Clonality Analysis of the Neoplastic Diseases in Cats

(ネコにおける腫瘍性疾患のクローン性解析)

望月　浩之

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General introduction

Clonal cell proliferation is a fundamental nature of neoplastic proliferation because neoplastic cells are generally derived from a single transformed cell. On the other hand, normal or reactive tissue is composed of polyclonal cell population. Assessment of the clonality of cell population can provide useful information for understanding the pathogenesis, supporting the accurate diagnosis, and monitoring of the diseases. Therefore, precise assessment of the proliferating cells would lead to optimal therapeutic strategies.

In human medicine, several analytical systems to assess the cell clonality have been reported. Those systems are based on the aberrant phenotype, specific chromosome/gene abnormality, antigen receptor gene rearrangement, or X-chromosome inactivation pattern (XCIP). Each method can be applied to certain kinds of neoplastic diseases but also has its limitations.

Phenotypic clonality analysis identifies clonal cell population using changes in protein expression patterns of neoplastic cells. Hematopoietic malignancies such as lymphoma and leukemia are usually diagnosed on the basis of cytological and histopathological findings. However, discrimination between reactive proliferation and neoplastic one is difficult in some cases such as well-differentiated lymphoma especially when a small amount of samples are available. In such cases, flow cytometry can be a useful additional diagnostic tool. Aberrant expression of cell surface antigens or marked expansion of a certain subset of cells was shown to be useful to indicate a clonal origin of the disease (Kaleem, 2007). Analysis of antigen expression pattern is also useful to apply some specific antibody therapeutics such as the use of anti-CD20 antibody for human lymphoma (Illidge *et al*., 2010). However, application of flow

cytometry has been limited for the clonality analysis in veterinary medicine due to the paucity of the monoclonal antibodies specific to each species (Comazzi et al., 2011).

Clonality of neoplastic cells can also be assessed by detection of specific genetic abnormalities. The most widely used approach is the chromosomal analysis in metaphase cells. Certain types of hematopoietic malignancies are known to have chromosomal abnormalities, many of which are specific for a particular disease such as 9q+:22q- translocation (Philadelphia chromosome) in human chronic myelogenous leukemia (Tefferi., 2007). When a chromosomal abnormality involves known genes, Southern blot and PCR analyses could also be used to identify the specific genotypic abnormality. Although genotypic clonality analysis is useful in identifying clonally derived cell population, such analysis can be available only when some specific abnormality has been identified.

In lymphoid malignancies, assessment of antigen receptor gene rearrangement can be used to demonstrate clonal cell expansion originated from a single lymphocyte. Rearrangement of antigen receptor genes, immunoglobulin (*Ig*) and T-cell receptor (*TCR)* genes, is a recombination process of their variable (V), diversity (D), and joining (J) region genes, in the early stages of lymphoid differentiation (Jung *et al*., 2006). Because the length and nucleotide sequences of the rearranged antigen receptor gene vary between each lymphocyte, normal and reactive populations of lymphocytes exhibit polyclonal patterns of *Ig* or *TCR* gene rearrangements. On the other hand, lymphoid malignancies are composed of a clonally expanded cell population fundamentally derived from a single neoplastic lymphocyte; virtually all of the cells contain a unique clonal antigen receptor gene rearrangement. These rearrangements could be detected by Southern blot or PCR analyses. In human medicine, analysis of antigen receptor gene

rearrangement has been used as a useful additional diagnostic tool as well as monitoring of the minimal residual disease (van Dongen *et al*., 2003). Studies on the PCR analysis for antigen receptor gene rearrangement were recently reported in dogs and cats (Burnett el al., 2003; Moore *et al*., 2005; Werner *et al*., 2005; Valli *et al*., 2006; Henrich *et al*., 2009; Weiss *et al*., 2011). However, in cats, low sensitivity of the assay to detect the gene rearrangement disturbs its clinical application.

Unlike these methods to assess cell clonality, XCIP analysis is applicable to any types of neoplastic diseases, even those in which no tumor-specific phenotype or genetic abnormality has been identified. In mammalian females, either the paternal or maternal X chromosome generally undergoes random inactivation in each cell through the epigenetic changes during embryogenesis. This event is stably inherited by the daughter progeny of each cell, resulting in the theoretical expectation of a 1:1 inactivation ratio between the paternally and maternally derived X chromosomes in normal or reactive tissues. By contrast, most neoplastic tissues are composed of a population clonally expanded from a single cell, generally exhibiting a uniform XCIP, and resulting in its significant deviation. Therefore, demonstration of a nonrandom XCIP (skewed XCIP) infers the clonal origin of cells (Chen *et al*., 2007). Although XCIP analysis can be applied to only females, it has been widely used for the assessment of clonality in various kinds of neoplasms, especially in which the morphology of abnormally proliferating cells is indistinguishable from that of normal cells. However, to date, there has been no XCIP analysis available in veterinary medicine.

The present study was conducted in order to construct several analytical systems for evaluation of cell clonality in cats. In chapter 1, a PCR analysis using immunoglobulin

heavy chain gene rearrangement was constructed for the evaluation of B-cell clonality. In chapter 2, another PCR analysis using T-cell receptor γ gene rearrangement was constructed for the evaluation of T-cell clonality. Finally, in chapter 3, a PCR analysis to detect XCIP was constructed to assess the clonality of cells originated from various kinds of tissues.

Chapter 1

Multiplex PCR and GeneScan analysis to detect immunoglobulin heavy chain gene rearrangement in feline B-cell neoplasms

Abstract

Lymphoid neoplasms are usually diagnosed on the basis of cytological and histopathological findings. However, in some cases, discrimination of lymphoid neoplasms from reactive lymphoid proliferation is difficult. PCR amplification of complementarity-determining region 3 (CDR3) of the immunoglobulin heavy-chain variable region (*IGHV*) gene can be used to assess clonality of B-cell populations as a supportive diagnostic tool for B-cell neoplasms. Because of the sequence variation and possible somatic hypermutation of the *IGHV* gene, sensitivity of the PCR-based assay to detect clonal *IGHV* gene rearrangement largely depends on the sequences and numbers of primer sets. Prior to the development of an efficient assay, 97 *IGHV* complementary DNAs (48 *IGHV-1* and 49 *IGHV-3* clones) from normal cat spleens were cloned and sequenced. On the basis of these sequences, 6 forward primers at the variable region and 5 reverse primers at the joining region were designed. Using each of 6 forward primers and a mixture of 5 reverse primers, CDR3 of IGH*V* genes were amplified and analyzed the PCR products by conventional PAGE and GeneScan analyses using fluorescence-labeled primers. Twenty-six feline B-cell neoplasms diagnosed by histopathological and immunohistochemical examinations were subjected to the newly developed analysis of *IGHV* gene rearrangement. Clonal *IGHV* gene rearrangement was detected in 22 of 26 (84%) samples by both PAGE and GeneScan analyses. To reduce the number of PCR reactions, a multiplex PCR analysis system was constructed using a mixture of *IGHV-1*– and *IGHV*-3–specific primers as forward primers and a mixture of 5 joining region reverse primers. Results of the multiplex PCR were 100% concordant with those obtained by each of the singleplex PCRs. The multiplex PCR-based assay and GeneScan analysis developed in the present study would be useful and practical

tools to detect clonal *IGHV* gene rearrangement in feline B-cell neoplasms.

1. Introduction

Lymphoid neoplasms are the most common tumors in cats and are known to be as a result of clonal expansions of lymphoid cells. They are generally diagnosed on the basis of the results of cytological and/or histopathological examinations. However, discrimination between reactive lymphoproliferative lesions and lymphoid neoplasms is sometimes difficult in some cases such as well-differentiated lymphoma and early stages of neoplasms and when only cytology samples are available. In such cases, PCR-based clonality assessment for immunoglobulin and T-cell receptor gene rearrangements can be a useful additional diagnostic tool. The PCR-based assay requires only small amounts of samples, which can be obtained from fine needle aspiration or accumulated fluids. The assay also provides information regarding the T- and B-cell lineages of the tumors, which has been demonstrated to correlate with the prognosis of canine lymphoid neoplasms (Teske *et al*., 1994).

In B-cells, rearrangement of the immunoglobulin heavy-chain variable region (*IGHV*) gene, which is a recombination process of many different variable region (VH), diversity region (DH), and joining region (JH) genes, occurs in the early stages of lymphoid differentiation (Jung *et al*., 2006). In addition to random recombination of VH-DH-JH segments, junctional diversity is generated from deletion and random insertion of a few nucleotides. Furthermore, somatic hypermutation after antigenic stimulation introduces point mutations throughout the *IGHV* gene, generating further diversity. For these reasons, the length and nucleotide sequences of *IGHV* genes, especially in complementarity-determining region 3 (CDR3), vary between each B-lymphocyte. Therefore, PCR amplification of CDR3 using forward primers at VH segments and reverse primers at JH segments results in many differently sized PCR

products in normal or reactive lymphoid tissues. On the other hand, B-cell lymphoid neoplasms are composed of a population of clonally expanded cells fundamentally derived from a single neoplastic B-cell; virtually all of the cells contain a unique clonal *IGHV* gene rearrangement (van Dongen *et al*., 2003).

PCR assays to detect clonal *IGHV* gene rearrangement in feline lymphoid neoplasms have been described by Werner *et al*. (2005) and Henrich *et al*. (2009). In these reports, the authors described that they could detect clonal *IGHV* gene rearrangement in 60–70% of feline B-cell neoplasms; however, they pointed out the need to improve the sensitivity of the PCR-based assay to detect *IGHV* gene rearrangement. They explained that the VH and JH primers used in their studies might not cover the full range of segments within the feline *IGHV* locus, resulting in the failure to amplify CDR3 in some cases. Single random nucleotide changes induced throughout the *IGHV* genes by somatic hypermutation may result in changes in the primer annealing sequences, which also can lead to false-negative results. One strategy to reduce the frequency of false-negative results is the use of greater numbers of primer sets covering all of the major and minor VH and JH genes. However, simply increasing the number of primer sets would complicate the assay and decrease its cost-effectiveness. In human medicine, a multiplex PCR technique utilizing multiple primer sets in the same PCR tube has been developed to increase the sensitivity of the assay and reduce the number of PCR reactions (Meier *et al*., 2001; van Dongen *et al*., 2003).

In the present study, a number of *IGHV* complementary DNAs (cDNAs) from normal cat spleens were cloned and sequenced to develop new primer sets to efficiently amplify *IGHV* CDR3. Each primer set was evaluated in terms of the ability to detect

clonal *IGHV* gene rearrangements in feline B-cell neoplasms. Furthermore, multiplex PCR and GeneScan analyses can be successfully developed to reduce the number of PCR reactions without decreasing the overall detection rate.

2. Materials and methods

2.1. Establishment of feline B-cell lymphoma cell line

A feline B-cell lymphoma cell line, MS4, was established from neoplastic pleural effusions of a 9-year old male Abyssinian cat with cutaneous B-cell lymphoma cell line. The MS4 cells were cultured in RPMI1640 supplemented with 15–20% f*et al* bovine serum (Biowest, Nuaille, France), 100 IU/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma–Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere of 5% CO2. MS4 cells were positive for B-cell markers, CD20 and CD79α, and were negative for a T-cell marker CD3.

2.2. Cases

All samples included in the present study were from cats with B-cell neoplasms that were diagnosed by histopathological and immunohistochemical examinations at the Veterinary Medical Center of the University of Tokyo between 2004 and 2010.

After fixation in 10% neutral buffered formalin, tissues obtained by biopsy or autopsy were processed by routine procedures for preparation of hematoxylin and eosin–stained sections for light microscopy.

Immunophenotyping was performed on 10% buffered formalin-fixed and paraffin-embedded (FFPE) tissue sectioned at 4 μm using an antibody against human CD3 (1:50; DAKO, Glostrup, Denmark) for a T-cell marker, and those against human

CD20 (1:400; Neomarkers, Fremont, CA) and human CD79α (1:50; Clone: HM57; Dako) for B-cell markers. Normal lymph node and spleen samples obtained from a cat that had died of a disease unrelated to lymphoid neoplasm were used as control tissues.

A total of 26 cats with B-cell neoplasms were included in the present study: 22 cats with B-cell lymphomas, 3 cats with myeloma-related disorders (MRD), a disease group proposed by Mellor *et al*. (2008), and 1 cat with B-cell acute lymphoblastic leukemia (B-ALL). The breeds were mixed (20 cats), American shorthair (4), Abyssinian (1), and Persian (1). Ages of the 26 cats ranged from 2 to 17 years (median, 9 years). The sex distribution was 11 spayed females, 11 castrated males, 2 reproductively intact females, and 2 reproductively intact males. Anatomic forms of the 22 lymphoma cases included nasal (6 cats), multicentric (4), alimentary (3), cutaneous (3), hepatic (2), laryngeal (1), ocular (1), renal (1), and tracheal (1) forms. In all tumor specimens from these cats, immunohistochemical analysis revealed positive reactions for CD20 and/or CD79α and negative reactions for CD3.

2.3. Cloning of feline *IGHV* genes

To clone feline *IGHV* genes, total RNA samples were extracted from normal spleens of 2 cats that had been euthanized for unrelated to lymphoid neoplasms; extraction was performed using an RNAqueous kit (Ambion, Austin, TX). To synthesize cDNA, total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers after treatment with DNase I (Invitrogen).

To amplify the entire *IGHV* genes, 2 primer sets (IgHV1L and FeIgMrV primers for amplification of the feline *IGHV-1* gene, and IgHV3L and FeIgMrV primers for amplification of the feline *IGHV-3* gene) were used; sequences of these primers were

previously described by Henrich *et al*. (2009). PCR for amplification of *IGHV* genes was performed using KOD FX high-fidelity DNA polymerase (Toyobo, Osaka, Japan). Cycle conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min.

The PCR products were cloned using a Zero Blunt PCR Cloning Kit (Invitrogen). Plasmid DNAs were isolated and purified using NucleoSpin1 Plasmid Kit (Macherey-Nagel, Düren, Germany). A total of 48 and 49 clones of *IGHV-1* and *IGHV-3* genes, respectively, were subjected to automated sequencing (ABI 3130xl Genetic Analyzer; Applied Biosystems), using primers complementary to the sequences of the plasmid.

2.4. Primer design for multiplex PCR

Forty-eight cDNA clones of feline *IGHV-1* genes and 49 cDNA clones of feline *IGHV-3* genes were sequenced after PCR amplification with the primer sets described. On the basis of the alignment of these sequences, a total of 6 forward primers (V1F1 and V1F2 for amplification of *IGHV-1* genes, and V3F1, V3F2, V3F3, and V3F4 for *IGHV-3* genes) were designed. For GeneScan analysis, forward primers complementary to the *IGHV-1* sequence (V1F1 and V1F2) and those complementary to the *IGHV-3* sequence (V3F1, V3F2, V3F3, and V3F4) were labeled with VIC and 6-FAM, respectively. As shown in Fig. 1, the V1F1 and V1F2 primers bind to framework region (FR) 1 and FR3 of the *IGHV-1* genes, respectively. The V3F1 and V3F2 primers bind to FR1 of the *IGHV-3* genes, and V3F3 and V3F4 bind to FR2 and FR3, respectively. Compared with the *IGHV-3* cDNA clones, the *IGHV-1* cDNA clones showed higher

degrees of sequence homology, which allowed for reduced numbers of *IGHV-1*–binding forward primers to anneal to most of the *IGHV-1* gene segments.

A total of 5 reverse primers were designed on the basis of the sequences of the JH of the *IGHV-1* and *IGHV-3* genes. All of the reverse primers were positioned at the same 5′-end to enable the use of these primers in a mixture. To facilitate A-addition to each PCR product, all of the reverse primers underwent the addition of a G to the 5′-end (Magnuson *et al*., 1996). Sequences of primers for amplification of CDR3 of the feline *IGHV* gene are shown in Table 1.

2.5. PCR analysis of *IGHV* gene rearrangement

DNA was extracted from fresh tissue or FFPE tissue by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).

Before the setting of multiplex PCR, all forward primers were tested by singleplex PCR. For singleplex PCR, PCR reactions were carried out in a final volume of 25 μL. The PCR mixture was composed of 1× Amplitaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA), each forward primer (V1F1, V1F2, V3F1, V3F2, V3F3, or V3F4) (200 nM), a mixture of reverse primers (JR1, JR2, JR3, JR4, and JR5) (JR1, JR2, and JR3, 2 μM; JR4 and JR5, 200 nM), and fresh tissue/cells (50 ng) or FFPE tissue samples (250 ng) as DNA templates. Cycle conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 90 s, and extension at 72°C for 30 s, and final extension at 72°C for 30 min to facilitate A-addition to each PCR product. Each PCR reaction was performed in duplicate.

For multiplex PCR, I prepared 2 tubes: (1) the *Multiplex FR1 tube,* which contained

2 forward primers (V1F1 and V3F1, complementary to FR1 of the *IGHV-1* and *IGHV-3* genes, respectively) and a mixture of 5 reverse primers (JR1, JR2, JR3, JR4, and JR5), and (2) the *Multiplex FR3 tube,* which contained 2 forward primers (V1F2 and V3F4, complementary to FR3 of the *IGHV-1* and *IGHV-3* genes, respectively) and a mixture of 5 reverse primers (JR1, JR2, JR3, JR4, and JR5). The amount of DNA used was increased (100 ng of fresh tissue/cells or 500 ng of FFPE tissue samples) for the multiplex PCR; however, the other PCR conditions were identical to those in the singleplex PCR.

The quality of DNA was evaluated using PCR amplification of an approximately 400-bp fragment of the feline β-actin gene (GeneBank: AB051104.1) and a 200-bp fragment of the feline androgen receptor gene (GeneBank: AJ893545.1). The primers for these genes were generated using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are shown in Table 1. PCR was performed using forward and reverse primers (200 nM each) for these control genes under the conditions used for amplification of CDR3 of the *IGHV* gene. DNA of the feline B-cell lymphoma cell line MS4, and 2 normal spleen samples were used as monoclonal and polyclonal controls, respectively.

2.6. PAGE and GeneScan analysis

All of the PCR products were analyzed by both PAGE and GeneScan analysis. For PAGE analysis, PCR products were heated at 95°C for 10 min and then cooled to 4°C for 1 h to allow reannealing (heteroduplex analysis). The PCR products were analyzed through 8% PAGE in Tris-borate-EDTA and stained with ethidium bromide. A reaction was considered monoclonal (or biclonal) if 1 (or 2) discrete and reproducible band(s) of

PCR products was observed in the duplicate samples within the expected size range on the gel after electrophoresis.

GeneScan analysis was carried out on the ABI 3130xl Genetic Analyzer (Applied Biosystems) with a G5 filter. Calibration of the G5 filter was performed using a DS-33 Matrix Standard Kit (Applied Biosystems). One microliter of a 10-fold diluted PCR product was mixed with 8.5 μL of formamide (Applied Biosystems) and 0.5 μL of 600 LIZ size standard (Applied Biosystems) in an optical 96-well plate. The products were denatured at 95°C for 5 min, immediately placed on ice for 15 min, and then subjected to the analysis. The data were analyzed with GeneMapper software (Applied Biosystems). When 1 (or 2) reproducible and identically sized distinct peak was observed in duplicate samples within the expected size range, it was judged as monoclonal (or biclonal). Polyclonal patterns were defined by the presence of multiple differently sized peaks frequently arranged in a Gaussian distribution (Fig 2B, Normal spleen).

3. Results

3.1. Cloning and sequencing of *IGHV* cDNAs from normal cat spleens

Forty-eight clones of feline *IGHV-1* genes and 49 clones of feline *IGHV-3* genes were sequenced after PCR amplification with primer sets as described above. The PCR products of the *IGHV-1* and *IGHV-3* genes were 558–594 bp and 550–592 bp in length, respectively. The sequences have been submitted to the DNA databank of Japan: AB609606–609702. The sequences were aligned and analyzed using the CLC Sequence Viewer (CLC bio, Katrinebjerg, Denmark).

3.2. Clonality analysis by singleplex PCR

DNA samples were extracted from primary tumor specimens before formalin fixation (20 tumor samples and 2 normal spleens) or FFPE samples (6 tumor samples). Assessment of DNA quality was performed by showing the amplification of control genes (β-actin gene, 400 bp; androgen receptor gene, 200 bp) before analysis of *IGHV* gene rearrangements. All of the samples from neoplastic and normal tissues yielded the PCR products of the 2 control genes. DNA samples from 26 B-cell neoplasms [22 lymphoma cases (Cases 1–22), 3 MRD cases (Cases 23–25), and 1 B-ALL case (Case 26)] were used for clonality analysis. Singleplex PCR amplifications of the CDR3 of DNA from 26 B-cell neoplasms, the MS4 cell line, and 2 normal spleens were performed in duplicate using each of the 6 VH forward primers (V1F1, V1F2, V3F1, V3F2, V3F3, and V3F4) and the mixture of 5 JH reverse primers (JR1, JR2, JR3, JR4, and JR5).

In the PAGE analysis, amplification of the DNA of the feline B-cell lymphoma cell line MS4 generated a single reproducible band using 3 *IGHV-3* primers (V3F1, V3F2, and V3F4) within the expected size range, indicating that the cells were a clonal cell population (Fig. 2A, MS4, the result with the V3F1 primer). On the other hand, amplification of splenic DNA produced only faint bands (Fig. 2A, normal spleen). Tumor samples obtained from 22 of 26 (84%) cases with B-cell neoplasms yielded a distinct band (1 exception, Case 26: 2 distinct bands) by PCR using at least 1 VH primer (V1F1, 2/26; V1F2, 2/26; V3F1, 12/26; V3F2, 12/26; V3F3, 15/26; V3F4, 9/26) (Table 2, Fig. 2A).

In GeneScan analysis, a distinct peak was detected in the amplification of MS4 DNA (Fig. 2B, MS4), whereas a Gaussian distribution curve was observed in the

samples of normal splenic DNA (Fig. 2B, normal spleen). All samples that had monoclonal *IGHV* gene rearrangements in PAGE analysis showed a distinct peak (Fig. 2B, Case 1) that was sometimes accompanied by multiple small peaks (Fig. 2B, Case 7) by GeneScan analysis. A biclonal (biallelic) *IGHV* gene rearrangement was found in Case 26 as indicated by 2 distinct peaks of 331 bp and 353 bp with the V3F1 primer (Fig. 2B, Case 26). Similarly, PCR amplification using the *IGHV-3* primers V3F2 and V3F3 generated 2 distinct peaks (300 bp and 321 bp with the V3F2 primer, and 237 bp and 258 bp with the V3F3 primer) in the sample of Case 26. Another example of biclonal rearrangement was found in Case 22 in which both *IGHV-1*– and *IGHV-3*–specific primers detected monoclonal *IGHV* gene rearrangement. The negative control (water) showed no PCR products in both PAGE (Fig. 2A, water) and GeneScan (Fig. 2B, water) analyses. Results of the analysis of the *IGHV* gene rearrangements by PCR amplification with each forward primer and a mixture of reverse primers are shown in Table 2. The results obtained by conventional PAGE and those by GeneScan analysis were 100% concordant.

Clonal *IGHV* gene rearrangement was detected in 22 of the 26 feline cases with B-cell neoplasms, but not in the other 4 cases. Further PCR analyses using primer sets reported by Werner *et al*. (2005) and Henrich *et al*. (2009) was carried out for evaluation of the clonal rearrangement of the *IGHV* gene in the 4 samples, but did not obtain any positive results to show clonal rearrangement in these samples.

3.3. Clonality analysis by multiplex PCR

To reduce the number of reaction tubes, multiplex PCR was performed in the *Multiplex FR1 tube* and *Multiplex FR3 tube*. PCR amplification obtained in the

Multiplex FR1 tube (with a mixture of V1F1 and V3F1 primers) revealed the same clonal *IGHV* gene rearrangements as those obtained by singleplex PCR with the V1F1 and V3F1 primers. Likewise, PCR amplification obtained in the *Multiplex FR3 tube* (with a mixture of V1F2 and V3F4 primers) revealed the same clonal *IGHV* gene rearrangements by singleplex PCR as those obtained with the V1F2 and V3F4 primers in all samples. The results obtained by multiplex PCR amplification were 100% concordant with those obtained by singleplex PCR. Fig. 3 shows simultaneous detection of VIC-labeled *IGHV-1* PCR products and 6-FAM–labeled *IGHV-3* PCR products in the same PCR reaction. GeneScan analysis and PAGE confirmed that no nonspecific products were amplified within the expected size range. Using the multiplex PCR technique, the number of PCR reactions could be reduced from 6 (6 singleplex PCRs) to 4 (2 multiplex and 2 singleplex PCRs) without decreasing sensitivity.

Discussion

In the present study, a multiplex PCR assay using fluorescent-labeled primers was constructed to detect clonal *IGHV* gene rearrangement in feline B-cell neoplasms. This assay enabled the detection of clonal *IGHV* gene rearrangements in 22 of 26 (84%) tissue samples of feline B-cell neoplasms by both singleplex and multiplex PCR.

Clonal rearrangement of the *IGHV* gene was detected in 20 feline B-cell neoplasms by PCR using *IGHV-3*–specific primers; however, rearrangement was demonstrated in only 2 samples by PCR with *IGHV-1*–specific primers (in Case 22, both *IGHV-1*– and *IGHV-3*–specific primers detected clonal rearrangement of *IGHV* gene). Similar results were reported in a study by Henrich *et al*. (2009). Frequent usage of the *IGHV-3* gene was also demonstrated in human normal lymphocytes and human B-ALL (Mortuza *et*

al., 2001) compared to *IGHV-1* gene. Other possible explanations for the less frequent detection using *IGHV-1*–specific primers compared with *IGHV-3*–specific primers may be attributed to the smaller number of *IGHV-1*–specific primers. Because *IGHV-1* genes showed higher homology than did *IGHV-3* genes in the present study, 2 *IGHV-1*–specific primers and 4 *IGHV-3*–specific primers were designed to cover most of these sequences. Use of the smaller number of *IGHV-1*–specific primers can be more susceptible to somatic hypermutation at primer binding sites, which results in failure of CDR3 amplification and a less frequent detection rate than the actual frequency in usage of the *IGHV-1* gene.

The greater number of primer sets in the present study resulted in the detection of clonal *IGHV* gene rearrangements in 22 of 26 (84%) cats with feline B-cell neoplasms. However, clonal *IGHV* gene rearrangement was not detected in 4 feline B-cell lymphoma samples. There are several possible explanations for these false-negative results. Although 6 primer sets were designed to cover most of the VH and JH segments, including minor ones, and to avoid failure of detection due to somatic hypermutation at the primer binding sites, the presence of unidentified VH and JH segments or somatic hypermutation cannot be completely ruled out. The phenomenon that different primer sets applied to the same sample detected different clonal statuses (e.g., in Case 1, primer sets of V3F1 detected clonal *IGHV* gene rearrangement, whereas V3F2, V3F3, and V3F4 did not) was frequently observed. In some of these samples, clonally rearranged *IGHV* genes were sequenced. Somatic hypermutation frequently occurred not only in the CDRs but also in the FRs (data not shown) in these clonally rearranged *IGHV* genes. Werner *et al*. (2005) also reported somatic hypermutation at the primer binding sites. From these observations, it was concluded that the use of a large number of VH primers

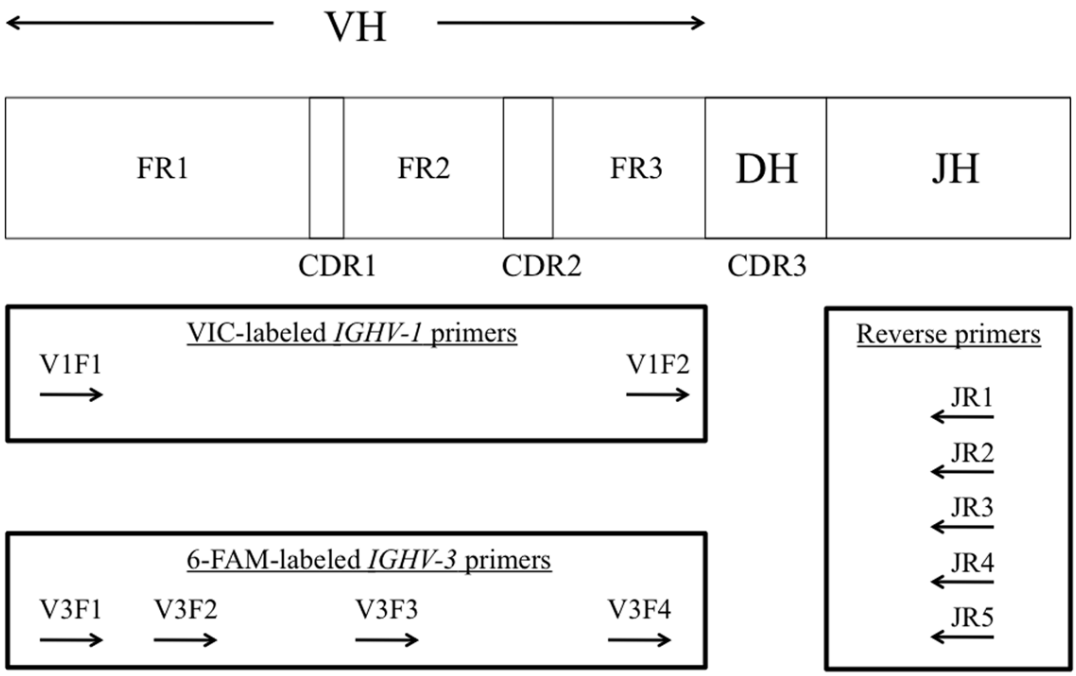
was necessary to increase the sensitivity of detecting clonal *IGHV* rearrangement by PCR in feline B-cell neoplasms, as well as in the diseases in other species.

Another possible explanation of failure of the detection of *IGHV* gene rearrangement is chromosomal translocation involving the *IGHV* gene, which could not be detected by the present assay. In human B-cell neoplasms, t(11;14)(q13;q32), which results in the generation of a *BCL1-IGHV* fusion gene, was detected in 60–70% of mantle cell lymphoma patients and small populations of other B-cell neoplasms (de Boer *et al*., 1997). Another well-known translocation in B-cell lymphoid neoplasms, t(14;18), which produces a *BCL2-IGHV* fusion gene, was present in up to 90% of patients with follicular lymphoma and approximately 20% of patients with large B-cell lymphoma (Weiss *et al*., 1987). Although chromosomal translocations found in human B-cell neoplasms have not been identified in feline B-cell neoplasms, they might also be responsible for the failure of *IGHV* gene rearrangements in feline B-cell neoplasms.

Although the results obtained by the conventional PAGE and GeneScan analyses were 100% concordant, it is conceivable that GeneScan analysis has several advantages over conventional PAGE analysis. First, GeneScan analysis employs an automated, rapid, and relatively simple technique, which leads to less laboratory work and human error. Second, GeneScan analysis enables exact size determination of each of the clonal *IGHV* PCR products. For example, PCR of the MS4 cell line DNA reproducibly generated 330-, 298-, and 148-bp products with primers V3F1, V3F2, and V3F4, respectively. Precise size determination of each rearranged *IGHV* gene can be used as a specific marker (fingerprint) of neoplastic B cells in each case. Therefore, GeneScan analysis can be used to monitor the same neoplastic B-cell proliferation during or after treatment and to determine whether multiple masses in the same patient have the same

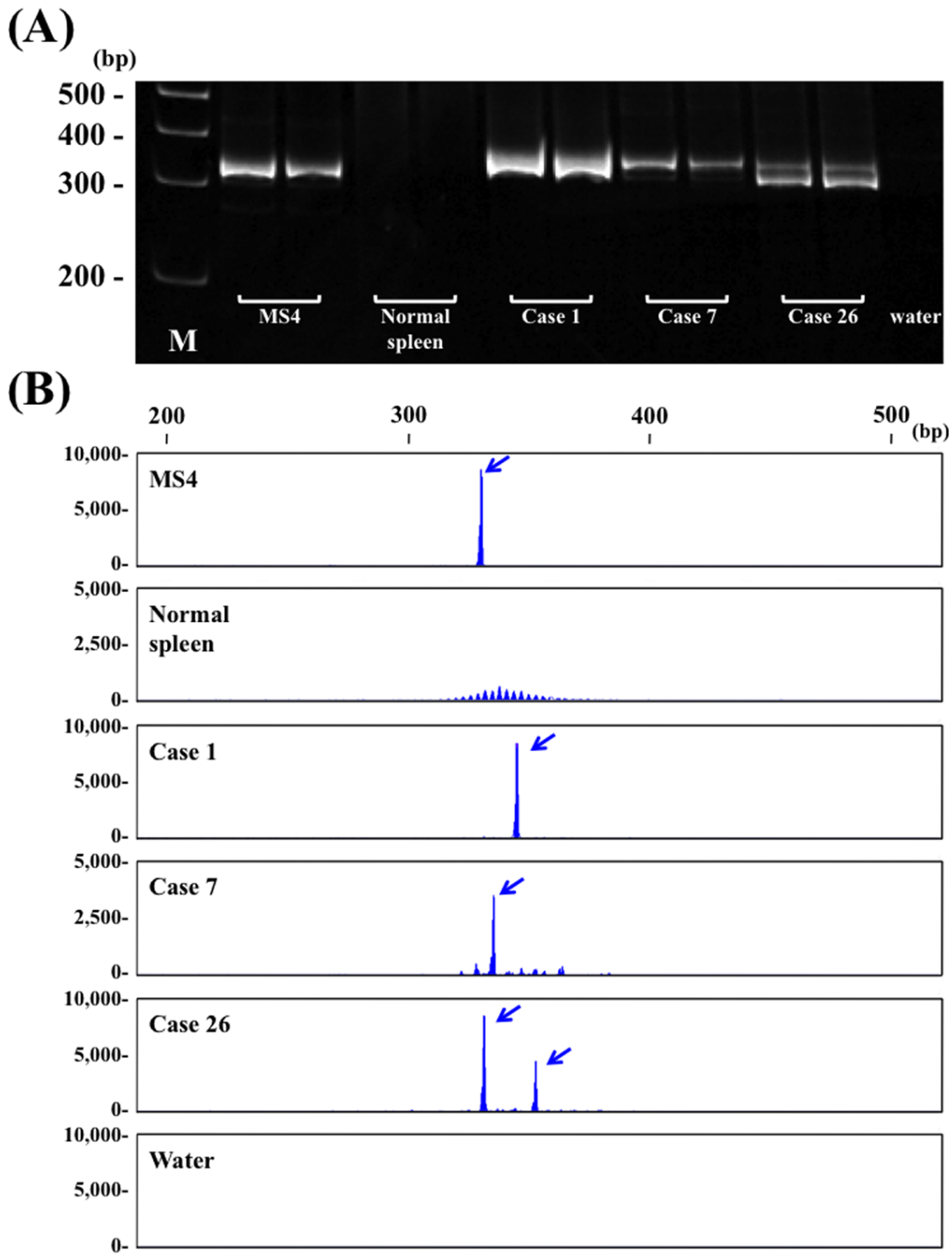
origin. In addition, precise size determination could facilitate the interpretation of abnormal samples, such as biclonal rearrangements. A biclonal (biallelic) *IGHV* gene rearrangements were detected in a B-ALL sample (Case 26) with 3 *IGHV-3*–specific forward primers. The size difference between 2 peaks obtained by 3 primers was always 21 bp, which strongly indicates that these PCR products reflected the biclonal *IGHV* gene rearrangements rather than being a result of PCR errors. Third, the combination of the fluorescent dyes 6-FAM and VIC used in this study, as well as several other fluorescent dyes, can be used simultaneously in GeneScan analysis. Therefore, GeneScan analysis enables judgment of the result of each in multiplex PCR with different fluorescence-labeled primer pairs, leading to a highly sensitive PCR assay without increasing the number of reaction tubes. In the present assay, it was actually possible to reduce the number of PCR reactions from 6 to 4 by labeling the 2 primers (*IGHV-1*– and *IGHV-3*–specific primers) with 2 different fluorescent dyes, respectively, resulting in the construction of a less complicated assay. In conclusion, a multiplex PCR-based assay was constructed to detect clonal *IGHV* gene rearrangement in feline B-cell neoplasms. The assay developed here would be a useful supportive diagnostic tool of feline B-cell neoplasms with high fidelity.

Fig. 1.



Schematic representation of rearranged immunoglobulin heavy-chain variable region (*IGHV*) genes and locations of primers designed in this study. V1F1 and V1F2 primers were designed to bind to framework region (FR) 1 and FR3 of the *IGHV-1* gene, respectively. For the *IGHV-3* gene, V3F1 and V3F2 (FR1), V3F3 (FR2), and V3F4 (FR3) were designed. *IGHV-1*– and *IGHV-3*–specific primers were labeled with VIC and 6-FAM, respectively. Five reverse primers were positioned at the same 5′-end and were used as a mixture. VH, variable region; DH, diversity region; JH, joining region; FR, framework region; CDR, complementarity-determining region.

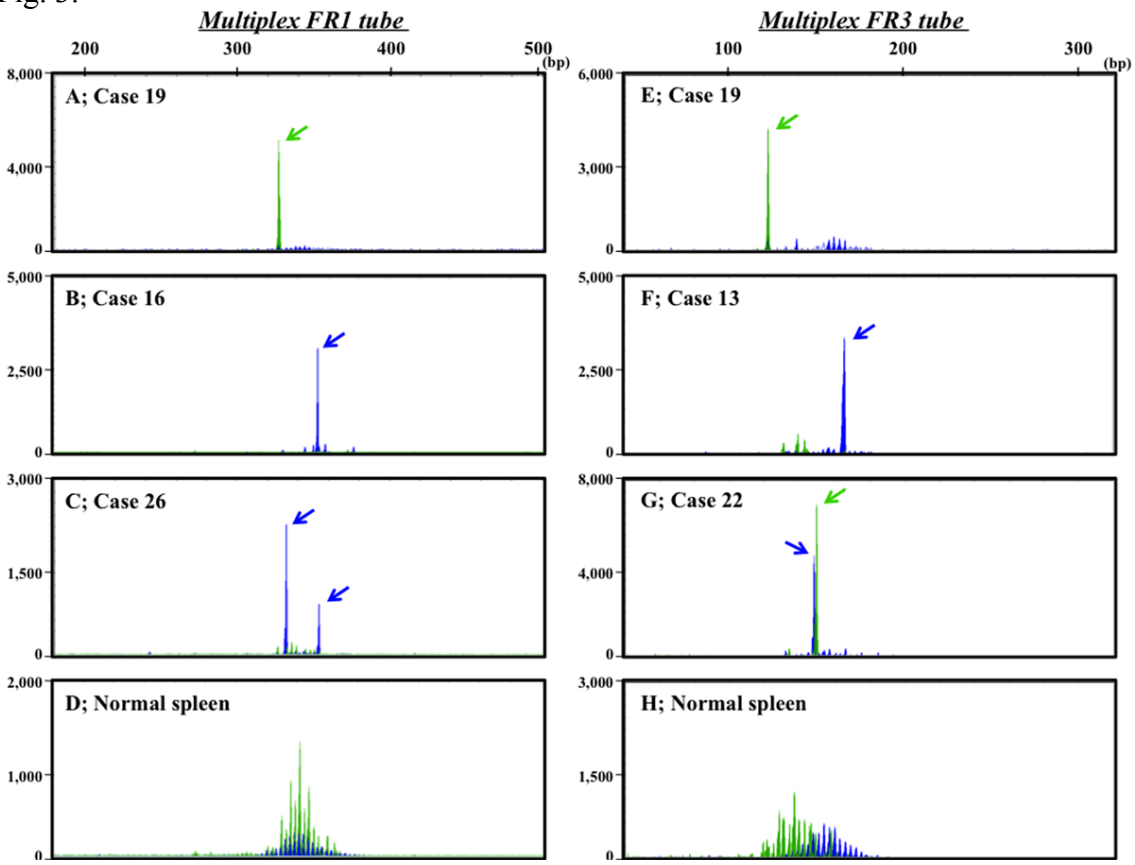
Fig. 2.



Examples of the PCR analysis of *IGHV* gene rearrangement using a 6-FAM–labeled

V3F1 forward primer and reverse prime mixture (JR1, JR2, JR3, JR4, and JR5). (A) A representative result of PAGE. MS4 cells, normal spleens, and water were used as monoclonal, polyclonal, and negative controls, respectively. (B) Results of the Genescan analysis for the same samples used for PAGE. Blue peaks represent 6-FAM–labeled PCR products. Samples of MS4 cells and normal spleen cells generated a distinct peak and multiple small peaks, respectively. Each blue arrow indicates a clonal peak.

Fig. 3.



Representative results with multiplex PCR analyzed by Genescan analysis showing simultaneous detection of PCR products of VIC-labeled *IGHV-1* gene (green peaks) and 6-FAM–labeled *IGHV-3* gene (blue peaks). Results from the *Multiplex FR1 tube* [multiplex PCR tube containing V1F1 and V3F1 forward primers and reverse primer mixture (JR1, JR2, JR3, JR4, and JR5)] for Cases 19, 16, and 26 and normal spleen cells are shown in the left panel (A–D). The *Multiplex FR3 tube* [multiplex PCR tube containing V1F2 and V3F4 forward primers and reverse primer mixture (JR1, JR2, JR3, JR4, and JR5)] for Cases 19, 13, and 22 and normal spleen cells are shown in the right panel (E–H). Green and blue arrows indicate *IGHV-1* and *IGHV-3* clonal peaks, respectively.

Table 1. Primers to amplify feline IGHV gene and control genes

Primer	Binding site	Sequence	Product size (bp)	Labeling
V1F1	*IGHV-1 VH FR1*	5'- GCTGGTGCAGTCTGGGGCTG -3'	310—380	VIC
V1F2	*IGHV-1 VH FR3*	5'- GCAGACACATCCACAAACACAGCCTAC -3'	100—170	VIC
V3F1	*IGHV-3 VH FR1*	5'- GGTGGAGTCTGGGGGAGACCTG -3'	310—380	FAM
V3F2	*IGHV-3 VH FR1*	5'- GGGGGTCCCTGAGACTCACCTG -3'	270—340	FAM
V3F3	*IGHV-3 VH FR2*	5'- GGGTCCGCCAGGCTCCAGG -3'	210—280	FAM
V3F4	*IGHV-3 VH FR3*	5'- GGCCGATTCACCATCTCCAGAGAC -3'	120—190	FAM
JR1	*IGHV JH*	5'- GCYSTCACCAGGRYTCCYBGGC -3'	-	-
JR2	*IGHV JH*	5'- GCTGYGACHMTDGTTCCAYGGCCC -3'	-	-
JR3	*IGHV JH*	5'- GCGRTGAYCWGGGTRYCYTGGC -3'	-	-
JR4	*IGHV JH*	5'- GCGGTGACCAGGGTCCCGGGGCCC -3'	-	-
JR5	*IGHV JH*	5'- GCCGTCACCAGGGTTCCGACGCC -3'	-	-
ACTB-f	*β-actin*	5'- CCAGGCCGTGCTGTCTCTGTACG -3'	400	-
ACTB-r	*β-actin*	5'- CCAGGAAGGAAGGCTGGAAGAGC -3'	-	-
AR-f	*Androgen receptor*	5'- CACAATGCCGCTACGGGTTTTCCA -3'	200	-
AR-r	*Androgen receptor*	5'- CACAATGCCGCTACGGGGACCT -3'	-	-

Table 2. Results of PCR detection of IGHV gene rearrangement in 26 cats with B-cell neoplasms

Case Number	Forward primer					
	IGHV-1		IGHV-3			
	V1F1	V1F2	V3F1	V3F2	V3F3	V3F4
1	P	P	M	P	P	P
2	P	P	M	M	M	M
3	P	P	M	P	M	P
4	P	P	M	M	M	P
5	P	P	P	P	P	P
6	P	P	P	M	M	M
7	P	P	M	P	M	P
8	P	P	P	P	P	P
9	P	P	P	M	P	P
10	P	P	P	M	M	P
11	P	P	M	M	P	P
12	P	P	M	M	P	M
13	P	P	M	M	M	M
14	P	P	M	P	M	P
15	P	P	P	P	P	P
16	P	P	M	M	M	M
17	P	P	P	P	M	P
18	P	P	P	P	P	P
19	M	M	P	P	P	P
20	P	P	P	P	M	P
21	P	P	P	P	P	M
22	M	M	M	M	M	M
23	P	P	P	M	M	P
24	P	P	P	P	M	P
25	P	P	P	P	P	M
26	P	P	B	B	B	M

*M, monoclonal; B, biclonal; P, polyclonal

Chapter 2

GeneScan analysis to detect clonality of T-cell receptor γ gene rearrangement in feline lymphoid neoplasms

Abstract

Lymphoid neoplasms are usually diagnosed on the basis of cytological and histopathological findings. However, in some cases, discrimination of lymphoid neoplasms from reactive lymphoid proliferation is difficult. PCR amplification of the complementarity-determining region (CDR) 3 of the *T-cell receptor (TCR) γ* gene can be used to assess clonality of T-cell populations as a supportive diagnostic tool for T-cell neoplasms. However, because the length variation in the *TCRγ* CDR3 is relatively small, false positive results may occur in non-neoplastic T-cell populations in the absence of high-resolution analytical methods for PCR products. In the present study, a PCR assay system was developed to detect clonal *TCRγ* gene rearrangement in feline lymphoid cells using GeneScan analysis. Thirty T-cell neoplasms, 27 B-cell neoplasms, and 34 non-neoplastic tissues were subjected to the newly developed *TCRγ* gene rearrangement analysis. Clonal *TCRγ* gene rearrangement was detected in 26 of 30 (87%) T-cell neoplasms, 2 of 27 (7%) B-cell neoplasms, and 1 of 34 (3%) non-neoplastic tissues. To compare GeneScan analysis with conventional PAGE and heteroduplex analysis, 20 clonal and 20 polyclonal samples were subjected to both analyses. Most of the results were concordant between the 2 analyses; however, several clonal peaks (bands) appeared as a single band when analyzed via conventional PAGE with heteroduplex analysis in 4 of the 20 (20%) clonal samples as a result of the difference in resolution. The PCR assay system to detect clonal *TCRγ* gene rearrangement in feline lymphoid cells, using GeneScan analysis, would be a useful molecular diagnostic tool for feline T-cell neoplasms, with high fidelity.

1. Introduction

Lymphoid tumor is the most common neoplasm in cats, and is generally diagnosed on the basis of cytological and/or histopathological findings. However, objective discrimination between reactive and neoplastic lesions with infiltration/proliferation of lymphoid cells is sometimes difficult based on histopathological findings; for example, discrimination between low-grade alimentary lymphoma and severe lymphoplasmacytic enteritis (LPE) is sometimes controversial, particularly when endoscopic biopsy specimens are evaluated (Evans *et al*., 2006; Briscoe *et al*., 2011; Kiupel *et al*., 2011). In such cases, PCR-based clonality assessment of *immunoglobulin* (*Ig*) and *T-cell receptor* (*TCR*) gene rearrangements can be a useful additional diagnostic tool.

The TCR is a complex of integral membrane proteins and is composed of 2 different types of peptides: α and β chains in αβ T-cells and γ and δ chains in γδ T-cells. The gene loci encoding these 4 molecules, *TCRα*, *TCRβ*, *TCRγ*, and *TCRδ*, contain variable (V), [diversity (D)], joining (J), and constant region genes within the loci. During T-cell development, rearrangement of *TCR* genes through recombination of V, (D), and J regions occurs in the early stages of lymphoid differentiation (Blom *et al*., 1999). In addition to the random recombination of V, (D), and J segments, junctional diversity is generated by the deletion and random insertion of a few nucleotides. For these reasons, the length and nucleotide sequences in the complementarity-determining region (CDR) 3 of *TCR* genes vary among T-cells (Davis *et al*., 1988; Lafaille *et al*., 1989). Therefore, PCR amplification of CDR3 using forward primers at V segments and reverse primers at J segments results in many PCR products of various sizes in both normal and reactive lymphoid tissues. On the other hand, T-cell lymphoid neoplasms comprise a population

of clonally expanded cells fundamentally derived from a single neoplastic T-cell; virtually all of the cells contain a unique *TCR* gene rearrangement.

Of these *TCR* genes, assessment of *TCRγ* gene rearrangement can be a useful marker for the detection of T-cell clonality because the rearrangement of the *TCRγ* gene occurs in most T-cells, including both αβ and γδ T-cell lineages; whereas rearrangements of the other *TCR* genes are restricted to a particular lineage (Blom *et al*., 1999). PCR assays to assess *TCRγ* gene clonality in cats have been described in 2 studies (Moore *et al*., 2005; Weiss *et al*., 2011). Although the value of clonality assessment using *TCRγ* gene rearrangement is known, the assay can result in false negative (indicating polyclonality in neoplastic samples) and false positive (indicating clonality in non-neoplastic samples) results. To avoid false negative results, suitable primers should be designed to be able to anneal to as many of the V and J segments in *TCRγ* genes as possible. False positive results would be derived from the limited diversity of the sizes in *TCRγ* CDR3. Because the *TCRγ* gene lacks the D region, diversity is generated mainly due to the insertion and deletion of random nucleotides at the VJ junction, and it tends to be smaller than other antigen receptor genes containing the D region, such as the *Ig heavy chain* (*IgH*) gene (van Dongen *et al*., 2003). Furthermore, a previous study indicated that the size diversity of PCR products derived from *TCRγ* CDR3 was limited, particularly when the J region primers were designed from the sequences of J2 and J3 segments, leading to false positive results in non-neoplastic samples (Weiss *et al*., 2011).

It is thought that advanced analytical methods with higher resolution can be a solution for this problem. In the present study, capillary electrophoresis using fluorescently labeled primers (GeneScan analysis) to separate different PCR products

that differ in size by 1 bp (van Dongen *et al*., 2003), a high-resolution fragment analysis method, was employed for the PCR-based clonality assessment of feline T-cells.

The purpose of the present study was to develop a PCR assay system to detect clonal *TCRγ* gene rearrangement in feline lymphoid cells using GeneScan analysis. The PCR assay was evaluated in terms of the ability to detect clonal *TCRγ* gene rearrangement in feline T- and B-cell neoplasms, in contrast to the polyclonality of non-neoplastic tissues.

2. Materials and methods

2.1. *Cases*

The samples used in the present study were tissues or cells of lymphoid neoplasms (57 samples) or non-neoplastic tissue samples (34 samples) obtained from feline patients referred to the Veterinary Medical Center of the University of Tokyo between 2004 and 2011.

The ages of the 57 cats with lymphoid neoplasms ranged from 10 months to 17 years (median, 11 years). The sex distribution was as follows: 28 spayed females, 18 castrated males, 4 reproductively intact females, and 7 reproductively intact males. Mixed (45 cats), American shorthair (8), Abyssinian (1), Birman (1), British shorthair (1), and Persian (1) breeds were included. The anatomic forms of the lymphoma in the 51 cases were as follows: alimentary (27 cats), nasal (6), cutaneous (5), multicentric (4), mediastinal (3), hepatic (3), laryngeal (1), renal (1), and tracheal (1) forms.

The 57 lymphoid neoplasm samples comprised lymphoma (51 cats), myeloma-related disorder [MRD; a disease group proposed by Mellor *et al*. (2008)] (3),

chronic lymphocytic leukemia (CLL) (2), and acute lymphoblastic leukemia (ALL) (1).

Of the 57 samples from cats with lymphoid neoplasms, 30 (28 lymphoma and 2 CLL) and 27 samples (23 lymphoma, 3 MRD, and 1 ALL) were shown to have T- and B-cell phenotypes, respectively, as determined by immunohistochemistry (52 samples) or flow cytometry (5 samples). For histopathological analysis, 86 tissue samples (52 lymphoid neoplasms and 34 non-neoplastic tissues) obtained by biopsy or necropsy were fixed in 10% neutral buffered formalin, stained with hematoxylin and eosin, and analyzed by light microscopy. Immunohistochemistry was performed on 10% buffered formalin-fixed and paraffin-embedded (FFPE) tissues sectioned at 4 μm using an antibody against human CD3 (1:50; DAKO, Glostrup, Denmark) as a T-cell marker, and antibodies against human CD20 (1:400; Neomarkers, Fremont, CA) and human CD79α (1:50; Clone: HM57; Dako) as B-cell markers. Normal lymph node and spleen samples obtained from a cat that had died of a disease unrelated to lymphoid neoplasm were used as controls. For each flowcytometric analysis, 1×10^6 cells were incubated with the 5 mouse monoclonal antibodies against feline CD4 (Clone: FE1.7B12), feline CD5 (Clone: FE1.1B11), feline CD8 (Clone: FE1.10E9), canine CD21 (Clone: CA2.1D6), and feline CD22 (Clone: FE2.9F2) for 30min on ice. Antibodies were purchased from the Leukocyte Antigen Biology Laboratory (Davis, CA). After a washing step, the samples were labeled with FITC-conjugated anti-mouse IgG1 antibody (BD Biosciences, San Jose, CA) and incubated for an additional 30 min on ice. Finally, labeled cells were washed twice and analyzed on a flow cytometer (FACSCalibur; BD Biosciences). Cells were judged to be of T-cell origin if cells were positive for CD5 and CD4 and/or CD8α, and B-cell origin if cells were positive for CD21 and/or CD22.

Thirty-four tissue specimens diagnosed by histopathological examination were used as non-neoplastic tissues in the present study. The 34 non-neoplastic samples consisted of 15 small intestines (12 lymphoplasmacytic enteritis (LPE) and 3 normal duodenum), 14 livers (6 lymphocytic cholangitis/hepatocholangitis and 8 normal liver), and 5 lymph nodes (4 lymph node hyperplasia and 1 lymphadenitis).

2.2. PCR analysis of TCRγ gene rearrangement

DNA specimens were extracted from fresh cells and tissues (47 neoplastic and 12 non-neoplastic samples) or FFPE tissues (10 neoplastic and 24 non-neoplastic samples), using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The quality of DNA was evaluated by PCR amplification of an approximately 200-bp fragment of the feline *androgen receptor* gene, as described in Chapter 1.

It has been reported that the feline *TCRγ* locus consists of 2 V gene subgroups containing 5 members (*TCRγ-V1–V5*) and 3 J gene subgroups (*TCRγ-J1–J3*) (Weiss *et al*., 2008; Weiss *et al*., 2010; Weiss *et al*., 2011). On the basis of the previously published feline *TCRγ* gene sequences, 4 forward primers (ftcrgv1-2, ftcrgv3, ftcrgv4, and ftcrgv5) and 3 reverse primers (ftcrgj1, ftcrgj2, and ftcrgj3) were synthesized for the amplification of feline *TCRγ* CDR3: 4 forward primers complementary to *TCRγ-V* segments and 3 reverse primers complementary to *TCRγ-J* segments (Fig. 4). The ftrgv1-2 primer binds to *TCRγ-V1* and *TCRγ-V2*, while the ftrgv3, ftrgv4, and ftrgv5 primers were designed to bind to *TCRγ-V3*, *TCRγ-V4*, and *TCRγ-V5*, respectively. To facilitate A-addition to each PCR product, all of the forward primers underwent the addition of a guanine nucleotide to the 5′-end (Magnuson *et al*., 1996). The ftcrgj1,

ftcrgj2, and ftcrgj3 primers were designed to bind to *TCRγ-J1*, *TCRγ-J2*, and *TCRγ-J3*, respectively. For GeneScan analysis, the ftcrgj1, ftcrgj2, and ftcrgj3 primers were labeled with 6-FAM, NED, and PET, respectively. Sequences of the primers for the amplification of feline *TCRγ* CDR3 are shown in Table 3.

In the present study, 3 PCR reactions (*fTCRγ-J1*, *fTCRγ-J2*, and *fTCRγ-J3*) were prepared for the analysis of *TCRγ* gene rearrangement: each tube contained a mixture of 4 *TCRγ-V* primers and each of the 3 *TCRγ-J* primers. PCR reactions were carried out in a final volume of 25 μL. The PCR mixture was composed of 1× Amplitaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA), a mixture of 4 forward primers (ftcrgv1-2, ftcrgv3, ftcrgv4, and ftcrgv5) (200 nM), each of the reverse primers (FAM-labeled-ftcrgj1 in *fTCRγ-J1*; 20 nM, NED-labeled-ftcrgj2 *fTCRγ-J2*; 40 nM, and PET-labeled-ftcrgj3 in *fTCRγ-J3*; 10 nM), and fresh tissue or cell DNA samples (50 ng) or FFPE tissue DNA samples (250 ng) as DNA templates. Cycle conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 90 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 30 min to facilitate A-addition to each PCR product. Each PCR reaction was performed in duplicate.

DNA samples extracted from 2 feline T-cell lymphoma cell lines, FT-1 (Miura *et al*., 1987) and 3201 (Snyder *et al*., 1978), were used as clonal controls for the *fTCRγ-J1* and *fTCRγ-J2* PCR reactions, respectively.

GeneScan analysis was carried out using the ABI 3130xl Genetic Analyzer (Applied Biosystems) with a G5 filter. Calibration of the G5 filter was performed using a DS-33 Matrix Standard Kit (Applied Biosystems). GeneScan analysis was performed as a multiplex run. Equal volumes of the 3 PCR reactions (*fTCRγ-J1*, *fTCRγ-J2*, and

fTCRγ-J3) were mixed and subjected to capillary electrophoresis. One microliter of a 10-fold diluted PCR product mixture was mixed with 8.5 μL of formamide and 0.5 μL of 600 LIZ size standard (Applied Biosystems) in an optical 96-well plate. The products were denatured at 95°C for 5 min, immediately placed on ice for 15 min, and then subjected to the analysis. The data were analyzed with the GeneMapper software (Applied Biosystems). Although each well contained 3 different PCR products obtained from 1 DNA sample, the 3 PCR products were labeled with different dyes and could be detected separately (multiplex run). When 1–3 reproducible and identically sized, distinct peaks within the expected size range were observed in duplicate samples, it was judged as clonal. Polyclonal patterns were defined by the presence of multiple peaks of different size.

To compare the results between GeneScan analysis and conventional non-denaturing PAGE analysis, 20 clonal and 20 polyclonal PCR products were randomly selected from the sample population and were analyzed by PAGE analysis. For PAGE analysis, PCR products were heated at 95°C for 10 min and then cooled to 4°C for 1 h to allow reannealing (heteroduplex analysis). The PCR products were analyzed through 15% PAGE in Tris-borate-EDTA buffer and stained with ethidium bromide. A reaction was considered clonal if 1–3 discrete and reproducible band(s) of PCR products were observed within the expected size range in duplicate samples on the gel after electrophoresis.

3. Results

3.1. GeneScan analysis of the TCRγ gene to detect clonality in feline neoplastic and

non-neoplastic tissue/cell samples

The assessment of DNA quality was performed by demonstrating the amplification of a control gene (the feline *androgen receptor* gene, 200 bp) before analysis of *TCRγ* gene rearrangements. All of the samples from neoplastic and non-neoplastic tissues yielded PCR products of the *androgen receptor* gene. DNA samples from 30 T-cell neoplasms, 27 B-cell neoplasms, and 34 non-neoplastic tissues were used for the clonality analysis.

Typical clonal and polyclonal patterns are shown in Fig. 5. GeneScan analysis of a DNA sample from a feline T-cell lymphoma cell line, FT-1, generated a 101-bp PCR product in the *fTCRγ-J1* PCR reaction (Fig. 5A). Similarly, a DNA sample from 3201 cells generated a 102-bp PCR product in the *fTCRγ-J2* PCR reaction (Fig. 5B). The DNA samples of these 2 feline T-cell lymphoma cell lines were used as clonal controls for the *fTCRγ-J1* and *fTCRγ-J2* reactions. Samples judged to be polyclonal showed peaks of multiple sizes within the expected size range (Fig. 5C and D). Samples judged to be clonal showed 1 (Fig. 5E), 2 (Fig. 5F), and 3 (Fig. 5G) reproducible distinct peak(s). A small polyclonal peak, of which the peak was 91–94 bp in size, was found in most of the PCR products in the *fTCRγ-J1* reaction (indicated as "*P*" in Fig. 5C, F, and G).

The *fTCRγ-J1* reaction yielded clonal results (a single peak in 6 cases, 2 peaks in 11 cases, and 3 peaks in 3 cases) in 20 of 30 lymphoid neoplasms with a T-cell phenotype, 2 of 27 (a single peak in 1 case and 2 peaks in 1 case) lymphoid neoplasms with a B-cell phenotype, and 1 of 34 non-neoplastic samples (a single peak). The *fTCRγ-J2* reaction yielded clonal results in 15 of 30 lymphoid neoplasms with a T-cell phenotype (a single

peak in 10 cases and 2 peaks in 5 cases), 2 of 27 lymphoid neoplasms with a B-cell phenotype (a single peak in both cases), and 1 of 34 polyclonal samples (2 peaks). The *fTCRγ-J3* reaction did not yield clonal results in any of the neoplastic or non-neoplastic samples. Comprehensive results of the GeneScan analysis are shown in Table 4.

Clonal *TCRγ* gene rearrangements were detected in 87% (26/30) of the T-cell neoplasms by the use of a combination of the *fTCRγ-J1* and *fTCRγ-J2* reactions. Of the 26 T-cell neoplasms showing clonal *TCRγ* gene rearrangements, 11, 6, and 9 samples were shown to be clonal by PCR in the *fTCRγ-J1* reaction only, *fTCRγ-J2* reaction only, and both *fTCRγ-J1* and *fTCRγ-J2* reactions, respectively. In 27 B-cell neoplasms, 7% (2/27) of the samples were shown to have clonal *TCRγ* gene rearrangements in both of the *fTCRγ-J1* and *fTCRγ-J2* reactions. Of the 2 B-cell neoplasms with a clonal rearrangement of the *TCRγ* gene, one was a multicentric B-cell lymphoma and the other was a B-cell hepatic lymphoma. Clonal rearrangement of the *IgH* gene was detected in the DNA sample from the hepatic B-cell lymphoma, but not in that of the multicentric B-cell lymphoma (data not shown). Of the 34 non-neoplastic samples, 1 DNA sample from the duodenum of a cat with LPE showed clonal results in both of the *fTCRγ-J1* and *fTCRγ-J2* reactions. The sample was further analyzed by a different *TCRγ-J1*-targeted primer set that was reported previously (Moore *et al*., 2005) for evaluation of the clonal rearrangement, and this primer s*et al*so revealed a clonal result (data not shown).

3.2. Comparison between GeneScan analysis and PAGE with heteroduplex analysis

To compare the results of GeneScan analysis with those of the conventional PAGE method with heteroduplex analysis, 20 clonal and 20 polyclonal samples were randomly

selected from the present study population and were subjected to both analyses. All samples with clonal *TCRγ* gene rearrangement detected by GeneScan analysis were shown to be clonal by PAGE with heteroduplex analysis. Similarly, all samples with polyclonal *TCRγ* gene rearrangement detected by GeneScan analysis were revealed to be polyclonal by PAGE with heteroduplex analysis (Fig. 6). However, discrepancies in the number of bands (peaks) were observed in 4 of the 20 clonal samples. For example, DNA from Cases 4 and 25 yielded 2 and 3 reproducible distinct peaks in GeneScan analysis, respectively; however, these PCR products appeared as a single band in the PAGE analysis (Fig. 6). Discrepancies between the 2 analytical methods were observed when the sizes of the 2 or 3 clonal peaks differed by less than 6 bp, as detected in GeneScan analysis.

3.3. Sensitivity of GeneScan analysis in serially diluted samples

DNA samples of 2 feline T-cell lymphoma cell lines, FT-1 and 3201, were serially diluted with DNA from histologically normal liver, spleen, or duodenum. Clonal peaks could be detected when DNA of the malignant clones was diluted to as little as 20%, 10%, and 5% with the liver or duodenum DNA (Fig. 7). When the neoplastic DNA samples were diluted to 2% or 1% with normal liver or duodenum DNA, the clonal peak was effaced by polyclonal backgrounds. When neoplastic DNA was diluted with normal spleen DNA, the lower limit for detection of the clonal peaks was 10%.

4. Discussion

In the present study, GeneScan analysis to detect clonal feline *TCRγ* gene

rearrangement was developed. The system developed was highly sensitive (87%) and was able to detect clonal *TCRγ* gene rearrangement in specimens from 30 cats with T-cell neoplasms.

In the present study, clonal rearrangement of the *TCRγ* gene was detected in 67% of feline T-cell neoplasms by PCR using a primer complementary to *TCRγ-J1*. This is in approximate accordance with the results of previous reports on *TCRγ* gene rearrangement analysis in feline lymphomas: 2 previous studies reported the sensitivity of the PCR assay using a primer complementary to *TCRγ-J1* to be as high as 89% and 68%, respectively (Moore *et al*., 2005; Weiss *et al*., 2011).

The primer complementary to *TCRγ-J2* yielded clonal results in 50% of feline T-cell neoplasms and 3% of non-neoplastic tissues. The result in this study was different from that of a previous study (Weiss *et al*., 2011), where PCR amplification using a *TCRγ-J2*-targeted reverse primer generated clonal results in 44% of non-neoplastic tissues. The difference between the present study and the previous study (Weiss *et al*., 2011) may be explained by differences in PCR product size and the analytical methods used. In their report, the PCR products were analyzed by non-denaturing PAGE with heteroduplex analysis, and the sizes of the PCR products were larger than 250 bp. They observed that there were only slight differences in the sizes of the CDR3 PCR products when the *TCRγ-J2*-targeted reverse primer was used. Slight variation in the sizes of such relatively large PCR products would make it difficult to interpret PCR results in the PAGE method with heteroduplex analysis that was used in their study. In contrast, the primers used in the present study were designed to amplify the *TCRγ* fragments that were 80–120 bp. A smaller PCR product size was considered to be suitable for better separation. Moreover, the GeneScan analysis employed here reflects a higher resolution

analysis to discriminate PCR products with a size difference of 1 bp.

The primer complementary to *TCRγ-J3* did not yield clonal results in any of the lymphoid neoplasms or non-neoplastic lymphoid cell/tissue samples. Because the *TCRγ-J3* sequence lacks a conserved nucleotide sequence typical of J regions (Weiss *et al*., 2008), the functional recombination of *TCRγ-J3* may be rare. The rare usage of *TCRγ-J3* in normal and neoplastic lymphocytes may lead to a lack of clonal results. Considering the frequency of usage of *TCRγ-J3* in comparison to *TCRγ-J1* and *TCRγ-J2*, a combination of the *fTCRγ-J1* and *fTCRγ-J2* reactions would be sufficient to maximize the sensitivity of the assay, and the use of the *fTCRγ-J3* reaction would be unnecessary.

Biclonal or triclonal *TCRγ* gene rearrangements were frequently observed in T-cell neoplasms analyzed in the present study: 47% (14/30) of T-cell neoplasms showed 2 or 3 peaks in the *fTCRγ-J1* reaction, and 17% (5/30) of T-cell neoplasms showed 2 peaks in the *fTCRγ-J2* reaction. There are several possible explanations for this unexpected finding. Tumor tissues might contain several neoplastic T-cell clones, which have different clonal *TCRγ* rearrangements. A previous study reported that 2 independent neoplastic T-cell clones were detected in 2 different sites in the same cat with a low-grade alimentary lymphoma (Moore *et al*., 2005). The other possibility is that the rearrangement of *TCRγ* occurs frequently on both chromosomes (biallelic rearrangement) because of the failure of allelic exclusion in these neoplastic T-cells.

Although the results of the T-cell clonality analyzed by GeneScan analysis agreed well with the histopathological findings in cats with neoplastic and non-neoplastic diseases, the current assay revealed clonal *TCRγ* gene rearrangement in 1 of 34 non-neoplastic tissues. The sample was a duodenum tissue sample obtained from a cat diagnosed with LPE by histopathological examination. The cat was euthanized because

of the progression of the symptoms due to hypertrophic cardiomyopathy, and the duodenum sample was collected by necropsy. In the tissue sample, a large number of small lymphocytes and plasma cells, admixed with a small number of eosinophils and neutrophils, were observed in the lamina propria. Infiltration of these cells was restricted to the mucosa. Immunohistochemistry revealed that the infiltrated lymphocytes were a mixed population of CD3-positive T-cells and CD20- and/or CD79α-positive B-cells. Taken together, these pathological and immunohistochemical findings indicated a diagnosis of LPE. It is uncertain whether the T-cell population with clonal characteristics corresponds to some specific reactive T-cell population, or some histologically unidentifiable neoplastic T-cell population in the lesion.

Several discrepancies in the number of bands (peaks) of PCR products between GeneScan analysis and non-denaturing PAGE analysis were observed in 20% of samples showing clonality in the present study. The discrepancy between the 2 analytical methods seemed to occur when 2 or more PCR products differed in size by <6 bp. In such cases, 2 or more PCR products of different sizes could not be efficiently separated, and appeared as a single band in the non-denaturing PAGE analysis. In contrast, GeneScan analysis successfully separated PCR products with a slight difference in size, even resolving products with a difference of 1 bp. Although the clonality status of each sample ("clonal" or "polyclonal") was 100% concordant between the 2 analytical methods, the degree of resolution between the 2 methods was quite different.

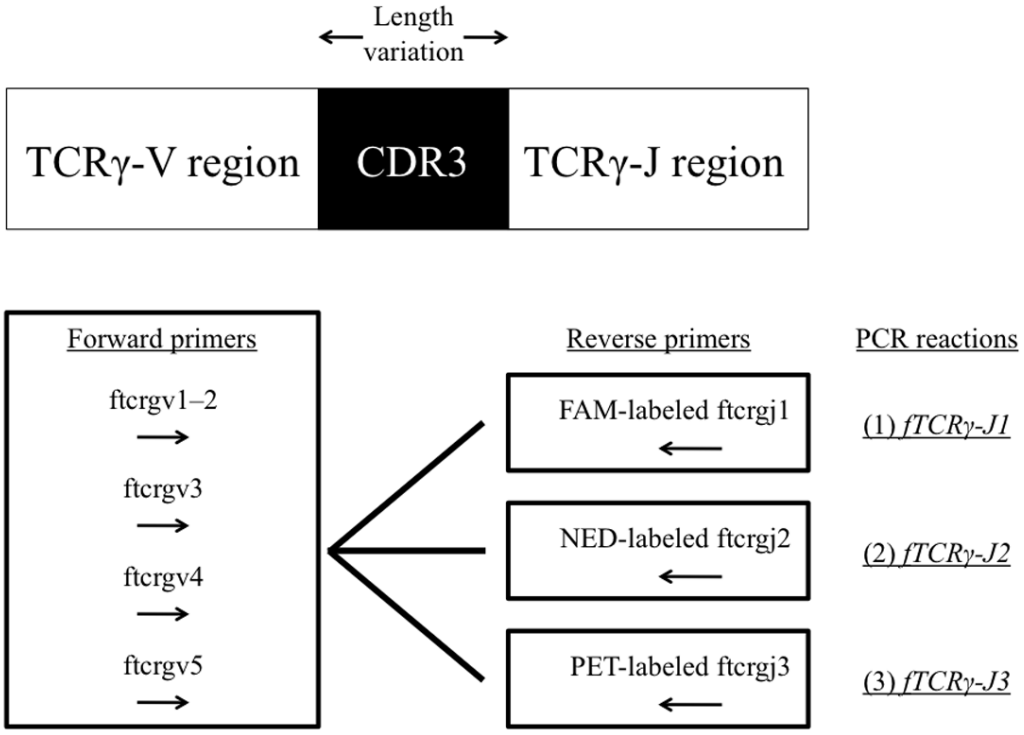
GeneScan analysis can separate PCR products with a 1-bp resolution, and may lead to more precise interpretation of *TCRγ* rearrangement patterns. Precise size determination of rearranged *TCRγ* PCR products also minimizes the risk of human error,

such as contamination, and allows for a comparison of T-cell clones from different sites or time-course monitoring of minimal residual disease in the same patients, without sequencing.

A combination of the fluorescent dyes 6-FAM, NED, and PET used in the present study, as well as several other fluorescent dyes, can be used simultaneously in GeneScan analysis. In the present study, 3 differently labeled PCR products from the same sample were analyzed in a single GeneScan analysis (multiplex run), thereby reducing time and cost. Therefore, GeneScan analysis is considered a simple, rapid, and more objective analysis for the assessment of the clonality of feline lymphoid cells.

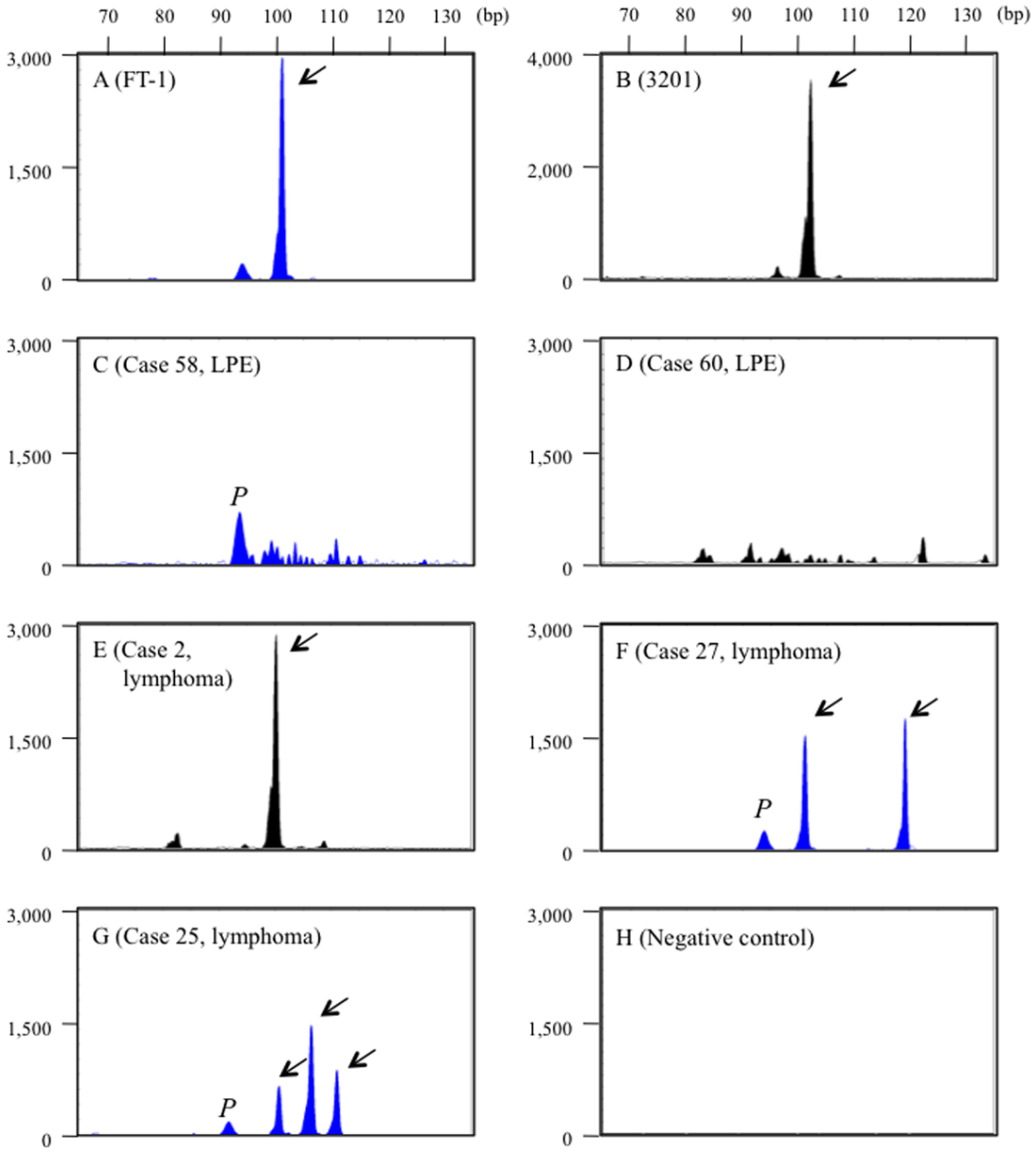
In conclusion, a GeneScan analysis system was developed to detect clonal *TCRγ* gene rearrangement in feline lymphoid neoplasms. The assay developed here would be a useful, high fidelity molecular diagnostic tool for feline T-cell neoplasms.

Fig. 4



Schematic representation of the rearranged *T-cell receptor (TCR) γ* gene and the locations of the primers designed in this study to amplify the *TCRγ* complementarity determining region (CDR) 3. The 4 forward primers, ftcrgv1–2, ftcrgv3, ftcrgv4, and ftcrgv5, were designed to bind to *TCRγ-variable (V)* regions. The 3 reverse primers, ftcrgj1, ftcrgj2, and ftcrgj3, were designed to bind to *TCRγ-joining (J)* regions. PCR was performed in 3 separate PCR reactions: the *fTCRγ-J1*, *fTCRγ-J2*, and *fTCRγ-J3* reactions. Each PCR tube contains a mixture of 4 forward primers and each of the 3 reverse primers: FAM-labeled ftcrgj1 primer, NED-labeled ftcrgj2 primer, and PET-labeled ftcrgj3 primer.

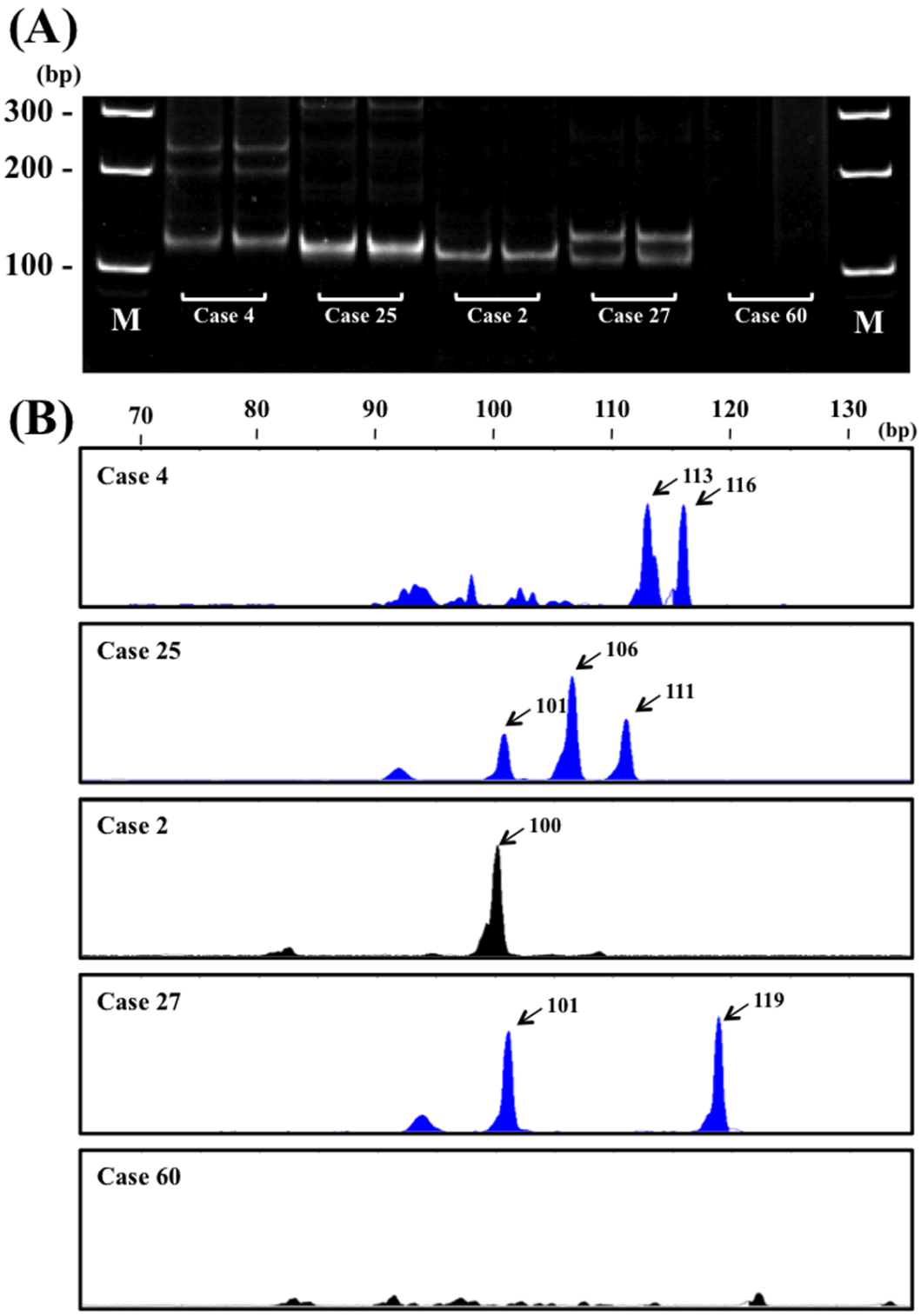
Fig. 5



Examples of the GeneScan analysis of *TCRγ* gene rearrangement. Blue and black peaks represent PCR products in the *fTCRγ-J1* and *fTCRγ-J2* reactions, respectively.

(A) Clonal control of *fTCRγ-J1* reaction: DNA from a feline T-cell lymphoma cell line, FT-1. (B) Clonal control of *fTCRγ-J2* reaction: DNA from a feline T-cell lymphoma cell line, 3201. (C) A representative result showing polyclonal *TCRγ* gene rearrangement in the *fTCRγ-J1* reaction: DNA from a duodenum sample from a cat with lymphoplasmacytic enteritis (LPE) (Case 58) (D) A representative result showing polyclonal *TCRγ* gene rearrangement in the *fTCRγ-J2* reaction: DNA from a duodenum sample from a cat with LPE (Case 60). (E) A representative result showing clonal *TCRγ* gene rearrangement (single peak) in the *fTCRγ-J2* reaction: the DNA sample from the duodenum of a cat with low-grade alimentary T-cell lymphoma (Case 2). (F) A representative result showing clonal *TCRγ* gene rearrangement (2 peaks) in the *fTCRγ-J1* reaction: DNA from a cutaneous mass sample from a cat with cutaneous T-cell lymphoma (Case 27). (G) A representative result showing clonal *TCRγ* gene rearrangement (3 peaks) in the *fTCRγ-J1* reaction: DNA from lymphoma cells from a cat with mediastinal T-cell lymphoma (Case 25). (H) Non-template control. A small polyclonal peak of 91–94 bp was found in most of the PCR products in the *fTCRγ-J1* reaction, and was indicated as "*P*".

Fig. 6

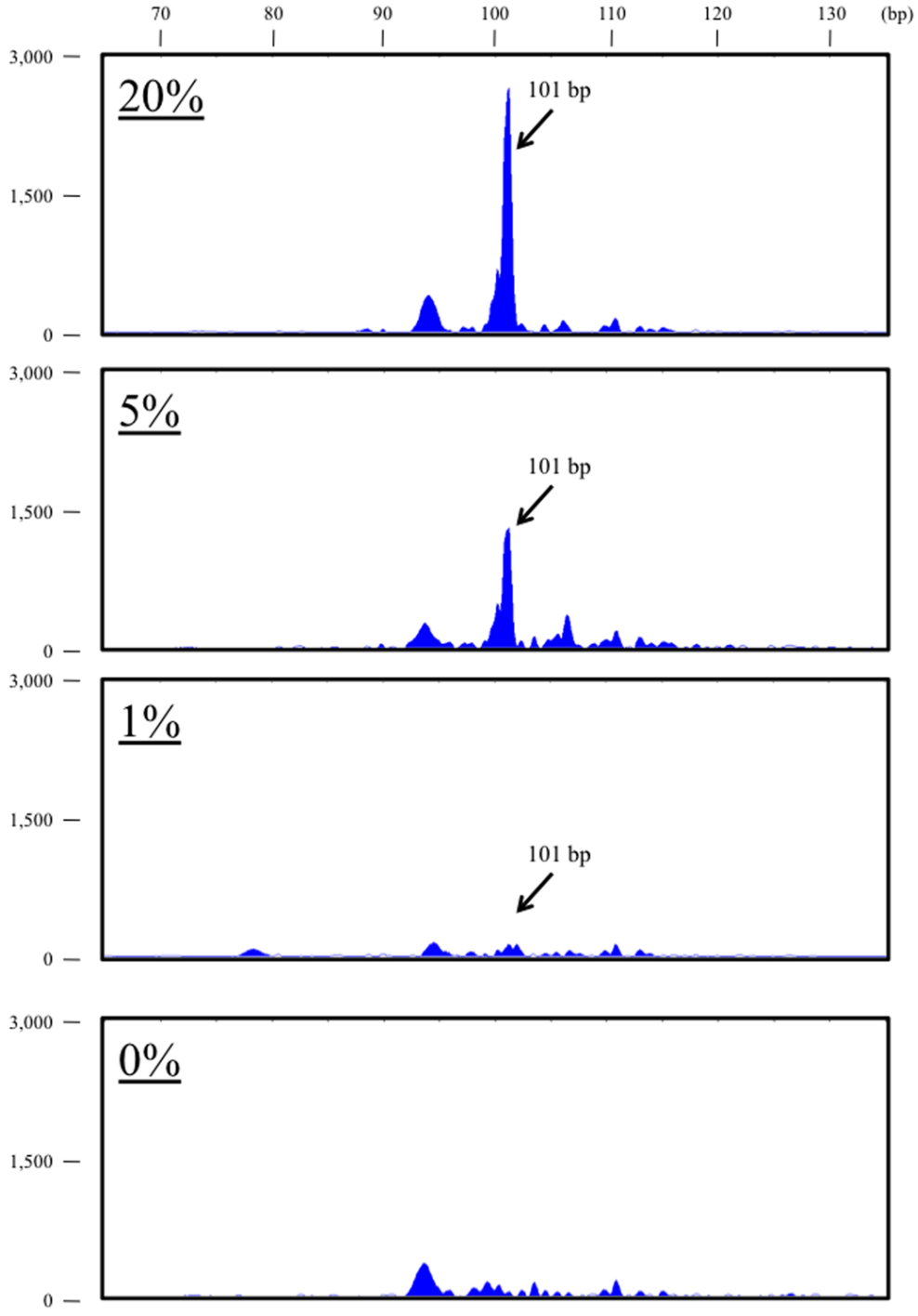


Comparison of GeneScan analysis to PAGE with heteroduplex analysis. Examples of discordant (Cases 4 and 25) and concordant (Cases 2, 27, and 60) results in 2 analytical

methods.

(A) PAGE with heteroduplex analysis and (B) GeneScan analysis were performed for samples showing clonal rearrangement (Cases 4, 25, 2, and 27) and those showing polyclonal rearrangements (Case 60) of *TCRγ* gene. Blue and black peaks represent PCR products in the *fTCRγ-J1* and *fTCRγ-J2* reactions, respectively. Each arrow indicates clonal PCR products. The numbers next to the arrows indicate the size of the PCR products from neoplastic clones. M, size marker.

Fig. 7



Sensitivity of the GeneScan analysis using tumor DNA samples serially diluted with DNA from normal tissues. DNA sample from a feline T-cell lymphoma cell line, FT-1,

was serially diluted with DNA from a histologically normal duodenum. The final content of neoplastic cell DNA was adjusted to 20%, 5%, 1%, and 0%, by mixing with normal duodenum DNA. The arrows indicate a PCR product of a neoplastic clone (101 bp). The proportions of the neoplastic cell DNA in the normal tissue DNA are indicated in the panels.

Table 3. Primers to amplify feline *TCRγ* genes used for the Genescan analysis to detect T-cell clonality in cats

Primer	Complementary to	Nucleotide sequence	Fluorescent dye used for labeling
ftcrgv1-2	*TCRγ–V1* and *–V2*	5'–GGSAGAAGAGCGACGAGGGCGTG–3'	–
ftcrgv3	*TCRγ–V3*	5'–GGGCGAAGAGCGATGAGGGAGTG–3'	–
ftcrgv4	*TCRγ–V4*	5'–GTAGTGAGGAGRATGCTGGTCTG–3'	–
ftcrgv5	*TCRγ–V5*	5'–GGCAGAAGCATGACAAGGGCATG–3'	–
ftcrgj1	*TCRγ–J1*	5'–CCCTGAGCAGTGTGCCAGSAC–3'	6-FAM
ftcrgj2	*TCRγ–J2*	5'–GGGGGAGTTACKATGASCTTARTTCC–3'	NED
ftcrgj3	*TCRγ–J3*	5'–ATCCAGATCTCAGGTTTGGGAGGAGG–3'	PET

Table 4. Results of the Genescan analysis of *TCRγ* gene rearranement in neoplastic and non-neoplastic samples from cats

Diagnosis		*fTCRγ-J1* reaction		*fTCRγ-J2* reaction		*fTCRγ-J3* reaction		Comprehensive results* from 3 PCR reactions	
	N	Clonality (+)	Clonality (–)	Clonality (+)	Clonality (–)	Clonality (+)	Clonality (–)	Clonality (+)	Clonality (-)
T-cell neoplasms	30	20 (67%)	10 (33%)	15 (50%)	15 (50%)	0	30 (100%)	26 (87%)	4 (13%)
B-cell neoplasms	27	2 (7%)	25 (93%)	2 (7%)	25 (93%)	0	27 (100%)	2 (7%)	25 (93%)
Non-neoplastic tissues	34	1 (3%)	33 (97%)	1 (3%)	33 (97%)	0	34 (100%)	1 (3%)	33 (97%)

*When clonal result was obtained in at least 1 of the 3 PCR reactions, the sample was interpreted as clonal.

Chapter 3

X-chromosome inactivation pattern analysis for the assessment of cell clonality in cats

Abstract

X-chromosome inactivation pattern (XCIP) analysis has been widely used to assess cell clonality in various types of neoplasms in humans. In the present study, a polymerase chain reaction-based feline XCIP analysis using the feline *androgen receptor* gene was developed. To construct the system of the analysis, polymorphism in CAG tandem repeats within the feline *androgen receptor* gene was explored using somatic DNAs from 50 male and 103 female cats. A CAG tandem repeats in exon 1 of the feline *androgen receptor* gene was found to be polymorphic, containing 15–22 CAG repeats. Of the 103 female cats, 70 (68%) were heterozygous for the number of CAG repeats, indicating the possible usefulness of XCIP analysis in cats. Application of the feline XCIP analysis to 3 feline mammary gland adenocarcinoma cell lines revealed distinctly skewed XCIPs in these cell lines, indicating their clonal origins. Twelve (80%) of the 15 primary tissue/cell samples obtained from cats with various neoplastic diseases showed skewed XCIPs. Moreover, bone marrow samples from 3 cats with myelodysplastic syndrome were also found to have skewed XCIPs. The polymerase chain reaction-based XCIP analysis developed in this study can provide information on cell clonality in female cats, potentially facilitating the differential diagnosis of various disorders in cats such as hypereosinophilic syndrome and progressive histiocytosis.

1. Introduction

Discrimination between neoplastic and nonneoplastic proliferation of cells is sometimes difficult in cats when diagnosing diseases such as hypereosinophilic syndrome (Lilliehöök *et al*., 2003), chronic myeloproliferative disorders (Raskin *et al*., 1996), and histiocytic proliferative disorders (Affolter *et al*., 2006). In such disorders, assessment of the clonal origin of the cells would enable a more accurate diagnosis.

Assessment of the clonality of cell populations has been recently developed for lymphoid tumors in cats by detection of antigen receptor gene rearrangements (Moore *et al*., 2005; Werner *et al*., 2005; Henrich *et al*., 2009; Weiss *et al*., 2011). However, to date, there is no assay system for evaluating the clonality of cells other than lymphoid cells in cats. In human medicine, X-chromosome inactivation pattern (XCIP) analysis has been utilized for the assessment of the clonal origin of the cells in various organ systems in female individuals.

In mammalian females, either the paternal or maternal X chromosome generally undergo random inactivation in each cell through the epigenetic changes during embryogenesis. This event is stably inherited by the daughter progeny of each cell, resulting in the theoretical expectation of a 1:1 inactivation ratio between the paternally and maternally derived X chromosomes in normal or reactive tissues. By contrast, most neoplastic tissues are composed of a population clonally expanded from a single cell, generally exhibiting a uniform XCIP, and resulting in a significant deviation from the expected 1:1 ratio. Therefore, demonstration of a nonrandom XCIP (skewed XCIP) infer the clonal origin of cells (Chen *et al*., 2007).

Although XCIP analysis is applicable only in female patients, it has been widely used for assessment of clonality in several neoplastic and preneoplastic conditions in

humans: e.g., chronic myeloid leukemia (Fialkow *et al*., 1967), acute myeloid leukemia (Wiggans *et al*., 1978), myelodysplastic syndrome (MDS) (Cermák *et al*., 2005), lymphoma (Peng *et al*., 1997), and certain nonhematopoietic tumors (Peng *et al*., 1997; Zhang *et al*., 2006; Jones *et al*., 2005). A major advantage of the XCIP clonality test is its potential application to any neoplastic diseases, even where a tumor-specific marker has not been identified.

A widely used method in humans for determining clonality using the X-chromosome inactivation principle is the human *androgen receptor* (*AR*) gene assay (HUMARA) (Chen *et al*., 2007). Transcription of human *AR* gene on the inactive X chromosome is strictly inhibited by methylation of CpG island containing its promoter (Jarrard et al., 1998). Similar epigenetic event is known on a region containing *Hpa*II sites near the polymorphic CAG tandem repeat (CAGr) in the exon 1 of *AR* gene (Allen et al., 1992). The *Hpa*II sites on the inactive X chromosome are methylated, whereas those on the active X chromosomes are unmethylated. Differences in the methylation status can be used to differentiate the inactive X chromosomes from the active one. A difference in the number of CAG repeats in the *AR* gene between 2 X chromosomes enables their distinction from each other.

Similar to the human *AR* gene, the feline *AR* gene is known to be located on the X chromosome and contains 2 clusters of CAGr in its exon 1 (GenBank accession number: AJ893545.1 and ACBE01496690.1). However, as yet, there has been no report of polymorphism of the CAGr in the feline *AR* gene. In this study, to develop a polymerase chain reaction (PCR)-based XCIP assay in cats, polymorphism of the CAGr in the feline *AR* gene was characterized by using nonneoplastic somatic DNA samples from male and female cats. By using the sequence data obtained in the present study, an

XCIP assay to assess clonal origin of cells in female cats was constructed, and its ability to detect cell clonality was examined in cultured tumor cell lines and primary tissue/cell samples obtained from cats with various diseases.

2. Materials and Methods

2.1. Nonneoplastic somatic DNA samples from cats

Peripheral blood samples were obtained from 50 male and 103 female cats; they included 24 healthy blood donors and 129 patients referred to the Veterinary Medical Center of the University of Tokyo for the diagnosis and treatment of various diseases unrelated to hematopoietic neoplasms. Use of the blood samples from the patients for research purposes was approved by the owners in written statements.

The breeds of the 153 cats were mixed (115 cats), American Short Hair (11), Scottish Fold (6), Maine Coon (4), Persian (4), Abyssinian (3), British Short Hair (2), Himalayan (2), Norwegian Forest Cat (2), Somali (2), Ragdoll (1), and Russian Blue (1).

2.2. Feline tumor cell lines

Three feline mammary gland adenocarcinoma cell lines, FMCp2, FMCm, and FYMp (Uyama *et al*., 2005) were kindly provided by Dr. N. Sasaki (Department of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo) and were used. FMCp2 and FYMp were established from primary mammary gland masses resected from 2 female cats with mammary gland adenocarcinoma. FMCm was established from a metastatic lymph node of the same cat from which FMCp2 was obtained. These cells were cultured in RPMI1640 (Invitrogen, Carlsbad,

CA) supplemented with 10% f*et al* bovine serum (Biowest, Nuaille, France) and antibiotics (100 IU/mL of penicillin and 0.1 mg/mL of streptomycin) (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Primary neoplastic tissue/cell samples

Twenty-one neoplastic tissue or cell samples diagnosed by histopathology or cytology were used. The neoplastic tissue/cell samples were obtained from female cats referred to the Veterinary Medical Center of the University of Tokyo between 2009 and 2011. Diagnosis in these cats included lymphoma (7 cats), epithelial tumors (6) [ceruminous adenocarcinoma (2), lung adenocarcinoma (2), mammary gland adenocarcinoma (1), and meningioma (1)], mesenchymal tumors (4) [leiomyomas (2), fibrosarcoma (1), and leiomyosarcoma (1)], mast cell tumor (2), acute lymphoblastic leukemia (ALL) (1), and chronic lymphocytic leukemia (CLL) (1). Of the 21 cats, 5 cases with hematological malignancies [lymphoma (3), ALL (1), and CLL (1)] were diagnosed by cytology, and remaining 16 cats were diagnosed by histopathology. The ages of the 21 cats with neoplastic diseases ranged from 2 to 20 years (median, 10 years). Eighteen cats were spayed and 3 cats were intact. The breeds were mixed (18 cats), American Shorthair (2), and Maine Coon (1).

Three female cats with MDS and 5 female cats with immune-mediated hematological disorders [immune-mediated neutropenia (3), immune-mediated hemolytic anemia (1), and pure red cell aplasia (1)] were diagnosed by the hematological examination including bone marrow cytology. Specimens of the bone marrow aspirates were subjected to the feline XCIP analysis. These 8 cats were mixed-breed spayed female cats with ages ranging from 2 to 13 years. The 3 cats with

MDS could be further subclassified into refractory cytopenia with multilineage dysplasia (RCMD) (1) and refractory anemia with excess blasts (RAEB) (2) according to the diagnosis and classification system of MDS in cats (Weiss *et al*., 2010).

2.4. DNA isolation

Genomic DNA samples were isolated from cells or tissues by using DNeasy Blood and Tissue Mini Kit (QIAGEN, Hilden, Germany). Concentration of the extracted genomic DNA samples was calculated on the basis of absorbance at 260 nm measured with a spectrophotometer.

2.5. Sequencing of CAGr in the feline AR gene in male cats

Primer pairs were designed according to the sequence of the feline *AR* gene (GenBank accession number: AJ893545.1) by using Primer3Plus, (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify a DNA fragment spanning both the first [GenBank accession number: AJ893545.1; nucleotide (nt) 91–165] and second CAGr (AJ893545.1; nt 475–531) in its exon 1 (AR-P1F: 5′-CGCGAAGTGATCCAGAACC-3′, nt 18–37; AR-P1R: 5′-AACTGTCCTTGGAGGAGGTG-3′, nt 588–607). Each reaction mixture contained 50 ng of genomic DNA, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 1× PCR buffer, and 0.5 units of ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) in a total volume of 20 μL. Cycle conditions consisted of an initial denaturation step at 95 °C for 1 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 C° for 30 s, and extension at 72 C° for 1 min; and a final extension at 72 °C for 7 min. PCR products were purified using a DNA purification kit (SUPREC PCR; Takara Bio

Inc., Otsu, Japan). Sequencing analyses of the PCR products were carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the Genetic Analyzer 3130xl (Applied Biosystems) by using the primers AR-P1F and AR-P1R. The repeat number of the 2 CAGr in each cat was counted with Sequence Scanner Software v1.0 (Applied Biosystems).

2.6. Examination of the heterozygosity of CAGr in the feline AR gene in female cats

Primer pairs were designed using Primer3Plus to amplify a region encompassing 2 *Hpa*II digestion sites and the polymorphic CAGr, as shown in Fig. 8 (AR-P2F: 5′--CTGGAGAGAGGCTGTGTTCC-3′, nt 271–290; AR-P2R: 5′-CTTGTTCTCCCGCTGCTG-3′, nt 552–569). The PCR mixture consisted of 50 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer, 0.2 μM FAM-labeled forward primer (AR-P2F), 0.2 μM reverse primer (AR-P2R), and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total volume of 20 μL.

Cycle conditions consisted of an initial denaturation step at 95 °C for 5 min; followed by 28 cycles of denaturation at 95 °C for 15 s, annealing at 64 °C for 15 s, and extension at 72 °C for 20 s; and a final extension at 72 °C for 7 min. One microliter of the 10-fold diluted PCR product was mixed with 8.5 μL formamide and 0.5 μL 600LIZ size standard (Applied Biosystems) in a 96-well plate. These samples were denatured at 95 °C for 5 min and immediately placed on ice for 15 min. The length of the PCR product was determined by capillary electrophoresis using the Genetic Analyzer 3130xl and evaluated by GeneMapper software (Applied Biosystems).

2.7. XCIP analysis

DNA samples (500 ng) were incubated with 5 units of a methylation-sensitive endonuclease, *Hpa*II (New England Biolabs Inc., Ipswich, MA), in 1× buffer 1 in a total volume of 50 μL. Undigested controls of each sample were incubated in 1× buffer 1 (total volume, 50 μL) without *Hpa*II. Both the digested and undigested samples were incubated for 16 h at 37 °C followed by inactivation for 20 min at 65 °C.

A 50 ng aliquot of the digested and undigested DNA samples was subjected to subsequent PCR amplification with the primers AR-P2F and AR-P2R.

As shown in HUMARA, because *Hpa*II is a methylation-sensitive endonuclease and its cleavage sites near the polymorphic CAGr in *AR* gene on the inactive feline X chromosome DNA is considered to be methylated, the *Hpa*II sites would not be cleaved by this enzyme. Tsherefore, the DNA with methylation of the sites can be conceivably amplified with the AR-P2F and AR-P2R primers (spanning the *Hpa*II sites) even after *Hpa*II digestion. In contrast, the *Hpa*II sites on the active feline X chromosome are considered to be unmethylated; therefore, *Hpa*II digestion of the DNA of active X chromosome leads to the failure of the PCR amplification because of the cleavage of the *Hpa*II sites. As a control for *Hpa*II digestion, the same amount of DNA from a male nonneoplastic peripheral blood sample was used because male somatic cells contain only 1 active X chromosome.

Results of the XCIP analysis were evaluated after confirming the absence of gene amplification from the completely digested male somatic DNA control. The degree of skewness (imbalanced inactivation) was determined by comparing the area under the curve (AUC) for each allele of the digested and undigested samples using the results of the capillary electrophoretogram. The corrected inactive allele ratio (CIAR) was defined

as the allele ratio of the digested sample divided by the allele ratio of the undigested sample, to avoid the influence of the potential preferential amplification of either allele.

Stutter peaks (shadow bands), generated by slippage synthesis of short tandem repeats (Walsh *et al*., 1996), are observed as minor peaks 3 bp and 6 bp shorter than the real peaks. When the CAGr in one allele is longer than that in another allele by 3 bp (1 CAG repeat) or 6 bp (2 CAG repeats), the longer allele (Fig. 9C, allele 2) affects the measured AUC of the shorter allele (Fig. 9C, allele 1). To avoid the influence of the shadow bands, the percentage of shadow bands for each repeat length was calculated from the data obtained from DNA samples of male cats. When the size of allele 1 was shorter than that of allele 2 by 1- or 2-repeat lengths, 27% or 7% respectively of the AUC of allele 2 was subtracted from that of allele 1. Thus, the CIAR was calculated as follows:

$$CIAR = \frac{\left[digested\ \ Allele\ 1 - (shadow\ \ bands)\right] / digested\ \ Allele\ 2}{[undigested\ \ Allele\ 1\ \ - (shadow\ \ bands)] / uudigested\ \ Allele\ 2}$$

As proposed in previous reports on XCIP analysis in humans (Mitterbauer *et al*., 1999; Levine *et al*., 2006), the sample population was judged to be clonal when the CIAR > 3.0. For ease of comparison, where CIAR < 1, the corrected ratio was calculated as the inverse value of the CIAR.

Statistical analysis

Pearson's chi-square test was used for comparing the allele frequency between male and female sample populations. The significance level was set at $P < 0.05$. Statistical

testing was performed using JMP version 8.0.1 (SAS Institute, Cary, NC).

3. Results

3.1. Sequencing of the first and second CAGr in exon 1 of the feline AR gene

The number of CAG trinucleotide repeats in 2 CAGr in exon 1 of the feline *AR* gene was examined in 50 male cats. There was no polymorphism in the first CAGr; however, the number of CAG trinucleotide repeats in the second CAGr varied between 16 and 20. Of the 50 male cats, 9 (18%), 5 (10%), 12 (24%), 20 (40%), and 4 (8%) cats had 16, 17, 18, 19, and 20 repeats of CAG, respectively.

3.2. Heterozygosity of the second CAGr of the feline AR gene in female cats

PCR amplification of the second CAGr followed by capillary electrophoresis was performed in 103 female cats. In homozygotes for the second CAGr, a single major peak was observed (Fig. 9A), similar to the result for male cats (Fig. 9B). Heterozygotes for the second CAGr were identified by the occurrence of 2 major peaks of different lengths after PCR amplification with the AR-P2F and AR-P2R primers (Fig. 9C and D). In all of the samples, stutter peaks (shadow bands indicated as "*S*") were observed as minor peaks, shorter than the major peaks by 3 or 6 bp (Figs. 9 A–D). Of the 103 female cats, 70 (68%) were found to be heterozygous for the second CAGr. Heterozygosity of the second CAGr was shown in 57 of 79 (72%) mixed breed cats and 13 of 24 (54%) purebred cats. The CAG repeat numbers in the second CAGr were 15 (3 alleles; 1%), 16 (37 alleles; 18%), 17 (30 alleles; 15%), 18 (37 alleles; 18%), 19 (79 alleles; 38%), 20 (18 alleles; 9%), and 22 (3 alleles; 1%). Allele frequency with respect to the number of CAG repeat was compared between females and males and there was no significant

difference between the 2 sample populations in this study.

3.3. Accuracy, reproducibility, and specificity of XCIP analysis using the feline AR gene

Before analyzing the samples from cats with neoplastic diseases, the performance of the feline XCIP analysis constructed in this study was evaluated. First, to confirm that the PCR amplification ratio corresponds to the amount of template DNA, DNA-mixing experiments were performed. Two DNA samples from male cats containing 16 or 19 CAG trinucleotides in the second CAGr were mixed in various proportions as follows: 1:10 (the ratio of the amount of DNA containing 16 CAG oligonucleotides to the amount of DNA containing 19 CAG oligonucleotides), 1:5, 1:3, 1:1, 3:1, 5:1, and 10:1. PCR was performed in triplicate using the same conditions as the feline XCIP analysis without digestion with *Hpa*II, and the allele ratios were calculated. To compensate for amplification bias towards the shorter allele, all allele ratios were divided by the allele ratio obtained in a sample of 1:1 DNA mixture. The allele ratios calculated from the capillary electrophoretic analysis were 1:10.0 (for the 1:10 mixture), 1:5.23 (1:5), 1:2.94 (1:3), 1:1.00 (1:1), 3.08:1 (3:1), 5.31:1 (5:1), and 12.3:1 (10:1).

Second, blood samples from healthy female cats heterozygous for the second CAGr were used to determine the reproducibility of the analysis. The entire assay was performed in triplicate on the same DNA samples of 10 healthy female cats, resulting in a coefficient of variation for CIAR of 13%.

Finally, specificity of the feline XCIP analysis was examined using DNA samples of peripheral blood from 10 healthy female cats. CIAR values obtained from these 10 samples ranged from 1.01 to 2.62 (median: 1.18) (Figs. 10B and 11), less than the

threshold value 3.0 (indicative of clonal origin) reported in XCIP analysis in humans (Mitterbauer *et al*., 1999; Levine *et al*., 2006).

3.4. Feline XCIP analysis in feline mammary gland adenocarcinoma cell lines

DNA samples from 3 feline mammary gland adenocarcinoma cell lines (FMCp2, FMCm, and FYMp) were analyzed by XCIP analysis using the feline *AR* gene. FMCp2 and FMCm, which are derived from the same cat, showed identical results in XCIP analysis (Fig. 10C). CIARs of FMCp2 and FMCm samples were calculated as ∞, because *Hpa*II digestion completely inhibited PCR amplification of the active (unmethylated) allele with a shorter CAGr, causing the AUC of the shorter allele to approach 0. FYMp also showed a distinctly skewed XCIP (CIAR: 12.6).

3.5. Feline XCIP analysis in clinical samples

Prior to the feline XCIP analysis, 21 primary neoplastic tissue/cell samples from cats with neoplastic disorders and 8 bone marrow aspirates from cats with MDS or immune-mediated diseases were examined for the heterozygosity of the second CAGr in *AR* gene. Of the 21 primary tissue/cell DNA samples from cats with neoplastic disorders, 15 samples (71%) [lymphoma (5), leiomyoma (2), ceruminous adenocarcinoma (1), lung adenocarcinoma (1), mammary gland adenocarcinoma (1), meningioma (1), leiomyosarcoma (1), fibrosarcoma (1), ALL (1), and CLL (1)] were shown to be heterozygous for the second CAGr, being appropriate for the feline XCIP analysis. Similarly, of the 8 bone marrow DNA samples, 5 samples (63%) [MDS (3), immune-mediated hemolytic anemia (1), and pure red cell aplasia (1)] were shown to be heterozygous for the second CAGr, and could be evaluated by feline XCIP analysis.

Of the 15 samples of neoplastic disorders, CIARs in 12 samples exceeded 3.0 (3.87–∞, median: 8.58), indicating skewed XCIPs (Figs. 10D and 10E); however, those of the remaining 3 samples [lymphoma (2) and mammary gland adenocarcinoma (1)] were < 3.0 (1.48, 1.79, and 1.90) (Fig. 11).

Bicytopenia (case No. 16: neutropenia and thrombocytopenia and case No. 17: anemia and thrombocytopenia) or pancytopenia (case No. 18) was a characteristic feature in the peripheral blood of the 3 cats with MDS. Bone marrow specimens of these cats were characterized by varying degrees of dysplastic features in myeloid, megakaryocytic, and erythrocytic cell lineages including neutrophils with a ring-shape nucleus, small megakaryocytes with a single nucleus, and megaloblastoid change of erythroblastic cells (Figs. 12A and 12B, case No. 1). Cats with immune-mediated hematological disorders were devoid of such dysplastic changes. Of the 5 bone marrow samples, 3 bone marrow samples from cats with MDS showed skewed XCIPs (CIARs: 3.29, 8.95, and ∞) (Fig. 12C, case No. 1), whereas 2 samples from cats with immune-mediated hematological disorders did not show skewed XCIPs (CIARs: 1.01 and 1.76).

4. Discussion

In order to construct the feline XCIP analysis, I first investigated the repeat number polymorphism of the 2 CAGr in exon 1 of the feline *AR* gene and revealed that 70 of 103 (68%) female cats were heterozygous for the polymorphic second CAGr, which contains 15–22 CAG trinucleotides. It has been reported that the *AR* genes of other mammals, such as humans and dogs, contain CAGr (Edwards *et al*., 1992; Shibuya *et al*., 1993; Lai *et al*., 2008). One of the CAGr in the human *AR* gene is polymorphic and

contains 11–31 repeats. (Edwards *et al*., 1992) Canine *AR* gene contains 2 polymorphic CAGr (the first CAGr containing 10–12 repeats and the second CAGr containing 10–13 repeats) (Shibuya *et al*., 1993; Lai *et al*., 2008). Heterozygosity for the CAGr in the *AR* gene was shown in 90% of human female individuals (Allen *et al*., 1992; Gale *et al*., 1996) and 40% of female dogs (Shibuya *et al*., 1993). In the present study, 68% of female cats were demonstrated to be heterozygous. Therefore, XCIP analysis for the *AR* gene would be applicable in approximately two-thirds of female cat patients in clinical settings.

The performance of the feline XCIP analysis constructed in this study was evaluated in terms of accuracy, reproducibility, and specificity. The allele ratio after PCR corresponded well with the amount of DNA template used for PCR. In addition, the coefficient of variation was shown to be relatively low. None of the nonneoplastic blood cell samples examined from female cats had a skewed XCIP, and all 3 of the mammary gland adenocarcinoma cell lines had distinctly skewed XCIPs. From these results, it is conceivable that the feline XCIP analysis using the *AR* gene developed in this study can be used as an assay to examine the clonality of cells with reasonable accuracy.

In primary cell/tissue samples from cats with neoplastic diseases, 12 of 15 neoplastic samples (80%) showed skewed XCIP, indicating clonal cell proliferation in these samples. However, 3 of 15 neoplastic samples (20%) did not show skewed XCIPs. There are several possible explanations for these results. First, success in the detection of unbalanced XCIPs largely depends on the proportion of tumor cells in the samples used for the assay. The tissue or cell samples usually contain certain numbers of nonneoplastic cells of the original organs, inflammatory cells, fibroblasts, and blood cells. When the percentage of the number of nonneoplastic cells in the neoplastic

tissue/cell samples is high (theoretically more than 50%), false negative results would be obtained. Tumor cell isolation methods such as microdissection and flowcytometric cell sorting may help to improve the sensitivity. Second, because the present assay is based on differences in the methylation status between active and inactive X chromosomes, the CIAR would not reflect the actual ratio of the inactivation pattern of the 2 X chromosomes when abnormal methylation occurs in the *AR* locus. Abnormal hypermethylation of the *AR* locus is reported in human lymphoma patients (McDonald *et al*., 2000).

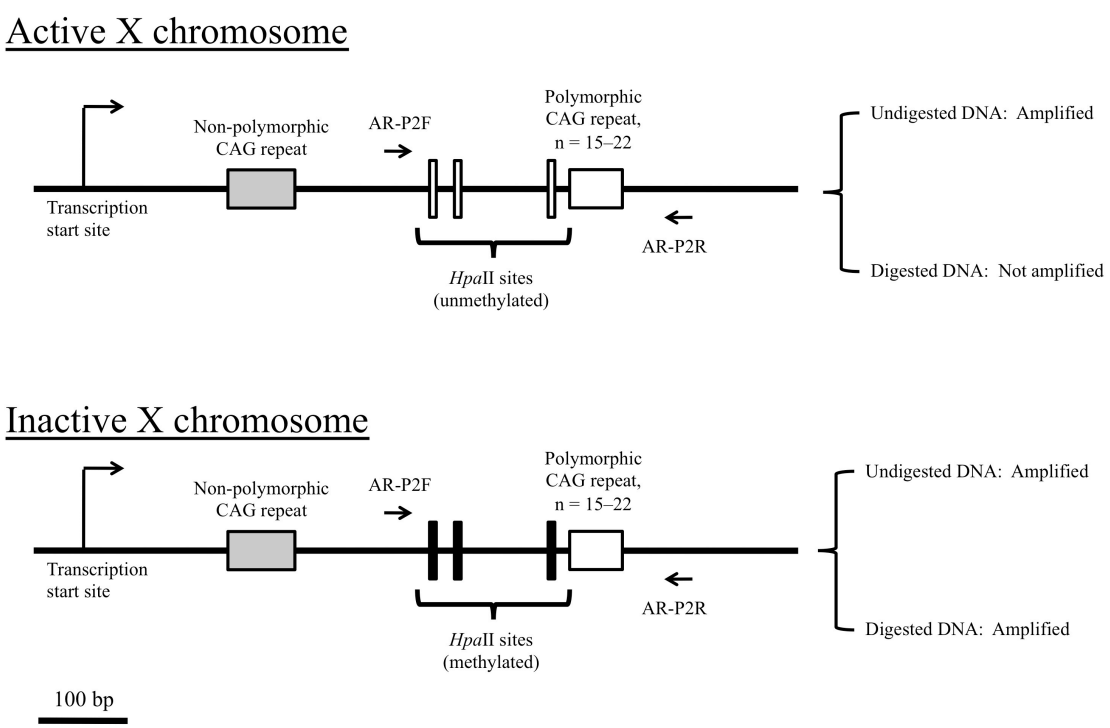
MDS is a group of diseases characterized by peripheral blood cytopenia due to dysplasia of hematopoietic cells. Occurrence of MDS in cats is associated with feline leukemia virus infection in cats (Hisasue *et al*., 2001). Although the pathogenesis of MDS has not been elucidated, it is thought to be caused by clonally expansion of bone marrow cells from aberrant pluripotent hematopoietic stem cells in humans. Progression to acute myeloid leukemia from MDS has been reported in both humans (Liesveld *et al*., 2010) and cats (Hisasue *et al*., 2001). Although the number of feline MDS patients examined in this study was small, distinctly skewed XCIPs were demonstrated in bone marrow obtained from cats hematologically diagnosed with MDS, indicating the clonal nature of bone marrow cells in cats with MDS. The present study supports the hypothesis that MDS is a preleukemic state resulting from the clonal expansion of abnormal hematopoietic stem cells in cats as well as humans.

In humans, although application of XCIP analysis is restricted to females, it has several advantages over other clonality assessment procedures such as cytogenetic analysis, mutation analysis of specific genes, or viral integration analysis. Most importantly, XCIP analysis can be utilized in a variety of diseases, even where no

disease-specific marker has been identified. By using the XCIP analysis, Langerhans cell histiocytosis and hypereosinophilic syndrome in humans were shown to be disorders resulting from clonal proliferation of the respective cell lineages (Yu *et al*., 1994; Chang *et al*., 1999). As feline counterparts of these diseases, feline progressive histiocytosis (Affolter *et al*., 2006) and hypereosinophilic syndrome (Lilliehöök *et al*., 2003) have been identified; however, the clonal origin of these diseases has not been revealed. Feline XCIP analysis could be a useful tool for evaluating clonality of these disorders as well as other conditions where clonal origins are suspected, thereby allowing precise understanding of their pathogenesis and accurate diagnosis.

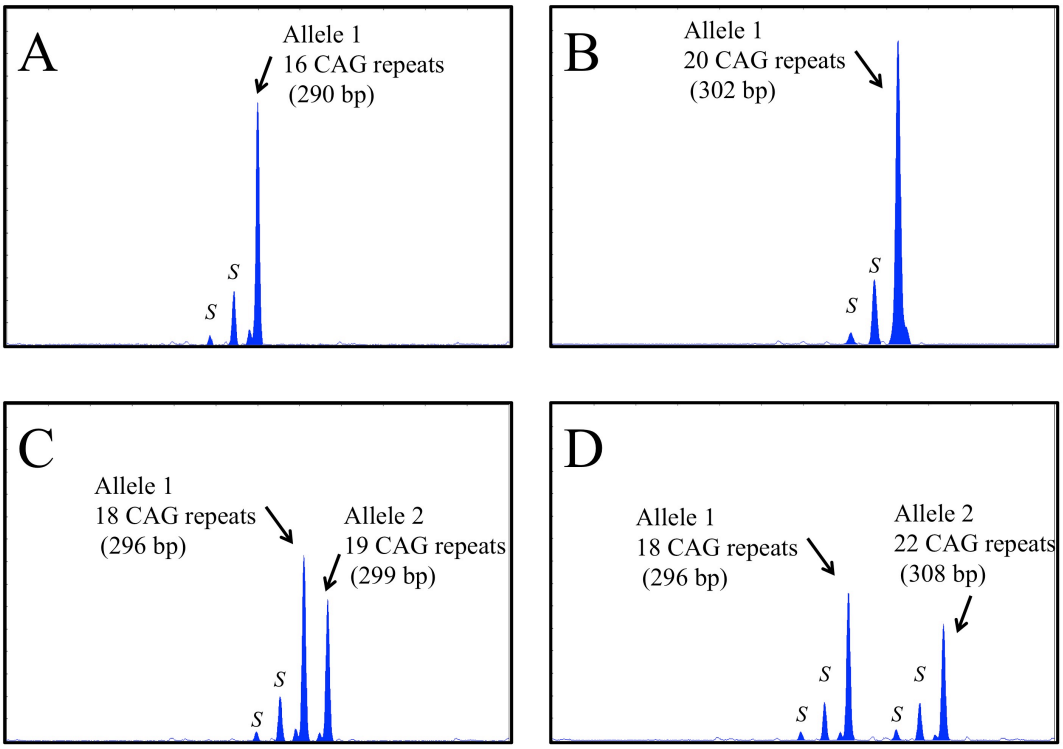
In conclusion, polymorphic CAGr in the feline *AR* gene was identified and a simple PCR-based feline XCIP clonality assay was constructed. This assay can be used as a practical tool for the assessment of cell clonality in various feline diseases such as hypereosinophilic syndrome and progressive histiocytosis.

Fig. 8



Schematic structure of the feline AR gene exon 1 and a PCR strategy to distinguish between active and inactive X chromosomes by amplification of a polymorphic CAG tandem repeat (CAGr) within its exon 1. A DNA fragment can be amplified by PCR using AR-P2F and AR-P2R primers from both active (unmethylated) and inactive (methylated) X chromosomes. After *Hpa*II digestion, the DNA fragment on the active (unmethylated) X chromosome cannot be amplified whereas that on the inactive (methylated) X chromosome can be amplified because of the failure of cleavage by *Hpa*II.

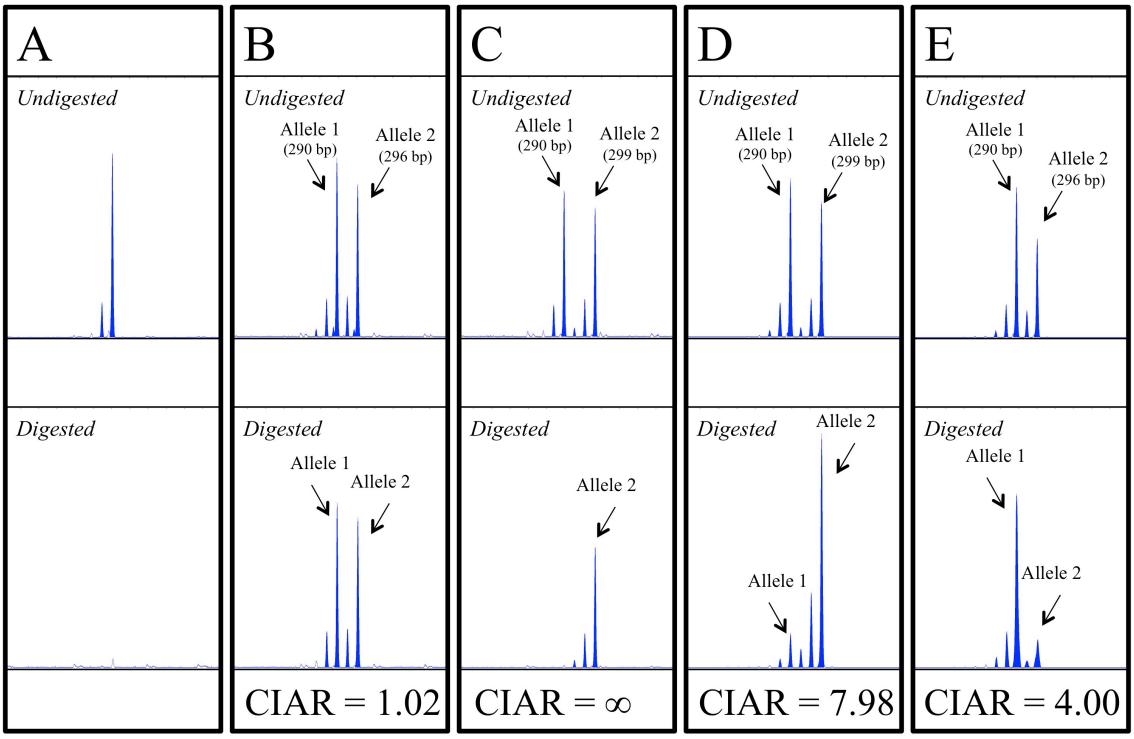
Fig. 9.



Representative capillary electrophoretograms after PCR to amplify the polymorphic CAG tandem repeat in the feline AR gene.

(A) A nonneoplastic blood DNA sample from a female cat homozygous for the repeat number of CAG trinucleotides (16 CAG repeats). (B) A nonneoplastic blood DNA sample from a male cat (20 CAG repeats). (C, D) Nonneoplastic blood DNA samples from female cats heterozygous for the repeat number of CAG trinucleotides (C: 18 and 19 CAG repeats, D: 18 and 22 CAG repeats). Two major peaks are observed in heterozygous female cats (C, D), whereas only 1 major peak is observed in a homozygous female cat (A) and a male cat (B). Stutter peaks (shadow bands, indicated as "*S*") are observed as minor peaks shorter than the major peaks by 3 or 6 bp.

Fig. 10.

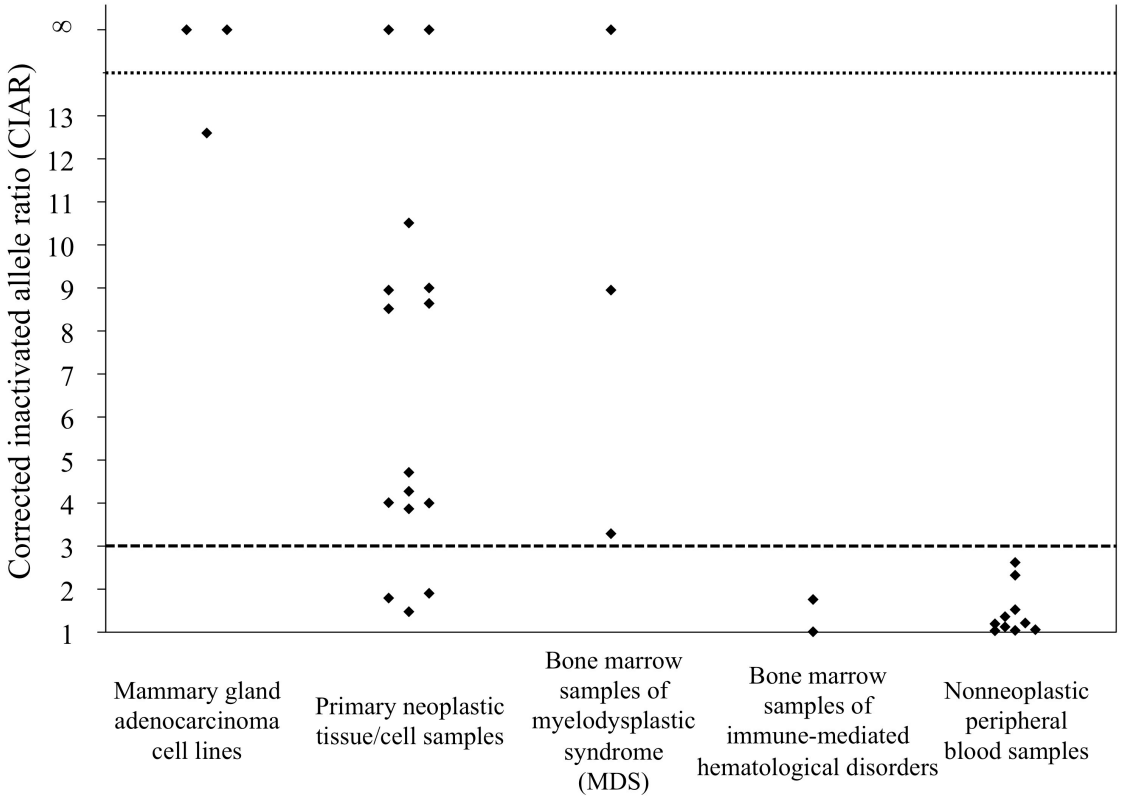


Representative results of XCIP analysis of the AR gene in nonneoplastic and neoplastic cell samples obtained from female cats.

(A) A nonneoplastic peripheral blood cell sample from a male cat. A peak corresponding to the PCR-amplified AR gene is observed in an undigested sample but not in the *Hpa*II-digested sample, indicating complete digestion with *Hpa*II. (B) A nonneoplastic peripheral blood cell sample from a female cat heterozygous for the CAG repeat number of the second CAGr in the AR gene. Because the cells are polyclonal, the AR gene on both alleles (allele 1 and allele 2) was almost equally amplified in the undigested and *Hpa*II-digested samples [corrected inactivated allele ratio (CIAR): 1.02]. (C) A feline mammary gland adenocarcinoma cell line, FMCp2. One of 2 X

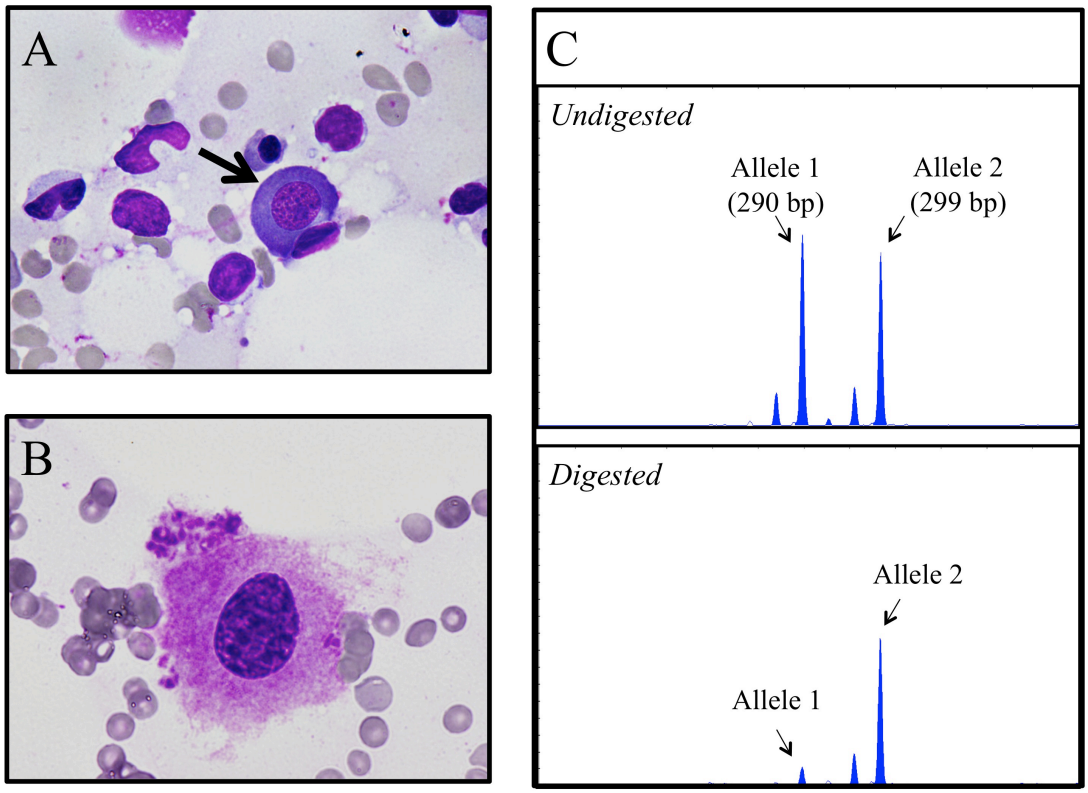
chromosomes (allele 1) was not amplified in the *Hpa*II-digested sample, whereas 2 X chromosomes were almost equally amplified in the undigested sample. The CIAR of FMCp2 was calculated as ∞, indicating clonal origin of the cells. (D, E) Primary neoplastic tissue samples (D: Case 6, ceruminous adenocarcinoma, E: Case 4, lung adenocarcinoma). The AR gene on both alleles (allele 1 and allele 2) was similarly amplified in the undigested samples; however, the amount of PCR product from one allele (allele 1 in D, allele 2 in E) was markedly reduced in the *Hpa*II-digested samples. The CIARs of these samples were 7.98 (D) and 4.00 (E), exceeding the threshold value 3.0, indicating clonal cell proliferation in these tissue samples. Stutter peaks are observed as peaks 3 or 6 bp shorter than the major peaks in all of the electrophoretograms.

Fig. 11.



Corrected inactivated allele ratios (CIARs) obtained from the XCIP analysis in 3 mammary gland adenocarcinoma cell lines, 15 primary neoplastic tissue/cell samples, 3 bone marrow samples of myelodysplastic syndrome, 2 bone marrow samples of immune-mediated hematological disorders, and 10 nonneoplastic peripheral blood samples. The threshold value of CIAR was set as 3.0.

Fig. 12.



(A) Bone marrow, cat, case No. 1, cytology of bone marrow aspirates from a cat with MDS-refractory anemia with excess blasts. Megaloblastoid change of a prorubricyte (arrow) (Wright's Giemsa stain, ×1000). (B) Bone marrow, cat, case No. 1, cytology of bone marrow aspirates from a cat with MDS-refractory anemia with excess blasts. A small megakaryocyte with a single nucleus (Wright's Giemsa stain, ×1000). (C) X-chromosome inactivation pattern analysis of bone marrow aspirates from a cat with MDS-refractory anemia with excess blasts (case No. 1). The AR gene on both alleles (allele 1 and allele 2) was similarly amplified in the undigested samples; however, the amount of PCR product from allele 1 was markedly reduced in the *Hpa*II-digested sample (CIAR: 8.95

Conclusion

It has been well accepted that clonality analysis can serve an important diagnostic tool for a variety of neoplastic diseases. To achieve the assessment of cell clonality, I explored various kinds of analytical strategies among those reported in humans such as examinations of aberrant phenotypes (Kaleem, 2007), specific chromosomal and/or genetic changes (Tefferi, 2007), rearrangement of antigen receptor genes (van Dongen *et al*., 2003), and XCIP (Chen *et al*., 2007). However, because tumor-specific phenotype, chromosomal aberration, or genetic change has not been identified in cats, I focused on the studies to detect the rearrangement of antigen receptor genes and XCIP. A series of studies in this thesis were carried out for the purpose of construction of clonality analytical methods and their application to the clinical cases in cats.

The study in Chapter 1 of the present thesis was carried out to construct an analytical system of the rearrangement of *IGHV* gene for the evaluation of clonality in feline B-cell lymphoid neoplasms. Prior to the development of an efficient assay, 97 *IGHV* complementary DNAs from normal cat spleens were cloned and sequenced. On the basis of these sequences, 6 forward primers at the variable region and 5 reverse primers at the joining region were designed. Using these primers, CDR3 of *IGHV* genes were amplified and the PCR products were subjected to the analyses by conventional PAGE and GeneScan analyses. Clinical samples from 26 feline B-cell neoplasms diagnosed by histopathological and immunohistochemical examinations were analyzed for the presence of clonal *IGHV* gene rearrangement. The multiplex PCR and GeneScan analysis disclosed clonal *IGHV* gene rearrangement in 22 of the 26 (84%) samples, indicating its high sensitivity in clinical settings.

Next, in Chapter 2, I constructed a clonality assay system to detect the rearrangement of *TCRγ* gene for the evaluation of T-cell clonality in cats. GeneScan

analysis newly developed here was applied to 30 T-cell neoplasms, 27 B-cell neoplasms, and 34 non-neoplastic tissues derived from cat patients. Clonal *TCRγ* gene rearrangement was detected in 26 of 30 (87%) T-cell neoplasms, 2 of 27 (7%) B-cell neoplasms, and 1 of 34 (3%) non-neoplastic tissues. Irrespective to the problems in recently reported studies in cats (Moore *et al*., 2005; Werner *et al*., 2005; Henrich *et al*., 2009; Weiss *et al*., 2011), the GeneScan analysis developed here was shown to be clinically useful to support the diagnosis of T-cell malignancies in cats.

In Chapter 3, I established an X-chromosome inactivation pattern (XCIP) analysis for the assessment of clonality of various cell lineages in cats. Prior to the construction of the assay, I characterized the polymorphism of the feline *AR* gene and revealed the polymorphism of the number of CAG tandem repeats within its exon 1. Of the 103 female cats, 70 (68%) were heterozygous for the number of CAG repeats, indicating the possible application of XCIP analysis in cats. The feline XCIP analysis developed here revealed distinctly skewed XCIPs in 3 feline mammary gland adenocarcinoma cell lines, indicating their clonal origins, although normal white blood cells showed balanced XCIPs. Twelve (80%) of the 15 feline primary neoplastic tissue/cell samples and bone marrow samples from 3 cats with myelodysplastic syndrome showed skewed XCIPs, indicating their clonal origins. XCIP analysis has never utilized in veterinary field, but the novel clonality analysis developed here would provide useful information to understand the pathogenesis of several diseasec in which the neoplastic nature has been argued (Lilliehöök *et al*., 2003; Affolter *et al*., 2006).

In small animal practice, importance of the management of neoplastic diseases is generally increasing because dogs and cats now benefit from advanced health care and tend to live longer lives with increased incidence of cancers. The clonality analyses

developed in the present study are an outcome of the translational research in which advanced molecular biological methods were utilized for progress of small animal practice. I believe that the clonality analysis of the tumors will undoubtedly provide new insights into pathogenesis, diagnosis, and prognostic markers in a diverse range of diseases in animals.

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