Studies on the pathogenesis of myelodysplastic syndromes in the cat

(猫における骨髄異形成症候群の病態に関する研究)

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CONTENTS

General Introduction

Myelodysplastic syndromes (MDS) are characterized by peripheral bicytopenia or pancytopenia derived from dysplastic changes of hematopoietic cells in the bone marrow (45). Since, the bone marrow is usually nomocellular or hypercellular, peripheral cytopenia is caused by deficiencies of differentiation or maturation of hematopoietic cells in bone marrow (17). Furthermore, mild to moderate increases of blast cell counts are sometimes observed in these cases with MDS (9, 11, 44). But progression to acute myeloid leukemia (AML) has been observed frequently, MDS is recognized as a kind of preleukemic stage of AML. Since Jain *et al* proposed the concept and criteria of MDS as a preleukemic disorder in dogs and cats (45), a number of cases with MDS have been reported in veterinary medicine (9, 11, 45, 57, 72, 73, 77) . Furthermore, it was demonstrated that 39 (21.5%) of 181 cats with myeloproliferative disorders were classified into MDS (44), thus MDS is thought as one of the hematopoietic disorders which are frequently observed in cats. In human medicine, MDS is recognized as one of the important hematological disorders. The pathogenesis of MDS is considered as a process of multi-step tumorigenesis of leukemia (74), therefore the analysis of its pathogenesis is considered to provide a useful finding which reveals the mechanism of tumorigenesis in hematopoietic cells . Although, there have been many reports describing feline MDS (9, 11, 44, 45, 57, 72, 73, 77), its pathogenesis has not been documented in detail. Therapies for feline MDS have been usually ineffective (11, 44). To establish more effective therapies for MDS, analysis of the pathogenesis in MDS is required.

In veterinary medicine, hematological disorders similar to human MDS are frequently observed, especially in cats naturally infected with feline leukemia virus (FeLV) (11, 44). In humans, FAB co-operative group proposed a classification of MDS into 5 subtypes primarily based on the morphologic features and the blast cell counts in the bone marrow and peripheral blood: *i.e.* refractory anemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB in T) and chronic myelomonocytic leukemia (CMMoL)(10). Similar subtypes of MDS were found in cats with MDS (9, 77). Furthermore, it is recognized that FAB classification is a useful

system to predict the prognosis and to evaluate the stages in human patients (88). Therapies for MDS in humans have been chosen based on the subtypes of MDS in FAB classification (14). To obtain better therapeutic effect for feline MDS, assessment of the state of MDS as well as development of new therapeutic protocol will be required. In humans, a number of dysplastic changes have been described to diagnose MDS (10, 87), but it has not been defined what dysplastic changes are important for the diagnosis feline MDS. Furthermore, it has been reported that survival durations of MDS are influenced by a variety of hematological values as risk factors, hemoglobin concentration, platelet count, lactate dehydrogenase (LDH) value, and bone marrow blasts percentage.

Feline MDS are considered as a kind of pre-leukemic disorders, but the status of proliferation of hematopoietic cells has not been demonstrated yet. Because neoplastic disease usually has monoclonal cell growth, analysis of clonal composition of hematopoietic cell population is important for investigation of hematological disorders (42). In human medicine, Raskind *et al.* suggested that MDS was a clonal hematopoietic disorder originating from a pluripotent stem cell as shown by the G6PD isozyme analysis (74). It is recognized that the cytopenia and deficiency of maturation or differentiation of human MDS depend on clonal proliferation of hematopoietic cells in bone marrow. But several reports indicated that polyclonal hematopoiesis were found in some of the patients with MDS (32, 49, 55, 67), so there has been a controversy of the pathogenesis of MDS. To confirm the status of clonality in feline MDS should be required to undestand the pathogesis .

It has been shown that most of cats with MDS are infected with feline leukemia virus (FeLV) (7, 11, 44). FeLV is a type-C retrovirus horizontally transmitted among outbreed domestic cat populations in natural conditions (27, 65). Persistent infection of FeLV is associated with induction of various proliferative and degenerative diseases of the hematopoietic cells in cats (1, 11, 16, 39, 52, 58). Although it has been shown that most cats that developed MDS are infected with FeLV (11, 44), the characteristics of the viruses associated with MDS have not been investigated so far. FeLV variants which induce specific types of disease have been described, and several characteristic genetic

changes in the viral genomes responsible for their pathogenicity have been identified (6, 7, 21, 25, 27, 58, 65, 69, 75). The U3 region of retroviral long terminal repeat (LTR) designated U3 contains the transcriptional promoter and enhancer elements necessary for viral gene expression (6, 7, 27, 63). These regulatory sequences not only provide signals for the transcription of viral genes but also may provide signals in the appropriate target cell for the transcription of adjacent cellular proto-oncogenes. It has been indicated that FeLV proviruses derived from thymic lymphomas contain duplication of the enhancer sequences containing enhancer elements, and the repeated structure of the enhancer sequences may have a possible role for leukemogenesis (27, 58, 61). Furthermore, Nishigaki *et al.* indicated that FeLV LTRs derived from naturally occurring AML frequently contained direct repeats in the upstream region of the enhancer (URE) (66).

A series of the present studies were carried out to provide novel insights for understanding the clinical and hematological features, molecular basis for the pathogenesis, and characteristics of the etiological agent of MDS in cats. This thesis is composed of 3 chapters as follows. In chapter I, I investigated the hematological abnormalities and prognoses in 16 cats with MDS, and analyzed the significance of each dysplastic change. Furthermore, human FAB classification system and Dusseldorf scoring system were employed to evaluate the prognoses of 16 feline MDS cases. In Chapter II, I investigated the clonalities of the hematopoietic cells in cats with various hematological disorders including MDS, AML and pure red cell aplasia (PRCA). In Chapter III, I analyzed structure of LTRs of FeLV derived from cats with MDS.

(Chapter I)

Hematological abnormalites and prognoses in 16 cats with myelodysplastic syndromes

Abstract

I investigated the hematological abnormalities and prognoses in 16 cats with myelodysplastic syndromes (MDS). Non-regenerative anemia and thrombocytopenia were seen in most of the cats with MDS, but neutropenia was less common in these cats. Megaloblastoid rubricytes, hyposegmentation of neutrophils, nuclear abnormality of rubricytes and neutrophils, and micromegakaryocytes were frequently seen in the cats with MDS. Furthermore, the 16 cats with MDS were subclassified into refractory anemia (RA) (8 cats), RA with excess of blasts (RAEB) (5 cats), RAEB in transformation (RAEB in T) (1 cat), and chronic myelomonocytic leukemia (CMMoL) (2 cats), according to the human French-American-British (FAB) classification. Half of the cases with RAEB, RAEB in T, and CMMoL developed acute myeloid leukemia (AML), however, only 1 of 8 cats with RA developed AML. Based on the Dusseldorf scoring system for the prognosis of human MDS, the survival durations of the cats showing high scores ($\geq$ 3 points) were significantly shorter than those of the cats with low scores ($<$ 3 points). These FAB classification and Dusseldorf scoring system were considered to be useful for predicting the prognosis of feline MDS. Furthermore, 15 of the 16 cats with MDS investigated in this study were found to be infected with feline leukemia virus, indicating its etiological role in the pathogenesis of feline MDS.

Introduction

Myelodysplastic syndromes (MDS) have been characterized by peripheral bicytopenia or pancytopenia derived from dysplastic changes of erythroid, myeloid, and megakaryocytic cells in the bone marrow. The bone marrow is usually normocellular or hypercellular. Mild to moderate increases of blast cell counts are sometimes observed in these cases with MDS, and the blast cell counts in bone marrow and peripheral blood do not exceed 30% and 5% of the bone marrow and peripheral blood nucleated cells, respectively, based on the French-American-British (FAB) classification system for the diagnosis of acute leukemia and MDS (45). Because progression to acute myeloid leukemia (AML) has been frequently observed in cats with MDS, MDS is considered as a kind of preleukemic state of AML (11, 44, 77). Occurrences of MDS have been reported in humans and dogs as well as in cats (9, 18, 35, 45, 57, 62, 73). Most of human MDS patients are old peoples (88), however, most of cats with MDS are young or middle-aged and are infected with feline leukemia virus (FeLV) (11, 44).

In humans, FAB co-operative group proposed a classification of MDS into 5 subtypes primarily based on the morphologic features and the blast cell counts in the bone marrow and peripheral blood: *i.e.* refractory anemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB in T) and chronic myelomonocytic leukemia (CMMoL) (10). RA and RARS with low blast cell counts are considered as low risk types, because the peripheral cytopenia is a chief feature and long survival durations have been observed in the patients with these two types. RAEB, RAEB in T and CMMoL with high blast cell counts are recognized as high risk types, because progressions to AML have been often observed in the patients with these types (88). Jain *et al* (1991,1993) (44, 45), proposed a classification system of acute myeloid leukemia in dogs and cats, and showed a criteria to diagnose MDS and MDS-Er (MDS with erythroid predominance). However, subclassification system of MDS has not been established in dogs and cats.

In humans, megaloblastoid rubricytes, ringed sideroblasts, hyposegmentation of neutrophils (pseudo-Pelger Huet anomaly), and micromegakaryocyte are widely accepted as significant and specific dysplastic changes of MDS (10, 87, 88) Many dysplastic changes indicating MDS have been reported in cats (9, 35, 57, 72, 73), however, it has not been defined what kind of dysplastic changes are frequent or diagnostic for feline MDS. Furthermore, the prognosis of MDS such as survival duration and rate of progression to AML has not been investigated in cats. In humans, it has been reported that survival durations of MDS are influenced by a variety of hematological values as risk factors, hemoglobin concentration, platelet count, lactate dehydrogenase (LDH) value, and bone marrow blasts percentage as indicated in Dusseldorf scoring system (8, 34).

Infection with FeLV is very common in the domestic cats in Japan (77), and a certain proportion of the cats infected with FeLV are diagnosed as MDS. From the clinical importance of MDS in small animal practice, the present study was carried out to provide useful informations for the accurate diagnosis and prediction of prognoses in cats with MDS. In this study, I investigated the hematological findings of the peripheral blood and bone marrow in 16 cats with MDS and subclassified the diseases by based on human FAB classification system. Furthermore, by using the Dusseldorf scoring system, the survival durations of the cats with MDS were compared.

Materials and methods

Cases

Sixteen cats referred to the Veterinary Medical Center, Faculty of Agriculture, the University of Tokyo, were diagnosed as MDS from the history and the findings by physical examination and hematological examination of the peripheral blood and bone marrow. Hematological abnormalities and prognoses of these 16 cats with MDS were analyzed.

Hematological examination

Counts of erythrocytes, leukocytes and platelets, and hemoglobin concentration were determined with an automated hemocytemeter (Sysmex Model CM-5) (Sysmex, Kobe, Japan). Peripheral blood smears stained with Wright and Giemsa solutions were used to examine the morphology of blood cells. Bone marrow specimens were collected by aspiration biopsy of the femur. At least 500 nucleated cells in the bone marrow smears stained with Wright-Giemsa were counted for preparing the myelograms in each case. In some cases, peroxidase and alpha-naphthyl butyrate esterase stainings were carried out to identify granulocytic and monocytic lineages, respectively. Iron staining by Berlin blue staining was applied for the bone marrow samples of 7 cats. Plasma lactate dehydrogenease (LDH) levels were measured with a serum chemistry examination apparatus (Fuji Drychem 5000) (Fuji Medical Film, Tokyo, Japan).

Virological examination

FeLV antigen (p27) and antibody against feline immunodeficiency virus in the plasma samples were examined with a commercial test kit (Snap FIV/FeLV combo) (IDEXX, Portland, ME).

Diagnostic criteria

The diagnosis of MDS was made by hematological examination of the peripheral blood and bone marrow. Criteria for the diagnosis MDS was substantially based upon the FAB classification for dogs and cats (10); *i. e.* the presence of bicytopenia or pancytopenia in the peripheral blood, dysplastic changes in two or more hematopoietic cell lineages in the bone marrow and peripheral blood, and blast cell counts less than 30% of all nucleated cells (ANC) or non-erythroid cells (NEC) in the bone marrow. In cases that the percentage of the nucleated erythroid cells in the bone marrow was less than 50% of ANC, the percentage of blast cell count was shown as the percentage of blasts in ANC. When the nucleated erythroid cells in the bone marrow were more than 50% of ANC, the percentage of blast cells was shown as the percentage of blasts in NEC.

Classification of the subtypes of MDS has not been reported in cats, therefore, it was based on the FAB classification for human acute leukemia and MDS (10). Based on the criteria for classification of MDS in humans, the cats diagnosed as MDS in this study were classified into RA, RARS, RAEB, RAEB in T, and CMMoL.

Evaluation of the prognoses

Survival duration was recorded as a period from the date of diagnosis of MDS to the date of death. In cases which developed unrelated fatal disease such as lymphoma, the end point was defined as the date of diagnosis of such disease. The 16 cats diagnosed as MDS were classified into two groups, MDS with low blast cell counts (RA and RARS) and MDS with high blast cell counts (RAEB, RAEB in T and CMMoL). A prognosis scoring system proposed in human MDS, Dusseldorf scoring system, (8) was also applied to the 16 cats with MDS in this study. In this systems, one point is allocated to each of the following parameters: hemoglobin concentration $\leq$9g/dl, platelet count $\leq 100 \times 10^3/\mu l$, LDH level >200IU, and bone marrow blasts $\geq$5%. The 16 cats with MDS in this study were divided into two groups, group A with 1 or 2 points and group B with 3 or 4 points in the Dusseldrof scoring system, to compare the survival durations. Survival curves were prepared by Kaplan-Meier method (48). Survival

durations in each group were analyzed by non-parametric test statistics (Log rank test). Differences in the rate of leukemic progression were tested by chi-square method. In these statistical analyses, the significance was defined as a P value <0.05.

Results

Cases

The 16 cats diagnosed as MDS in this study were 9 months to 14 years old, and many of them were young adult cats (median age, 1.5 years old) (Table 1). The cats examined in this study consisted of 10 males (including neutered 4 males cats) and 6 females (including 3 neutered females). Of these cats, 15 were Japanese domestic cats and 1 was Somali. The clinical signs in most of the cases at the referral were non-specific signs such as weakness and anorexia. A few cases had diarrhea, vomiting or signs of upper respiratory disease. Abnormal findings at the physical examination of these 16 cats with MDS included pale mucous membranes (13 cats, 81.5%), splenomegaly (8 cats, 50%), hepatomegaly (3 cats, 18.8%), lymphadenopathy (3 cats, 18.8%), pleural effusion (3 cats, 18.8%), and fever (2 cats, 12.5%) .

Complete blood cell counts

Moderate to marked anemia was seen in most of the cats with MDS (15/16, 93.5%) (Table 2). One cat that did not show anemia at the first admission also developed marked anemia at the terminal stage. Most of these cats did not show reticulocytosis in the peripheral blood irrespective of the profound anemia. Leukopenia and neutropenia were found in 5 cats (31.3%) and 4 cats (25%), respectively. Two cats with CMMoL had monocytosis (2825/μl and 2670/μl, respectively). Thrombocytopenia less than $300x10^3/\mu l$ was found in 13 cats of 16 cats (81.6%), and severe thrombocytopenia less than $100x10^3/\mu l$ was found in 9 of 16 cats (56.3%).

Morphological features of the blood cells

All of the cats with MDS in this study showed at least one of the morphological abnormalities indicating dyserythropiesis in the bone marrow and/or peripheral blood: *i.e.* megaloblastoid rubricytes (9 cats, 56.3%), circulating rubricytes without polychromasia (10 cats, 62.5%), macrocytosis (9 cats, 56.3%), abnormal nuclear shape such as unequal

division or multinuclei (9 cats, 62.5%), and nuclear fragmentation (9 cats, 62.5%) (Table 3, Figure 1). Of the 7 cats (Cases 2, 3, 5, 6, 7, 8, and 16) examined for iron staining in this study, only 1 cat (Case 12) had ringed sideroblasts in the bone marrow, but they did not exceed 15 % of ANC. Three cats (Cases 2, 5 and 6) had increased number of bone marrow sideroblasts.

All of the 16 cats with MDS also showed one or more morphological features of dysmyelopoiesis such as neutrophil hyposegmentation (pseudo-Pelger-Huet nuclear anomaly) (7 cats, 43.7%), neutrophil hypersegmentation (10 cats, 62.5%), nuclear abnormality such as ring-shaped nucleus or multinuclei (13 cats, 81.3%), cell giantism (8 cats, 56.3%), and abnormal cytoplastic granulation such as absence of granules or negative staining for peroxidase (6 cats, 37.5%) (Table 3, Figure 1). Auer rod, one of the important dysplastic changes in human MDS, was not found in any of the cases with MDS examined in this study.

Twelve of the 16 MDS cases also showed one or more findings indicating dysthrombopiesis : *i.e.* micromegakaryocytes (10 cats, 62.5%), large mononuclear megakaryocytes (10 cats, 62.5%) and large platelets (11 cats, 68.7%) (Table 3, Figure 1). In most of the cats with MDS, normally differentiated promegakaryocytes and/or megakaryocytes were very few in the bone marrow.

Diagnosis

In this study, the 16 cats were diagnosed as MDS based on the criteria in FAB classification: *i.e.* bicytopenia or pancytopenia in the peripheral blood, dysplastic changes in two or more cell lineages in the bone marrow and/or peripheral blood, and blast cell counts less than 30% of bone marrow cells (ANC or NEC) (Table 4) (10). Subtypes of MDS in these 16 cats were determined according to the criteria in FAB classification for human MDS. Of the 16 cats with MDS, 8 cats (Cases 1 - 8) were categorized into RA from the blast cell count in peripheral blood less than 1 % and that in bone marrow less than 5%. In iron staining examination for 7 cats (Cases 2, 3, 5, 6, 7, 8, and 16) in this study, since no case had ringed sideroblasts more than 15 % of ANC in the bone

marrow, there was no case diagnosed as RARS. Five cats (Cases 9 - 13) were categorized into RAEB based on the blast cell count less than 5% in the peripheral blood and that between 5% and 20% in the bone marrow. One cat (Case 14) was diagnosed as RAEB in T from the presence of blasts more than 20% of the bone marrow cells. Cases 15 and 16 were diagnosed as CMMoL based on the hematological findings such as the blast cell counts between 5% and 20% in the bone marrow and an absolute monocytosis more than 1000/μl in the peripheral blood.

Treatments and clinical outcomes

Several different treatments which have been used MDS patients in humans were employed for the control of diseases in the 16 cats with MDS in this study. Frequent whole blood transfusions were carried out in 15 of the 16 cats with MDS. When the cases showed moderate to marked anemia, improvement of clinical signs were found in most of the cases after transfusions, but repeated transfusions were required in most cases. Predonisolone (1 ~ 4 mg/kg, IM or PO, SID) was administered to all of the 16 cases, and it was often effective to improve the peripheral blood cytopenia especially in the early phase in the cases with RA. Low dose cytarabine therapy (cytarabine, 0.7 ~ 1.4mg/kg, SC, SID or cytarabine ocfosfate, 2 ~ 4mg/kg, PO, SID) in combination with predonisolone was employed for the treatment of 9 cases (Cases 1, 2, 4, 5, 8, 10, 12, 14 and 15). Then 2 of the 9 cases (Cases 1 and 10) with RA and RAEB, respectively, showed obvious clinical and hematological improvement after initiation of the therapy, however, other 7 cases did not show obvious improvement in the clinical and hematological features. Therapy with cyclosporin A (5mg/kg, PO, SID) in combination with predonisolone was used for 1 cat (Case 3) with RA, and induced marked improvement in the hematological values and clinical symptoms. But this case died of progression to AML 1 month after initiation of the therapy. Combination chemotherapy composed of daunorubicin (20mg/m^2, IV, 1 ~ 3days), cytarabine (100mg/m^2, IV or SC, 1 ~ 4days), vincristine (0.025mg/kg, IV, weekly), and predonisolone (1 ~ 4 mg/kg, IM or PO, SID) was employed for the treatment of 1 case

with RA (Case 8) and 2 cases with RAEB (Cases 11 and 13). In these cases, partial response by the chemotherapy was observed as shown by the decrease of blasts in the bone marrow, however, these cases died 1 week to 3 months after initiation of the chemotherapy because of the progression to AML or other reasons.

Of the 8 cases with RA, only 1 cat (Case 3) showed progression to AML (M0) 1 month after the diagnosis (Table 5). On the other hand, of the 6 cases with RAEB, RAEB in T, and CMMoL, 3 cases (Cases 11, 13 and 15) showed progression to AML (M2, M6, and monocytic leukemia, respectively) 1 week to 4 months after the diagnosis. An RA case (Case 8) developed RAEB 11 months after the diagnosis. An RA case (Case 4) and an RAEB case (Case 10) developed lymphoma 2 to 7 months after the diagnosis.

The survival durations of the 16 cats with MDS were 1 week to 73.5 months (median, 2.1 months). The survival durations in 8 cats with RA (low blast count group) were 10 days to 73.5 months (median, 4.7 months), whereas those in 8 cats with RAEB, RAEB in T or CMMoL (high blast count group) were 1 week to 6 months (median, 2 months), however, the survival durations were not significantly different ($p=0.06$) between these two groups (Figure 2). Progression to AML was observed in 1 of 8 (12.5%) cats with RA, and in 3 of 6 cats with RAEB, RAEB in T or CMMoL, but statistical analysis did not show significant difference ($P=0.35$) on the rate of leukemic progression between these two groups.

According to the Dusseldorf scoring system, the 16 cats with MDS were divided into 2 groups: *i.e.* group A (<3 points) and group B ($\geq$ 3 points). The survival durations in group A (7 cats, Cases 1, 4, 6, 7, 8, 10 and 15) were 10 days to 73.5 months (median, 7.2 months), whereas those in group B (9 cats, Cases 2, 3, 5, 9, 11, 12, 13, 14, and 16) were 1 week to 6 months (median 2.5 months). The survival duration of group A was significantly longer ($p=0.01$) than that of in group B (Figure 3).

Discussion

MDS is characterized by the cytopenias in the peripheral blood and the dysplastic changes of the hematopoietic cells. In this study, 4 of the 16 cats with MDS showed progression to AML 1 week to 5 months after the diagnosis. The leukemic progressions of MDS in cats have been also described in several reports (9, 35, 57, 72, 73). These findings are indicative the nature of MDS as a preleukemic state of AML.

Since most of the cats with MDS in this study as well as those in previous reports were found to be infected with FeLV, it has been that FeLV infection is associated with the development of MDS in cats (11, 14). FeLV transmitted among domestic cats are associated with a variety of neoplastic and non-neoplastic diseases (6, 7, 21, 25, 27, 58, 65, 69, 75). Some peculiar FeLV strains were shown to induce specific diseases such as immunodeficiency and pure red cell aplasia (1, 2, 40). Cats with naturally occurring thymic lymphoma were shown to be infected with FeLVs with repetition of the enhancer sequence in the long terminal repeats (LTRs) (27, 58, 61). Toth *et al* indicated that some of the kittens experimentally infected with FeLV-AB/GM-1 strain developed MDS and AML (82). We previously reported that LTRs of FeLVs from cats with AML frequently had unique deletion and tandem direct repeats in the upstream region of enhancer (URE) in the LTRs (66). In my preliminary data, the structures of FeLV LTRs from cats with MDS were found to be very similar to those from cats with AML (data not shown). These findings indicate that several types of FeLV-associated diseases including MDS are presumably caused by specific FeLVs to each type of the diseases in cats.

In hematological examination, the most common cytopenia in cats with MDS in this study was anemia, which was found in 15 of the 16 cats (93.8%). A previous report also showed that 20 of 21 (95.2%) cats with MDS had moderate to severe non-regenerative anemia (11). Thrombocytopenia was seen in 13 of 16 cats (81.3%) in this study, and in 14 of 21 (66.7%) cats with MDS in a previous report (11). Incidence of leukopenia in cats with MDS was 5 of 16 cats (31.3%) in this study and in 9 of 21 cats (42.9%) with MDS in a previous report (11). Therefore, in feline MDS, it is

conceivable that the order of the frequency of cytopenia is : first, anemia; second, thrombocytopenia; and third, leukopenia. In human medicine, Linman *et al* . indicated that first hematological and pathogenetic abnormalities were found in the erythroid cell lineage, second abnormalities were found in the megakaryocytic cell lineage and third abnormalities were found in the myeloid cell lineage, thus the early pathogenesis of MDS seems to occurr in the erythroid cells (53, 54). Therefore, the pattern of the cytopenia in feline MDS was considered to be similar to that in human MDS (8, 87, 88).

A variety of dysplastic changes showing dyserythopoiesis, dysmyelopoiesis, and dysthrombopoiesis were found in the 16 cats with MDS in this study. Previous reports documented that cats with MDS had various dysplastic changes such as megaloblastoid rubricytes, circulating rubricytes without polychromasia, macrocytosis, abnormal nuclear shape, nuclear fragmentation, and ringed sideroblasts in the erythroid cell lineage; abnormal cytoplasmic granulation, hypersegmentation, hyposegmentation, cell giantism, and monocytoid neutrophils in the myeloid cell lineage; and large mononuclear megakaryocytes, micromegakaryocytes, large platelets, multiple separate nuclei, abnormal cytoplasmic granularity, and platelet vacuolation in the megakaryocytic cell lineage (9, 11, 35, 57, 72, 73). Many of these morphological features indicating dyshematopoiesis were also found in the 16 cats with MDS in this study. However, ringed sideroblasts was observed only in 1 cat, and monocytoid neutrophils were observed in only 2 cats in this study. Multiple separate nuclei, abnormal cytoplasmic granularity and platelet vacuolation in the megakaryocytic lineage were not seen in any of the cats with MDS in this study. It was thought that these dysplastic changes might be uncommon findings in feline MDS. Although the neutrophil staining negative for peroxidase has not been reported in cats, so far, this finding was shown in 5 of the 16 cases in this study. Therefore it can be considered as one of the common dysplastic changes in feline MDS. In human MDS patients, megaloblastoid rubricytes, ringed sideroblasts, hyposegmentation of neutrophils, nuclear abnormality, and micromegakaryocyte are considered as important dysplastic changes to diagnose MDS (10, 87, 88). It is conceivable that these morphological abnormalities diagnostic for

human MDS are also important to diagnose MDS in cats. Moreover, in the present study, I showed the frequencies of a variety of dysplastic changes in cats with MDS, which will be useful for the diagnosis of feline MDS.

In the present study, the prognoses of 16 cats were relatively poor and the survival durations were short, however, the survival durations in some cats with MDS in this study were longer than those in cats with MDS in previous reports. Blue *et al*. reported that 18 of 21 cats with MDS were euthanized or died within one week after diagnosis, and only one cat survived for nine months (11). Other reports indicated that the survival durations in the cats with MDS were from a few days to several weeks (9, 35, 73). Reasons for the relatively long survival durations in this study might be due to the treatments with predonisolone, cyclosporin A, low dose cytarabine, or other several chemotherapeutic agents as well as frequent blood transfusion. The cats that survived for more than 1 year in this study were categorized into low blast count group (RA) or low point group in the Dusseldorf scoring system. In some cats with such low risk-type MDS, long time survival might be expected.

The rate of leukemic progression in low blast count group (RA) was lower than that in high blast count group (RAEB, RAEB in T, and CMMoL) in this study. The result of the statistical analysis indicated that the rate of leukemic progression was not significantly different between these two groups, however, the number of cats with known clinical outcomes were only 14 in this study. Further study for a large number of cases with MDS will be required. In veterinary medicine, the risk of MDS for leukemic progression has not been discussed in detail. In human medicine, it has been indicated that patients in the high blast count group show poorer prognosis and higher rate of leukemic progression in comparison with patients in the low blast count group (88). Diagnosis of the subtypes of MDS based on the FAB classification for human MDS is desired to be applied to the cat cases with MDS.

Dusseldorf scoring system is considered as a reliable system for risk assessment of MDS in human (8). In this system, among the patients belonging to different prognostic groups (group A, score 0; group B, score 1 or 2; group C, score 3 or 4),

significant difference of the survival durations has been observed: *i.e.* 2 years cumulative survival was 86% in group A, 57% in group B and 14% in group C. In the cats with MDS in this study, there was no cat categorized into a group with score 0, however, survival durations were significantly different between the group with score 1 or 2 and the group with score 3 or 4. Dusseldorf scoring system is conceivably a useful system to predict the prognosis of the cats with MDS.

Few reports have described chemotherapy for cats with MDS. In the present study, many of the cats with MDS were treated with low dose cytarabine therapy or aggressive combination chemotherapy. Two of the 3 cats treated with the combination chemotherapy achieved partial remission, however, they died within 4 months. Only 1 of 9 the cats treated with low dose cytarabine therapy achieved complete remission. Recent studies indicated that in human MDS patients both of the low dose chemotherapy and aggressive chemotherapy did not show good results to improve the clinical outcomes in the patients (14, 76). Therapies for MDS in humans have been chosen based on the subtypes of MDS in FAB classification (14). For the patients with RA or RARS, in conjunction with supportive therapy, administration of vitamin D3, granulocyte-colony stimulating factor (G-CSF), erythropoietin or cyclosporin A is used (14). For the patients with RAEB, RAEB in T, and CMMoL, low dose chemotherapy, aggressive chemotherapy, G-CSF, or bone marrow transplantation is chosen (14, 30, 76). To obtain better therapeutic effect for feline MDS, assessment of the disease stage of MDS as well as development of new therapeutic protocol will be required.

(Chapter II)

Clonality analysis of hematopoietic disorders in cats naturally infected with feline leukemia virus

Abstract

Clonality analysis of the bone marrow cells by detecting feline leukemia virus (FeLV) integration was applied to know the pathogenesis of hematological disorders in cats. Bone marrow cells from 4 cases with acute myeloid leukemia (AML), 9 cases with myelodysplastic syndromes (MDS), and 2 cases with pure red cell aplasia (PRCA) in cats infected with FeLV were subjected to Southern blot analyses using an exogenous FeLV probe. Clonal hematopoiesis was found in all cases with AML and in 6 of the 9 cases with MDS, but not in the 2 cases with PRCA and 3 healthy carriers infected with FeLV. In the 2 cases with MDS, the same clone of the hematopoietic cells was found before and after the progression of the disease irrespective of the change of the hematological diagnoses. It was noteworthy that clonal proliferation of hematopoietic cells was demonstrated in MDS, which could be recognized as a pre-leukemic state of AML.

Introduction

It is well recognized that erythrocytes, granulocytes and platelets originate from a single common hematopoietic stem cell such as a pluripotential stem cell in bone marrow. Since substantial numbers of the hematopoietic stem cell are present in normal bone marrow, they constitute a polyclonal population in general. By contract, because tumor cells generally derive from a single cell acquired malignant phenotype, monoclonal or oligoclonal cell proliferation is usually observed in neoplastic disorders. Therefore, analysis of the clonal composition of hematopoietic cell population is important for investigation of hematopoietic disorders (3, 4, 32, 46, 50, 51, 55, 56, 59, 67, 89). In human medicine, clonality analysis using the inactivation/methylation analysis in female patients heterozygous for X-linked DNA polymorphism, such as the CAG repeat of the human androgen receptor (HUMARA) gene, can be used to explore the presence of clonally expanded cell population in various hematopoietic disorders including polycythemia vera, primary myelofibrosis, acute leukemia, and myelodysplastic syndromes (MDS), and aplastic anemia (AA) (5, 23, 24, 42).

In veterinary medicine, various hematopoietic disorders are frequently observed in cats infected with feline leukemia virus (FeLV) (16, 39, 52). FeLV is a type-C retrovirus horizontally transmitted among domestic cat populations in natural conditions (39). Persistent infection of FeLV is associated with induction of proliferative and degenerative diseases of various hematopoietic cell lineages in cats (65). The predominant form the neoplasia is thymic lymphoma, but a variety of acute myeloid leukemia (AML) and MDS are also frequently observed. It is well known that pure red cell aplasia (PRCA) and aplastic anemia (AA) are also associated with FeLV infection (1, 2). Since most of field strains of FeLV do not contain oncogene transduced from the host genome, the tumorigenesis by FeLV infection is considered to be mediated through the interaction of FeLV with host genome (39, 52). In the initial infection, integration of FeLV in the target cells for FeLV infection such as lymphoid and myeloid cells occurrs at random sites in the host genome. On the contrary, in the cats that developed maligancies such as lymphoma and leukemia, clonally expanded cell population can be

discovered by Southern blot analysis for T-cell receptor and immunoglobulin genes or integration of FeLV proviral genome (26). Furthermore, in these tumors, clonal cell populations with proviral integrations adjacent to oncogenes such as *myc, bim-1,* and *myb,* have been observed (61, 83, 84).

In small animal practice, one of the major problems in FeLV infection is nonregenerative anemia. Many of the cats with such nonregenerative anemia in FeLV infection can be diagnosed as PRCA, AA, MDS, and AML (11, 45, 44). Among these diseases, MDS is a controversial disease, characterized by peripheral blood cytopenia due to dysplastic changes of hematopoietic cells. Furthermore, a certain number of cats with MDS develop AML several months after the diagnosis of MDS (9, 73, 77). Therefore, it has been required to investigate the pathogenesis of MDS, especially on its relation to AML. Based on these findings, it was conceivable that clonality analysis of hematopoietic cells should provide useful informations on the pathogenesis of these hematological disorders especially on that of MDS. Because most of the cats with these diseases are infected with FeLV, the clonality analysis of hematopoietic cells can be performed by detecting the proviral integration of FeLV. The purpose of this study is reveal the presence of clonal proliferation of hematopoietic cells in various feline hematopoietic disorders including neoplastic and non-neoplastic diseases by using the analysis of FeLV proviral integration in the bone marrow cells.

Materials and methods

Cases

Bone marrow cells were obtained from 18 cats infected with FeLV (Table.1). Three cats (Case 1–3) were healthy asymptomatic carriers which had been experimentally inoculated with FeLV-A PJ7E2 strain (61). These cats were clinically healthy and had no abnormal finding in their bone marrow and peripheral blood. Other 15 cats were patients referred to the Veterinary Medical Center, the University of Tokyo for diagnosis and treatment. The diagnoses were primarily based on the examinations of complete blood cell counts and cytology of the peripheral blood counts and bone marrow aspirates. The cases examined in this study included 2 cats with PRCA, 9 cats with MDS and 4 cats with AML. The diagnoses of AML and MDS were based on the criteria of French-British-American (FAB) classification for human AML and MDS(10). Furthermore, the MDS cases were subclassified into refractory anemia (RA) (Cases 6–9), refractory anemia with excess of blasts (RAEB) (Cases 10 and 11), refractory anemia with excess of blasts in transformation (RAEB in T) (Case 12), chronic myelomonocytic leukemia (CMMoL) (Cases 13 and 14), and the AML cases were subclassified into myeloblastic leukemia (M2) (Cases 15 and 16), monocytic leukemia (M5) (Case 17), erythroleukemia (M6) (Case 18) according to the FAB classification(10).

Three (Cases 9, 11, and 13) of the 9 cases with MDS developed AML 1 week to 1 month after diagnosis of MDS. In Case 9, bone marrow sample at the diagnosis of MDS and that at subsequent AML could be evaluated for the clonality of bone marrow cells. In Case 12 with RAEB in T, a bone marrow sample 3 months prior to the diagnosis of MDS could be also obtained for this study.

All of the 18 cats examined for the clonality of bone marrow cells in this study were shown to be infected with FeLV. Infection with FeLV was evaluated by the presence of FeLV antigen in plasma samples by using an enzymed-linked immunosorbent assay-based commercial test kit (CITE Combo Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit) (IDEXX, Portland, ME).

Probe

To detect FeLV proviral genemes in the chromosomal DNA, a probe specific to exogenous FeLV was prepared from a plasmid clone of FeLV-A pJ7E2 (61). The probe specific to exogenous FeLV corresponding to the U3 region of LTR was prepared by polymerase chain reaction (PCR). PCR kit, Gene amp (Perkin-Elmer Cetus, Norwalk, CT) was used for PCR amplification. The amplimer sequences were 5'-TTACTCAAGTATGTTCCCATG-3' and 5'-CTGGGGAGCCTGGAGACTGCT-3', complementary to the sequences upstream and downstream of the enhancer sequence, respectively(58). Reaction mixtures contained 1x reaction buffer (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin), 200 mM dNTP, 2.5 units of Ampli*Taq* DNA polymerase, 1 μg of genomic DNA, and 0.5 μg of each primer. Amplification was achieved by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min. PCR products were directly cloned into a cloning vector (TA cloning kit) (Invitrogen, San Diego, CA), and sequenced by the dideoxy chain termination method using an Auto Read Sequencing kit (Pharmacia, Uppsala, Sweden). The DNA fragment of 165bp corresponded to U3 region of PJ7E2 clone was used as a probe specific to exogenous FeLV.

Southern blot hybridization

Bone marrow tissues were obtained from the cats by bone marrow aspiration biopsy or necropsy. High-molecular-weight cellular DNAs extracted from the bone marrow tissues were analyzed by Southern blot hybridization. Samples of the cellular DNAs (10 μg/lane) were digested with restriction endonuclease, *Eco*RI or *Bam*HI, and subjected to 0.8% agarose gel electrophoresis. The DNAs were then transferred to nylon membranes filters and hybridized with the ^{32}P-labeled exogenous FeLV-specific probe under relatively stringent conditions in buffer containing 5 x SSC (1xSSC is 0.15M NaCl plus 0.015M sodium citrate), 5 x Denhardt's solution, 1% sodium dodecyl sulfate at 65°C for 18h. The filters were washed 2 times in a solution containing 1x SSC and 0.1% SDS at 55°C and then exposed to X-ray films at -80°C for 3~4 days.

Results

Integration of proviral DNA

For detection of the integration of FeLV provirus in bone marrow cells, high-molecular-weight cellular DNA samples were digested with *Eco*RI which dose not cut the proviral genome of most FeLV strains, or *Bam*HI which has a single cutting site in *pol* region of most FeLV strains . The digests were examined by Southern blot analysis with the FeLV probe containing LTR U3 region. In this analysis, the presence of bands indicate the clonally expanded cell population with FeLV provirus integration at a certain site of the host genome.

In asymptomatic healthy cats (Cases 1~3) infected with FeLV, both *Eco*RI and *Bam*HI digests did not give any discrete band hybridized with the FeLV probe, but showed indistinct smears (Figure. 4 A, B). In 2 cats (Cases 4 and 5) with PRCA, there was no distinct band observed in the Southern hybridization analysis, the results similar to those in the asymptomatic carrier cats. These results indicated that there was no detectable clonally expanded cell population infected with FeLV in the bone marrow from asymptomatic carrier cats and cases with PRCA in this study.

Of the 9 cats with MDS, 4 cats (Cases 6~9) were diagnosed as RA belonging to low blast count group (blast cell counts less than 5% in bone marrow cells and less than 1% in peripheral blood nucleated cells), and 5 cats (Cases 10~14) were diagnosed as RAEB, RAEB in T, and CMMoL belonging to high blast count group (blast cell counts more than 5% in bone marrow cells and more than 1% in peripheral blood nucleated cells) in MDS. In the sample from 2 (Cases 8 and 9) of the 4 cases with RA, there were 5.3 kb-band and 3.2 kb-band in each case in the *Eco*RI digests and 3 bands and 1 band in each case in the *Bam*HI digests. However, there was no detectable band hybridized with the FeLV probe in 2 cases (Cases 6 and 7) with RA. In the samples from 4 of 5 cases with MDS of high blast count group, 3 to 6 discrete bands of 4.9~25.0 kb in the *Eco*RI digests and 5 to 10 bands of 2.0~12.5kb in the *Bam*HI digests were shown in the Southern hybridization. In one (Case 13) case with CMMoL, there was no distinct

band hybridized with the FeLV probe in both of the *Eco*RI and *Bam*HI digests. These results indicated an evidence that there were clonally expanded cell populations infected with FeLV in the bone marrow from 6 of 9 cases with MDS.

The Southern blot analysis of the samples from 4 cases with AML (Cases 15~18) revealed 2~4 bands of 5.7~11.0kb in their *Eco*RI digests and 3~9 bands of 1.7~17.0kb in their *Bam*HI digests.

Case 9 was diagnosed as RA at the referral, however, there remained some question about the diagnosis because the bone marrow was hypocellular and the number of the cells with dysplastic changes was small in the bone marrow (Figure. 5 a). The case showed rapid progression to AML/M0 1 month after the diagnosis (Figure. 5 b). In the Southern blot analysis using FeLV probe, the bone marrow sample at the diagnosis of MDS/RA showed a band of 3.0-kb *Bam*HI fragment, and that at the diagnosis of AML/M0 revealed 5 bands of 2.4~5.6kb including a band of 3.0kb in the *Bam*HI digest (Figure. 5 c). The results indicated that the same clone existed at both stages diagnosed as MDS/RA and AML/M0 and the increased number of the bands detected at the AML/M0 stage corresponded to the superinfection of the same clone with FeLV or clonal expansion of another bone marrow cells infected with FeLV.

Case 12 was diagnosed as MDS/RAEB in T in this study based on the cytology of the bone marrow showing the increased number of blast cells and dysplastic changes (Figure. 6 b). This case had been examined for the bone marrow cytology 3 months prior to the diagnosis of MDS/RAEB in T. At that time, the case had been suspected to have AA based on the hypocellular bone marrow and apparent lack of dysplastic change (Figure. 6 a). However, the DNA sample of this case at the diagnosis of suspected AA showed 7 *Bam*HI bands of 2.7~12.0 kb in the Southern blot hybridization for FeLV integration. Interestingly, the band pattern at the diagnosis of AA was much similar to that at the diagnosis of MDS/RAEB in T (Figure. 6 c) indicating that the hematopoietic cell clone infected with FeLV shown at the stage of MDS/RAEB in T had already existed at the stage of suspected AA.

Discussion

In the present study, provirus integration of FeLV was investigated in primary bone marrow cells, from cats infected with FeLV. In our analysis, proviral integration of FeLV was random in the population of bone marrow cells derived from asymptomatic FeLV carrier cats and the cats with PRCA, indicating the absence of clonally expanded cell population in the asymptomatic carrier state and PRCA. It was also indicated that PRCA in cats was a disease of polyclonal cell population using an analysis for cellular mosaicism of glucose-6-phosphate dehydrogeneas (G-6-PD) (4). But the method was only available in cats heterozygous for G-6-PD, therefore, the reports which referred to the clonality in PRCA were limited to the cats experimentally infected with FeLV. In this study, it was indicated that the cats with naturally occurring PRCA had polyclonal hematopoiesis, as shown in the cats experimentally infected with FeLV.

Southern blot analysis in this study showed that leukemic cells derived from typical AML cases were clonally expanded cells in which the provirus genomes were integrated into certain site of the cat genome. Furthermore, similar clonal hematopoiesis was proved in 6 of 9 cats with MDS in the present study. The results showed that the dyshematopoiesis found in MDS was caused by clonal proliferation of abnormal hematopoietic cells in the bone marrow, indicating a highly possible explanation that MDS is a preleukemic state of AML. Especially, even 2 of the 4 MDS cases with low blast counts had clonal hematopoiesis, therefore, it was thought that dyshematopoiesis at the early stage of MDS might be also due to expansion of abnormal cell clones. In 3 of 9 cats with MDS, however, clonal hematopoiesis was not detected by Southern blot analysis using the FeLV probe, although they had severe dyshematopoiesis. The reason why the clonal hematopoiesis was not detected in these MDS cases may be the polyclonal hematopoiesis as a mechanism for development of MDS or undetectable clonal hematopoiesis due to the apoptosis, small number of cells, or lack of FeLV infection.

In human medicine, it was recognized that increased apoptosis of hematopoietic cells was found in the bone marrow from patients with MDS (12, 13, 33, 37, 70, 71, 91).

Furthermore, monoclonal hematopoiesis was shown in most of MDS patients by X-inactivation/methylation analysis in female MDS patients heterozygous for X-linked DNA polymorphism of G-6-PD, phosphoglycerate kinase, hypoxanthine phosphoribosyl transferase or androgen receptor (5, 32, 51, 67). Therefore, human MDS is now considered as a clonal hematopoietic disorder originated from a pluripotent stem cell having an ability to differentiate into erythrocytes, granulocytes and platelets. But, some of human MDS patients were shown to have polyclonal hematopoiesis at the diagnosis of MDS (24, 32, 49, 55). These findings indicate that MDS in cats resemble that in humans with respect to the clonal origin of the abnormal hematopoietic cells although the possible etiologic agent, FeLV, can be found only in cats.

Although it was difficult to confirm dysplastic changes in the hypocellular bone marrow samples in Cases 9 and 12 at some stages of their diseases, Southern hybridization analysis of the bone marrow cells showed clonal population of the hematopoietic cells in this study . In veterinary medicine, AA is generally recognized as disorder with in depletion of hematopoietic cells in the bone marrow (60, 78, 89). However, case 12 with suspected AA was found to progress to MDS/RAEB in T, and the pattern of FeLV provirus integration at at the stage of AA was similar to that at the stage of MDS/RAEB in T. Therefore, it was suggested that some cases with AA may have a feature similar to those with MDS. In human medicine, it was reported that some AA patients underwent MDS or AML and that the bone marrow cells in AA had a molecular evidence of clonal hematopoiesis (78, 89). Further research is required to investigate the pathogenesis of AA in relation to the clonal hematological disorders including MDS and AML.

In 7 cases with MDS or AML (Case 8, 9, 10, 11, 12, 14, and 18), discrete bands shorter than 8.0kb corresponding to the length of common FeLV were detected in the Southern blot analysis for FeLV using the *Eco*RI digests. Because *Eco*RI dose not have a cutting site in most of the common FeLV proviruses, these short bands may be due to some genetic change of FeLV in these cats with MDS or AML. These short bands might be derived from FeLV proviruses which had some restriction site of *Eco*RI

in its genome or those with large deletion of the genome. Some deleted FeLV proviruses have been reported in the cats with AML. FeLV-GM1/AB strain isolated from the cat with AML had a large deletion in its *gag* and *pol* gene (85). It was previously showed that the FeLVs from AML frequently contained deletion and direct repeats in the upstream region of enhancer (URE) in LTR (66). However, such genetic change can not explain the mechanism to generate the short proviral genomes. Further effort is needed to characterized the proviral genome of FeLV in the bone marrow from cats with MDS and AML.

The present study disclosed the existence of clonal hematopoiesis in FeLV-infected cats with hematopoietic disorders such as MDS and AML. The clonality analysis employed in this study will provide useful informations not only for the diagnosis of the hematopoietic disorders but also for the further investigation on the pathogenesis of these diseases associated with FeLV infection.

(Chapter III)

Structure of the long terminal repeats of feline leukemia viruses derived from myelodysplastic syndromes in cats

Abstract

Myelodysplastic syndromes (MDS) primarily characterized by peripheral blood cytopenias and dysplastic changes of the bone marrow are frequently observed in cats naturally infected with feline leukemia virus (FeLV). To characterize the FeLV strains associated with MDS, the U3 region in the long terminal repeat (LTR) was amplified by polymerase chain reaction and sequenced. In 10 of 13 cats with MDS, I found 22-bp deletion and/or 2 to 5 copies of tandem direct repeat of upstream region of enhancer (URE) in the LTR, a region between the inverted repeat and enhancer. Furthermore, I detected deletions and/or point mutations of the enhancer sequences of the LTR in 5 of 13 cats with MDS. The FeLV LTRs derived from MDS were structurally very similar to those which had been obtained from acute myeloid leukemia in our previous report, providing a virological evidence that MDS is a pre-leukemic state of AML.

Introduction

The myelodysplastic syndromes (MDS) are a group of hematological disorders characterized by combined cytopenias in the peripheral blood including anemia, leukopenia, and thrombocytopenia due to dysplastic changes of hematopoietic cells of two or more lineages in the bone marrow (11, 45, 72, 73). The disease entity of MDS was first established in humans, as diagnosed from the criteria in French-American British (FAB) classification for acute myeloid leukemia (AML) and MDS (10). In veterinary medicine, hematological disorders similar to human MDS are frequently observed, especially in cats naturally infected with feline leukemia virus (FeLV) (11, 44). Diagnostic criteria for MDS have been also reported in cats and dogs (45), which are based on the FAB classification in humans. Furthermore, the MDS in humans are now categorized into 5 subtypes (10), refractory anemia (RA), refractory anemia with ring sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), RAEB in transformation (RAEB in T), and chronic myelomonocytic leukemia (CMMoL). Similar subtypes of MDS can be found in cats with MDS (9, 77). AS shown in MDS in humans, a proportion of cats with MDS are considered to undergo progression to AML.

It is well known that FeLV infection is associated with a variety of neoplastic and non-neoplastic diseases in cats (52). FeLV variants which induce specific types of disease have been described, and several characteristic genetic changes in the viral genomes responsible for their pathogenicity have been identified. Major structural determinants of the aplastic anemia-inducing FeLV-C were identified in the N-terminus of *gp70* of *env* gene (2, 75). Furthermore, a replication-defective variant with mutations in the *env* gene was shown to be associated with feline acquired immunodeficiency syndrome (FAIDS) (21).

It has been indicated that FeLV proviruses isolated from thymic lymphoma contain duplication of the enhancer sequences containing enhancer elements such as leukemia virus factor b (LVb), simian virus (SV) 40-like core (CORE), nuclear factor 1 (NF1), glucocorticoid responsive element (GRE), and FeLV-specific binding site (FLV-1) (27,

58, 61). However, LTRs from other types of tumors and those of weakly pathogenic strains contain only a single copy of the enhancer (65). Recently, we reported that LTRs of FeLVs derived from naturally occurring AML frequently contained a 22-bp deletion and direct repeats of 40 to 74 bp in the upstream region of the enhancer (URE) and several mutations in the enhancer sequences (66).

Although it has been shown that most cats that developed MDS are infected with FeLV (11, 44), the characteristics of the viruses associated with MDS have not been investigated so far. In our previous report, two cats infected with FeLV in the same household developed MDS (77). In our experience, MDS are frequently diagnosed in more than 1 cat from a litter and cats kept in the same house. In the present study, to identify the specific structure of FeLV associated with MDS, I cloned and analyzed the FeLV LTRs from 13 cats with naturally occurring MDS.

Materuals and methods

Cases

Twelve cats referred to the Veterinary Medical Center, Faculty of Agriculture, the University of Tokyo, and a cat admitted to the Veterinary Teaching Hospital, Faculty of Agriculture, the University of Miyazaki were used for characterization of FeLV LTRs.

Diagnosis

The 13 cats were diagnosed as MDS from the complete blood cell counts and the findings of bone marrow aspiration biopsy primarily based on the criteria of FAB classification for AML and MDS in humans (10). The MDS were subclassified into RA (Case 1, 2, 3, 4, 5 and 6), RAEB (Case 7, 8, 9 and 10), RAEB in T, (Case 11) and CMMoL (Case 12 and 13). FeLV infection was detected by presence of FeLV p27 antigen in the plasma (Pet check, IDEXX, Portland, ME).

Tissue and cell samples

Bone marrow cells were obtained from 12 of the 13 cats with MDS. Since, in 1 cat (Case 5), enough bone marrow cells could not be obtained, peripheral blood mononuclear cells (PBMC) were used for the experiment. The bone marrow cells were obtained at bone marrow aspiration biopsy or necropsy and then rapidly frozen in liquid nitrogen and stored at -80^{o}C for subsequent DNA extraction. The tissue homogenates prepared in liquid nitrogen and PBMC suspension were incubated with an extraction buffer containing 10mM Tris-HCl (pH8.0), 0.1M EDTA, 20μg of pancreatic RNAase per ml and 0.5% sodium dodecyl sulfate (SDS) at 37^{o}C for 1 hour and then treated with 100μg proteinase K per ml at 37^{o}C overnight. The DNA samples were extracted with phenol and chloroform-isoamyl alcohol (24:1), and then precipitated with ethanol.

PCR amplification of FeLV LTRs

For analysis of the FeLV LTR structure from cases with naturally occurring MDS associated with FeLV infection, PCR was employed for amplification of DNA fragments containing the full length U3 region in the 3'LTR. The sequence of the 5' primer is specific to the fragment between *env* and 3'LTR of exogenous FeLV: Fe-1S, 5'-GAGAGCTCTCAATACGATTCGGACCGACCATG-3' (nt.1968-1989 in FeLV A/Glasgow-1(80), the *Sac*I linker is underlined). The 3' primer was synthesized based on the sequence downstream of capping site of FeLV LTR: Fe-1R, 5'-GGTACCCGGGGCGGTCAAGTCTCGGCAAAG-3' (nt. 539-510 in pJ7E2 strain (61) containing a conserved restriction endonuclease site of *Kpn*I underlined). The sequence of the 3' primer is common to most of exogenous and endogenous FeLV isolates. PCR reaction mixtures contained 1x reaction buffer (10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, and 0.001% gelatin), 200mM dNTP, 2.5 units of *AmpliTaq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), 1μg of genomic DNA and 0.5μg of each primer. Amplification was achieved by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 1 min.

Cloning and sequencing

The PCR products were directly cloned into a cloning vector (TA cloning kit; Invitrogen, San Diego, CA) and sequenced by the dideoxy chain termination method using a BigDye Primer Cycle Sequencing Ready Reaction Kit (Pharmacia, Uppsala, Sweden).

Results

Detection of FeLV LTR by PCR

The PCR strategy was employed to obtain FeLV LTR fragments from bone marrow and peripheral blood in cats with MDS. Figure 7 shows the PCR products from 13 cases with MDS. The PCR using Fe-1S and Fe-1R was found to amplify a band of 468 bp containing the whole U3 region in of a standard strain FeLV/Glasgow-1 strain (80) with 1 copy of enhancer. PCR-amplified fragments larger than that from the standard strain (468bp) were observed in 9 (Cases 2, 4, 5, 6, 7, 8, 9, 10, and 12) of 14 cases with MDS. PCR products similar to that from the standard strain in size were observed in 3 cases (Cases 2, 3, and 10). PCR products smaller than that from the standard strain were also obseved in 8 cases (Case 1, 4, 5, 7, 8, 11, 12, and 13).

Nucleotide sequence of the U3 from 3' FeLV LTR

In order to characterize the LTR of proviruses derived from MDS, the obtained PCR fragments were cloned and sequenced. The sequences of 28 clones from 13 cases with MDS were determined (Figure. 8). Major differences of the sequences between the clones from MDS and the standard FeLV were observed in a region between the inverted repeat and enhancer, designated as upstream region of enhancer (URE). Two to 5 times direct repeats of URE with a 22-bp (nt -296 to -274) deletion were found in 7 (Case 4, 5, 6, 7, 9, 10, and 12) of the 13 cases with MDS. One unit of the direct repeat of URE was 39 to 70 bp. The 5' and 3' termini of the repeat of URE were nt -345 to -241 and nt -261 to -241, respectively, from the presumptive RNA cap site in the FeLV/Glasgow-1 LTR. In 3 cases (Case 1, 8, and 13), the same deletion of 22 bp was observed, however, there was no repetitive structure of URE in the clones obtained in this study. In clone 10-1, a five copies of 49-bp sequence of URE (nt -319 to -251) was obseved. In clones 10-2 and 6-1, four copies of 37-bp and 47-bp sequence of URE (nt-312 to -256 and nt-313 to -257) were obseved, respectively. In clones 9-1, 10-3, 10-4, and 12-1, three copies of 37-bp to 47-bp sequence were found (from nt -323

to nt -251). In 3 cases (Case 4, 5, and 7), two copies of 39 to 70-bp sequence of URE (from nt -343 to -255) repeated.

In the present study, deletion of enhancer was found in 3 (Case 10, 11, and 12) of the 13 cases with MDS. Clones 10-3 and 10-4, which contained triplicated direct repeats and deletion of URE, had deletion of 31-bp sequence corresponding to NF-1 and GRE binding motives (nt -191 to -161) and deletion of 20-bp sequence including GRE binding motif (nt -183 to -163), respectively. Clone 11-1 had deletion of 65-bp sequence in enhancer element (nt -229 to -164). Clone 12-1, which had triplicated direct repeat and deletion of URE, was found deletion of 97-bp sequence between URE to enhancer element (nt -256 to -159).

A point mutation of enhancer elements was found in 2 cases (Case 2 and 6). Clone 2-1, in which deleted and repeated sequence of URE was not found, had A to G transition (CAGGATA - CAGGATG) in LVb binding motif and A to G transition (CCGGCTTGAGGCCAAG - CCGGCTTGGGGCCAAG) in NF1 binding motif. Clone 6-4 , which did not have deleted and repeated sequence of URE, and had a A to G transition (CAGGATA - CAGGATG) in LVb binding motif, A to G transition (CCGGCTTGAGGCCAAG - CCGGCTTGGGGCCAAG) in NF1 binding motif and A to G transition (AGAACAG - AGAGCAG) in GRE binding motif. In clones 8-1 and 8-2 from Case 8 with MDS (RAEB) and lymphoma, repeated sequence was not found in URE, but three and two copies, respectively, were found in the enhancer element. Furthermore, clone 9-2 with deletion of URE had T to C transition (CCAAT - CCAAC) in CAT box of promoter region.

Discussion

In the present study, FeLV LTRs derived from naturally occurring MDS frequently contained two to five times repeated sequence of URE with 22-bp deletion. The deleted sequences in the URE and the elements of the repeated URE were very similar among a number of LTR clones from MDS in this study. The characteristic structures of LTRs from MDS were very similar to the those derived from AML as shown in our recent paper (66). Because FeLVs with unique structures such as direct repeat of URE with 22-bp deletion have never been detected in other disease including lymphoma, pure red cell aplasia, and immunodeficiency, these viruses may have a specific relation to induce MDS and AML in cats.

In previous report on the function of FeLV LTRs derived from AML in cats, transcriptional activities of LTRs with URE repeats were higher in myeloid cells than in T-cells (66). Furthermore, the enhancer activities of the LTRs were dependent on the number of URE repeats in myeloid cells, but not in T-cells. From these findings, it was hypothesized that the formation of direct repeats of URE accelerated the tumorigenic potential in myeloid cells. Because the structure of LTRs from MDS in this study was quite similar to those from AML in our previous report, they should have functions of transcriptional activation upon expression as shown in LTRs from AML. These findings indicate that MDS is a preceeding state of AML which might be caused by viruses with strong transcriptional activity in myeloid cells.

It is impossible to know the structure of the initial FeLV inoculum in these cats with naturally occurring MDS. In the 6 cases (Case 4, 6, 7, 9, 10, and 12) in which FeLVs with repetitive URE were detected, the sequences of the clones with repetitive URE were almost identical to those without URE repeat derived from the same case in the regions other than the URE repeats. Furthermore, in the cases from which 2 or more clones with repetitive URE (Case 6, 11, and 13) were detected, the sequences in the regions except the repetitive URE were almost the same between the clones with different number of URE repeats. From these findings, it can be considered that the animals were originally infected with a virus with a single URE sequence, and then LTRs with two to

five copies of URE could have arisen *de novo* in each infected cat. I am now carrying out an experimental infection of kittens with an FeLV clone having 3 URE repeats to investigate its pathogenicity to induce MDS and subsequent AML. In my preliminary data, in newborn kittens experimentally inoculated with a cloned FeLV with three copies of URE, viruses with 4 or 5 copies of URE could be detected three months after infection (data not shown).

In murine leukemia virus system, studies on the leukemogenicity of chimeric viruses generated from leukemogenic and nonleukemogenic viruses have shown that LTRs are the major genetic determinants for the pathogenic potential, (15, 79). Moreover, similar studies on the chimeric viruses generated with thymic lymphoma-inducing virus and erythroid leukemia-inducing virus have shown that LTRs determine the tissue type of disease caused by the virus (7). In Moloney murine leukemia virus, mutations of the motives in the enhancer region were shown to influence disease specificity and latent period of disease induction (79). It is notable that the viruses with mutations in the LVb or SV40 CORE binding motif were found to induce erythroleukemia. In addition to this event, deletions and mutations of protein binding motives such as LVb, SV40 CORE, NF-1 and GRE within enhancer may accelerate induction of MDS and AML and suppress induction of thymic lymphoma. Similar mutations of these protein binding motives were reported in FeLVs derived from various hematopoietic disorders including lymphoma, leukopenia, anemia and AML (43). However, the significance and the role of pathogenicity in each disease have been unclear. In the present study, deletion and mutation of protein binding motifs such as LVb, NF-1 and GRE in the FeLV clones derived from MDS may be also associated with the induction of MDS and AML in conjunction with the repetition and deletion of URE. The analysis of enhancer activities of these LTRs were not determined.

In spite of many reports on the induction of lymphoma by experimental infection with FeLV (39), there have been only a few reports on experimentally induced AML in cats. The GM-1 strain of FeLV (FeLV-AB/GM1) was isolated from naturally occurring case of myeloid leukemia and induced severe hematopoietic abnormalities in cats (82).

Furthermore, FeLV-AB/GM1 was shown to decrease the ability of granulocyte-macrophage colony formation of bone marrow cells (85). FeLV-AB/GM1 was composed of exogenous FeLV A and mutant FeLV B, but the structure of URE region was not determined. Therefore, it is impossible to compare the structure of FeLV-AB/GM1 strain with those of the viruses obtained from AML and MDS in my studies.

In the present study, I indicate that feline MDS may be induced by specific FeLV variants which have deletion and tandem direct repeats of URE in LTR, and that MDS and AML are associated with the same type of FeLV variant. From the standpoint of virology, the present study in conjunction with our previous study suggested that MDS is a preleukemic state of AML, which has been indicated from the hematological aspect. To clarify the etiologic role of such FeLV variants in the development of MDS and AML in cats, further investigation on the experimental infection with molecularly cloned FeLV with the characteristic LTR structures is highly required.

Conclusion

Myelodysplastic syndromes (MDS) are a kind of hematopoietic disorder, characterized by combined cytopenia in peripheral blood including anemia, leukopenia, and thrombocytopenia derived from dysplastic changes of erythroid, myeloid, and megakaryocytic cells in the bone marrow. Because progression to acute myeloid leukemia (AML) has been frequently observed in cats with MDS, MDS is considered as a kind of preleukemic state of AML (11, 44, 77). Most of cats with MDS are naturally infected with feline leukemia virus (FeLV), which is associated with the pathogenesis of MDS in cats (11, 44). In a series of the present studies, I investigated the clinical, hematological, and virological features of MDS in cats, to understand the pathogenesis of MDS.

In Chapter I, I investigated the hematological abnormalities in 16 cats with MDS. Non-regenerative anemia and thrombocytopenia were seen in the most of cats with MDS, but neutropenia was less common. The dysplastic changes of the hematopoietic cells such as megaloblastoid rubricytes, neutrophil hyposegmentation, nuclear abnormality and micromegakaryocytes were frequently seen in most of cats with MDS, and these findings were similar to those in human patients with MDS. In humans, French-American-British (FAB) co-operative group proposed a classification of MDS into 5 subtype based on the morphologic features and the number of blast cells: refractory anemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB in T), and chronic myelomonocytic leukemia (CMMoL) (10). The 16 cats with MDS were classified into 8 cats with RA, 5 cats with RAEB, 1 cat with RAEB in T, and 2 cats with CMMoL (88). The rate of leukemic progression were relatively low in cats with RA (12.5%) but high in cats with RAEB, RAEB in T and CMMoL (50.0%), thus human FAB classification system was shown to be useful to determine the stage of feline MDS. According to human Dusseldorf scoring system (8), which is based on the number of blasts in bone marrow, plasma lactate dehydrogenease value, hemoglobin concentration, and platelet counts, the survival durations of cats having high scores (≥ 3points) were significantly shorter than those of cats having low scores (< 3points), indicating that the survival durations of feline MDS

were influenced by such risk factors as shown in human MDS patients. The results obtained in this chapter were considered to be useful make a diagnosis and predict the prognoses in feline MDS.

In Chapter II, clonality analysis of the bone marrow cells by detecting FeLV proviral integrations was applied to know the pathogenesis of hematological disorders in cats with MDS and other hematological disease. Clonal hematopoiesis was found in all of the 4 cases with AML and 6 of the 9 cases with MDS, but not in 2 cases with PRCA and 3 asymptomatic FeLV carriers. Since the clonal proliferation of hematopoietic cells was demonstrated in feline MDS, the pathogenesis of feline MDS was revealed to be a kind of pre-leukemic stage. Especially, in some cases with MDS, the same clone of the hematopoietic cells was found before and after the progression of the disease irrespective of the change of the hematological diagnoses, therefore the clonality analysis will provide useful informations for diagnosis of hematological disorders, but not in 2 cases with PRCA and 3 asymptomatic FeLV carriers.

In Chapter III, I investigated the molecular characteristics of FeLV strains associated with MDS. The U3 region in the long terminal repeat (LTR) was amplified by polymerase chain reaction and sequenced. In 10 of 13 cats with MDS, I found 22-bp deletion and/or 2 to 5 copies of tandem direct repeats of upstream region of enhancer (URE) in the LTR, a region between the inverted repeat and enhancer. Furthermore, I detected deletions and/or point mutations of the enhancer sequences of the LTR in 5 of 13 cats with MDS. The FeLV LTRs derived from MDS were structurally very similar to those which had been obtained from AML in a previous report (66), providing a virological evidence that MDS is a pre-leukemic state of AML. Experimental infection system using such FeLV variants will be useful to establish an experimental system to induce MDS and AML.

In the present study, I showed that the degree of hematological abnormalties in cats with MDS were related to their prognoses, and the pathogenesis of MDS was based upon the clonal ploriferation of abnormal hematopoietic cells. Furthermore, I showed that specific FeLV variant strains might have a pathogenic potential to induce feline MDS.

These findings can be considered as useful informations for understanding the pathogenesis of feline MDS.

Mechanism of the pathogenesis of MDS has been studied mainly in human with MDS. It has been indicated that the dyshematopoiesis of MDS in the early stage of the disease is caused by apoptosis of hematopoietic cells (12, 25, 31, 33, 37). When the hematopoietic clone, which underwent apoptosis, acquires another genetic changes to induce uncontrolled proliferation, such clone will have a neoplastic feature leading to AML (13, 19, 20, 22, 29, 71, 81, 90, 91). In human MDS, has been recognized that abnormalities of several oncogenes, and tumor-suppress genes such as *ras, p53, p15, WT-1, Evi-1, flt-1* and *IRF-1* (36, 38, 41, 47, 64, 68, 86), are related with the progression to AML. But pathogenesis of MDS in its early stage has not been documented yet. Any useful animal model for MDS has not been established, so it is difficult to analyze the pathogenesis of MDS and to examine the therapeutic protocol for MDS. There have been a few studies referred to the animal model for MDS in and mice. For example, transgenic mice harboring SV 40 large T antigen under an immunoglobulin enhancer were shown to develop MDS, but were quite different from MDS in humans (28). There are a number of spontaneous cat case with MDS, it is considered that feline MDS may be one of the useful animal model of MDS. In the present study, I showed that the clinical, hematological and molecular biological features of feline MDS were very similar to those of human MDS, and indicated a presence of unique FeLV variant from feline MDS. If animal model for MDS is established using this FeLV variant, finding derived from the animal model system for MDS will provide useful informations to understand the pathogenesis of multi-step tumorigenesis of hematopoietic cells and to develop an effective therapeutic protocol for MDS and AML.

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Table 1. Profile of 16 cats with MDS

Case No.	Age (Years old)	Sex	Breed	Chief complaints	Findings in physical examination
1.	1	NM	Somali	Lymphadenopathy	Pale mucous membranes, Lymphadenopaihy
2.	3	M	J.D.C	Weakness, Anorexia, Diarrhea	Pale mucous membranes, Splenomegaly
3.	2	NM	J.D.C	Weakness, Anorexia	Pale mucous membranes, Hepatomegaly
4.	1	M	J.D.C	Weakness	-
5.	6	F	J.D.C	Anorexia	Pale mucous membranes, Pleural effusion
6.	0.7	F	J.D.C	Weakness, Anorexia	Pale mucous membranes, Fever, Splenomegaly
7.	1	M	J.D.C	Weakness, Anorexia, Diarrihea, Vomiting	Pale mucous membranes, Lymphadenopathy, Splenomegaly, Hepatomegaly, Fever
8.	1	M	J.D.C	Weakness, Anemia	Pale mucous membranes, Splenomegaly
9.	14	NM	J.D.C	Tachypnea	Pale mucous membranes
10.	1	M	J.D.C	Weakness, Anorexia, Vomiting	Pale mucous membranes, Splenomegaly
11.	1	NF	J.D.C	Weakness	Pale mucous membranes
12.	1	F	J.D.C	Weakness, Anorexia	Pale mucous membranes, Splenomegaly
13.	14	NF	J.D.C	Weakness, Anorexia	Pale mucous membranes, Dehydration, Emaciation, Pleral effusion
14.	6	NF	J.D.C	Anemia	Splenomegaly, Hepatomegaly
15.	5	NM	J.D.C	Weakness, Anorexia	Splenomegaly
16.	3	M	J.D.C	Upper respiratory disease, Weakness Anorexia	Pale mucous membranes, Lymphadenopathy

M, Male; F, female; NM, neutered male; NF, neutered female; J.D.C, Japanese domestic cat

Table 2. Results of the peripheral blood and bone marrow examination in 16 cats with MDS

Case No.	Subtype of MDS	RBC ($x10^6/\mu l$)	PCV (%)	Hb (g/dl)	WBC ($x10^3/\mu l$)	Neutrophils ($x10^3/\mu l$)	PLT ($x10^3/\mu l$)	LDH (IU)	Blasts in PB(%)	Blasts in NEC(%)	Blasts in ANC(%)	M/E ratio	FeLV antigen	FIV antibody
1.	RA	2.79	16	5.6	11.3	3.7	8	166	0	0.9	3.0	0.2	+	-
2.	RA	2.81	17	6.5	7.7	5.7	8	5600	0	0.5	0.4	1.7	+	-
3.	RA	3.79	13	3.7	3.1	2.4	50	262	0	1.5	1.5	5.8	+	-
4.	RA	5.44	25	8.8	9.6	8.3	73	98	0	0.4	2.5	0.2	+	-
5.	RA	2.13	11	4.1	20.9	20.3	4	1480	0	4.9	5.2	7.5	+	-
6.	RA	6.49	18	5.7	5.5	1.9	487	245	0	2.8	1.9	1.1	-	-
7.	RA	3.17	21	7.1	2.1	1.1	140	158	0	1.2	3.4	0.1	+	-
8.	RA	1.82	7	2.8	5.5	3.8	99	85	0	1.1	1.6	0.7	+	-
9.	RAEB	1.65	10	3.9	3.3	1.7	88	260	0	6.6	16.0	0.4	+	+
10.	RAEB	2.12	11	4.0	15.2	12.0	128	140	2	5.2	8.8	0.9	+	-
11.	RAEB	2.37	14	4.5	25.0	21.8	57	239	0	12.0	2.7	0.6	+	-
12.	RAEB	1.00	9	2.0	68.4	17.8	23	459	1	13.0	19.0	0.3	+	-
13.	RAEB	1.46	9	6.5	37.0	27.8	328	303	1	9.6	9.4	0.9	+	-
14.	RAEB in T	4.60	26	7.1	35.7	31.4	507	403	0	21.2	25.1	5.1	+	-
15.	CMMoL	9.54	39	13.9	17.8	10.2	139	711	6	18.6	2.6	8.5	+	-
16.	CMMoL	3.89	22	5.5	11.3	5.3	109	569	11	5.5	0.7	11.5	+	+

Table.3 Dysplastic changes in the peripheral blood and/or bone marrow in 16 cats with MDS

Dysplastic changes	Number of cases (%)	
Dyserythropoiesis		
Megaloblastid rubricyte	9/16	(56.3)
Ringed sideroblasts	1/7	(14.3)
Nuclear fragmentation	10/16	(62.5)
Abnormal nuclear shape	10/16	(62.5)
Circulating rubricytes without polychromasia	10/16	(62.5)
Macrocytosis	9/16	(56.3)
Dysmyelopoiesis		
Neutrophil hyposegmerntation (psudo-Pelger-Huet nuclear amomaly)	7/16	(43.7)
Neutrophil hypersegmentation	10/16	(62.5)
Cell giantism	8/16	(50.0)
Nuclear abnormality	13/16	(81.2)
Abnormal cytoplasmic granulation	6/16	(37.5)
Dysthrombopoiesis		
Micromegakaryocytes	10/16	(62.5)
Large mononuclear megakaryocytes	10/16	(62.5)
Large platelets	11/16	(68.8)

Table 4. Results of the diagnosis and prognosis in 16 cats with MDS

Case No.	Diagnosis		Survival durations (Months)
	First admitted	Final stage	
1.	RA	RA	+73.5
2.	RA	RA	2.2
3.	RA	M0	1
4.	RA	RA and lymphoma	7.2
5.	RA	RA	1.3
6.	RA	RA	+0.3
7.	RA	RA	+24
8.	RA	RAEB	15.5
9.	RAEB	ND	2.5
10.	RAEB	RAEB and lymphoma	2
11.	RAEB	AML/M6	6
12.	RAEB	ND	1
13.	RAEB	AML/M2	0.2
14.	RAEB in T	RAEB in T	3.5
15.	CMMoL	AML	1
16.	CMMoL	CMMoL	+2

ND, not detemine the diagnosis in final stage

Table.5 Leukemic progression in 16 cats with MDS

	Progression to acute leukemia		
	(+)	(-)	Unknown
Low blast count group (n=8)	1	7	0
High blast count group (n=8)	3	3	2

Table.6

Clonality analysis in cats with hematopoietic disorders

Case No.	Diagnosis	Clonality Status
1	Healthy	-
2	Healthy	-
3	Healthy	-
4	PRCA	-
5	PRCA	-
6	MDS-RA	-
7	MDS-RA	-
8	MDS-RA	+
9	MDS-RA	+
10	MDS-RAEB	+
11	MDS-RAEB	+
12	MDS-RAEB in T	+
13	CMMoL	-
14	CMMoL	+
15	AML-M2	+
16	AML-M2	+
17	AML-M5	+
18	AML-M6	+

+, detectable clonal hematopoiesis
-, absence of detectable clonal

Table. 7

Structure of LTRs from Cats with MDS

Case No	Diagnosis	Deletion of URE	Repetitive URE	Deletion of enhancer	Mutation of enhancer	Repetitive enhancer
1	RA	+	-	-	-	-
2	RA	-	-	-	+	-
3	RA	-	-	-	-	-
4	RA [c]	+	+(2)[a]	-	-	-
5	RA	+	+(2)[a]	-	-	-
6	RA	+	+(4,2)[a]	-	+	-
7	RAEB	+	+(2)[a]	-	-	-
8	RAEB[c]	+	-	-	-	+ (3,2)[b]
9	RAEB	+	+(3)[a]	-	-	-
10	RAEB	+	+ (5,4,3)[a]	+	-	-
11	RAEB in T	-	-	+	-	-
12	CMMoL	+	+ (3,2)[a]	+	-	-
13	CMMoL	+	-	-	-	-

a, copy number of URE.
b, copy number of enhancer element.
c, with lymphoma.

Legends for figures

Figure. 1 Morphological abnormalities indicating dyshematopoiesis in cats with MDS. A megaloblastoid rubricytes (a) in bone marrow from a cat with RAEB (Case 2). Nuclear fragmentation of a rubricyte (b) and abnormal nuclear shape of an erythroid progenitor cell (c) in bone marrow from a cats with RAEB (Case 12). Hyposegmentation (psudo-Pelger Huet nuclear anomaly) (d) in bone marrow from a cat with CMMoL (Case 16). Nuclear abnormalty (ring-shaped nucleus) (e) in bone marrow from a cat with RA (Case 5). Abnormal cytoplasmic granulation (peroxidase-negative mature neutrophils) (f) in bome marrow from a cat with RAEB in T (Case 14). Micromegakaryocytes (g) and large mononuclear megakaryocytes (h) in bone marrow from a cat with CMMoL (Case 16). Blast cells of monocytic lineage (i) in peripheral blood from a cat with CMMoL (Case 15) at the progression to AML. Wright-Giemsa stain. x650.

Figure. 2 Cumulative survival curves of 16 cats with low blast cell count and high blast cell count MDS. The cats that developed lymphoma or died of other disorder other than MDS and AML are censored (as shown by | on the survival curve). Low blast cell count group includes RA, and high blast cell count group include RAEB, RAEB in T and CMMoL. Survival duration of the low blast count MDS is relatively longer than that of high blast count MDS (P=0.06).

Figure. 3 Cumulative survival of 16 cats with MDS divided into two groupd A and B according to Dusseldorf scoring system. The cats that developed lymphoma or died of disorders other than MDS and AML are other disorders are censosored (as shown by |on the survival curve). Survival durations between group A (point 1 or 2) and group B (point 3 or 4) are significantly different (P=0.01).

Figure.4. Southern blot analysis for the integration of proviral genome of FeLV in the bone marrow cells from cats with various hematological disorders. Cellular DNAs extracted from the bone marrow tissues were digested with *Eco*RI (A) or *Bam*HI (B) and subjected to Southern blot analysis with a specific probe to exogenous FeLV. Numbers of lanes show the case numbers. Cases 1~3, asymptomatic healthy carriers; Cases 4 and 5, PRCA; Case 6~9, MDS with low blast counts (RA); Cases 10~14, MDS with high blast counts (RAEB, RAEB in T and CMMoL), Cases 15~18, AML. The DNA size maker of lambda DNA digested with *Hin*dIII is shown at the left of the panel.

Figure. 5. Progression from MDS/RA to AML/M0 in Case 9. Bone marrow aspiration cytology at the diagnosis of MDS/RA (a) and that at the diagnosis of AML/M0 (b). Wright-Giemsa staining. x 450. Southern blot analysis for FeLV integration using the bone marrow DNA sample digested with *Bam*HI (c). Lane 1, DNA sample obtained at the diagnosis of MDS/RA; lane 2, DNA sample obtained at the diagnosis of AML/M0.

Figure. 6. Progression from suspected AA to MDS/RAEB in T in Case 12. Bone marrow aspiration cytology at the diagnosis of suspected AA (a) and that at the diagnosis of MDS/RAEB in T (b). Wright-Giemsa staining. x 450. Southern blot analysis for FeLV integration using the bone marrow DNA sample digested with *Bam*HI (c). Lane 1, DNA sample obtained at the diagnosis of MDS/RA; lane 2, DNA sample obtained at the diagnosis of MDS/RAEB in T.

Figure. 7. PCR amplification of U3 region of FeLV LTR from bone marrow and peripheral blood mononuclear cells from cats with MDS. PCR using the primer pairs Fe-1S and Fe-1R was performed using 1 mg of following genomic DNA samples: lane 1 (Case 1 RA), lane 2 (Case 2 RA), lane 3 (Case 3 RA), lane 4 (Case 4 RA), lane 5 (Case 5 RA), ane 6 (Case 6 RA), lane 7 (Case 7 RAEB), lane 8 (Case 8 RAEB),

lane 9 (Case 9 RAEB), lane 10 (Case 10 RAEB), lane 11 (Case 11 RAEB in T), llane 12 (Case 12 CMMoL), ane lane 13 (Case 13 CMMoL), and lane 14 (an uninfected feline born marrow tissue as control). The PCR products were subjected to electrophoresis in 3.0% agarose gel and staind with ethidium bromide.

Figure. 8. Upstream region of enhancer (A), and enhancer region (B) and promoter region (C) of LTR of FeLV. Comparison of the nucleotide sequences of LTRs of various FeLV isolates. Sequences shown include FeLV-A/Glasgow-1, FeLV-B/GM-1, FeLV-C/Sarma, and FeLV LTRs derived from MDS in the present study. The sequences are compared with that of FeLV-A/Glasgow-1, and only the different nucleotides are shown. Dashes indicate gaps which are introduced to optimize the alignment because of the insertions and deletion. The inverted repeats, transcriptional initiation (CCAAT, TATAAAA) sequences, presumed site for 3' capping (GCGCG), and nuclear protein binding site in the FeLV LTRU3 are indicated (LVb, SV40CORE, NF1, GRE and FLV1).

Figure. 1

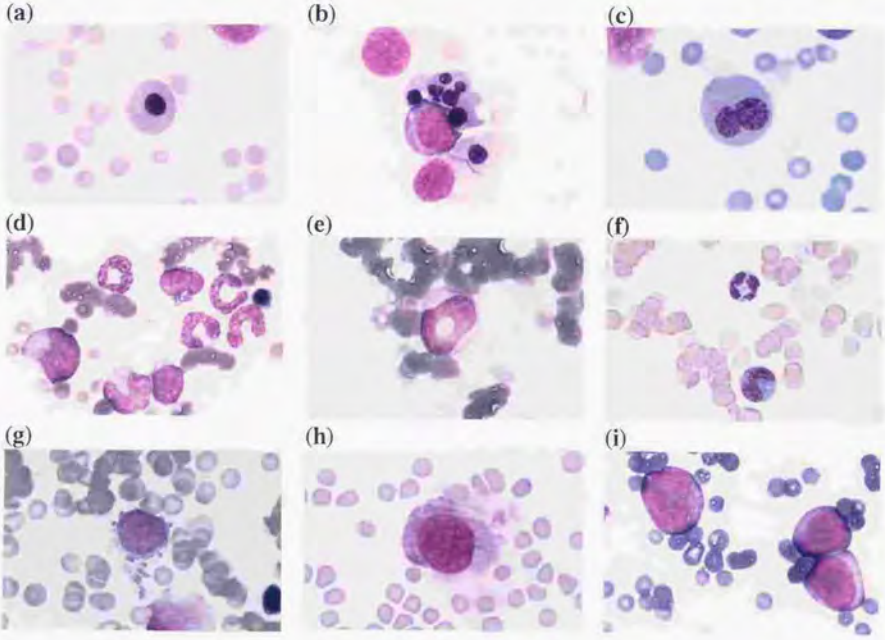
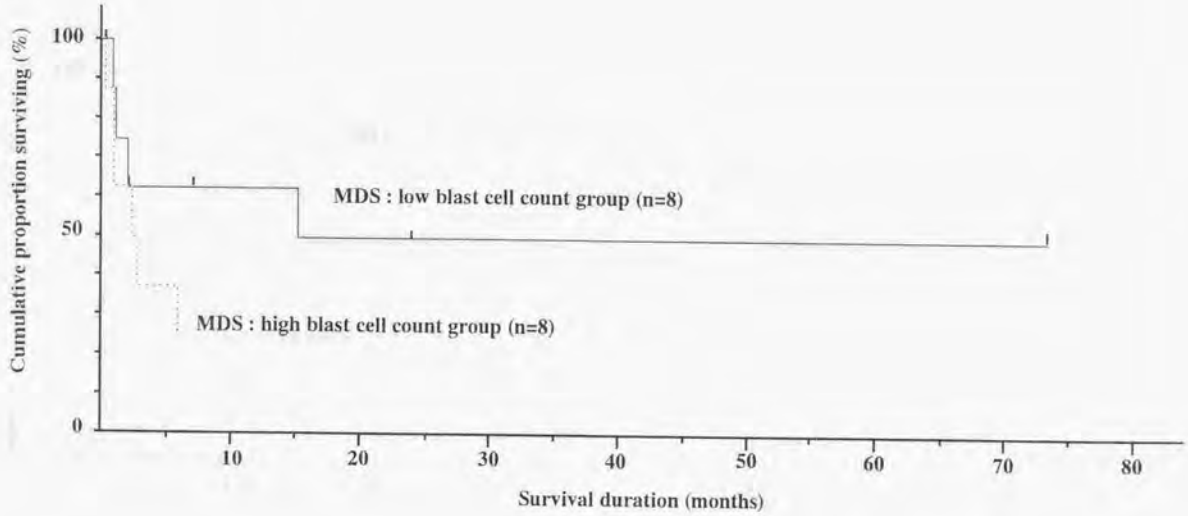


Figure. 2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MDS : low blast cell count group (n=8)
MDS : high blast cell count group (n=8)
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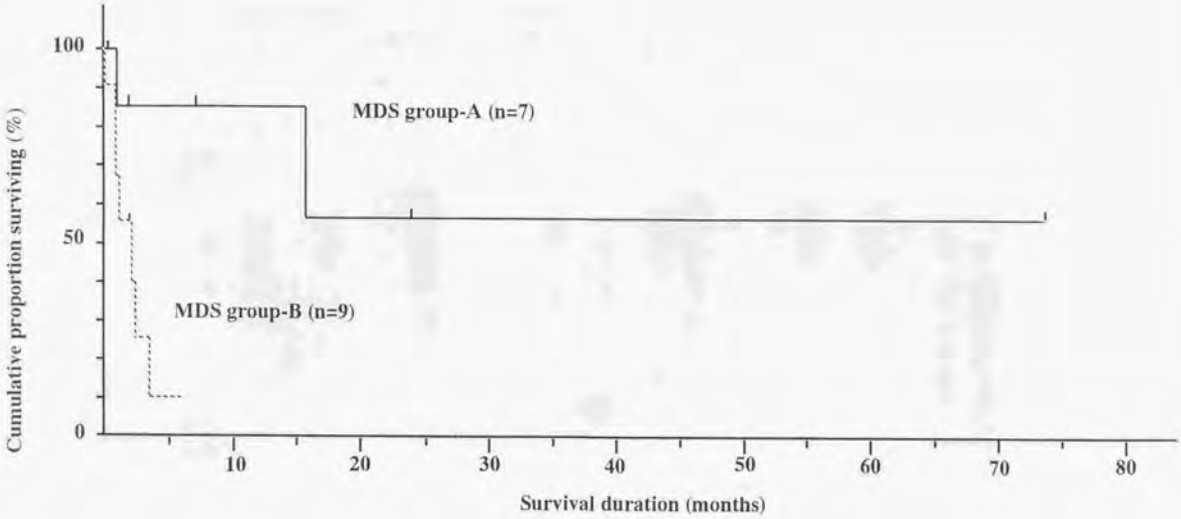
50
60
70
80
Survival duration (months)

Figure. 3


100
50
0
Cumulative proportion surviving (%)
MDS group-A (n=7)
MDS group-B (n=9)
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20
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40
50
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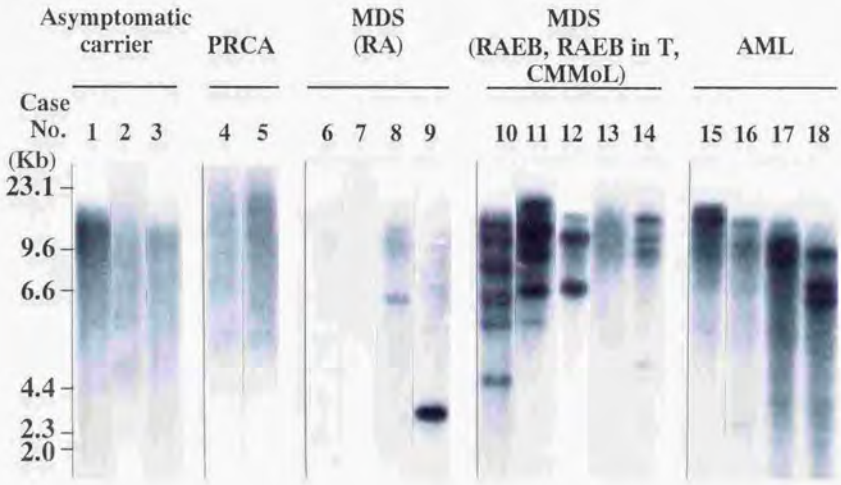
80
Survival duration (months)

Figure.4. (A)


Asymptomatic carrier
PRCA
MDS (RA)
MDS (RAEB, RAEB in T, CMMoL)
AML
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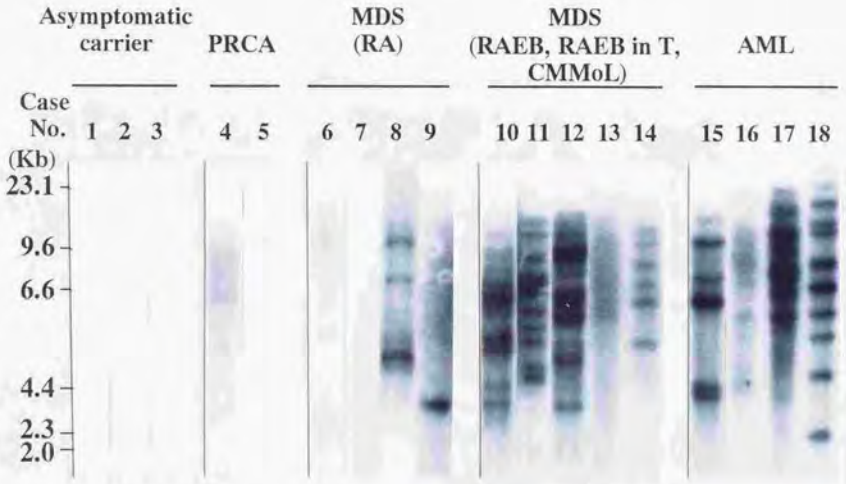
6.6
4.4
2.3
2.0

Figure.4. (B)


Asymptomatic carrier
PRCA
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MDS (RAEB, RAEB in T, CMMoL)
AML
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(Kb)
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9.6
6.6
4.4
2.3
2.0

Figure. 5

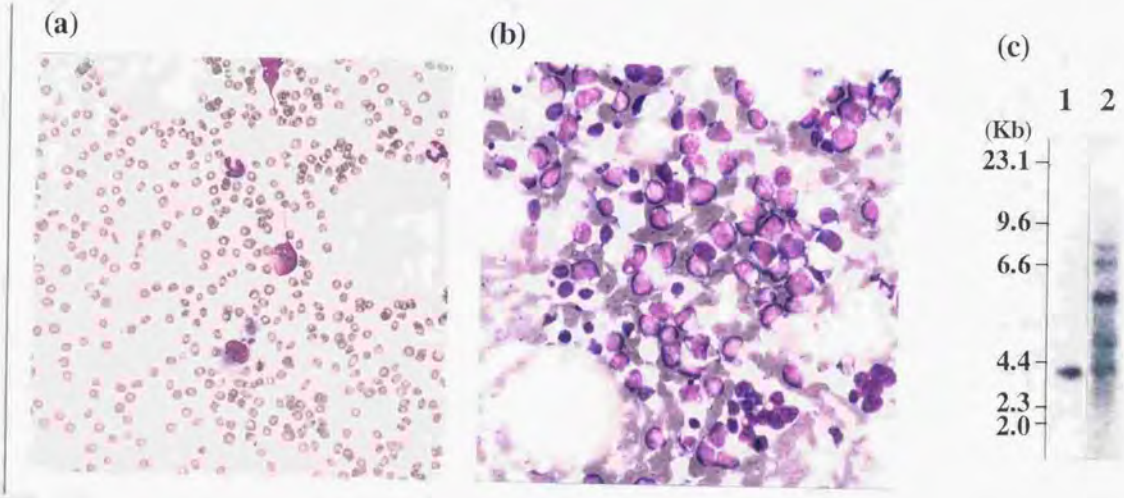


Figure. 6

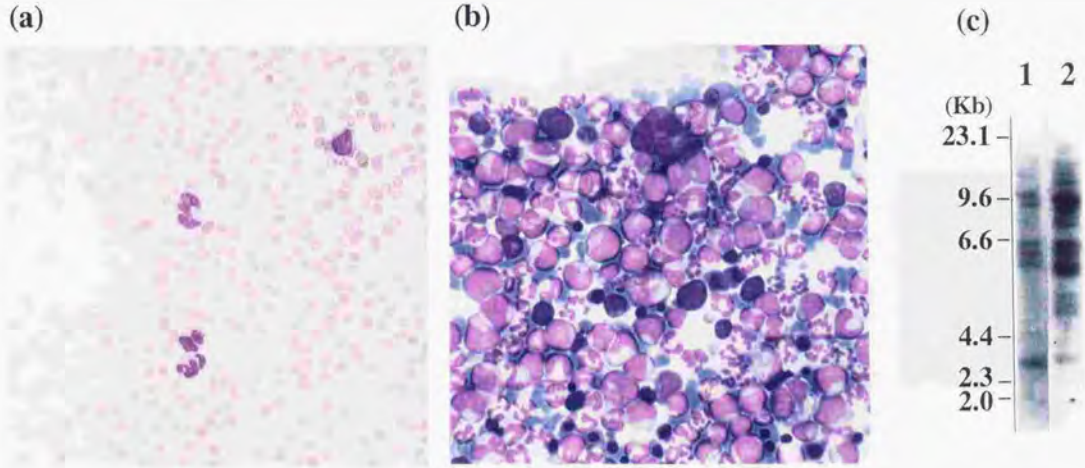


Figure. 7

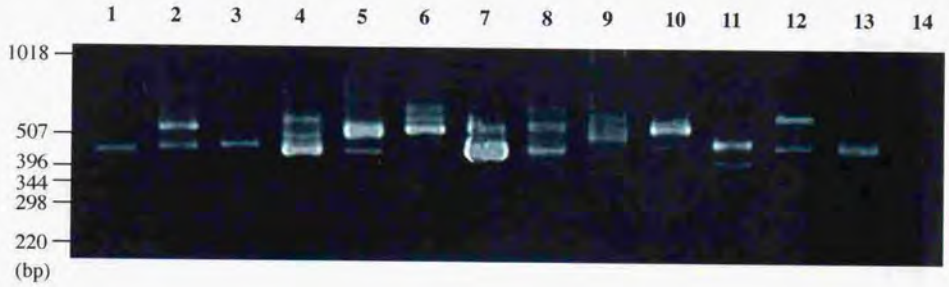
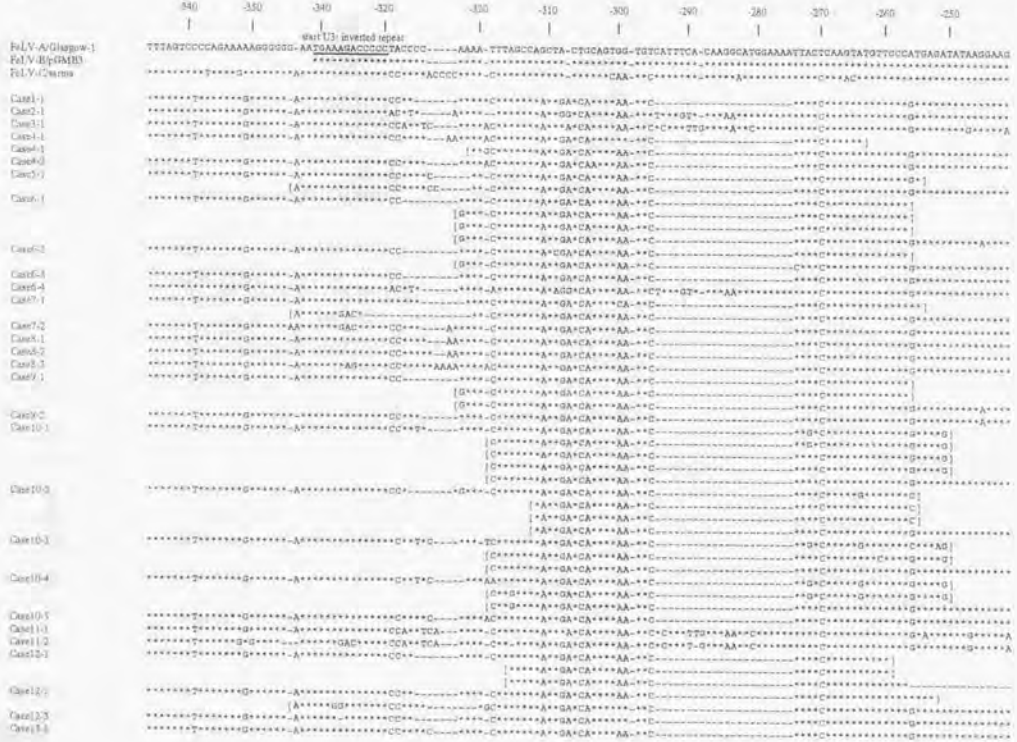


Figure. 8 (A)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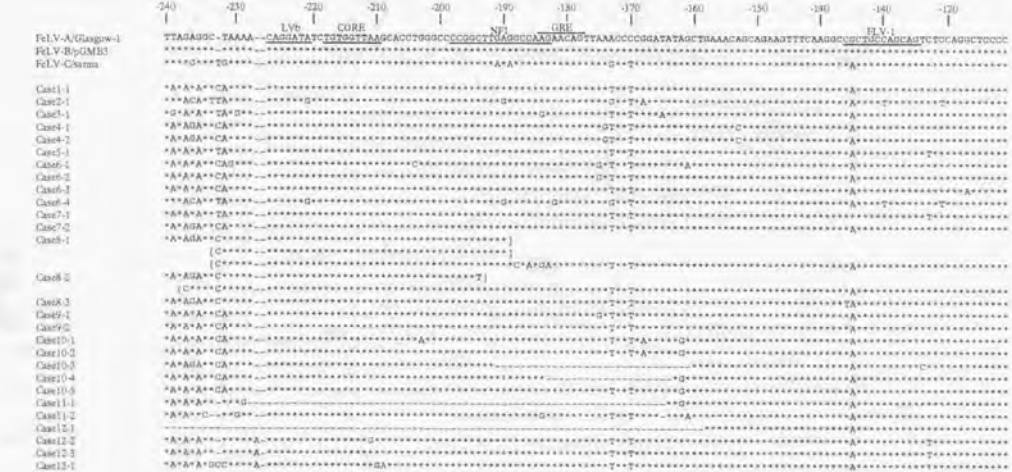
FeLV-B/pGMB3
FeLV-C/Sarma

Figure. 8 (B)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LVb
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NF1
GRE
FLV-1
FeLV-A/Glasgow-1
FeLV-B/pGMB3
FeLV-C/Sarma
Case1-1
Case2-1
Case3-1
Case4-1
Case4-2
Case5-1
Case6-1
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Case6-3
Case6-4
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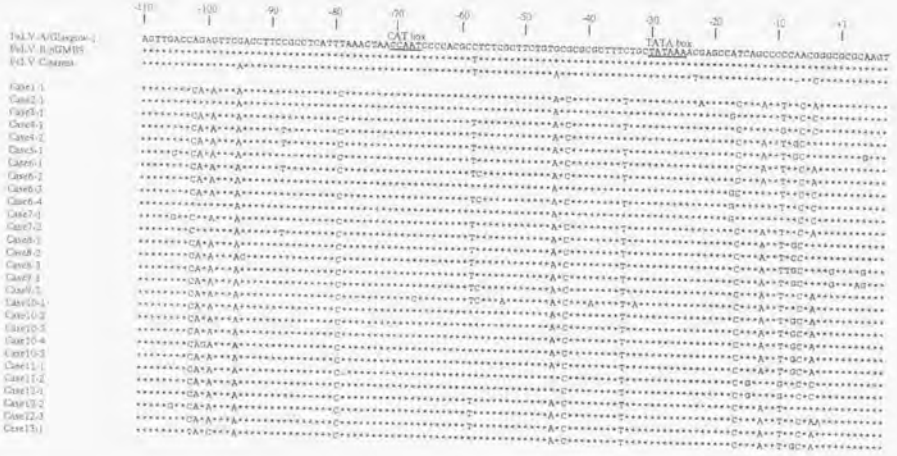
Figure. 8 (C)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+1
CAT box
TATA box
TnLV-A/Glasgow-1
PnLV-B/GMHS
PnLV-C/anma
Case1-1
Case2-1
Case3-1
Case4-1
Case4-2
Case5-1
Case6-1
Case6-2
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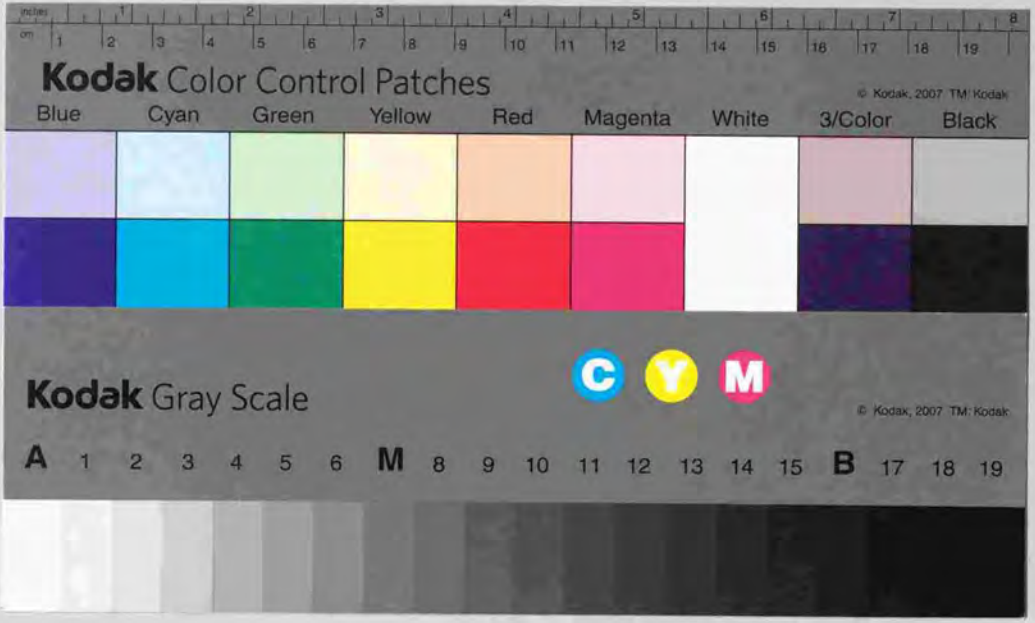
inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
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Magenta
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C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19