博　士　論　文

Application of X-chromosome inactivation pattern (XCIP) analysis in canine haematological disorders for detection of their clonalities

（犬の造血器疾患のクローン性判定における X 染色体不活化パターン (XCIP)解析の応用）

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General introduction

Neoplastic cells are generally considered to be derived from a single transformed cell. Assessment of clonal cell population is a key to the strategies of diagnosis, monitoring, and treatment of neoplastic diseases. Several procedures to assess the clonality have been used in human medicine such as chromosomal translocation (Philadelphia chromosome) in chronic myelogenous leukaemia[1] and specific gene mutations: JAK2 V617F gene mutation in polycythemia vera[2-5], KIT D816V gene mutation in mastocytosis[6,7] etc. In veterinary medicine, rearrangement of antigen receptor genes has been used to demonstrate the clonality in lymphoproliferative diseases[8-11]; however, it can be only applied to the diagnosis of lymphoma or lymphoid leukaemia for diagnostic purpose. Furthermore, consistent chromosomal abnormalities or gene mutations have not been detected in most of canine neoplasms.

X-chromosome inactivation pattern (XCIP) analysis is a molecular biological method to detect cell clonality and has been generally known as human androgen receptor assay (HUMARA) in human medicine[12]. Mammalian females have 2 X-chromosomes in their somatic cells and either paternal or maternal X-chromosome undergoes inactivation in each cell during embryogenesis via epigenetic alterations such as DNA methylation[12]. This event generally occurs randomly and is stably inherited by the daughter cells, therefore, almost equal ratio of inactivation between the 2 X-chromosomes is maintained in normal, inflammatory, or hyperplastic tissues (Figure A). On the other hand, tumours are generally clonal origin and methylation status is almost stable, therefore, uniform XCIP is a characteristic finding in neoplastic tissues, and this situation suggests the existence of clonal cell population (Figure A)[12].

XCIP analysis can be fundamentally applied to any kinds of neoplastic diseases including haematopoietic tumours and solid tumours in female patients. In veterinary medicine, XCIP analyses have been previously constructed by using androgen receptor (AR) gene in dogs and cats[13-15]. In order to use XCIP analysis, 2 X-chromosomes and methylation status must be distinguished. In a report by Mochizuki et al. (2015)[15], discrimination of 2 X-chromosomes were archived using the difference of CAG trinucleotide repeats in AR gene (Figure B), and the methylation statuswas revealed using methylation-sensitive endonuclease, *Hpa* II, which could only cut its unmethylated restriction sites of chromosomes (Figure C). However, since the frequency of heterozygosity in the number of CAG trinucleotide repeats in AR gene is relatively low in dogs[14,15] probably due to the high frequency of purebred populations, clinical application of the XCIP analysis has been limited in dogs.

Polycythemia vera (PV) is classified as one of the myeloproliferative neoplasms (MPNs) together with chronic myelogenous leukaemia (CML) and essential thrombocythemia (ET)[16]. PV is characterised by autonomous proliferation of erythroid lineage cells derived from an aberrant haematopoietic progenitor clone, and JAK2 V617F gene mutation is demonstrated in most of human PV patients[2-5,17,18]. Canine patients suffered from PV are sometimes encountered in small animal practice[19-29]; however, its diagnosis is generally performed by exclusion of other disease conditions causing erythrocytosis. JAK2 V617F gene mutation has been identified only in 1 canine PV case[19], and its frequency in canine PV has not been identified yet.

Myelodysplastic syndromes (MDS) are characterised by ineffective

haematopoiesis derived from the abnormal haematopoietic progenitor clone in bone marrow[30]. Morphological changes suggesting dysplasia have been identified in the peripheral blood and bone marrow cells[31-33]. MDS is known to be a pre-leukaemic condition although the rate of progression to acute leukaemia is different among subtypes[34]. Unlike PV, consistent gene mutation has not been found in human MDS[35]. Therefore, exclusion of other disease conditions causing dysplasia of bone marrow/blood cells in conjunction with single or multiple cytopenia(s) is a fundamental procedure. It has been reported that clonal cytogenetic abnormalities are observed in ~50% of MDS cases in humans[34,36]. Meanwhile, MDS is sometimes diagnosed in dogs[37-44]; however, there has been no report to demonstrate the genetic abnormality or assess the cell clonality in dog patients with MDS.

To aid the diagnosis of haematopoietic diseases by application of clonality assessment, I carried out a series of studies to detect the clonally expanded cell populations in canine PV and MDS cases. In Chapter 1, I performed a work to improve canine XCIP analysis by incorporation of a new locus on X-chromosome to a previously reported XCIP analysis using androgen receptor gene. In Chapter 2, I applied the improved canine XCIP analytical system to detection of cell clonality in canine PV cases and explored the existence of JAK2 V617F gene mutation shown in human PV. In Chapter 3, I examined cell clonality in canine MDS cases by the XCIP analysis, and compared the haematological characteristics between the dogs harbouring monoclonal and polyclonal cell populations.

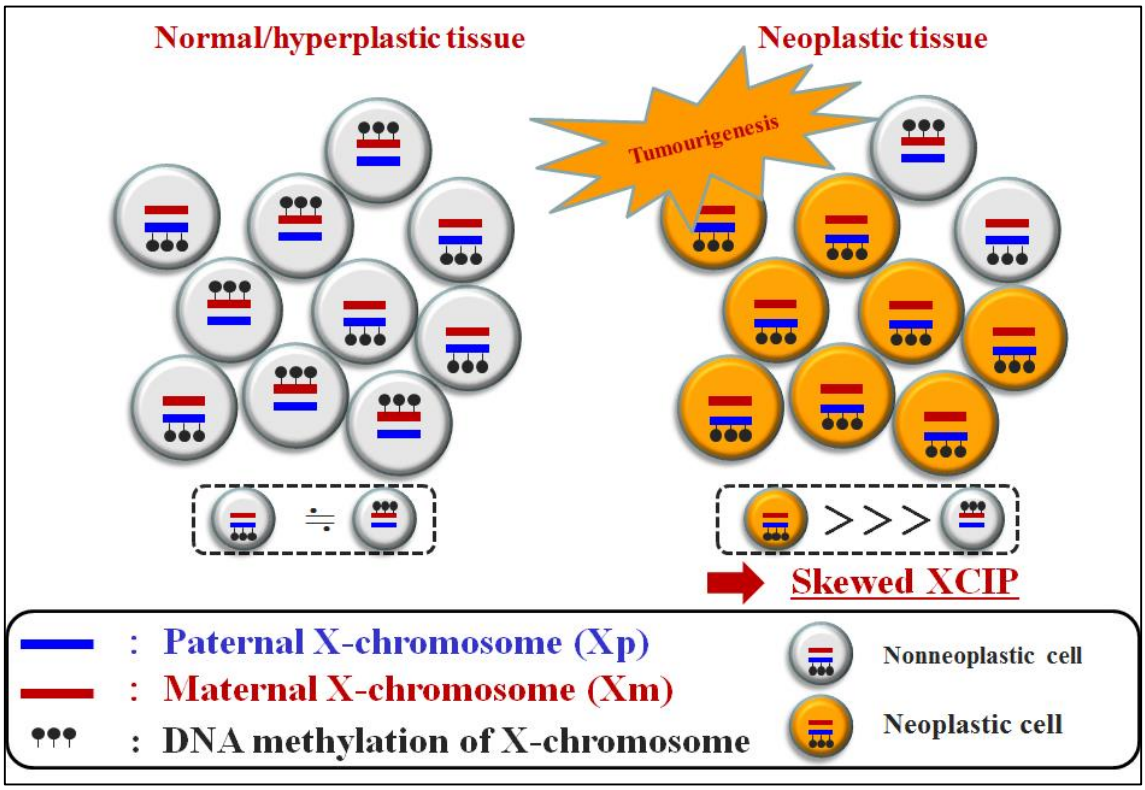


Figure A. Concept of X-chromosome inactivation pattern (XCIP) analysis

Mammalian females have 2 X-chromosomes in their somatic cells, and either paternal or maternal chromosomes is inactivated by DNA methylation during embryogenesis. This event is occurred randomly and the methylation status is strictly inherited by their daughter cells. Therefore, almost equal number of methylated paternal and maternal X-chromosomes exist in normal or hyperplastic tissues. On the other hand, methylation status in neoplastic cells is almost the same, so the numbers of methylated paternal and maternal X-chromosomes become apparently different in neoplastic tissues, resulting in skewed XCIP.

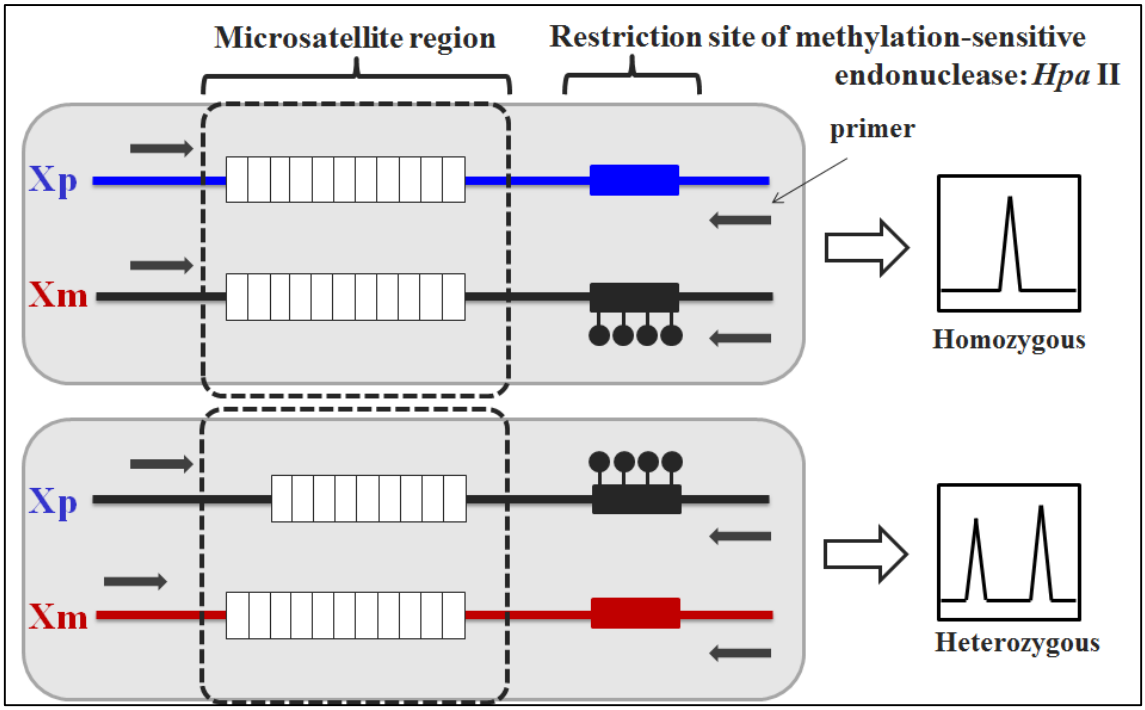


Figure B. Discrimination of 2 X-chromosomes by XCIP analysis

In order to discriminate 2 chromosomes, 2 loci of CAG trinucleotide tandem repeats in AR gene are analysed. Both microsatellite regions are amplified by PCR and then analysed by capillary electrophoresis. When both chromosomes have the same repeat length (upper schematic diagram), only one peak is detected by electrophoresis (homozygous pattern). On the other hand, when the 2 chromosomes have different repeat lengths (lower schematic diagram), two peaks are observed after electrophoresis, resulting in successful discrimination of 2 X-chromosomes (heterozygous pattern).

Xp: Paternal X-chromosome, Xm: Maternal X-chromosome

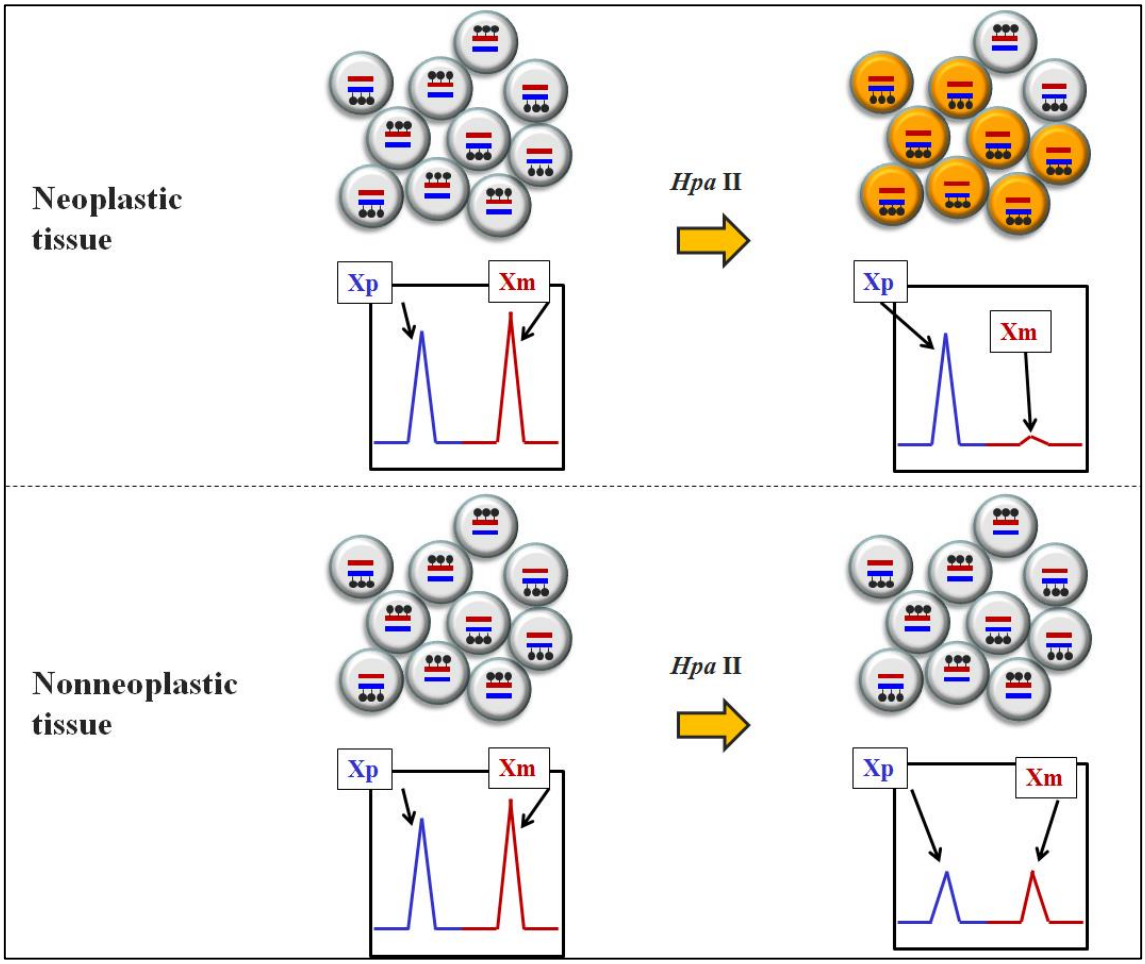


Figure C. Detection of methylation status by a methylation-sensitive endonuclease, *Hpa* II.

A methylation-sensitive endonuclease, *Hpa* II, can only cut its unmethylated restriction site. Therefore, after digestion with *Hpa* II, only methylated chromosomes can be amplified by PCR. These methylation status can be evaluated by comparison of the capillary electrophoretic patterns before and after digestion with *Hpa* II.

Xp: Paternal X-chromosome, Xm: Maternal X-chromosome

Chapter 1

Development of canine X-chromosome inactivation pattern analysis for detection of cell clonality by incorporation of the examination of SLIT and NTRK-like family, member 4 (SLITRK4) gene

Abstract

X-chromosome inactivation pattern (XCIP) analysis can be used to assess the clonality of cell population of various origins by distinguishing the methylated X chromosome from the unmethylated one. In this study, I carried out a study to improve the availability of XCIP analysis by incorporation of the examination of AC dinucleotide repeats in SLIT and NTRK-like family, member 4 (SLITRK4) gene into the previously reported CAG repeat examination of androgen receptor (AR) gene in dogs. The rate of heterozygosity (125/150, 83.3%) when both 2 genes were analysed was higher than that when only AR gene was examined (57.3 %). Tissue samples obtained from heterozygous dogs in either *AC-1* or *AC-2* of SLITRK4 gene were examined for the corrected inactivation allele ratio (CIAR), resulting in the reference range of CIAR < 4.0 in nonneoplastic cell/tissue samples. Even in the normal dog population, s small proportion (16/236, 6.8%) of the dogs showed skewed XCIP (CIAR > 4.0) and there was no tendency to be higher in older dogs. Through the present study, availability of canine XCIP analysis was improved by incorporation of the examination of SLITRK4 gene, providing more useful laboratory examination system to detect the clonality of various cell/tissue samples in dogs; however, it might be noted that a certain level of skewed XCIP can be observed in some proportion of normal individuals.

Introduction

Assessment of the clonality of cells can provide useful information to identify neoplastic and nonneoplastic proliferations in dogs as well as in humans. In human medicine, several analytical methods to indicate the cell clonality have been introduced by employing the examinations of specific chromosome/gene aberration, antigen receptor gene rearrangement, and X-chromosome inactivation pattern (XCIP).

In mammalian females, either paternal or maternal X-chromosome is inactivated by random fashion in each cell through the epigenetic changes during embryogenesis. This inactivation is precisely maintained by transmitting to the daughter cell. Therefore, nearly 1:1 inactivation ratio between paternal and maternal X-chromosomes is detected in normal, inflammatory, or hyperplastic conditions. On the other hand, neoplastic tissues are formed by a clonally expanded population from a single cell and share the same inactivation pattern of X-chromosome as in the cell of origin, resulting in apparently skewed XCIP[12].

XCIP analysis has been applied to various neoplastic disorders of various origins including hepatocellular carcinoma[45], ovarian cancer[46], renal cell carcinoma[47], leiomyosarcoma[48], and several haematopoietic disorders such as polycythaemia vera[49-51], myelodysplastic syndrome[52] and essential thrombocythaemia[53] in humans. XCIP analysis was recently introduced to assess the clonality of cells derived from dogs with cutaneous histiocytoma[14] and acute and chronic myelogeneous leukaemias in addition to high-grade

lymphoma[15]; however, since the frequency of heterozygosity in the number of CAG trinucleotide repeats in androgen receptor (AR) gene is relatively low in the dog populations examined, there remains a problem to apply the analysis to broader clinical specimens. The frequency of heterozygosity in canine AR gene was reported as 31.1-49.3%[14,15], lower compared to 68% in cats[13] and approximately 90% in humans[12,53-55].

To obtain higher heterozygosity rate in dogs, I tried to look for additional gene loci on the X-chromosome which would be used for XCIP analysis. In a report on human XCIP analysis, a new locus harbouring SLIT and NTRK-like family, member 4 (SLITRK4) gene, which includes AC dinucleotide repeat in its promoter region [56] was listed up as a candidate. Like human SLITRK4 gene, canine SLITRK4 gene locates on the X-chromosome and contains 2 clusters of AC dinucleotide repeats immediately downstream of the coding region. Differences in the numbers of AC dinucleotide repeats was shown to be able to discriminate two X-chromosomes in heterozygotes. I considered that it might be possible to use the SLITRK4 AC repeat for XCIP analysis by distinguishing the methylation status using a methylation-sensitive endonuclease, *Hpa II*[54,56].

Initial work of the present study was carried out to characterise canine SLITRK4 gene and examine its usefulness for the XCIP analysis in dogs. Then, I constructed an improved canine XCIP analysis using AR and SLITRK4 genes to examine the cell clonality in clinical specimens from dogs.

Materials and Methods

Nonneoplastic peripheral blood DNA samples

For characterisation of canine SLITRK4 gene, peripheral blood samples were obtained from 50 male and 150 female dogs. These dogs comprised 7 healthy dogs kept for research purposes and 193 dog patients referred to the Veterinary Medical Center of the University of Tokyo for diagnosis and treatment. These dogs included Miniature Dachshund (n=39), Shih Tzu (16), Chihuahua (14), mixed (14), Toy Poodle (14), Pomeranian (10), Beagle (9), Yorkshire Terrier (9), Pembroke Welsh Corgi (8), Border Collie (7), Cavalier King Charles Spaniel (6) and other 25 purebred dogs. Dogs affected with haematopoietic tumours and immune-mediated diseases were excluded before sampling.

In order to evaluate the age-related XCIP skewness, blood samples were obtained from another 236 healthy female dogs with heterozygous in at least one locus of various ages who admitted to the private animal clinics for prophylactic reasons. Use of the blood samples for research purposes was approved by their owners with written statements.

Further, nonneoplastic tissue samples were obtained in conjunction with blood samples to compare their CIARs in 28 dogs who were referred to the Veterinary Medical Center of the University of Tokyo.

Tissue samples

Neoplastic (n=54) and nonneoplastic (n=42) samples were obtained from female dog patients referred to the Veterinary Medical Center of the University of Tokyo. Diagnoses and tissue types of the 54 neoplastic samples are listed in Table 1-1. Forty-two nonneoplastic samples comprised 29 normal tissue samples and 13 samples showing hyperplasia. Twenty-nine normal tissues included intestine (n=12), liver (n=4), muscle, spleen (each 3), mammary gland, mesometorium (each 2), lung, skin and subcutis (each 1). Thirteen samples showing hyperplasia included inflammatory colorectal polyp (n=12) and multiple hepatic cyst (n=1) (Table 1-2). The tissue samples were collected by surgical or endoscopic resection and stored immediately at -80 °C until use. Veterinary pathologists in the University of Tokyo (MT and KU) defined the histopathological diagnoses of these samples. The neoplastic and nonneoplastic regions were separately sampled at the surgical resection or macroscopically observation of the resected tissues. Of the 54 cases with neoplasia, surrounding normal tissue samples from the same organ systems were obtained simultaneously from 18 cases. Further, 28 tissue samples including oral mucosa (n=12), intestinal mucosa (6), skin (5), lung, mammary gland, mesometrium, muscle and spleen (each 1) were also obtained simultaneously with peripheral blood samples for the purpose of comparison to blood samples.

DNA extraction

Genomic DNA samples were extracted from neoplastic or nonneoplastic tissue samples or peripheral blood by using QIAamp Blood and Tissue Mini Kit (QIAGEN, Hilden, Germany). Concentrations of the obtained DNA were calculated from the absorbance measured by a spectrophotometer.

Sequence analysis of canine SLITRK4 gene AC repeats in male dogs

Primer pairs were designed to amplify SLITRK4 gene (gene accession no. NC_006621.3) by using Primer3 Plus (http://bioinfo.ut.ee/primer3-0.4.0/). Primers seqAC-1-F and seqAC-1-R were used to analyse the first AC repeat (*AC-1*) and primers seqAC-2-F and seqAC-2-R were used to analyse the second AC repeat (*AC-2*) (Table 1-5).

Each reaction mixture contained 50 ng of genomic DNA, 0.2 μM of each primer, 1× GC enhancer, 1× AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 15 μl. PCR cycle conditions consisted of an initial denaturation step at 95 °C for 7 min; followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min; and the final extension at 72 °C for 7 min. After purification of the PCR product (SUPREC PCR; Takara Bio Inc., Otsu, Japan), sequencing analysis was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Amplification of CAG-1 and CAG-2 repeats in AR gene and AC-1 and AC-2 repeats in SLITRK4 gene in female dogs

Forward primers used in PCR amplifications for female dogs were labelled with 6-FAM at 5' end for capillary electrophoresis. PCR amplifications for 2 loci in AR gene were performed with CAG-1F and CAG-1R primers for *CAG-1* and CAG-2F and CAG-2R primers for *CAG-2* (Table 1-5). Amplification of *CAG-1* and *CAG-2* in AR gene were performed according to our method described previously[15]. 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 2 units of ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) in a total volume of 20 μl, Cycle conditions included initial denaturation step at 95 °C for 1min; followed by 28 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec; and a final extension at 72 °C for 7 min.

PCR amplifications for the 2 loci in SLITRK4 gene were performed with AC-1-F and AC-1-R primers for *AC-1* and AC-2-F and AC-2-R primers for *AC-2* (Table1-5). PCR condition for amplification of *AC-1* and *AC-2* was identical to the PCR condition for sequencing analysis described above except for the amplification cycles changing to 28 cycles.

One microliter of the 10-fold diluted PCR product was mixed with 8.5 μl formamide and 0.5 μl 600 LIZ size standard (Applied Biosystems) in a 96-well plate. These samples were denatured at 95 °C for 5 min followed by incubation at 4 °C for 15 min. The length of the PCR product was determined by capillary

electrophoresis using the Genetic Analyzer 3130xl and evaluated by PeakScanner Software (Applied Biosystems).

XCIP analysis using AR and SLITRK4 genes

XCIP analysis was performed based on the previously reported method[15] with some modification. DNA samples (500 ng) were incubated with 5 units of a methylation-sensitive endonuclease, *Hpa II* (New England Biolabs Inc., Ipswich, MA, USA) in a total volume of 50 µl. Undigested control of each sample was incubated without *Hpa II*. Both digested and undigested samples were incubated at 37 °C for 16 hr followed by inactivation at 80 °C for 20 min. Fifty ng of digested or undigested genomic DNA was subjected to PCR amplification for *CAG-1*, *CAG-2*, *AC-1* and *AC-2*, as described above.

Inactivated allele ratio (IAR) was calculated from obtained AUC by using the following formula as previously described[15].

$$\text{IAR} = \frac{[\text{digested allele 1} - (\text{shadow bands})] \,/\, [\text{digested allele 2}]}{[\text{undigested allele 1} - (\text{shadow bands})] \,/\, [\text{undigested allele 2}]}$$

In the analysis of *AC-1* and *AC-2* repeats in SLITRK4 gene, shadow bands generated by slippage synthesis of short tandem repeats are observed as minor peaks 2 base pairs shorter than real peaks[15,57]. When the AC dinucleotide repeats in one allele is longer than that in another allele by 2 base pairs (1 AC repeat), the longer allele affects the measured AUC of the shorter allele. To avoid the influence of shadow bands, the percentage of shadow bands for *AC-*

1 and *AC-2* were determined from the calculation of male dogs. If the size of 2 alleles at *AC-1* differed by 1- or 2-repeat length, 18% or 1.9% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. Similarly, when the difference of each allele was 1-, 2- or 3-repeat length at *AC-2*, 43%, 13% or 4.7% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. Likewise, at *CAG-1* and *CAG-2*, when the difference of each allele was 1 repeat length, 15% and 20% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. IAR was inversed when it was lower than 1.0 and defined as corrected IAR (CIAR). The highest CIAR of CIARs determined from 4 loci was used, and skewed XCIP was determined when CIAR deviated from normal range.

Results

Polymorphism of the repeat number in AC-1 and AC-2 repeats of SLITRK4 gene in male dogs

The number of AC dinucleotide repeats in *AC-1* and *AC-2* loci were examined by using peripheral blood samples from 50 male dogs. The numbers of AC dinucleotide repeats in *AC-1* locus were 10 (14/50, 28%), 12 (26/50, 52%), 13 (3/50, 6%) and 7 (7/50, 14%). In *AC-2* locus, they were 15 (1/50, 2%), 16 (29/50, 58%), 18 (5/50, 10%), 19 (12/50, 24%), 20 (1/50, 2%) and 21 (2/50, 4%).

Heterozygosity of CAG-1 and CAG-2 repeats in AR gene and AC-1 and AC-2 repeats in SLITRK4 gene in female dogs

Using peripheral blood samples of 150 female dogs, 31% (47/150), 37% (55/150), 49% (73/150) and 41% (62/150) were shown to be heterozygous for *CAG-1*, *CAG-2*, *AC-1* and *AC-2*, respectively. As a result, 57% (86/150) were heterozygous for either *CAG-1* or *CAG-2* repeats in AR gene. On the other hand, 63% (94/150) of dogs were found to be heterozygous for either *AC-1* or *AC-2* repeats in SLITRK4 gene. By incorporation of the results of the 4 loci, heterozygosity of at least one locus was observed in 83% (125/150) of the female dogs examined.

Determination of the normal range of CIAR for AC-1 and AC-2 loci in SLITRK4 gene

Twenty-three neoplastic tissue samples and 24 nonneoplastic tissue samples for *AC-1* and 28 neoplastic tissue samples and 10 nonneoplastic tissue samples for *AC-2* were determined as heterozygous cases (Table 1-1). Cut-off points of CIAR using receiver operating characteristic (ROC) curve were 2.691 in *AC-1* and 2.762 in *AC-2*, respectively (Figure 1-1); however, in order to decrease false-positive results, cut-off points of CIARs in both loci were determined as 4.0 (Tables 1-3 and 1-4). In addition, in order to simplify the comparison, cut-off points of CIARs in CAG-1 and CAG-2 of AR gene were also determined as 4.0, although cut-off points of CIARs in CAG-1 and CAG-2 in the previous study were 3.0[15]. Therefore, CIAR result higher than 4.0 in any locus were determined as skewed XCIP indicating clonal cell populations. When multiple loci were analysed, the highest CIAR was employed as the result for each case. All XCIP analyses were run in duplicates to check the reproducibility.

XCIP analysis of neoplastic and nonneoplastic tissues

Fifty-four neoplastic and 42 nonneoplastic tissue samples were examined for heterozygosity prior to the XCIP analysis. Of the 54 neoplastic samples, 43 samples (80%) were heterozygous for at least 1 locus (Table 1-1). Similarly, 34 (81%) out of 42 nonneoplastic samples were heterozygous for at least 1 locus (Table 1-2).

Among the heterozygous 43 neoplastic tissues, CIAR higher than 4.0 was shown in 21 sample (49%), indicating the skewed XCIP (Table 1-1 and Figures 1-2 and 1-3). Among the 21 neoplastic cases with skewed XCIP, 2 or more loci were analysed in 14 of 21 cases. Five of the 14 cases showed skewed XCIP at all loci, but other 9 samples showed skewed XCIP at only a part of the heterozygous loci. All of the 34 heterozygous nonneoplastic samples including 24 normal tissues and 10 hyperplastic tissues showed balanced XCIP (Figures 1-2 and 1-3).

Of the 43 dogs with neoplastic disease, 18 dogs were subjected to the comparisons of CIARs of the neoplastic tissue and surrounding nonneoplastic tissue. Nine out of 18 neoplastic tissue samples (50%) showed skewed XCIP (CIAR > 4.0), while all of the nonneoplastic tissue samples showed balanced XCIP (CIAR < 4.0) (Table 1-6).

Evaluation of age-related XCIP skewness in peripheral blood from healthy females

Dogs were divided in 14 groups by their ages; under 6 months, 6 months to 1 year, each 1 year during 1 to 11 years and over 12 years. CIAR for each dog was obtained and compared among different age groups (Figure 1-4). 8.3% of dogs younger than 6 months showed CIAR > 4.0, and the rate of CIAR > 4.0 tended to be increased in groups over 10 years but no significant difference was observed when dogs older than 10 years were compared to dogs younger than 10 years. The groups of high rate of CIAR > 4.0 were 10, 11 and 4 year

old (25%, 20%, 12.5%, respectively). Overall rate of CIAR > 4.0 was 6.8% (range: 0-25%).

Comparison of CIAR between peripheral blood and normal tissue samples

CIARs were compared between peripheral blood and normal tissue samples in 28 dogs (Figure 1-5). All analysable loci were compared in this study (n=46). Skewed XCIP was detected in 6.5% (3/46) of the peripheral blood samples and 4.3% (2/46) of normal tissue samples. The two tissue samples showed CIAR > 4.0 were both oral mucosa. Correlation of CIAR was not observed between the peripheral blood and normal tissue samples (r=0.21).

Discussion

In this study, to obtain data of XCIP analysis in larger number of dog population, I incorporated the examination of SLITRK4 gene to the previously reported canine XCIP analysis using AR gene. Frequency of heterozygosity of at least one locus of the four loci was 83.3%, which was higher compared to our previous study using AR gene alone (49.3%)[15]. Although XCIP assay is applicable only to females, examination at more loci on X-chromosome enabled its higher utilization rate more than 80 % of the female dogs.

When using the 4 loci for XCIP analysis, *CAG-1* and *CAG-2* loci in AR gene and *AC-1* and *AC-2* loci in SLITKR4 gene, CIAR of each locus was different in many of the cases in which multiple loci could be used for XCIP analysis. The inconsistency among multiple loci may be due to the non-uniform pattern of X-chromosome methylation[58]. The methylation rate in each gene was reported to be 24:76 in AR gene, and 44:56 in SLITRK4 gene in a study using normal human tissues, indicating the inconsistent methylation pattern of the X-chromosome[56]. In order to obtain a reliable result in XCIP analysis, further increase of the number of loci would be important to avoid false negative result.

From the result of XCIP analysis for the solid tumours samples and nonneoplastic tissue samples in this study, the sensitivity and specificity of the assay in this study was determined to be 48.8% (21/43) and 100% (34/34), respectively. The sensitivity was low compared to our previous study using AR gene (90%)[15], but similar to the results in human samples (39%)[53]. The specificity was comparable to our previous data (100%) [15] and the data obtained

in human samples (94%)[53]. There are several possible factors affecting the sensitivity of XCIP analysis. First, the sensitivity of XCIP analysis depends on the proportion of tumour cells contained in the samples. Theoretically, when the nonneoplastic cells account for more than 40% of the tissue examined, XCIP analysis would result in CIAR lower than 4.0 even though the sample contains tumour cells of clonal origin. It might be possible that connective tissue and inflammatory cells included in the tissue samples used in the present study affected the results. It might be also possible abnormal hyper- or hypo-methylation which sometimes occurs at same loci on the X-chromosome in tumours could affect the results in XCIP analysis. In human medicine, abnormal hypermethylation at the AR locus has been reported in lymphoma patients[59].

In humans, skewed XCIP has been reported in a certain proportion of normal females, especially in older individuals. In such individuals with skewed XCIP, false-positive results may occur although they do not have any neoplastic lesion. Therefore, it has been recommended to analyse control nonneoplastic tissue samples sharing the common origin with the objective neoplastic tissue[60,61]. In this study, normal tissue sample derived from same stem cells were compared with their neoplastic counterparts. Regardless of the XCIP status in neoplastic tissues, all of the 18 surrounding nonneoplastic tissues did not show elevated CIAR more than 4.0. All of the 34 nonneoplastic tissues also showed balanced XCIP, therefore skewed XCIP in normal tissue may be a rare event in dogs.

On the other hand, 6.8% of peripheral blood samples from healthy female

dogs showed CIAR higher than 4.0. Frequency of the individuals showing skewed XCIP did not correlate with the age in the dog population in this study. In human females, it has been demonstrated that the rate of skewness tends to increase by aging. The discrepancy might be explained in 2 reasons. First, the sample sizes in this study were small compared to human studies. Other reason is the difference of lifetime between humans and dogs. In humans, the rate of skewness has been reported to be from 4.9% in new-borns, increasing up to 14.2% in adults whose ages were 30 to 55 years[62-66]. Exact reason for the skewed XCIP in healthy females is unclear; however, it is possible that the skewness is a result of a stochastic event during stem cell division[67]. Because life span of dogs is shorter than that of human, canine females might have less chance of excessive skewness compared to human females.

In the present study, I compared CIAR of normal tissue samples with those of peripheral blood cells to examine whether peripheral blood can be used as a substitute for the control tissue of the same origin. As a result, no relationship was observed between CIAR of peripheral blood and that of other normal tissues. Together with the data showing higher rate of skewed XCIP in peripheral blood compared to other tissues, it would not be suitable to use peripheral blood as a control tissue as indicated in humans[68]. Recent study in humans indicated the correlation of the results in XCIP analysis between oral mucosa and other tissues such as heart, liver, muscle or kidney[69]. Future study is needed to examine whether canine oral mucosa can be used as a substitute of the control tissue.

Conclusions

In this study, canine XCIP analysis system could be improved by incorporation of the examination of SLITRK4 gene, providing more useful detection tool of clonal population from various cell/tissue samples. However, it is also important to recognise the possibility of false-positive and false-negative results.

Table 1-1. Diagnoses and the results of XCIP analysis of neoplastic tissue samples.

Case No.	Diagnosis	Origin	IAR (CIAR)				
			CAG1	*CAG1*	*AC1*	*AC2*	Maximum CIAR
1	Adenocarcinoma	Adrenal gland	0.55 (1.82)	-	-	-	1.82
2	Adenocarcinoma	Adrenal gland	0.76 (1.32)	0 (> 10.0)	1.57	0.25 (4.03)	> 10.0
3	Adenocarcinoma	Adrenal gland	-	1.16	-	-	1.16
4	Adenocarcinoma	Adrenal gland	-	-	-	> 10.0	> 10.0
5	Adenocarcinoma	Mammary gland	0.58 (1.74)	1.05	-	1.26	1.74
6	Adenocarcinoma	Mammary gland	-	0.78 (1.28)	-	-	1.28
7	Adenocarcinoma	Mammary gland	-	0.92 (1.09)	2.79	-	2.79
8	Adenocarcinoma	Mammary gland	-	-	4.3	0.36 (2.76)	4.30
9	Adenoma	Adrenal gland	0.07 (> 10.0)	-	-	-	> 10.0
10	Adenoma	Mammary gland	0.3 (3.37)	1.75	-	0.42 (2.39)	3.37
11	Ameloblastoma	Mandibular subcutis	-	4.92	0.12 (8.21)	0.16 (6.19)	8.21
12	Anal sac gland carcinoma	Anal sac	-	-	-	0.25 (4.03)	4.03
13	Cholangiocarcinoma	Liver	-	0.18 (5.49)	0.12 (8.35)	5.76	8.35
14	Cholangiocarcinoma	Liver	-	-	7.66	9	9.00
15	Fibroma	Vagina	0.85 (1.17)	0.19 (5.13)	-	-	5.13
16	Fibroma	Vagina	-	0.17 (5.94)	-	4.77	5.94
17	Gastrointestinal stromal tumour	Intestine	0.83 (1.21)	-	0.5 (2)	-	2.00
18	Gastrointestinal stromal tumour	Intestine	-	2.05	-	-	2.05
19	Granulosa cell tumour	Ovary	-	-	-	0.16 (6.28)	6.28
20	Haemangiopericytoma	Carpal subcutis	1.15	-	-	-	1.15
21	Haemangiopericytoma	Shoulder subcutis	-	-	2.77	1.47	2.77
22	Haemangiopericytoma	Gluteal subcutis	-	-	-	0 (> 10.0)	> 10.0
23	Haemangiosarcoma	Liver	-	-	0.7 (1.42)	-	1.42
24	Hepatocellular adenoma	Liver	3.03	-	2.69	3.03	3.03
25	Hepatocellular adenoma	Liver	-	0.57 (1.75)	-	0.5 (1.99)	1.99
26	Heptocellular carcinoma	Liver	1.14	1.14	4.94	9	9.00
27	Heptocellular carcinoma	Liver	-	0.29 (3.39)	0 (> 10.0)	-	> 10.0
28	Heptocellular carcinoma	Liver	-	-	0 (> 10.0)	8.15	> 10.0
29	Leiomyoma	Abdominal	-	-	-	> 10.0	> 10.0
30	Lipoma	Vagina	-	0.96 (1.04)	0.57 (1.76)	0.27 (3.74)	3.74
31	Lymphangioma	Liver	-	-	0.49 (2.06)	0.43 (2.34)	2.34
32	Lymphangiosarcoma	Femoral subcutis	-	-	0.67 (1.49)	-	1.49
33	Malignant melanoma	Lip	1.63	-	0.97 (1.03)	1.02	1.63
34	Malignant melanoma	Mandibular subcutis	-	-	0.24 (4.09)	0.29 (3.39)	4.09
35	Malignant peripheral nerve sheath tumour	Oral	-	-	0.33 (3.05)	0.33 (3.07)	3.07
36	Mucinous carcinoma	Intestine	-	0.72 (1.39)	-	1.12	1.39
37	Renal cell carcinoma	Kidney	0.07 (> 10.0)	-	-	-	> 10.0
38	Sarcoma NOS	Elbow	-	0.88 (1.13)	-	-	1.13
39	Synovial cell sarcoma	Elbow	0.2 (4.89)	0.76 (1.31)	0.32 (3.09)	0.8 (1.25)	4.89
40	Transitional cell carcinoma	Kidney	1.77	-	> 10.0	0.04 (> 10.0)	> 10.0
41	Transitional cell carcinoma	Kidney	0.96 (1.04)	-	> 10.0	0.09 (> 10.0)	> 10.0
42	Transitional cell carcinoma	Kidney	-	-	1.01	-	1.01
43	Transitional cell carcinoma	Urethra	-	-	-	0.43 (2.32)	2.32
44	Adenocarcinoma	Mammary gland	-	-	-	-	-
45	Adenocarcinoma	Mammary gland	-	-	-	-	-
46	Adenocarcinoma	Mammary gland	-	-	-	-	-
47	Adenocarcinoma	Mammary gland	-	-	-	-	-
48	Adenoma	Mammary gland	-	-	-	-	-
49	Hepatocellular adenoma	Liver	-	-	-	-	-
50	Heptocellular carcinoma	Liver	-	-	-	-	-
51	Heptocellular carcinoma	Liver	-	-	-	-	-
52	Heptocellular carcinoma	Liver	-	-	-	-	-
53	Mucinous carcinoma	Intestine	-	-	-	-	-
54	Synovial cell sarcoma	Elbow	-	-	-	-	-

s

CIAR: corrected inactivated allele ratio. -: homozygous, Shaded box means CIAR > 4.0.

Table 1-2. Sample types of normal and hyperplastic tissues and results of XCIP analysis.

Sample type	Tissue	CIAR				
		CAG-1	CAG-2	AC1	AC2	Maximum CIAR
Normal	Intestine	1.01	-	-	-	1.01
Normal	Intestine	1.22	1	1.43	-	1.43
Normal	Intestine	1.25	1.06	1.07	-	1.25
Normal	Intestine	1.41	-	1.39	-	1.41
Normal	Intestine	2.06	-	1.66	-	2.06
Normal	Intestine	2.32	-	2.37	-	2.37
Normal	Intestine	1.23	-	1.31	-	1.31
Normal	Intestine	1.10	-	-	-	1.10
Normal	Liver	3.55	-	3.16	3.55	3.55
Normal	Liver	-	1.08	1.62	1.51	1.62
Normal	Liver	-	1.37	-	-	1.37
Normal	Liver	-	-	1.13	1.19	1.19
Normal	Lung	1.08	1.32	-	-	1.32
Normal	Mammary gland	-	1.03	3.92	-	3.92
Normal	Mammary gland	-	-	2.15	1.63	2.15
Normal	Mesometrium	1.48	-	1.76	1.68	1.76
Normal	Mesometrium	-	-	-	2.54	2.54
Normal	Muscle	-	1.21	1.05	1.12	1.21
Normal	Muscle	-	-	1.15	-	1.15
Normal	Muscle	-	-	2.1	1.57	2.10
Normal	Skin	1.72	2.14	2.55	2.58	2.58
Normal	Spleen	3.30	-	-	-	3.30
Normal	Spleen	2.09	-	1.28	-	2.09
Normal	Spleen	-	-	-	2.31	2.31
Normal	Intestine	-	-	-	-	-
Normal	Intestine	-	-	-	-	-
Normal	Intestine	-	-	-	-	-
Normal	Intestine	-	-	-	-	-
Normal	Subcutis	-	-	-	-	-
Multiple hepatic cyst	Liver	-	1.70	-	-	1.70
Rectal polyp	Rectum	1.05	1.09	1.09	-	1.09
Rectal polyp	Rectum	1.12	-	1.4	-	1.40
Rectal polyp	Rectum	-	-	2.27	-	2.27
Rectal polyp	Rectum	1.89	-	2.94	-	2.94
Rectal polyp	Rectum	1.04	-	1.84	-	1.84
Rectal polyp	Rectum	1.15	1.16	1.10	-	1.16
Rectal polyp	Rectum	1.01	-	1.08	-	1.08
Rectal polyp	Rectum	1.14	-	-	-	1.14
Rectal polyp	Rectum	1.03	-	-	-	1.03
Rectal polyp	Rectum	-	-	-	-	-
Rectal polyp	Rectum	-	-	-	-	-
Rectal polyp	Rectum	-	-	-	-	-

CIAR: corrected inactivated allele ratio. -: homozygous

Table 1-3. ROC table of CIAR in *AC-1*

CIAR	Sensitivity	Sensitivity-(1-Specificity)	True positive	True negative	False positive	False negative
> 10.0	0.1739	0.1739	4	24	0	19
8.346	0.217	0.217	5	24	0	18
8.213	0.261	0.261	6	24	0	17
7.664	0.304	0.304	7	24	0	16
4.944	0.348	0.348	8	24	0	15
4.302	0.391	0.391	9	24	0	14
4.085	0.435	0.435	10	24	0	13
3.918	0.435	0.393	10	23	1	13
3.156	0.435	0.351	10	22	2	13
3.087	0.478	0.395	11	22	2	12
3.046	0.522	0.438	12	22	2	11
2.939	0.522	0.397	12	21	3	11
2.791	0.565	0.44	13	21	3	10
2.77	0.609	0.484	14	21	3	9
2.691*	0.652	0.527	15	21	3	8
2.554	0.652	0.486	15	20	4	8
2.37	0.652	0.444	15	19	5	8
2.272	0.652	0.402	15	18	6	8
2.148	0.652	0.361	15	17	7	8
2.1	0.652	0.319	15	16	8	8
2.055	0.696	0.362	16	16	8	7
2.002	0.739	0.406	17	16	8	6
1.836	0.739	0.364	17	15	9	6
1.76	0.739	0.323	17	14	10	6
1.759	0.783	0.366	18	14	10	5
1.664	0.783	0.324	18	13	11	5
1.623	0.783	0.283	18	12	12	5
1.567	0.826	0.326	19	12	12	4
1.495	0.87	0.37	20	12	12	3
1.434	0.87	0.328	20	11	13	3
1.421	0.913	0.371	21	11	13	2
1.403	0.913	0.33	21	10	14	2
1.393	0.913	0.288	21	9	15	2
1.31	0.913	0.246	21	8	16	2
1.28	0.913	0.205	21	7	17	2
1.154	0.913	0.163	21	6	18	2
1.129	0.913	0.121	21	5	19	2
1.103	0.913	0.08	21	4	20	2
1.093	0.913	0.038	21	3	21	2
1.083	0.913	-0.004	21	2	22	2
1.075	0.913	-0.045	21	1	23	2
1.045	0.913	-0.087	21	0	24	2
1.03	0.957	-0.044	22	0	24	1
1.012	1	0	23	0	24	0
1.012	1	0	23	0	24	0

*: cut-off point

Table 1-4. ROC data of CIAR in *AC-2*

CIAR	Sensitivity	Sensitivity-(1-Specificity)	True positive	True negative	False positive	False negative
> 10.0	0.1786	0.1786	5	10	0	23
9.004	0.214	0.214	6	10	0	22
8.996	0.25	0.25	7	10	0	21
8.148	0.286	0.286	8	10	0	20
6.277	0.321	0.321	9	10	0	19
6.187	0.357	0.357	10	10	0	18
5.765	0.393	0.393	11	10	0	17
4.773	0.429	0.429	12	10	0	16
4.034	0.464	0.464	13	10	0	15
4.033	0.5	0.5	14	10	0	14
3.739	0.536	0.536	15	10	0	13
3.546	0.536	0.436	15	9	1	13
3.391	0.571	0.471	16	9	1	12
3.072	0.607	0.507	17	9	1	11
3.033	0.643	0.543	18	9	1	10
2.762*	0.679	0.579	19	9	1	9
2.578	0.679	0.479	19	8	2	9
2.545	0.679	0.379	19	7	3	9
2.393	0.714	0.414	20	7	3	8
2.345	0.75	0.45	21	7	3	7
2.323	0.786	0.486	22	7	3	6
2.309	0.786	0.386	22	6	4	6
1.993	0.821	0.421	23	6	4	5
1.676	0.821	0.321	23	5	5	5
1.633	0.821	0.221	23	4	6	5
1.573	0.821	0.121	23	3	7	5
1.509	0.821	0.021	23	2	8	5
1.469	0.857	0.057	24	2	8	4
1.262	0.893	0.093	25	2	8	3
1.251	0.929	0.129	26	2	8	2
1.187	0.929	0.029	26	1	9	2
1.121	0.929	-0.071	26	0	10	2
1.121	0.964	-0.036	27	0	10	1
1.022	1	0	28	0	10	0
1.022	1	0	28	0	10	0

*: cut-off point

Table 1-5. Primers used in this study

Primer name	Sequence (5'-3')	Accession number	Nucleotide number
seqAC1-F	TAAGGACACCGGATCTTTGG	(CFA) X	112,805,252-112,805,271
seqAC1-R	GGCGCGTGTATGTAAAGGTG	(CFA) X	112,805,767-112,805,748
seqAC2-F	CGGGCTGCACCTTTACATAC	(CFA) X	112,805,741-112,805,760
seqAC2-R	GAGCTACAAAGCCCAAGTGC	(CFA) X	112,806,332-112,806,313
AC1-F	TATGGTCGCTCTGGCCACTC	(CFA) X	112,805,329-112,805,348
AC1-R	CCGGAGGTGGGTGAATTTCCA	(CFA) X	112,805,542-112,805,522
AC2-F	CACGCACACTCGAGTCCTTTC	(CFA) X	112,806,048-112,806,068
AC2-R	TGCTCCTCAAAGGAACGCC	(CFA) X	112,806,163-112,806,145
CAG-1F	CGAAGTGATCCAGAACCCGG	NC_006621.3	93–112
CAG-1R	TTCCTCATCCAGAGCCAGGTAGC	NC_006621.3	294–272
CAG-2F	CCCATCCACATTGTCACTGCTG	NC_006621.3	435–456
CAG-2R	CATGGACACCGACACTGCCTT	NC_006621.3	750–730

Table 4. Comparison of CIARs between neoplastic and their nonneoplastic counterparts

Case No.	Origin	Diagnosis	IAR (CIAR)						IAR (CIAR)				
			CAG-1	CAG-2	AC-1	AC-2	Maximum IAR (CIAR)		CAG-1	CAG-2	AC-1	AC-2	Maximum IAR (CIAR)
2	Adrenal gland	Adenocarcinoma	0.76 (1.32)	0 (> 10.0)	0.64 (1.57)	0.25 (4.03)	>10.0	Normal	0.51 (1.98)	0.53 (1.89)	0.43 (2.35)	0.59 (1.7)	2.35
7	Mammary gland	Adenocarcinoma	-	0.92 (1.09)	2.79	-	2.79	Normal	-	1.72	3.45	-	3.45
14	Liver	Cholangiocarcinoma	-	-	7.66	9.00	9.00	Normal	-	-	1.19	0.82 (1.22)	1.22
15	Vagina	Fibroma	0.85 (1.17)	0.19 (5.13)	-	-	5.13	Normal	0.94 (1.07)	0.79 (1.27)	-	-	1.27
16	Vagina	Fibroma	-	0.17 (5.94)	-	4.77	5.94	Normal	-	1.23	-	0.78 (1.28)	1.28
20	Carpal subcutis	Haemangiopericytoma	1.15	-	-	-	1.15	Normal	1.47	-	-	-	1.47
21	Shoulder subcutis	Haemangiopericytoma	-	-	2.77	1.47	2.77	Normal	-	-	1.35	1.86	1.86
22	Gluteal subcutis	Haemangiopericytoma	-	-	-	0 (> 10.0)	>10.0	Normal	-	-	-	0.77 (1.29)	1.29
24	Liver	Hepatocellular adenoma	3.03	-	2.69	3.03	3.03	Normal	0.42 (2.36)	-	2.04	0.29 (3.45)	3.45
25	Liver	Hepatocellular adenoma	-	0.57 (1.75)	-	0.5 (1.99)	1.99	Normal	-	1.03	-	0.94 (1.06)	1.06
27	Liver	Hepatocellular carcinoma	-	0.29 (3.39)	0 (> 10.0)	-	>10.0	Normal	-	1.02	0.7 (1.44)	-	1.44
28	Liver	Hepatocellular carcinoma	-	-	0 (> 10.0)	8.15	>10.0	Normal	-	-	0.7 (1.44)	1.16	1.44
30	Vagina	Lipoma	-	0.96 (1.04)	0.57 (1.76)	0.27 (3.74)	3.74	Normal	-	1.2	0.71 (1.41)	0.79 (1.26)	1.41
33	Lip	Malignant melanoma	1.63	-	0.97 (1.03)	1.02	1.63	Normal	0.61 (1.63)	-	1.51	0.75 (1.34)	1.63
37	Kidney	Renal cell carcinoma	0.07 (> 10.0)	-	-	-	>10.0	Normal	0.34 (2.91)	-	-	1.93	2.91
38	Elbow	Sarcoma NOS	-	0.88 (1.13)	-	-	1.13	Normal	-	0.6 (1.67)	-	-	1.67
41	Kidney	Transitional cell carcinoma	0.96 (1.04)	-	>10.0	0.09 (> 10.0)	>10.0	Normal	2.91	-	1.24	2.76	2.91
42	Kidney	Transitional cell carcinoma	-	-	1.01	-	1.01	Normal	-	-	1.76	-	1.76

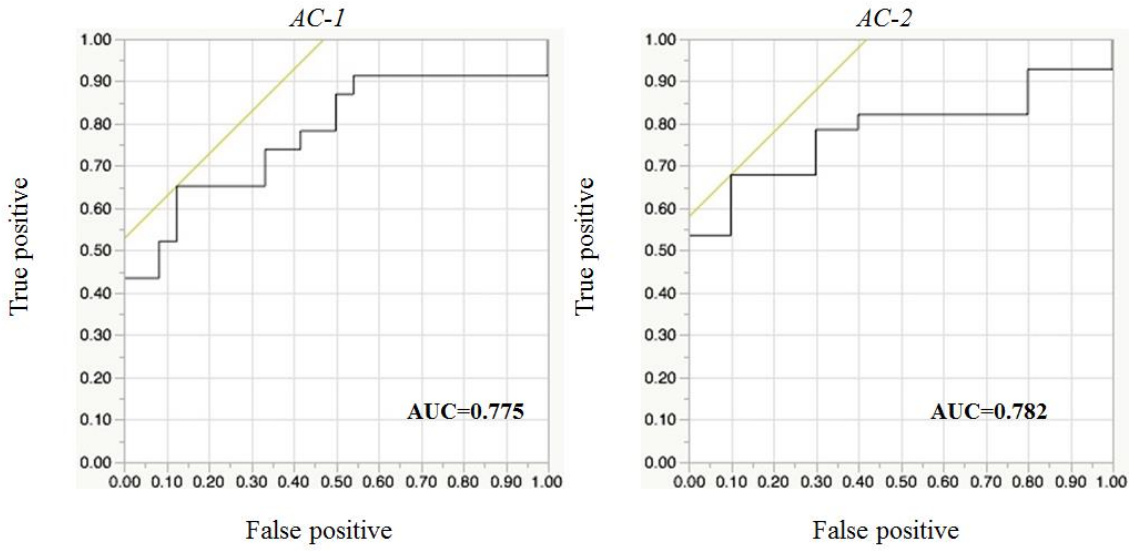


Figure 1-1. Receiver operating characteristic (ROC) curve for determination of cut-off points in CIARs of *AC-1* and *AC-2*

Cut-off points of CIARs in *AC-1* and *AC-2* were 2.691 and 2.762, respectively, and area under ROC were 0.775 and 0.782, respectively.

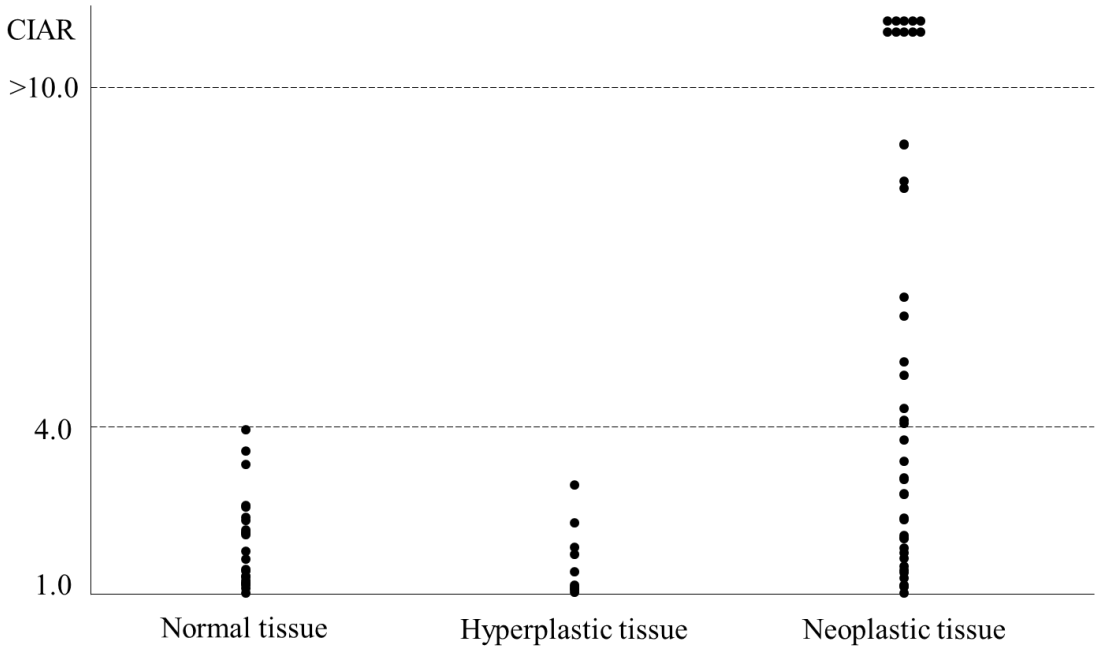


Figure 1-2. Results of XCIP analysis in nonneoplastic and neoplastic tissue samples.

CIARs obtained by XCIP analysis for the normal, hyperplastic and neoplastic tissues. Inactive allele ratio (IAR) was inversed when it was lower than 1.0, and indicated as corrective IAR (CIAR). CIAR higher than 4.0 was considered as skewed XCIP.

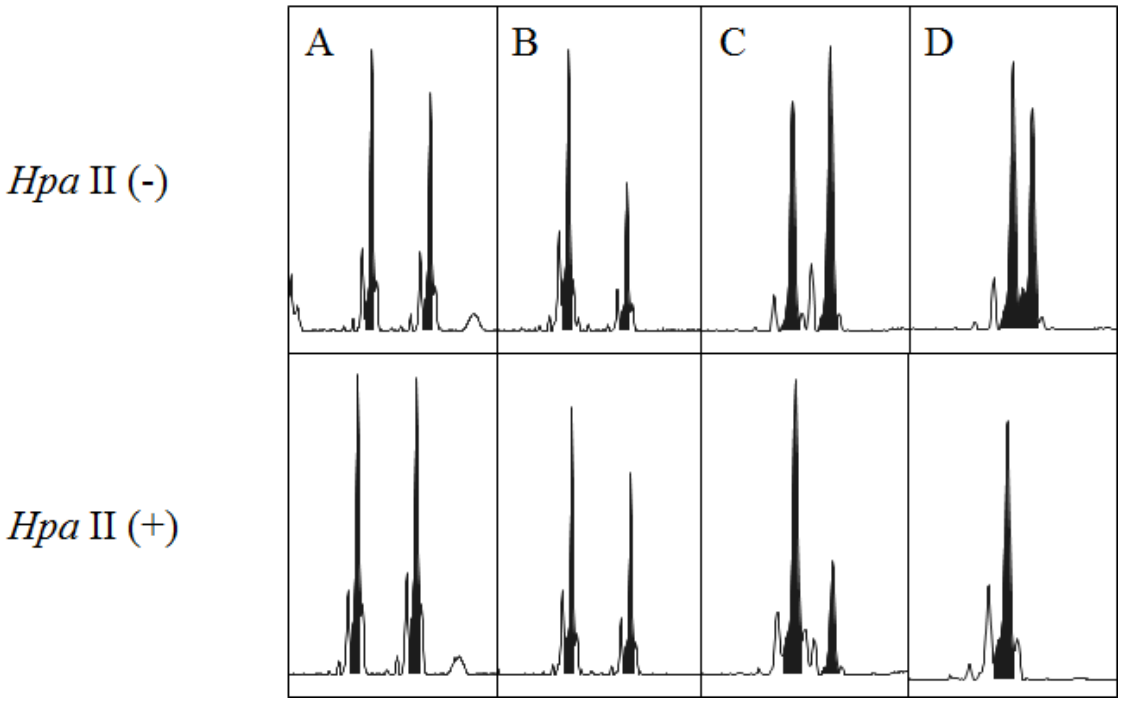


Figure 1-3. Results of XCIP analysis.

Upper illustrations showed XCIP results without *Hpa* II digestion and lower illustrations showed XCIP results with *Hpa* II digestion. Representative results for XCIP analysis of *AC-1* locus in SLITRK4 gene. **A**) Normal tissue (spleen) showed balanced XCIP (CIAR = 1.28). **B**) Hyperplastic tissue (rectal polyp) showed balanced XCIP (CIAR = 1.40). **C**) Neoplastic tissue (malignant melanoma, Case 34) showed skewed XCIP (CIAR = 4.09). **D**) Neoplastic tissue (transitional cell carcinoma, Case 41) showed skewed XCIP (CIAR > 10). Right allele (allele 2) was almost depleted after digestion by *Hpa II*.

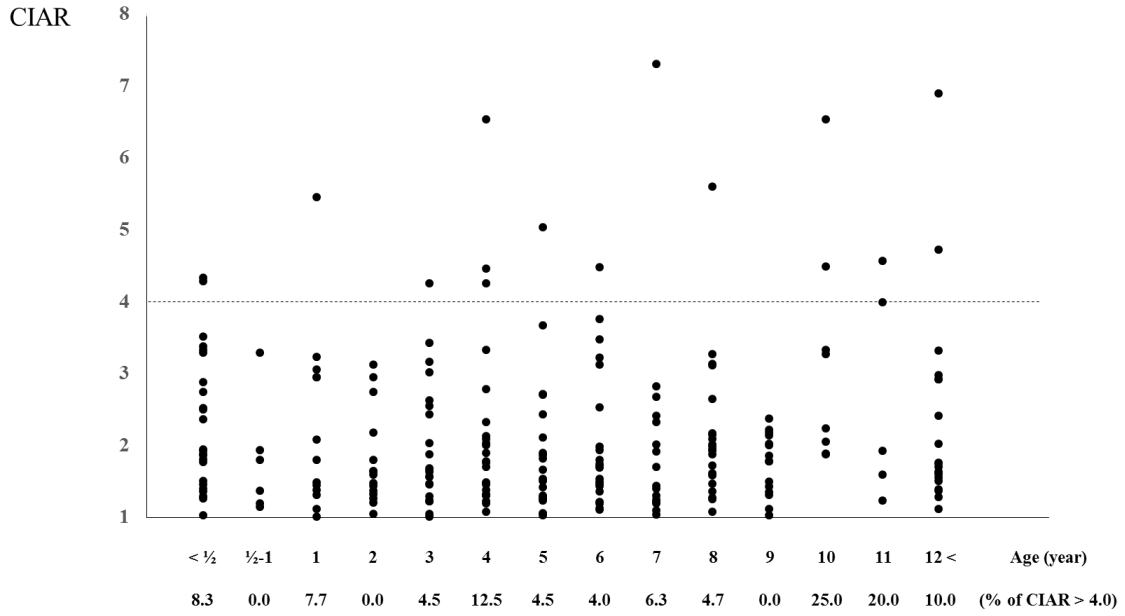


Figure 1-4. Results of XCIP analysis in various age females.

CIARs obtained by XCIP analysis of peripheral blood from healthy dogs of various age groups. The overall average rate of skewness was 6.8%. Rate of skewness was indicated below the figure for each group.

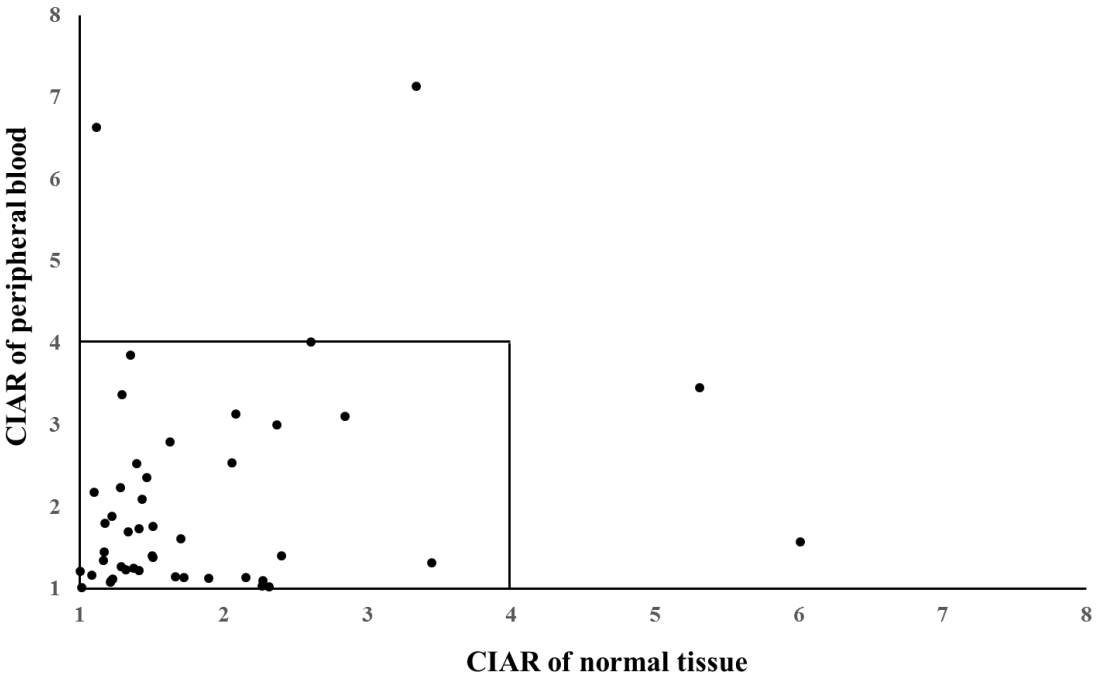


Figure 1-5. Comparison results of XCIP analysis in peripheral blood and tissue samples.

Comparison of CIARs obtained by XCIP between peripheral blood and other tissue samples from identical cases. CIAR higher than 4.0 was considered as skewed XCIP.

Chapter 2

Clonal assessment of blood and bone marrow cells in dogs with polycythemia vera by X-chromosome inactivation pattern analysis

Abstract

Polycythemia vera (PV) is one of the myeloproliferative neoplasms (MPNs) characterised by autonomous erythropoiesis without any underlying disease. Human PV is known to be caused by the proliferation of aberrant haematopoietic clone and many patient present pancytosis possibly derived from an abnormal progenitor clone with pluripotency. Further, JAK2 gene mutation (V617F) has been observed in most of the human PV patients, indicating that the JAK2 aberration is responsible for the disease development. On the other hand, in canine PV, pancytosis as shown in human PV is rarely observed and the JAK2 mutation has been reported only in one case. Diagnosis of PV has been generally based upon the exclusion of other disease conditions to induce erythrocytosis because there has been no analytical system to demonstrate the cell clonality in canine erythroid cells. In this study, I evaluated the haematological features of 8 dogs clinically diagnosed with PV. Of the 8 dogs with PV, leukocytosis and thromobocytosis were observed in 1 and 2 dogs, respectively. I examined the cell clonality in 6 of the 8 canine PV cases by using X-chromosome inactivation pattern (XCIP) analysis. The remaining 2 cases could not be evaluated by XCIP analysis because of the homozygosity of the loci examined. Five of the 6 PV cases were found to possess clonal populations in their peripheral blood and/or bone marrow, but not in T-lymphocyte populations. These results indicated that PV arose by clonal expansion of an aberrant haematopoietic progenitor clone with differentiation ability to both erythroid and myeloid cells in dogs. Eight canine

PV cases were subjected to the nucleotide sequence analysis of JAK2 gene, but none of them has V617F mutation.

Introduction

Polycythemia vera (PV) is defined by autonomous proliferation of erythroid lineage cells with maturation to erythrocytes in the absence of underlying disease to cause erythrocytosis, and classified as one of the myeloproliferative neoplasms (MPNs) in 2008 WHO classification[16]. Human PV is considered to be a disease originated from an abnormal haematopoietic stem cell, and about 60-100% of patients show clonality by X-chromosome inactivation pattern (XCIP) analysis in their peripheral blood leukocyte[49-51]. These reports also revealed the absence of clonality in T-lymphocyte population irrespective of the clonal nature of the myeloid, megakaryocytic and erythroid cell populations[49,51,53]. JAK2 V617F gene mutation has been observed in the peripheral blood in 65-97% of human PV patients [2,4,5,70-72]. Therefore, the JAK2 mutation is considered to be responsible for the development of PV and is a key to the diagnosis of PV in humans[16-18].

PV is also defined as a disease characterised by autonomous erythropoiesis without any underlying disease in dogs[19-29]; however, unlike human PV patients, leukocytosis or thrombocytosis in conjunction with erythrocytosis is uncommon. Pathophysiology of canine PV has not been well understood, resulting in the difficulty in its diagnosis. Recently, JAK2 gene mutation corresponding to V617F mutation was reported in a canine case with PV; however, it was detected in only 1 of the 5 cases examined[19].

X-chromosome inactivation pattern (XCIP) analysis is a molecular biological technique to assess cell clonality in various cell populations by

discrimination of 2 X-chromosomes with different DNA methylation pattern in female individuals[12]. Mammalian females have two X-chromosomes in their somatic cells and either of them is inactivated by DNA methylation. Because this event occurs randomly, the number of inactivated paternal and maternal X-chromosomes is almost equal in nonneoplastic tissues; however, the methylation balance is apparently different between the 2 X-chromosomes in neoplastic tissues, because methylation status are stably inherited to daughter cells. In Chapter 1 in this thesis, availability of canine XCIP analysis using androgen receptor (AR) gene[15] was improved by incorporation of the examination of SLIT and NTRK-like family, member 4 (SLITRK4) gene, resulting in that more than 80% of females could be applied to this assay. XCIP analysis was shown to be useful to indicate the clonal origin of neoplasms including haematopoietic tumours such as polycythemia vera, myelodysplastic syndrome and essential thrombocythemia in humans[2,4,5,52,70-72].

In this study, the improved canine XCIP analysis was applied to peripheral blood, bone marrow and T-lymphocyte samples derived from dogs clinically diagnosed with PV to assess its clonal origin. JAK2 mutation correspondent to its V617F mutation was also explored in these dogs clinically diagnosed with PV.

Materials and Methods

Cases

Eight female dogs were diagnosed as PV from the results of haematological examination indicating persistent and progressive erythrocytosis (PCV > 65%) and exclusion of the underlying diseases causing erytrhocytosis such as dehydration, cardio-vascular and respiratory disorders to induce hypoxia, and erythropoietin-producing tumours.

Values in complete blood cell count (CBC) including red blood cell (RBC) count, packed cell volume (PCV) value, haemoglobin (Hb) concentration, white blood cell (WBC) count, platelet count, and erythropoietin concentration at the day of diagnosis were recorded. Reticulocyte count was also recorded when the data were available (Table 2-1).

DNA extraction from peripheral blood or bone marrow

Peripheral blood samples were obtained via jugular vein or venous in the extremities by venipunctures, and bone marrow samples were obtained from humerus or femur by using Jamshidi's bone marrow biopsy needles under general anaesthesia. Genomic DNA samples were isolated from peripheral blood or bone marrow by using DNeasy Blood and Tissue Mini Kit (QIAGEN, Hilden, Germany). Concentrations of the obtained DNA samples were measured by a spectrophotometer.

Separation of CD3-positive T-lymphocyte

Dog anti-CD3 antibody (clone CA17, 2A12, Abd Serotec, Kidlington, UK) was conjugated with EasySep mouse IgG antibody (STEMCELL, Tokyo, Japan), and obtained mixture was then applied to peripheral blood sample to react to T-lymphocytes. Magnet beads were then applied to reacted sample to conjugate to EasySep mouse IgG antibody and conjugated sample was collected by Dynamag magnets. Genomic DNA were isolated as described above.

XCIP analysis

XCIP analysis was performed as described in Chapter 1 of this thesis. In brief, DNA samples (500 ng) were incubated with or without 5 units of a methylation-sensitive endonuclease, *Hpa II* (New England Biolabs Inc., Ipswich, MA, UDS) in a total volume of 50 µl. Both of the digested and undigested samples were incubated at 37 °C for 16 hr followed by inactivation at 80 °C for 20 min.

Fifty ng of both genomic DNA was subjected to PCR amplification for *AC-1*, *AC-2* in SLITRK4 gene and *CAG-1* and *CAG-2* in AR gene and the resulting PCR products were analysed by capillary electrophoresis using the Genetic Analyzer 3130xl and evaluated by PeakScanner Software (Applied Biosystems). Inactivated allele ratio (IAR) was calculated from AUC of each chromosome with or without *Hpa II* digestion using the following formula.

$$\text{IAR} = \frac{\text{[digested allele 1 – (shadow bands)] / [digested allele 2]}}{\text{[undigested allele 1 – (shadow bands)] / [undigested allele 2]}}$$

In the analysis in AR and SLITRK4 genes, shadow bands generated by slippage synthesis of short tandem repeats are observed as minor peaks 2 base pairs in SLITRK4 gene and 3 base pairs in AR gene shorter than real peaks[15,57]. When the size of 2 alleles at *AC-1* was differed by 1- or 2-repeat length, 18% or 1.9% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. In the same way, when the difference of each allele was 1-, 2- or 3-repeat length at *AC-2*, 43%, 13% or 4.7% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. When the difference was 1 repeat length in *CAG-1* and *CAG-2*, 20% of the AUC of the longer allele was subtracted from that of shorter allele.

IAR was inversed when it was lower than 1.0 and defined as corrected IAR (CIAR). CIAR higher than 4.0 was judged to show skewed XCIP.

Examination of JAK2 V617F mutation

Peripheral blood or bone marrow samples were used to examine JAK2 gene mutation correspondent to V617F mutation detected in human patients[2,19]. Corresponding valine residue was also predicted to locate in the 617th amino acid in canine JAK2 gene. Mutation analysis was performed based on the previous report[19] with some modifications. Each reaction mixtures for PCR

included 50 ng of genomic DNA and 0.2 μM of primer pair ; JAK-F: 5'-ATGCATTTCTCTTACAGCAGACAGTATC-3' and JAK-R: 5'-GTTTGGGCATTTTAACATATAGCATATT-3', 1× GC enhancer and 1× Amplitaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 15 μl. PCR cycle conditions consisted of an initial denaturation step at 95 °C for 7 min; followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min; and the final extension at 72 °C for 7 min. The PCR products were purified by using a DNA purification kit (SUPREC PCR; Takara Bio Inc., Otsu, Japan). Sequencing analysis of the purified PCR products was carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and primers JAK-F and JAK-R.

Results

Cases and clinical data

Eight female dogs with marked progressive erythrocytosis were included in this study. Breeds of the 8 dogs were Pembroke Welsh Corgi (n=3), Shiba Inu (n=2), Chihuahua (n=2), French Bulldog, Miniature Dachshund and Yorkshire terrier (each 1). The ages of these dogs ranged from 5 years to 13 years (median, 11 years).

Serum erythropoietin concentrations were normal to low in all 8 cases (< 0.6-15.0 mU/ml; mean, 3.2 mU/ml) (reference range, 2.8-17.2 mU/ml).

Values of CBC were summerised in Table 2-1. CBC values at the time of diagnosis were as follows; PCV, 61-88% (mean, 72%); Hb, 20.7-28.8 g/dl (mean, 24.5 g/dl); RBC, 9.77-16.06x 10^6 /μl (mean, 11.73 x 10^6 /μl); WBC, 5,600-26,900 /μl (mean, 13,900 /μl); platelet, 100,000-891,000 /μl (mean, 405,000 /μl) (Table 2-1). Reticulocyte counts were recorded in 5 cases; 32-177 x 10^3 /μl (mean, 153 x 10^3 /μl).

Case 5 had been previously diagnosed as PV 2 years before the first admission to our hospital. This cases was referred to our hospital for controlling chronic renal failure; however, died 1 month after initial consultation. Chronic renal failure was also observed in Case 3 during disease course. This case died 12 months after diagnosis of PV. Case 4 was diagnosed as PV during the observation period for protein losing enteropathy, and eventually developed alimentary lymphoma and died 12 months after diagnosis

of PV. Other 5 cases were alive at the time of data collection.

All cases were subjected to phlebotomy with intervals of about 2 weeks to 1 month for the control of PV.

XCIP analysis for the peripheral blood, CD3-positive T-lymphocyte and bone marrow

In 2 of the 8 cases (Cases 7 and 8), the 2 X-chromosomes could not be distinguished by the difference of microsatellite repeat length at any loci of *CAG-1* and *CAG-2* in AR gene and *AC-1* and *AC-2* in SLITRK4 gene. Thus, six female dogs clinically diagnosed with PV and having heterozygosity at least 1 of the 4 loci for the XCIP analysis in this study (Cases 1-6) could be evaluated for the cell clonality by XCIP analysis (Table 2-1).

In Cases 1 and 2 clinically diagnosed with PV, remarkably skewed XCIP (CIARs > 10.0) was observed in their bone marrow DNA samples. Since the cytology of the bone marrow smears in these cases revealed the predominant erythroid progenitor cells admixed with less numbers of myeloid and megakaryocytic cells, the result indicated the presence of the clonally expanded erythroid progenitor cells in the bone marrow. Moreover, high CIARs more than 10.0 were also found in the peripheral blood DNA samples of these 2 cases. The peripheral blood DNA was primarily derived from leukocytes; therefore, the data indicated that the leukocytes in these 2 cases also had skewed XCIP.

In Cases 3, 4 and 5, bone marrow samples were not obtained; however,

peripheral blood NDA samples could be subjected to XCIP analysis, resulting in skewed XCIP (CIARs > 4.0). The results also indicated the presence of clonality in the peripheral leukocyte population in these 3 cases.

In Case 6 who was also diagnosed with PV, CIARs of the peripheral blood and bone marrow samples were 1.93 and 2.34 (less than 4.0), respectively. The data did not indicate the presence of clonally expanded cell population in this case.

In Cases 1 and 3 who showed skewed XCIP in the peripheral blood (and bone marrow, Case 1), XCIP analysis could be performed for T-lymphocyte populations separated from their peripheral blood. CIARs in the T-lymphocyte populations from Cases 1 and 3 were 1.91 and 1.77 (less than 4.0), respectively, indicating that there was no clonally expanded cell population in the T-lymphocyte fractions in these cases suffered from PV (Table 2-1 and Figures 2-1).

Examination of JAK2 gene mutation

All of the 8 cases clinically diagnosed with PV were analysed for JAK2 gene mutation at V617F. None of the 8 cases showed gene mutation corresponding to V617F and its surroundings region in exon 13 of canine JAK2 gene (NC_006583.3, nt; 1789-1876) (Fig. 2-2).

Discussion

In this study, leukocytosis and thrombocytosis in conjunction with erythrocytosis were observed in 1 and 2 dogs, respectively. Human PV is haematologically characterised by marked erythrocytosis, while leukocytosis and thrombocytosis were observed in 37% and 72% of the patients, respectively[73]. Marked erythrocytosis is a characteristic haematological change in canine PV; however, leukocytosis and/or thrombocytosis are reported in only few cases[19-29]. Since the frequency of leukocytosis or thrombocytosis seems to be different between human PV and canine PV, differentiation level of the abnormal clone that acquired genetic change causing autonomous proliferation might differ between the 2 species.

Six of the 8 dogs clinically diagnosed with PV were shown to be heterozygous at least 1 locus and could be subjected to XCIP analysis. Skewed XCIP were detected in 5 out of 6 peripheral blood (83%) and 2 of 3 bone marrow (67%) samples examined. Because nucleated cells in the peripheral blood are almost exclusively leukocyte, the result indicating the skewed XCIP in peripheral blood means the presence of unbalanced cell population in the blood leukocyte population. Some human PV patients possess clonal population in their peripheral bloods and one study revealed the different teromerase activities between clonal and polyclonal patients[50]. Although PV is a kind of MPNs with characteristic erythrocytosis, some genetic change responsible for the autonomous proliferation of erythrocytes may be shared by leukocytes (mainly neutrophils). Thus, demonstration of skewed XCIP in peripheral blood leukocytes would support the diagnoses of PV in dogs having

haematological characteristics consistent with PV.

In this study, all of the 3 T-lymphocyte samples separated from peripheral blood did not show skewed XCIP (CIAR > 4.0) although 2 of them showed CIARs > 4.0 in peripheral blood samples (Cases 1 and 3). These results indicate that canine PV is caused by aberrant progenitor cells which have already committed to myeloid/erythroid lineage, rather than the abnormal pluripotent haematopoietic stem cells which also have a differentiation ability to T-lymphocytes.

One of the 6 cases, Case 6, showed CIAR < 4.0 in both of the peripheral blood and bone marrow samples, although this cases showed progressive erythrocytosis without elevation of serum erythropoietin concentration. Absence of the skewed XCIP in this case might be explained by 1) abnormal hyper- or hypomethylation occurred in aberrant clone[59], 2) other disease causing erythrocytosis without elevation of serum erythropoietin concentration such as primary familial congenital erythrocytosis in human medicine[74], or 3) a small proportion of aberrant clones in analysed samples. False-negative result would be obtained when more than 40% of nonneoplastic cells are included in the sample. Since low percentage of aberrant clone have been reported in a number of human PV patients[73,75], low rate of aberrant clone in the sample population might be the most likely possibility.

In this study, none of the 8 dogs with progressive erythrocytosis possessed the mutation corresponding to V617F mutation. JAK2 V617F gene mutation causes continuous activation of downstream signalling pathways such as signal transducer and activator of transcription 5 (Stat5)[3]. Several reports described

that mouse models with JAK2 V617F gene mutation show erythrocytosis with or without thrombocytosis and leukocytosis, and constitutive phosphorylation of Stat5[76-78]. Since about 90% of PV patients had JAK2 V617F mutation in their peripheral blood leukocyte, this mutation was incorporated to 2008 WHO diagnostic criteria of PV[16]. On the other hand, only 1 of the 5 dogs with PV was identified to have JAK2 V617F mutation in a previous report[19], and none of the 8 dogs suffered from PV was shown to have the JAK2 mutation. Considering these results, JAK2 mutation does not seem to be a key molecular event to develop PV in dogs.

In humans, 10 of 11 PV patients without JAK2 V617F gene mutation were shown to possess some mutations in another exon in JAK2 gene; exon 12 [79]. In the same study, these mutations in JAK2 exon 12 were not observed in 55 PV patients with JAK2 V617F mutation, nor in 25 essential thrombocythemia patients or 12 idiopathic myelofibrosis patients both without JAK2 V617F mutation. Although JAK2 exon 12 mutation is a rare feature in human, it might be a candidate to explain the molecular event in canine PV. Future study would be necessary to seek the causative gene mutation in canine PV.

Conclusion

In this chapter, I have indicated that canine PV is developed as a result of the clonal expansion of an abnormal myeloid/erythroid progenitor cell. XCIP analysis of the peripheral blood and bone marrow would be a supportive tool for the diagnosis of PV in dogs. Haematological abnormalities of PV are

different between dogs sand humans, and unlike human PV, JAK2 V617F mutation was a rare finding in dogs with PV.

Table 2-1 Haematological data of 8 PV cases.

Case No.	Breed	Age (years old)	PCV (%)	RBC (10^6/μl)	Hb (g/dl)	Reticulocyte (10^3/μl)	WBC (10^3/μl)	Platelet (10^3/μl)	Erythropoietin concentration (mU/ml)	CIAR calculated by XCIP analysis		
										Peripheral blood	Bone marrow	T-lymphocyte
1	Shiba Inu	5	88	16.06	28.6	177	10.8	184	2.1	> 10	> 10	1.91
2	Chihuahua	12	65	-	-	-	12.3	866	< 0.6	> 10	> 10	NT
3	Pembroke Welsh Corgi	11	70	10.71	23.1	154.2	26.9	891	1.5	5.26	NT	1.77
4	Yorkshire Terrier	13	68	10.98	24.6	-	13.0	266	15.0	5.59	NT	NT
5	Pembroke Welsh Corgi	12	61	10.85	20.7	111.8	16.6	244	3.2	4.05	NT	NT
6	Chihuahua	8	64	9.77	21.2	195	5.60	374	1.9	1.93	2.34	1.38
7	Shiba Inu	9	76	12.0	21.9	32	8.70	100	< 0.6	-	-	-
8	Pembroke Welsh Corgi	11	83	12.0	28.8	128	11.9	317	0.9	-	-	-
	Reference range		37-61	5.65-8.87	13.1-20.5	10.0-110.0	5.0-16.7	148-484	2.8-17.2			

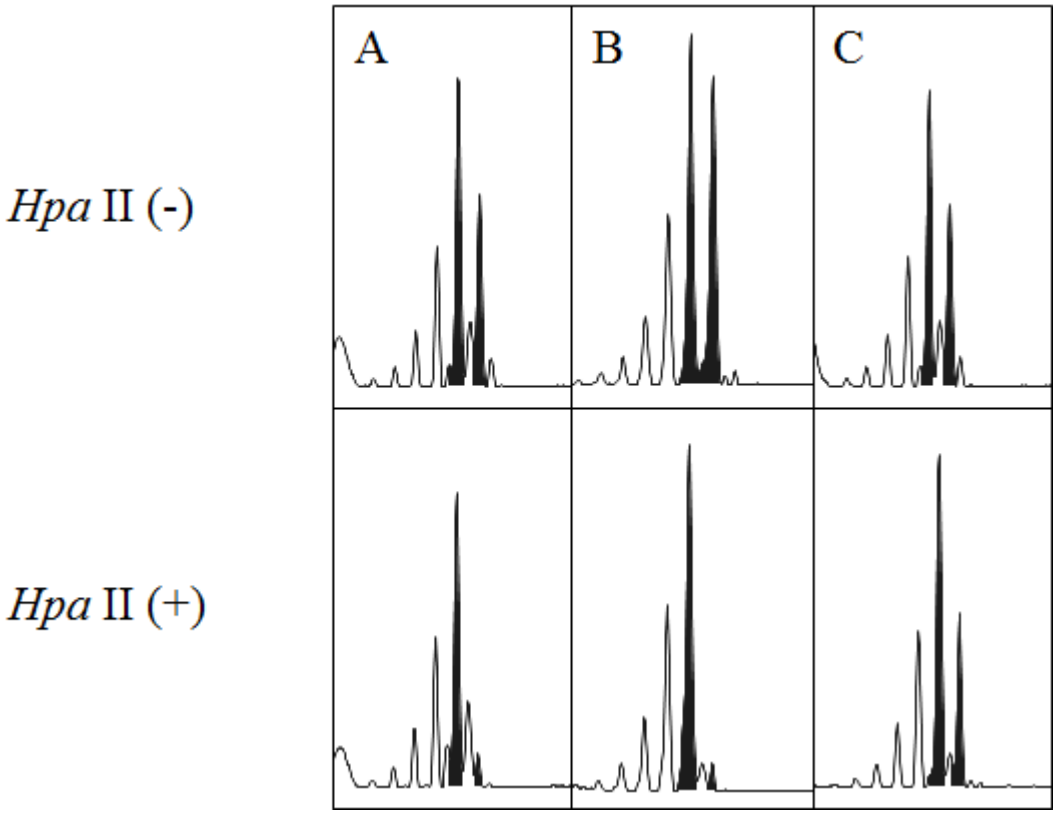


Figure 2-1. Representative results of XCIP analysis for a case with PV (Case 1).

Upper capillary electrophoretic patterns show the results of XCIP analysis without *Hpa* II digestion and the lower patterns show the results of XCIP analysis with *Hpa* II digestion. **A-C**. XCIP analysis at *AC-2* locus in SLITRK4 gene in Case 1.Peripheral blood (**A**) and bone marrow (**B**) show skewed XCIP: longer allele (right filled peak) was almost depleted after digestion with *Hpa II* (CIAR > 10 in both samples). T-lymphocyte population (**C**) shows balanced XCIP (CIAR = 1.27)

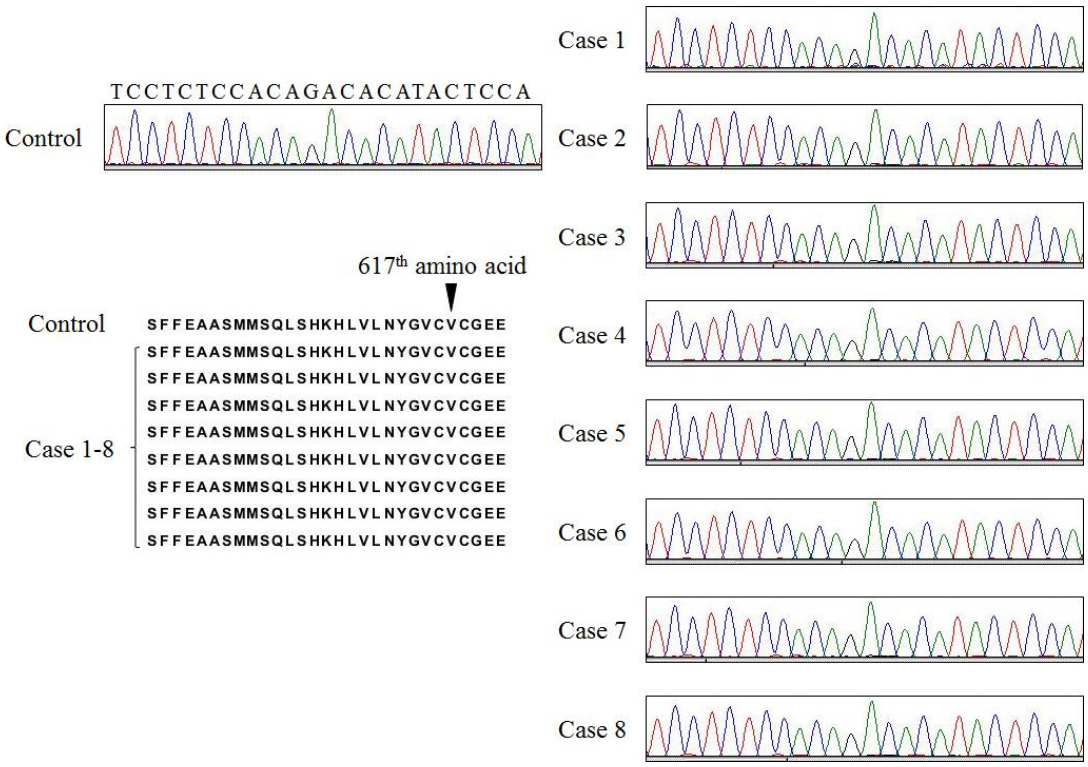


Figure 2-2. Results of sequence analysis of JAK2 gene in 8 dogs with PV.

None of 8 cases showed gene mutation at V617F in JAK2 gene (left) and also showed any other mutations around 617th amino acid (upper-left and right).

Chapter 3

Detection of clonally expanded blood and bone marrow cells in dogs with myelodysplastic syndrome by X-chromosome inactivation pattern analysis

Abstract

Myelodysplastic syndromes (MDS) are characterised by ineffective haematopoiesis and dysplasia of blood cells and their progenitors. In human medicine, 3 to 65% of MDS patients progress to acute myelogenous leukaemia (AML), suggesting that MDS is a kind of preleukaemic state. MDS in humans is considered to be derived from aberrant haematopoietic stem cells resulting in clonal cell population in bone marrow and/or peripheral blood. On the other hand, although several cases diagnosed with MDS have been reported in dogs and clinicopathological characteristics including the rate of progression to AML are not well understood in dogs. In this study, I evaluated the haematological features in 15 dogs suspected to be affected with MDS, and explored the existence of the clonally expanded cells in peripheral blood and/or bone marrow by X-chromosome inactivation pattern (XCIP) analysis. The most common clinicopathological finding was nonregenerative anaemia (12/15, 80%) and bicytopenia and pancytopenia were observed in 4 and 1 of the 15 cases, respectively. Two cases with nonregenerative anaemia in conjunction with marked thrombocytosis were diagnosed as myelodysplastic/myeloproliferative neoplasm (MDS/MPN). Frequently observed morphological changes indicating dysplastic features included nuclear separation of megakaryocyte (n=5) and megaloblastic change in erythroid cells (n=3). Twelve of the 15 cases with haematological characteristics indicating MDS were subjected to XCIP analysis by examining the androgen receptor (AR) gene and SLIT and NTRK-like family, member 4 (SLITRK4) gene, resulting in the demonstration of the clonally expanded cell

population in 6 of the 12 dogs. In 4 cases, T-lymphocytes populations separated from peripheral blood were subjected to XCIP analysis. Two of the 4 dogs also showed skewed XCIP in the T-lymphocyte populations. The present study revealed the presence of clonally expanded blood cell population in a proportion of dogs haematologically indicated as MDS; however, the proportion of clonal cells and/or the differentiation level of the clonal cell expansion seem to be variable among the dog patients.

Introduction

Myelodysplastic syndromes (MDS) are characterised by ineffective haematopoiesis caused by abnormal haematopoietic stem cell clone. The aberrant stem cells produce bone marrow cells with various morphological characteristics indicating dysplasia such as nuclear separation of megakaryocyte, megaloblastic change of erythroid cells or nuclear hyposegmentation of myeloid cells[30,31,80]. Peripheral blood cytopenia is a common finding in MDS patients. Since progression to acute myelogenous leukaemia (AML) is frequently observed, MDS is also known as a kind of pre-leukaemic condition[30,34,80-82].

In 1982, French-American-British (FAB) classification system was proposed by assessing morphological examination and clinicopathological characteristics. In this classification scheme, MDS were subdivided into 5 groups including refractory anaemia (RA), refractory anaemia with ringed sideroblasts (RARS), refractory anaemia with excess blasts (RAEB), refractory anaemia with excess blasts in transformation (RAEB-t) and chronic myelomonocytic leukaemia (CMML). Recently, haematopoietic neoplasms were classified by WHO classification in MDS, myeloproliferative neoplasms (MPN), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and AML[30,80]. In the WHO classification system, MDS are further classified into 7 subgroups, and CMML is classified as on subtype of MDS/MPN. Molecular biological features such as isolated del (5q) chromosome abnormality are included in diagnostic criteria in WHO classification[30], and X-chromosome

inacitivation pattern (XCIP) analysis or other clonal assessment techniques are also used to aid diagnosis[83]; however, any common molecular biological abnormalities in various MDS patients have not been detected[35].

In the veterinary field, several MDS cases have been reported in FeLV-positive cats[84-87]. Clonality of the blood cells was also shown in feline MDS using the integration site of FeLV[85] or X-chromosome inactivation pattern (XCIP) analysis[13], and 4 of the 14 cats with MDS were reported to progress to AML[86]. Meanwhile, MDS in dogs have been diagnosed in only a few reports[37-44] and in these studies MDS were diagnosed by the morphological assessment of bone marrow and peripheral blood as well as the exclusion of other diseases causing cytopenias. No uniform diagnostic criteria or classification systems have been defined in canine MDS so far[37-44]. Although clonal assessment of the bone marrow and peripheral blood cells might be a useful aid for the diagnosis of MDS, analysis to indicate the cell clonality has not been carried out for canine MDS. XCIP analysis is a molecular biological method to detect cell clonalities using female 2 X-chromosomes[15]. In Chapter 1 of this thesis, I revealed that canine XCIP analysis could be applied to about 83% of females. As its clinical application, I evaluated the clinicopathological findings in canine MDS and explored the clonality of blood and bone marrow cells by using the improved XCIP analysis in a study of this chapter.

Materials and Methods

Dogs diagnosed with myelodysplastic syndromes (MDS)

MDS were diagnosed fundamentally based upon the criteria described in the WHO classification system (2008) for the tumours of haematopoietic and lymphoid tissues[30]: cytopenia of 1 or more lineage, dysplastic features of blood cells, exclusion of other haematopoietic disorders. MDS/MPN were also diagnosed based on the same classification system: MDS plus increase of other lineage cell[80].

Clinical data including complete blood count, bone marrow cellularity, morphology of the peripheral blood and bone marrow cells, treatments and outcomes were collected from medical records.

DNA extraction

Genomic DNA samples were extracted from peripheral blood and bone marrow by using QIAamp Blood and Tissue Mini Kit (QIAGEN, Hilden, Gerimany). Concentrations of the obtained DNA samples were calculated by a spectrophotometer.

Separation of CD3-positive T-lymphocyte

Dog anti-CD3 antibody (clone CA17, 2A12, Abd Serotec, Kidlington, UK) was conjugated with EasySep mouse IgG antibody (STEMCELL, Tokyo, Japan), and obtained mixture was then applied to peripheral blood sample to react to T-lymphocytes. Magnet beads were then applied to reacted sample to conjugate to EasySep mouse IgG antibody and conjugated sample was collected by Dynamag magnets. Genomic DNA were isolated as described above.

XCIP analysis for peripheral blood, T-lymphocyte and bone marrow

XCIP analysis was performed as described in Chapters 1 and 2. Shortly, DNA samples (500 ng) were incubated at 37 °C for 16 hr with or without 5 units of a methylation-sensitive endonuclease, *Hpa II* (New England Biolabs Inc., Ipswich, MA, USA) and followed by inactivation at 80 °C for 20 min.

Fifty ng of both *Hpa II* digested and undigested genomic DNA was subjected to PCR amplification for *AC-1* and *AC-2* in SLITRK4 gene and *CAG-1* and *CAG-2* in AR gene. The resulting PCR products were analysed by capillary electrophoresis and evaluated by PeakScanner Software (Applied Biosystems). Inactivated Allele Ratio (IAR) was calculated from AUC of each chromosome with or without *Hpa II* digestion using the following formula.

$$\text{IAR} = \frac{[\text{digested allele 1} - (\text{shadow bands})]/\text{digested allele 2}}{[\text{undigested allele 1} - (\text{shadow bands})]/\text{undigested allele 2}}$$

Shadow bands generated by slippage synthesis of short tandem repeats are observed as minor peaks 2 base pairs in SLITRK4 gene and 3 base pairs in AR gene shorter than real peaks [15,57]. When the size of 2 alleles at *AC-1* was differed by 1- or 2-repeat length, and 1-, 2- or 3-repeat length at *AC-2*, 18% or 1.9% and 43%, 13% or 4.7% of the AUC of the longer allele was subtracted from that of shorter allele, respectively. When the difference was 1 repeat length in *CAG-1* and *CAG-2*, 20% of the AUC of the longer allele was subtracted from that of shorter allele.

IAR was inversed when it was lower than 1.0 and defined as corrected IAR (CIAR). CIAR higher than 4.0 was judged to show skewed XCIP.

Results

Case profiles

Fifteen female dogs with haematological findings indicating MDS were included in this study. Breeds of the 15 dogs were Miniature Dachshund (n=8), mixed breed (2), Labrador Retriever, Shiba Inu, Papillion, Shih Tzu, Toy Poodle and undetermined breed (each 1). Ages were 7 years 5 months to 14 years (median, 11 years) (Table 3-1).

Haematological findings

Cytopenia of various degrees were observed in the 15 cases. Of the 15 cases, anaemia, leukopenia and thrombocytopenia were noted in 12, 2 and 7 cases, respectively. Ten cases showed cytopenia in one blood cell lineage: anaemia in 8 cases and thrombocytopenia in 2 cases. Four cases showed bicytopenia: 3 were anaemia plus thrombocytopenia (Cases 4, 5 and 7) and other one case had leukopenia and thrombocytopenia (Case 11). One of 15 cases showed pancytopenia (Case 10). Two of 9 anaemic cases (Cases 2 and 3) showed concurrent marked thrombocytosis together with mild leukocytosis in one case (Case 2). These 2 cases were diagnosed as MDS/MPNs in WHO classification (2008). Clinical data were listed in Table 3-1.

Bone marrow examination

Bone marrow examination was performed in all 15 cases. The bone marrow was judged to be hyperplastic in 9 cases, normoplastic in 2 cases, and hypoplastic in 4 cases. Various dysplastic features were observed in all 15 cases. The most frequently detected abnormalities were nuclear separation of megakaryocyte (n=6), megaloblastoid change and abnormal karyokinesis of the erythroid cell lineage (each 3), abnormal karyokinesis, maturation arrest, peroxidase-negative neutrophils and ring-shaped nucleus of the myeloid cell lineage (each 2) (Table 3-2 and Figure 3-1)

Treatment and survival time

Most dogs were treated by oral administration of prednisolone, cyclosporine, mycophenolate mofetil or Vitamin K, and their combinations. Two cases having marked thrombocytosis (MDS/MPN) were further treated with hydroxyurea (Table 3-1). Overall survival times were 0.5 to 51 months when the day of diagnosis were set as day 1 (Table 3-1). Acute myelogenous leukaemia (AML-M6) was diagnosed at autopsy in Case 9 by the observation of proliferated blast cells in its bone marrow.

XCIP analysis for the peripheral blood, T-lymphocyte and bone marrow

Of the 15 dogs with haematological findings indicating MDS, 12 were found

to be heterozygous at least one of the 4 loci (*CAG-1* and *CAG-2* in AR gene and *AC-1* and *AC-2* in SLITRK4 gene) and could be examined by XCIP analysis; however, the other 3 dogs were homozygous at all of the 4 loci and could not be analysed by XCIP analysis.

From the 12 dogs, peripheral blood (n=11), bone marrow (n=9), and T-lymphocyte (n=4) samples were collected for the analysis by XCIP. Skewed XCIP (CIAR > 4.0) was frequently detected in the peripheral blood (4/11, 36.4%) and bone marrow (6/9, 66.7%). Four cases (Cases 1 to 4) showed skewed XCIP in both peripheral blood and bone marrow, and 2 of them (Cases 1 and 3) also showed skewed XCIP in T-lymphocyte population, while other 2 cases (Cases 2 and 4) did not show the skewed XCIP in T-lymphocyte population. Two cases were detected to have skewed XCIP in bone marrow (Cases 5 and 6), and latter case (Case 6) did not show skewed XCIP in peripheral blood. Six cases did not show skewed XCIP (CIAR > 4.0) in any of the samples analysed (Cases 7 to 12). A total of 8 cases were analysed in both peripheral blood and bone marrow samples, and 7 of them showed the same results with respect to the XCIP skewness; however, Case 6 showed different results between peripheral blood and bone marrow. This case was diagnosed with refractory anaemia with ring sideroblast whose dysplastic features were mainly detected in erythroid lineage cells. Two cases diagnosed with MDS/MPN (Cases 2 and 3) showed distinct skewness in both of the peripheral blood and bone marrow (Table 3-3).

Discussion

In this study, I evaluated the clinicopathological features and explored the presence of clonal cell population in 15 dogs diagnosed with MDS based upon the criteria described in the WHO classification in 2008. The most frequent clinicopathological feature was anaemia (n=12), while 7 dogs showed thrombocytopenia. Of note, 2 cases showed anaemia with concurrent marked thrombocytosis and were diagnosed as MDS/MPN (Cases 2 and 3). MDS/MPN is a rare disorder in the veterinary field, which has been reported only in one study[37] and should be differentiated from other myeloproliferative diseases such as chronic myelogenous leukaemia or essential thrombocythemia which usually show no to mild dysplasia in bone marrow and peripheral blood cells compared to MDS/MPN blood cells.

Of 15 MDS cases, 1 case progressed to AML (Case 9). Human MDS is considered as preleukaemic condition[30,34,80-82], and the progression rate to AML is 3-20% in low risk MDS and 44-65% in high risk MDS. Progression to AML is also reported in several canine MDS cases[37,43]; however, the rate of progression to AML in canine MDS is still unclear. As Case 9 poorly responded to the treatment, follow-up bone marrow aspiration might be important for poor-responsive cases.

In dogs with haematological findings consistent with MDS as well as skewed XCIP, the most frequently detected dysplastic features were nuclear separation of megakaryocyte (n=5) and megaloblastoid change of erythroid cells (n=3). One human study proposed to classify the dysplastic features into 2 categories,

features highly specific to MDS and those less specific[32] . In this proposal, micromegakaryocyte (megakaryocyte lineage), ring sideroblast (erythroid lineage) and hyposegmented mature neutrophils and degranulation neutrophils (myeloid lineage) were categorised as highly specific finding for the diagnosis of MDS. In our study, micromegakaryocyte and ring sideroblast were observed in 4 and 1 case(s), respectively. Of these 5 cases, 4 cases could be examined by XCIP analysis and all of them showed skewed XCIP. Morphologial changes highly associated with the diagnosis in human MDS would be helpful to propose the diagnostic criteria for canine MDS.

I could indicate cell clonalities in 4 of 11 peripheral blood samples (36.4%), 2 of the 4 T-lymphocyte samples (50.0%) and 6 of 9 bone marrow samples (66.7%) by using XCIP analysis. Of the 6 cases showing skewed XCIP in the peripheral blood and/or bone marrow, 4 cases showed consistent results of skewness in both samples, while Case 6 showed skewed XCIP in bone marrow and balanced XCIP in peripheral blood. This case was diagnosed with refractory anaemia with ring sideroblast whose dysplastic features were limited to erythroid cell lineage and most nucleated cells in peripheral blood were leukocyte rather than erythroid cells. Abnormal clone might be limited in erythroid lineage cells in this case, therefore, balanced XCIP was detected in peripheral blood.

Four of 6 skewed XCIP cases also analysed in T-lymphocyte population. Two of them showed balanced XCIP (Cases 2 and 4), while other 2 cases showed skewed XCIP also in T-lymphocyte. In human study, 18 of 19 MDS patients showed skewed XCIP in myeloid lineage, while only 8 of 19 patients showed

skewed XCIP in lymphoid lineage[88]. It is conceivable that the abnormal haematopoietic stem cells produce both clonal myeloid and lymphoid cells in some cases, while produce only myeloid cells in others. Alternatively, a reactive lymphoid cell clone might exist in some of the cases resulting in skewed XCIP of T-lymphoid cells[88].

Skewed XCIP could not be detected in 6 of 12 cases. It might be possible that the sensitivity of this assay was not enough. From the result of Chapter 1 of this thesis, the sensitivity of this analytical system is about 45% in solid tumours. Because the sensitivity of XCIP analysis depends on the proportion of clonal cells, sensitivity may be abated by contamination of inflammatory cells or normal connective tissues. Since incorporation of connective tissue is less possible in peripheral blood or bone marrow samples, sensitivity would be higher in haematopoietic tumours than solid tumours; however, the rate of abnormal clone might be less than 40%, which is the theoretical limit of this assay. In human medicine, karyotype analysis and genetic examination are used in conjunction with the haematological features to diagnose MDS[30,34,80,82,89]. Because there is no useful molecular information to help diagnosis in canine MDS, other disease might be misdiagnosed as MDS in these dogs.

In this study, maturation arrests of several bone marrow cells were observed in 4 of 6 balanced XCIP cases. Maturation arrest could also be observed in non-regenerative immune-mediated anaemia[90]. Therefore, it is possible that these dogs might have immune-mediated haematological diseases, although some of them also showed other dysplastic features, morphological differentiation between MDS and immune-mediated diseases might be difficult. In addition to

careful assessment of bone marrow and peripheral blood cell examination, molecular biological assay such as XCIP analysis would be necessary to exclude diseases such as immune-mediated anaemia, infection, auto-immune cytopenias or drug-administration.

Seven out of eight cases that were examined for both peripheral blood and bone marrow revealed high concordance rate between the two samples. Therefore, XCIP analysis might be used as a useful aid diagnostic tool to evaluate the existence of clonal population before performing bone marrow aspiration.

Conclusion

I showed the clinicopathological features in canine MDS cases, and also showed clonal population in some of them. These results indicated that at least some of the canine MDS cases possessed clonal population as well as human MDS. Further, MDS cases with clonal population showed lower haematocrit and higher leukocyte count compared to polyclonal cases. By selecting clonal cases using XCIP analysis, further study would be possible to establish MDS classification system to characterise canine MDS.

Table 3-1. Clinicopathological data in 15 dogs diagnosed with MDS

Case No.	Breed	Age (years old)	Ht (%)	RBC (10^6/μl)	Hb (g/dl)	WBC (10^3/μl)	Platelet (10^3/μl)	Treatment	Time to death (month)
1	Miniature Dachshund	12	17	2.45	5.7	22.6	214	P, M, V	10
2	Miniature Dachshund	12	25	1.84	4.4	33.6	1,085	P, HU	42
3	Miniature Dachshund	13	13	1.90	3.9	10.4	1,441	P, C, V, HU	5
4	Shiba Inu	10	14	1.86	4.2	15.7	106	P, C, V	21
5	Miniature Dachshund	13	9	-	-	13.5	8.00	P, M, V	½
6	Labrador Retriever	14	9	1.04	-	8.80	258	P, C, V	8+
7	Mixed breed	10	25	3.78	8.6	8.20	17	P, C, V	8
8	Miniature Dachshund	7	16	2.08	5.4	18.1	340	P, C, V	51
9	Miniature Dachshund	10	16	2.80	5.6	16.4	185	P, C, V	2
10	Toy Poodle	11	32	5,45	10.9	3.70	3	P, V	4
11	Miniature Dachshund	8	37	5.95	12.9	0.68	26	P, V	1+
12	Miniature Dachshund	11	52	8.05	18.1	6.40	27	P, C, V	30+
13	Miniature Dachshund	8	19	3.33	6.5	15.7	242	P, C, V	21
14	Shih Tzu	13	43	7.58	20.3	7.20	6.00	P, C, V	29
15	Papillion	12	22	2.03	5.0	8.40	294	P, C, V	4
Reference range			37-61	5.65-8.87	13.1-20.5	5.0-16.7	148-484		

P: Prednisolone, C: Cyclosporine A, V: Vitamin K, M: Mycophenolate mofetil, HU: Hydroxyurea

Table 3-2. Bone marrow features in 15 dogs diagnosed with MDS

Case No.	Cellularity	Dysplastic features		
		Erythroid lineage	Myeloid lineage	Megakaryocyte lineage
1	Hypoplastic	-	Giant myeloid cells	Micromegakaryocyte
2	Hyperplastic	-	Peroxydase-negative neutrophil	Micromegakaryocyte Nuclear separation
3	Hyperplastic	-	-	Nuclear separation Micromegakaryocyte
4	Normoplastic	Megaloblastic change Abnormal karyokinesis	Abnormal karyokinesis Hypersegmented neutrophils	Nuclear separation
5	Hyperplastic	Abnormal nuclear shape	-	-
6	Hyperplastic	Ring sideroblast	-	-
7	Normoplastic	-	Abnormal karyokinesis Maturation arrest	Abnormal nuclear shape Nuclear separation
8	Hypoplastic	Maturation arrest	-	-
9	Hypoplastic	Abnormal karyokinesis Megaloblastic change	-	Giant platelet
10	Hyperplasitc	Nuclear bridging	-	Maturation arrest Lack of platelet release
11	Hyperplastic	-	Maturation arrest	-
12	Hypoplastic	Abnormal karyokinesis Abnormal nuclear shape	Ringed nuclear of neutrophils	Mononuclear megakaryocyte Nuclear separation
13	Hyperplastic	Megaloblastic change Apoptosis	Maturation arrest	Anisokaryosis
14	Hyperplastic	-	-	Lack of platelet release
15	Hyperplastic	-	Ringed nuclear of neutrophils Peroxydase-negative neutrophil	Nuclear separation Micromegakaryocyte

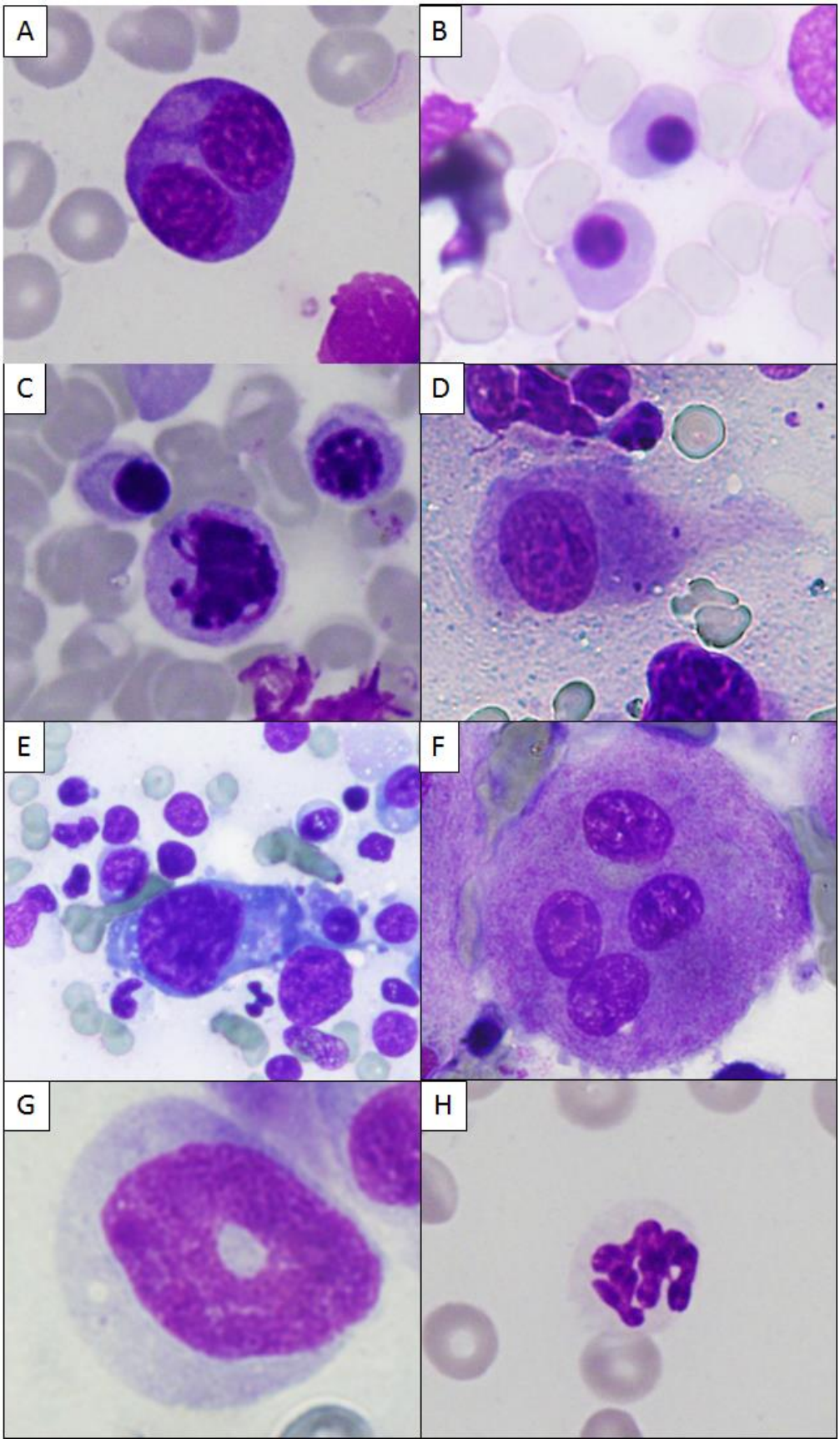
-: No corresponding morphological abnormality

Table 3-3. Results of XCIP analysis of canine MDS patients

	Peripheral blood	T-lymphocyte	Bone marrow
Case 1	4.60	9.95	4.34
Case 2	> 10	2.02	> 10
Case 3	> 10	4.20	6.93
Case 4	> 10	1.18	> 10
Case 5	NT	NT	> 10
Case 6	2.13	NT	5.22
Case 7	2.18	NT	2.54
Case 8	1.68	NT	1.63
Case 9	1.48	NT	2.18
Case 10	1.56	NT	NT
Case 11	1.84	NT	NT
Case 12	3.31	NT	NT
Case 13	-	-	-
Case 14	-	-	-
Case 15	-	-	-

NT: Not tested because the sample was not available.

-: XCIP analysis could not be applied because of the lack of heterozygosity at any loci of *AC-1* and *AC-2* in SLITRK4 gene and *CAG-1* and *CAG-2* in AR gene.


A
B
C
D
E
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H

Figure 3-1. Dysplastic features in bone marrow of the dog patients diagnosed with MDS

A. Abnormal karyokinesis observed in an erythroid lineage cell (Case 4). Binucleation was observed in the erythroid cell.

B. Megaloblastic change of erythroid lineage cells (Case 4). The differences of maturation status between nuclear and cytoplasm was indicated.

C. Abnormal karyokinesis of erythroid lineage cell (Case 12). Nuclei of erythroid lineage cells showed karyorrhexis.

D. Micromegakaryocyte (Case 3). Abnormally small megakaryocyte was observed.

E. Micromegakaryocyte (Case 2). Abnormally small megakaryocyte was observed.

F. Nuclear separation of megakaryocyte (case 12). Nuclei of a megakaryocyte are separated.

G. Ringed shape nucleus of a neutrophil (Case 12).

H. Hypersegmented nucleus of a neutrophil (Case 4).

Conclusion

In veterinary field, PV and MDS are generally diagnosed by exclusion of other diseases and careful assessment of morphological features in bone marrow and peripheral blood cells. At this moment, since there has been no known gene mutations associated with these 2 disorders, specific diagnostic markers are not available. Therefore, diagnostic accuracy depends on each clinician, and these facts make further research on these diseases difficult. In order to further understand about these diseases, assessment of the cell clonalities seemed to be useful. I tried to use XCIP analysis for detection of clonal cell populations in haematopoietic disorders derived from the expansion of abnormal cell clone.

In Chapter 1, I focused on the SLITRK4 gene[56] to be integrated into canine XCIP analysis, and could raise the rate of dogs that could be analysed by XCIP analysis to 83.3% (125/150) compared to 49.3% in a previous report[15]. This improved XCIP analysis system would be a useful tool for detecting clonal population in various cell/tissue samples, although attention should be paid to avoid false-negative and false-positive results.

Next, in Chapter 2, I evaluated cell clonalities in canine PV cases, and found out clonal population in 5 of 6 peripheral blood samples and 2 of 3 bone marrow samples, while all 3 cases analysed for T-lymphocyte showed balanced XCIP (CIAR < 4.0). These results indicates that canine PV arises by clonal haematopoiesis of an abnormal haematopoietic progenitor clone having a differentiation ability to both erythroid and myeloid lineage cells. Furthermore, the results obtained from peripheral blood (mainly leukocyte) were consistent with those from bone marrow in 3 PV cases, indicating that the

clonality analysis of peripheral blood seems to be useful for the diagnosis.

Several haematological differences were observed when compared to human PV[16,18]. The causative gene mutation in human PV, JAK2 V617F mutation[2,4,5], was not detected from any of 8 dog cases with PV. These results indicated the different disease nature between canine PV and human PV. Future study to investigating causative gene mutations in canine PV was strongly warranted.

Clonality and haematological features in canine MDS were evaluated in Chapter 3. XCIP analysis revealed their clonalities in 50% (6/12) of the cases in peripheral blood and/or bone marrow cells, and also showed the consistent results between peripheral blood and bone marrow in 7 of 8 cases examined. From results of T-lymphocyte population, MDS might be considered as complex disorders derived from abnormal haematopoietic stem cell clone or some differentiated progenitor clone.

Highly specific dysplastic features such as micromegakaryocyte or ring sideroblast proposed in human medicine[32] were detected only in cases of CIAR > 4.0. On the other hand, dogs diagnosed with MDS from the observation of maturation arrest only did not show high CIAR more than 4.0 in the blood and bone marrow samples. Latter results might indicate the possibilities of other diseases such as immune-mediated cytopenia[90]. Considering these results, further evaluation of cell morphology from clonal populations might lead to an opportunity to discuss classification of haematopoietic disorders in dogs.

Through this study, I could indicate the clonal cell populations in peripheral blood and bone marrow cells in canine PV and MDS by improved XCIP

analysis. I do believe that clonal assessment shown in this study must become a powerful tool for the diagnosis and better understanding of canine haematopoietic neoplasms.

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