIH 3T3 cells (MMTV-positive murine cell line) that expresses MMTV env and LTR sequences (28) was used as the positive control, and the negative control was the reaction performed without adding DNA template or with DNA isolated from muscle of other animal, swine. All PCR amplifications were conducted in 25 µl of mixture containing 1 µl of 5 µM of each sense and anti-sense primers, 2 µl of 2.5 mM of each dNTP, 2.5 µl of 10X Taq buffer and 1U of Taq DNA polymerase (New England Biolabs).

Primers for MMTV env gene amplification were designed according to the sequences obtained from a prototype of MMTV in GenBank (accession number AF228551.1). Approximately 200 ng of DNA template (2 ng for NIH 3T3 cells DNA) of normal and neoplastic mammary gland tissues was used in the first-run of PCR amplification with outer primers ENV-1F and ENV-1R (Table 2). The 2 µl of resulting DNA products (673 bp) were used as the template for the second round of amplification with the inner primer pair MMTV ENV-2F and ENV-2R (Table 2). The expected size of product by nested PCR is 449 bp (Fig. 1).

Thermocycle conditions of outer primers (ENV-1F and ENV-1R) PCR for MMTV env sequence were initiated with denaturing at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and then 72°C for 45 s, and ended
with 72°C for 7 min. Second run of PCR using inner primers (ENV-2F and ENV-2R) followed the same condition as the first run.

Primers for MMTV-like LTR amplification were designed according to sequences of a prototype MMTV in GenBank (accession number: AF228551.1) and the primer sequences were listed in Table 2. Approximately 200 ng of each DNA template was used for the nested PCR amplification with the outer primer pair LTR1 and LTR4, and the inner primer set LTR2 and LTR3 for the first-run and the second-run of PCR amplification, respectively. Two ml of the first-run PCR product with expected size of 663 bp were used as template for the second round PCR (expected size of 292 bp) (Fig. 1). The thermocycle conditions for MMTV LTR were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and ended with a final extension at 72°C for 7 min. Standard PCR precautions and procedures were used to avoid contamination.

PCR products were separated by 2% of agarose gel electrophoresis, and gels were stained with 0.05% HealthView Nucleic Acid Stain (Genomics BioSci & Tech Corp, Taiwan) and then visualized by UV illuminator.

**DNA sequencing**

To confirm the authenticity of the PCR products, MMTV env-like or LTR-like PCR products were purified from agarose gels by Gel and PCR Clean-Up Purification kit (GeneAid) and then sent for automated sequencing (Mission Biotech Co., Ltd, Taiwan).
Phylogenetic analysis

The resulting sequences (labeled with initial of DMGT or DBT in phylogenetic tree indicating those identified from canine mammary gland tumors or normal tissue, respectively; CMGT was from feline mammary gland tumors) were compared with each corresponding region of the prototype MMTV (HeJ strain), MMTV from NIH 3T3, HMTV, HERV-K10 sequences (GenBank accession numbers AF228551.1, AY52722.1, AF243039 and M14123, respectively), as well as other MMTV-like sequences submitted to GenBank (the accession number were shown on the phylogenetic tree). In addition, two sets of env sequences identified from cats (Cat1 and Cat2) were obtained from a previous report (24). Moreover, several env sequences identified from rodent (MTW1), and human (with initial HBC, HBT indicating those identified from breast cancer tissue or normal tissue of human) in Taiwan were also included.

The nucleotide sequences were aligned using Clustal W multiple alignment tool in the BioEdit Sequence Alignment Editor version 7.0.9.0. The phylogenetic tree was inferred based on the 410 nucleotide sequence of the partial env gene of MMTV. The neighbor-joining phylogenetic tree (21) was constructed after 1000 replicates using the PHYLIP package, version 3.68 (9) and drawn using FigTree software, version 1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Clinical and pathological analysis

The expression of ER (estrogen receptor) α, PR (progesterone receptor) and HER-2 (human epidermal growth factor receptor-2) in canine malignant
mammary tumors, collected from 2000 to 2006, have been investigated by immunohistochemical assay in our laboratory (5, 15). Scoring system utilized for exploring the expression status of ER\(\alpha\), PR was based on a previous report (1). Briefly, expression of specimen was considered positive when total score = sum of proportion score and intensity score \(\geq 3\). The HercepTest (DakoCytomation), approved by FDA, was used to evaluate HER-2 protein expression, and the criteria for staining intensity and pattern were based upon HercepTest scoring system. Score \(\geq 2\) was grouped as over-expression of HER2 protein.

Statistical analysis

Due to the small sample size of cat specimens, the statistical analysis was only conducted with dog samples. Chi-square test or Fisher’s exact test where was appropriate was applied to evaluate if there was a significant association between presence of MMTV-like sequences in tumors and various factors, including clinical and pathological status (age of tumor onset, tumor size, side of affected gland, location of affected gland, number of tumors, clinical stage, histological type and grade, lymph node status, observed time and overall survival time) and the archive of immunohistochemical results (ER\(\alpha\), PR, and HER-2). A p values \(\leq 0.05\) was considered statistically significant. All analyses were conducted with the commercial statistical software SPSS 15.0 statistical software (SPSS Inc, Chicago, Ill). For the animal with mammary gland tumors, the overall survival time was calculated from the date of surgical removal to the date of death or the last date of
follow-up. If the animal’s cause of death was irrelevant to malignant mammary
tumors, the date of death was used as the censored date for calculating survival
time. Kaplan-Meier method was used for survival curve analysis and category-
specific survival cures were further constructed. The log-rank test was used to
identify if there was significant association between the evaluated factor and long-
term survival.

Results

Animals and tissue specimens
Canine breeds of 108 patients with mammary tumors included 30 mixed-breed
dogs, 29 Maltese, 8 Toy poodles, 7 Pomeranians, 5 Shi-tzus, 4 Akitas, 4 Cocker
spaniels, 3 Husky, 3 Chihuahuas, 2 Dalmatians, 2 German shepherd dogs, 2
Shiba, 2 Spitz, 2 Yorkshire terriers, 1 Dachshund, 1 Lhasa, 1 Rottweiler, 1
Schnauzer and 1 Shetland sheepdog. The mean age of tumors initially identified
was 9.30 ± 0.26 years (ranging from 1.83-9.83 years). The mean observed time
was 17.11 ± 1.64 months (ranging from 0.25-72 months). In 26.85% (29/108) of
dogs, OHE was performed prior to the removal of mammary tumors.

Histologically, tumor type of 113 mammary tumors comprised of 27 (23.89%) of
benign tumors, 41 (36.28%) of simple carcinomas (including special types of
carcinoma), 31 (27.43%) of complex carcinoma, and 14 (12.39%) of sarcoma
(including carcinosarcoma).
Feline specimens were collected from 12 cats. The feline breeds included 7 domestic shorthaired (DSH) cats, 3 Persian cats, 1 Siamese cat and 1 chinchilla cat. The mean age of tumors initially identified was 7.89 ± 0.61 years (ranging from 1.79-9.33 years). The mean observed time was 4.40 ± 1.66 months (ranging from 0.5-18 months). In 7/11 (63.64%) of cats, OHE was performed prior to the removal of mammary tumors. Histologically, tumor type of 11 samples comprised of 2 (18.18%) of benign tumors, and 9 (81.82%) of simple carcinomas.

Detection of MMTV-like sequences and transcript by nested PCR

The integrity and quality of all the DNA samples were validated by PCR amplification of a 229-bp fragment of the GAPDH gene (data not shown).

Among 113 canine mammary tumors and 11 feline mammary tumors samples, amplification of env sequences was observed in 3 (3.49%) of canine and 2 (22.22%) of feline malignant mammary tumors, respectively (Table 3, 4) (Fig. 2). Moreover, this sequences were found in 3 (11.11%) of canine but in none of feline benign mammary tumors. Among the 56 normal canine mammary gland tissue (15 from the normal tissue away from tumors and 41 from healthy dogs), only one (1/56) of normal mammary tissues from healthy dog was detected as positive for specific MMTV-like env sequence (Table 3). In feline samples, 1 (50.00%) of normal mammary tissues was detected as positive (Table 4, Fig. 3).

MMTV-like LTR sequences were also detected in canine and feline specimens. Amplification of 292-bp MMTV-like LTR sequences was observed in 16 (18.60%) of dogs and 2 (22.22%) of cats with malignant mammary tumors (Fig. 2,
3), as well as 6 (22.22%) of canine benign mammary tumors. However, these sequences were present in 10 (24.39%) of normal mammary tissues from healthy dog as well as in normal mammary tissues away from tumors of 2 dogs (13.33%) and one cat (50%) (Table 3, 4). In the 19 MMTV-positive samples, expression of env gene was detected in one of the samples by reverse transcription-PCR (Fig. 4).

**Identification and analysis of MMTV-like sequences**

The sequences of the PCR products were identified and aligned with the corresponding region of NIH 3T3 (MMTV-positive murine cell line), the prototype MMTV (HeJ) and HMTV (human mammary tumor virus; or MMTV-like as described elsewhere) sequences. Results indicated sporadic nucleotide variations spanning across the LTR and env gene (Fig. 5, 6, respectively). Overall, our sequences shared 93–98% and 92-99% homology to MMTV and HMTV env sequences, respectively (supplementary Tab. I). In the conserved regions of LTR, these positive samples shared 88-89% and 95–98% homology with MMTV and HMTV, respectively (supplementary Table 2). Further, no significant similarity was found when our sequences were blasted or compared to the canine, feline and human genome sequences available in GenBank database with BLAST software in NCBI website, indicating that these amplified products were not of canine, feline and human genomic or endogenous retrovirus origin.

**Phylogenetic analysis of MMTV-like sequences**
The neighbour-joining phylogenetic analysis showed that the MMTV detected from different hosts, including mice, human, cat, and dog, were not classified into different clusters, indicating that the MMTV might possibly transmit between these hosts (Fig. 7).

Correlation between nucleotide sequences and pathological characteristics in canine malignant mammary tumors

Recently, Hachana et al. demonstrated that the presence of the MMTV-like sequences significantly correlates inversely with over-expression of progesterone receptor and HER-2 (13). We then conducted statistic analysis to explore the correlation between the pathological and immunohistochemical characteristics of canine malignant mammary tumors and MMTV-like sequences. Due to the small size of feline samples, only canine samples were included in this assay. Results indicated no significant difference was identified between the presence of MMTV-like sequences with patient’s age, tumor size, side of affected gland, location of affected gland, number of tumors, clinical stage, OHE status before surgery, histological type and grade, lymph node status, or observed time (data not shown). Immunohistochemically, the results indicated that ER-α, PR or HER2 expression was not significantly associated with presence, in comparison with absence, of MMTV-like sequences in malignant mammary tumors. However, the presence of MMTV-like sequences was more likely, but with no significant difference, to be identified in canine malignant mammary tumors with ER-α negativity (P = 0.056), HER2 positivity (P = 0.063) (Table 5), complex carcinoma
(P = 0.063) or low histologic grade (P = 0.069) (data not shown). The Kaplan-Meier survival curves further showed that presence of MMTV-like sequences in malignant mammary tumors was not significantly associated with one-year or two-year survival in dogs.

**Discussion**

Since the mid-1990s, several lines of evidence based on the detection of proviral DNA or the expression of specific viral genes implicated mouse mammary gland tumor virus (MMTV) is connected to human breast cancer (18, 28-30). Molecular evidence of its presence in human breast tumors supported it can be a human tumor-causing virus; however, viral entry, virus proliferation, viral tumorigenesis, and the transmission between mice to human remain to be studied.

Dogs and cats have been implied as the possible mediator for the MMTV transmission. Thus, the presence of MMTV-like sequences in dogs and cats is one of the important evidences to support this hypothesis. In this study, we identified sequences and expression of MMTV-like gene in canine and feline mammary tumors, indicating these pet animals possibly harbor MMTV-like virus. Moreover, as phylogenetic relationship of viruses from various host species provides evidence for predicting the transmission event (26), the phylogenetic relationship was further analyzed. As shown in Fig 7, the MMTV env sequences from mice, dog, cat, and humans were in separate clusters, indicating that the MMTV might be able to transmit between these hosts. In addition, the
transcription status of MMTV-like virus that is required for spreading viruses via exogenous route was also investigated. As illustrated in Fig 4, the env transcript was detected in one of the MMTV positive canine samples.

Regarding nonspecific PCR amplifications, such as from other retroviruses other than MMTV, contamination from positive control or between samples in human breast cancer (2), several evidences in our study can exclude this nonspecificity: (i) Direct sequencing of most PCR fragments (or RFLP for those with low yields) showed big nucleotides differences between samples (sequence identities of env and LTR between clinic samples ranged 91.7-99.5%, and 88.9-99.6%, respectively); more heterogeneity was obtained when sequences amplified from dogs were compared with positive control. (ii) The result of PCR assay using three porcine DNA samples was found to be negative for MMTV. (iii) The neighbor-joining phylogenetic analysis indicated that the human endogenous retroviral virus K-10 (HERV) was significantly different from MMTV, indicating that the detected-MMTV DNA is not likely to be HERV. It is worthy of emphasis that in the present study the possible contamination can be eliminated by the fact of no amplification of such sequences from negative controls, including reaction without template DNA or DNA isolated from other healthy animals, and also by the evidence of sequence polymorphisms of the resulting PCR fragments between specimens (Fig. 3, 4 and supplementary Table 1). As regard to spontaneous mutations resulted from PCR amplification, the genetic distances between the MMTV detected in this study were up to 7.9 %. The error rate of reverse transcriptase was estimated to be around $10^{-4}$ on a complex template. As referred
in other reports, the error rate of Taq polymerase was estimated to be between $3.9 \times 10^{-6}$ and $9.1 \times 10^{-6}$/nucleotide/cycle under most PCR conditions (17, 27). After 60 cycles of PCR amplification, the error frequencies were between $2.3 \times 10^{-4}$ and $5.5 \times 10^{-4}$, which together with the RT errors, are lower than the mean diversity (0.2-7.9 %) observed in our samples. Furthermore, the existence of MMTV-like sequences was also evidenced by Southern blot analysis (supplementary Fig 1). Considering the low copy number of MMTV integration, the first run PCR products of MMTV-positive DNA samples were detected by Southern blotting. As shown in supplementary Fig 1, env probe successfully detected the first run PCR products amplified from two canine samples. According to the signal intensity and the detection rate, the canine/feline harbors a low level of MMTV-like sequences. Although the detection rate was not the same between the methods of nested PCR and Southern blot analysis, this can be explained by the sensitivity; only the samples harbors higher copy number of MMTV-like sequences can be detected by a less sensitive method. Taken together, these results suggested that the detected-MMTV DNA was not resulted from contamination.

An association of MMTV-like sequences with severity of human BC has been demonstrated (8, 10). However, unlike in humans, the prevalence of MMTV-like sequences was no significant difference between normal and canine mammary gland tumor. Moreover, it was found that the presence of MMTV-like sequences was not significantly related with clinical stage, histological type and grade of canine mammary tumors (data not shown). It has been shown that MMTV
proviruses preferentially integrated in the vicinity of a number of host oncogenes, such as Wnt and Fgf family genes that could lead to oncogene expression and clonal outgrowth of the infected cell (4, 25). Thus, this apparent discrepancy between our results and previous reports based on humans can be speculated that proviral DNA might not integrate close to cellular proto-oncogenes involving in pathogenesis or MMTV infection might not be a significant risk factor for canine tumorigenesis.

Recently, it was proposed that intimate contacts between human and dogs could transmit MMTV or MMTV-like virus of mouse (16). Although results from the current study revealed that presence of MMTV has no significant effect on the tumor development, dogs themselves do not need to develop mammary tumors in order to spread the virus.

Noticeably, as described above, the MMTV-like fragment was not only present in malignant tumors, but actually amplified in the samples of benign tumors as well as of normal mammary gland. As shown in Table 3, 3 out of 86 MGT samples were detected positive for env, but none of their counter part of normal samples was positive for env, implicating the possibility of presence of an exogenous infection status of MMTV-like virus. Noticeably, LTR gene sequences were simultaneously amplified from both normal and tumor tissue of 2 cases (Benign MGT). However, in the case of an exogenous transmission, it is also possible to detect MMTV-like sequences in tissues further away from the tumor if this virus is replication competent. Taken together, this is the first report to provide evidence of the presence of MMTV-like sequences in canine and feline...
mammary tumors. The existence of env transcript and phylogenetic relation supply more information on the contribution of companion animals on transmission of MMTV.

Acknowledgments

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43  2001. Detection of MMTV-like LTR and LTR-env gene sequences in
Figure 1. Illustration of the MMTV genome and the location of primers used for amplification of env and LTR regions. Four sets of primers were designed to amplify env and LTR of MMTV-like virus. The expected sizes of resulting fragments are 292 base pair (bp) and 449 bp, respectively.

Figure 2. Detection of MMTV-like sequences of PCR products in canine mammary tumors. DNA samples isolated from four canine mammary tumors were analyzed by agarose gel electrophoresis. Results showed positive for 449 bp of MMTV-like env gene (E) and a 292 bp of LTR gene (L). Lane 1-4, canine mammary tumor samples. Lane N, negative control (the reaction performed with no adding DNA template). Lane M, 100-bp DNA size markers.

Figure 3. Detection of MMTV-like sequences in feline mammary tumors. The DNA samples were detected positive for 449 bp of MMTV-like env gene (A), or a 292 bp of LTR gene (B). Lane 1, feline normal mammary tissues. Lane 2-5, feline mammary tumors. Lane P, positive control using DNA from NIH3T3 cells as the template. Lane M, 100-bp DNA size markers.

Figure 4. Reverse-transcription PCR analysis of 19 MMTV-like positive clinical specimens. RNA extracted from 19 clinical specimens (lane 1-19) that were detected as MMTV-positive by nested PCR were reverse transcribed and
followed by detection of MMTV-like env sequences. Env-specific products were amplified from one dog sample (lane 17) and positive control (RNA from NIH3T3 cells labelled as +), but not from negative control (RNA extracted from swine muscle). Lane M, 100-bp DNA size markers.

**Figure 5. Multiple nucleotide alignment of the MMTV-like env gene**

The env sequences from six canine and two feline mammary tumors (indicated by their respective number) were aligned with NIH 3T3 (positive control for MMTV), mouse mammary tumor virus from HeJ and HMTV (accession number AF228551.1 and AF243039, respectively).

**Figure 6. Multiple nucleotide alignment of the MMTV-like LTR gene**

The LTR sequences from ten canine, one feline mammary tumors, and one canine normal mammary tissue (indicated by their respective case numbers) were aligned with that of MMTV identified from NIH 3T3 cell line (positive control), and MMTV from mouse (HeJ, AF228551.1), as well as human mammary tumor like virus (HMTV, accession number AF243039) retrieved from GenBank database.

**Figure 7. Determination of phylogenetic relationships of env sequences identified from various hosts.** A Neighbor-Joining phylogenetic tree from nucleotide sequences of env of MMTV viruses. The tree was rooted to HERV-
Robustness of individual nodes of the tree was determined by using bootstrap analyses at 1000 replicates.
Table 1. Animals and tissue samples used in this study included neoplastic and normal mammary glands of dogs and cats.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Number in the dog</th>
<th>Number in the cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant mammary tumor</td>
<td>86</td>
<td>9</td>
</tr>
<tr>
<td>Benign mammary tumor</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>Normal mammary gland</td>
<td>56</td>
<td>3</td>
</tr>
</tbody>
</table>

*a, b* Including histologically confirmed normal tissues of the mammary glands; 15 in dogs and 2 in cats respectively, away from those with mammary tumors.

Table 2. Sequences of primers from conserved regions and their corresponding locations in the MMTV genome.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequences (5’→3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENV-1F</td>
<td>CCTTCTGGGAGGGAGACGAGT</td>
<td>7274-7293</td>
</tr>
<tr>
<td>ENV-1R</td>
<td>AGCTCGAATTTAGTCTGTGCGTAG</td>
<td>7895-7919</td>
</tr>
<tr>
<td>ENV-2F</td>
<td>CCTTGGGTTACTTTGGGATTTTCTC</td>
<td>7361-7384</td>
</tr>
<tr>
<td>ENV-2R</td>
<td>TGATCGCTGCGATCAGTGGCAAG</td>
<td>7788-7809</td>
</tr>
<tr>
<td>LTR-1</td>
<td>GGAGTGGCGCTTGTCAAAATAGGAG</td>
<td>582-605</td>
</tr>
<tr>
<td>LTR-2</td>
<td>AGAGAAAGACGACATGAAACAACAACAC</td>
<td>805-828</td>
</tr>
<tr>
<td>LTR-3</td>
<td>ACTCAGAGCTCAAGATCAAACCTT</td>
<td>1073-1096</td>
</tr>
<tr>
<td>LTR-4</td>
<td>GGATGGGCAACAGACACAAACAC</td>
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Table 3. Statistics of MMTV-like env and LTR sequences in neoplastic and normal mammary tissues in dogs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No.</th>
<th>env gene sequences</th>
<th>LTR gene sequences</th>
<th>env or LTR&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Positive No.</td>
<td>Incidence rate</td>
<td>Positive No.</td>
</tr>
<tr>
<td>Malignant MT</td>
<td>86</td>
<td>3</td>
<td>3.49</td>
<td>16</td>
</tr>
<tr>
<td>Benign MT</td>
<td>27</td>
<td>3</td>
<td>11.11</td>
<td>6</td>
</tr>
<tr>
<td>NMG</td>
<td>41</td>
<td>1</td>
<td>2.44</td>
<td>10</td>
</tr>
<tr>
<td>NMG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MT: mammary tumor
NMG: normal mammary gland
ND: not detected

<sup>a</sup> Histologically normal tissue of the mammary gland away from those with mammary tumor
<sup>b</sup> Sequences of LTR present in 2 dogs (13.3%, 2/15), both in normal mammary tissues and also in benign mammary tumors.
<sup>c</sup> The number of samples were detected positive by MMTV-specific nested PCR using either env or LTR primer sets.

Table 4. Statistics of MMTV-like env and LTR sequences in neoplastic and normal mammary tissues in cats.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No.</th>
<th>env gene sequences</th>
<th>LTR gene sequences</th>
<th>env or LTR&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive No.</td>
<td>Incidence rate</td>
<td>Positive No.</td>
</tr>
<tr>
<td>Malignant MT</td>
<td>9</td>
<td>2</td>
<td>22.22</td>
<td>2</td>
</tr>
<tr>
<td>Benign MT</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NMG</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NMG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>1</td>
<td>50.00</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MT: mammary tumor
NMG: normal mammary gland
ND: not detected

<sup>a</sup> Histologically normal tissue of the mammary gland away from those with mammary tumor
<sup>b</sup> Sequences of LTR present in one cat (50.0%, 1/2), both in normal mammary tissue and also in mammary tumor.
<sup>c</sup> The number of samples were detected positive by MMTV-specific nested PCR using either env or LTR primer sets.
Table 5. Correlation between MMTV-like LTR sequences and immunohistochemical expression status of hormonal receptors and HER2 in canine malignant mammary tumors.

<table>
<thead>
<tr>
<th></th>
<th>Number of MMTV-like LTR negative cases (%)</th>
<th>Number of MMTV-like LTR positive cases (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER-α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (50.0)</td>
<td>4 (100.0)</td>
<td>0.056</td>
</tr>
<tr>
<td>Positive</td>
<td>19 (50.0)</td>
<td>0 (0.0)</td>
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<tr>
<td><strong>PR</strong></td>
<td></td>
<td></td>
<td>0.787</td>
</tr>
<tr>
<td>Negative</td>
<td>12 (31.6)</td>
<td>1 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26 (68.4)</td>
<td>3 (75.0)</td>
<td></td>
</tr>
<tr>
<td><strong>HER 2</strong></td>
<td></td>
<td></td>
<td>0.063</td>
</tr>
<tr>
<td>Negative</td>
<td>27 (71.1)</td>
<td>1 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11 (28.9)</td>
<td>3 (75.0)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 7