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Centrosome Amplification and Chromosomal Instability in Feline Lymphoma Cell Lines

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ABSTRACT. To evaluate the presence of centrosome amplification and the resulting chromosomal instability in cat tumors, a newly established feline lymphoma cell line and four already established feline lymphoma cell lines were examined using immunohistochemical analysis of centrosomes. The number of chromosomes were subsequently counted by metaphase spread. Moreover, to explore whether mutational inactivation of the *p53* gene or inactivation of the P53 protein caused by *mdm2* gene overexpression, occurred in the feline lymphoma cell lines, mutational analysis of the feline *p53* gene was carried out. The expression of feline *mdm2* mRNA was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Centrosome amplification and chromosomal instability was observed in three out of the five feline lymphoma cell lines. Of these three feline lymphoma cell lines, one had aberrations in the P53 amino-acid sequence, whereas the others had none. There was no significant difference in the expression of *mdm2* mRNA between peripheral blood mononuclear cells (PBMC) obtained from a normal cat and that of the five feline lymphoma cell lines. These findings indicate th at centrosome amplification also occurs in cat tumors and is strongly correlated with chromosomal instability, suggesting that the immunostaining of centrosomes could be an alternative method for the examination of the chromosomal instability. Furthermore, this study s uggests the presence of unknown mechanism that leads to the centrosome amplification in feline lymphomas.

KEY WORDS: centrosome, chromosome, feline, lymphoma, p53.

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A centrosome is a major microtubule organizing center of the animal cell and plays a vital role in spindle pole formation, which is essential for balanced chromosome segregation. Centrosome amplification (more than 3 centrosomes are contained in a cell) has been frequently observed in human and dog tumors [1, 32], and is thought to be a major cause of chromosomal instability [1, 19, 29]. Chromosomal instability and the resulting aneuploidy, like other types of genomic instability, is considered to be important for multistep tumorigenesis through the accumulation of alterations responsible for malignant phenotype [15, 22, 30]. Although a number of chromosomal abnormalities have been revealed in cat tumors [9, 10, 42], the centrosome amplification has yet to be explored.

An aberration of the *p53* tumor suppressor gene is one of the most frequently occurring gene mutations in a variety of human and dog tumors (more than 50% in human tumors) [14, 18, 33]. It has been shown that the P53 tumor suppressor protein is involved in the regulation of the centrosome checkpoint at both the G1/S [11, 16, 40] and the G2/M transition phases [4, 28, 36, 39] of the cell cycle, and loss or mutational inactivation of *p53* results in abnormal amplification of centrosomes [7, 8]. Moreover, mouse double minute 2 (MDM2) induced by P53 is known to be a negative-regulator of P53 function, and promotes P53 degradation [12, 17]. Several types of P53 inactivation, such as point mutation, deletion of the *p53* gene, and inactivation of the P53 protein induced by overexpressed MDM2, which contributes to centrosome amplification, have been frequently observed in human and dog tumors [1, 32].

Neoplastic diseases, especially those occurring of the hematopoietic system, are frequently encountered in cats [38] and are one of the major problems faced by veterinarians in small animal practice. Feline leukemia virus (FeLV) infection promotes tumorigenesis in cats by the activation of the *c-myc* gene [6, 24, 25]. However, activation of the *c-myc* gene alone is not sufficient in inducing tumors [27]; these feline hematopoietic tumors may be formed through a multi-step tumorigenesis process like human colon carcinomas [5]. In mouse erythroleukemias induced by murine leukemia virus, rearrangements and retroviral insertional inactivation of the *p53* gene have been noted [13]. However, these types of *p53* aberration have not been found in feline lymphomas [26]. Furthermore, it is known that mutational inactivation of the *p53* gene occurs less frequently in feline lymphomas as compared to human and dog tumors [26]. Because the abrogation of P53 function is one of the most important processes in human and dog tumors, other mechanism resulting in the loss of the P53 function might be involved in feline lymphoma tumorigenesis.

Recently, it has been shown that the centrosome amplification is reduced in prolonged cell cultures [3]. Therefore, we established a new feline lymphoma cell line (R96) for use in this study. Using the R96 cell line and 4 already established cell lines, we evaluated the presence of centrosome amplification, which results in chromosomal insta-

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bility in feline lymphoma cell lines. We also examined whether *p53* mutation or inactivation of P53 caused by *mdm2* gene overexpression occurs in the feline lymphoma cell lines.

MATERIALS AND METHODS

Cells: Tumor cells were isolated from a mix-breed cat with multicentric lymphoma that was referred to the Veterinary Hospital of Yamaguchi University. Mononuclear cells were isolated by gradient centrifugation (Lymphoprep, NYCOMED, Oslo, Norway) from the pleural effusion sample. The isolated cells were cultured in a tissue culture flask with RPMI1640 (GIBCO BRL, MD, U.S.A.) containing 10% fetal bovine serum (FBS, GIBCO BRL), and 100 U/m*l* Penicillin-Streptomycin (GIBCO BRL). The flask was incubated in a 37°C humidified chamber with 95 % air and 5% CO_2. The doubling time of the R96 cells was calculated by dividing the cells into 15 wells, diluting approximately 8 $\times 10^5$ cells/m*l*, and counting the number of cells 5 times (3 , 6, 9, 12 and 21 hr). At each counting, the R96 cells were counted 3 times, and the average was obtained. Other cell lines used in this study, KO-1 [20], 3201 [34], FT-1 [24], FL74 [21] were also cultured in the same medium as described above, and incubated under the same conditions. As a control, peripheral mononuclear cells (PBMC) were also isolated from a clinically healthy cat by gradient centrifugation, and cultured in a similar manner to the lymphoma lines.

FeLV antigen detection: FeLV virus antigen was tested from supernatant of the cell culture using Snap FeLV/FIV combo (IDEXX, ME, U.S.A.).

Indirect immunofluorescence: For all immunostaining except for co-immunostaining of γ-tubulin and β-tubulin, cells were washed three times in phosphate buffered saline (PBS, pH 7.2, 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$), and centrifuged on positively-charged glass slides (Fisher Scientific, PA, U.S.A.) for 5 min at 250 g at room temperature. After centrifugation, cells were immediately fixed in 10% formalin containing 10% methanol for 20 min at room temperature. The cells were then washed with PBS and permeabilized with 1% Nonident P-40 (Katayama, Tokyo, Japan) in PBS for 5 min at room temperature. Cells were first incubated with blocking solution (10% normal goat serum in PBS) for 1 hr and then probed with mouse anti-γ-tubulin monoclonal antibody (GTU-88, Sigma, MO, U.S.A.) for 1 hr. The antigen-antibody complex was detected after incubation for 1 hr at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probe, OR, U.S.A.). For co-immunostaining of anti-γ-tubulin antibodies, rhodamine-conjugated anti-γ-tubulin polyclonal antibody (C-20, SantaCruz, CA, U.S.A) was also used with the anti-γ-tubulin monoclonal antibody (GTU-88). The samples were washed three times with tris buffered saline (TBS, 9.9 mM tris, 0.15 M NaCl plus pH 7.4), and then counterstained with 4', 6-diamidino 2-phenylindole (DAPI) DNA dye. The number of centrosomes per cell were scored for >300 cells by three investigators.

For co-immunostaining of γ-tubulin and β-tubulin, cells on positively-charged glass slides were first placed on ice for 20 min to destabilize microtubules nucleated at the centrosomes. The cold-treated cells were then subjected to brief extraction (10 sec) with cold extraction buffer [0.75% Triton X-100, 5mM Pipes, 2mM EGTA (pH 6.7)], briefly washed in cold PBS, fixed and then incubated in blocking solution as described above. Cells were then incubated with rhodamine-conjugated anti-γ-tubulin polyclonal antibody (C-20) and mouse anti-β-tubulin monoclonal antibody (Tub 2.1, Sigma). The antigen-antibody complex was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probe) for γ-tubulin.

Metaphase spread and chromosome counting: Metaphase spread was prepared as described previously [8]. Briefly, cells were incubated in the presence of 0.5 μg/m*l* colcemid (Sigma) for 2 hr to enrich mitotic cells. The cells were pelleted by centrifugation. The cell pellet was gently resuspended in the hypotonic solution (0.075 M KCl) and allowed to stand for 10 min at 37°C. After removal of hypotonic solution, a methanol-acetic acid (3:1) fixative was added, and cells were allowed to stand for 5 min. After discarding the old fixative, fresh fixative was added. This procedure was repeated a total of three times. After the last change of fixative, a few drops were put onto a positively-charged glass slide (Fisher Scientific) and dried completely on a 37°C hot plate. The dried samples were subjected to Giemsa staining and analyzed under a light microscope. For each cell line and for the control PBMC, more than 300 cells were independently examined by three investigators.

Statistical analysis: Variance ratio of the chromosome numbers of the each cell line against that of the control PBMC was obtained using the test for equal varians (*F* test). The correlation between the percentages of centrosome amplification and the variance ratio was examined by simple linear correlation.

Sequence analysis of the p53 gene: Total RNA was extracted (RNeasy Mini Kit, QIAGEN, Hilden, Germany), and then reverse transcribed using an oligo-dT adapter primer (AP, 3' RACE System for Rapid Amplification of cDNA ENDs kit, GIBCO BRL). For PCR amplification, primers were designed based on the sequence of the feline p53 gene [26], which covered the entire coding region. The sequences of primers used for amplifying the 5' portion were cSP53–1 (corresponding to nt. 67–85 of cat p53, GenBank accession number D26608) and cAP53–1 (nt. 755–737). The 3' porton was amplified with primers, cSP53–2 (nt. 683–700) and cAP53–2 (nt. 1363–1346). The cDNA was amplified by PCR in a volume of 50 μ*l*, using each pair of primers (25 μM each), 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, U.S.A.) and the reagents as recommended by the manufacturer. After the initial hot start (94°C, 9 min), amplification was performed by 30 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), and polymerization (72°C, 1 min). The

resulting products were subjected to direct sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The nucleotide sequence of each fragment was determined for both DNA strands in opposite direction.

Expression of mdm2 mRNA in feline lymphoma cell lines: For the amplification of a part of feline *mdm2* and detection of its expression pattern in the cell lines, reverse transcription of 1 μg total RNA was performed (3' RACE System for Rapid Amplification of cDNA ENDs kit, GIBCO BRL). After the initial hot start (94°C 9 min), PCR amplification was performed by 20 or 30 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C. For PCR, gene-specific primers, cMDMS (corresponding to nt. 747–765 of cat *mdm2*, GenBank accession number AB099709) and cMDMA (nt. 951–933), were used. A primer pair, FBAIS (corresponding to nt. 284–307, GenBank accession number AB051104) and FBAIR (nt. 672–649) was used to amplify 389 bp feline β-actin as a control for cDNA synthesis. PBMC isolated from the blood of normal cat by gradient centrifugation was also used as a control.

RESULTS

Establishment of a new feline lymphoma cell line: Tumor cells in the pleural effusion of a cat with multicentric lymphoma were cultured. Four weeks after initiation of the culture, active proliferation of cells was observed, and the cells could be subcultured. This established cell line designated as R96, grew well in suspension with a doubling time of 31.8 hr. The R96 cells have been maintained in RPMI1640 medium with 10% FBS for more than a year.

FeLV antigen detection: FeLV antigen was detected from supernatant of R96, FT-1 and FL74 cell lines, but not from the other cell lines (KO-1 and 3201).

Centrosome amplification in feline lymphoma cell lines: To examine whether centrosome amplification is common in feline lymphoma cell lines, five feline lymphoma cell lines were immunostained for centrosomes, using anti-γ-tublin monoclonal antibody (GTU-88). The γ-tubulin is a major component of centrosomes, and immunostaining for γ-tubulin detects centrosomes throughout the cell cycle in human and murine cells [35, 44]. Although γ-tubulin is a highly conserved protein among species, to the best of our knowledge, no study has been conducted on the immunostaining of centrosomes using anti-γ-tublin antibody in feline cells. In this study, for the examination of immunohistochemical reactivity of the anti-γ-tublin monoclonal antibody (GTU-88) in feline cells, we performed co-immunostaining with anti-γ-tublin monoclonal antibody (GTU-88) and anti-γ-tubulin polyclonal antibody (C-20) using R96 cells (Fig. 1A, a-a''). The dot signals of immunostaining using each antibody were observed at the same component on or adjacent to the nuclear membrane where centrosomes are typically localized. Moreover, co-immunostaining with anti-γ-tubulin polyclonal antibody (C-20) and anti-β-tubulin monoclonal antibody (Tub 2.1) showed that most of the dot signals detected by the anti-γ-tubulin polyclonal antibody (C-20) contained two anti-β-tubulin-reactive dots representing a pair of centrioles at a high magnification (Fig. 1A, b and b'). This confirms that these anti-γ-tubulin antibodies specifically stain the centrosomes in feline cells.

Figure 1A (c and d) shows a representative immunostaining of γ-tubulin in R96 cells. Immunohistochemical analysis with anti-γ-tubulin antibody revealed that >30% of R96 cells and >20% of 3201 cells contained more than 3 centrosomes, indicating noticeable centrosome amplification in these two cell lines (Fig. 1B). Approximately 10% of the FL74 cells contained more than 3 centrosomes indicating the possibility of centrosome amplification in the FL74 cell line. Like the PBMC isolated from a clinically healthy cat, KO-1 and FT-1 cells with an abnormal number of centrosomes (≥3 centrosomes) were less common.

Chromosomal instability in the feline lymphoma cell lines: Figure 2A shows representative microphotographs of euploid, hypoploid and hyperploid chromosomes taken from the R96 cell line. In the R96 and 3201 cell lines, the number of chromosomes were extensively expanded, and deviated from the normal number (2N=38), indicating that the newly established R96 cells and 3201 cells have extensive chromosomal instability (Fig. 2B). Chromosome numbers of FL74 were moderately expanded, indicating that chromosomes were also unstable in the FL74 cell line (Fig. 2B). In the FT-1 cells, the distribution of the number of chromosomes per cell was limited in the culture (approximately 70% of FT-1 cells containing 37 chromosomes) (Fig. 2B). Furthermore, the distribution of the number of chromosomes per cell was even more limited in the KO-1 cell line (approximately 80% of KO-1 cells containing 41 chromosomes) (Fig. 2B).

Correlation between centrosome amplification and chromosomal instability: To explore the degree of chromosomal instability, the variance ratios of the chromosome numbers of each cell line against those of the control PBMC were calculated using the *F* test. The variance ratios of R96, KO-1, 3201, FT-1 and FL74 were 12.9, 1.62, 9.81, 1.73 and 8.35, respectively, indicating that the R96, 3201 and FL74 cell lines have greater chromosomal instability compared to the control PBMC. Next, we examined the correlation between the percentages of centrosome amplification and variance ratios by simple linear correlation. A significant correlation was observed between centrosome amplification and chromosomal instability in feline lymphoma cell lines ($r=0.91$, $P<0.05$).

Sequence analysis for cat p53: PCR amplifications for the 5' portion of *p53* using cSP53–1 and cAP53–1 resulted in the production of a DNA fragment of expected size in the five-lymphoma cell lines (689 bp). PCR for the 3' portion of *p53* using cSP53–2 and cAP53–2 also yielded major bands of expected size (681 bp) in all cell lines. These two DNA fragments had an overlapping sequence of 73 bp. The nucleotide sequence of each DNA fragment was determined by direct sequencing. Table 1 summarizes the results of the sequence analysis for cat *p53* in the five-lymphoma cell

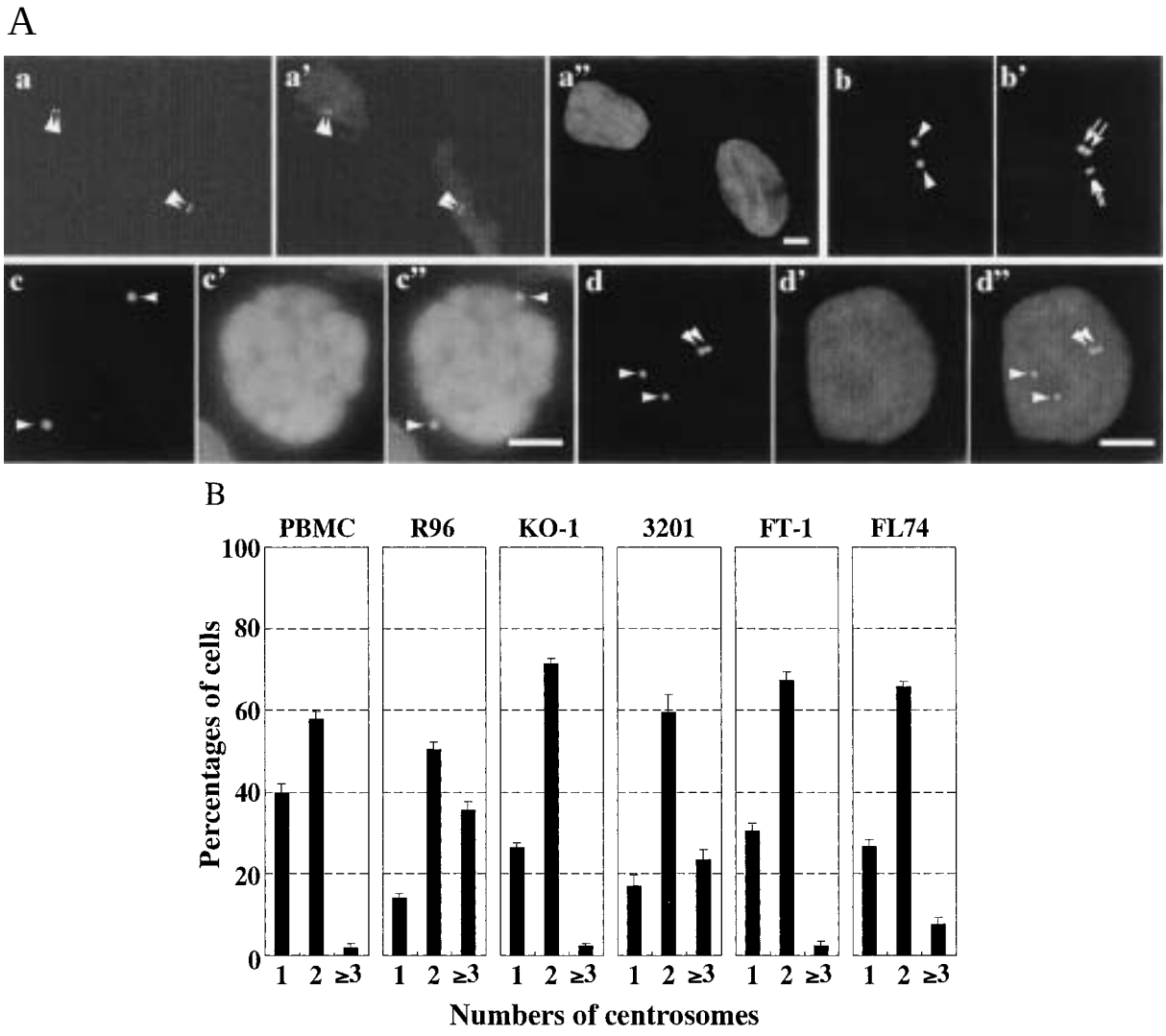

Fig. 1. Centrosome amplification in cat lymphoma cell lines. (A) Representative immunostaining of centrosomes in R96 cells. Panels a, a', and a'' reveal immunostaining of centrosomes using of anti-γ-tubulin monoclonal antibody (GTU-88, Sigma), anti-γ-tubulin polyclonal antibody (C-20, SantaCruz), and DAPI DNA dye counterstain in the same microscopic field, respectively. Panels b and b' sh ow immunostaining of centrosomes with anti-γ-tubulin polyclonal antibody (C-20), and of centrioles with anti-β-tubulin monoclonal antibody (Tub 2.1, Sigma) in the same microscopic field, respectively. For centrosome counting, cells were immunostained with anti-γ-tubulin antibody (GTU-88) (panels c and d), and were also counterstained with DAPI for visualization of DNA (panels c' and d').Panels c'' and d'' show the overlay images of the γ-tubulin immunostainings and DAPI stainings. Panels c and d show the R96 cells which contain two and four centrosomes, respectively. Bars = 3 μm. Arrowheads and arrows show centrosomes and centrioles, respectively. (B) Distribution of centrosomes in clinically healthy cat PBMC and five lymphoma cell lines. Centrosome numbers of each cell was examined by immunostaining of γ-tubulin and were counted under a fluorescence microscope. At least 300 cells were examined for the control PBMC and each cell line.

lines. In the R96 cell line, there was a silent point mutation at nt. 591 (from C to T). In the KO-1 cell line, there was a heterozygous point mutation at nt. 231 (from A to G), where guanine (mutant allele) and adenine (normal allele) waves were observed at nt. 231 (Fig. 3A). This confirms the fact that direct sequencing as used in this study can detect mutations on both alleles. In the 3201 cell line, there was a missense point mutation resulting in a substitution of cysteine[235] for alanine without heterozygosity (Fig. 3B), as has been previously reported [26]. In the FT-1 cell line, there was a silent point mutation at nt. 591 (from C to T), and in FL74 cell line, there were three silent point mutations at nt. 231 (from A to G, heterozygous), nt. 591 (from C to T) and nt. 1176 (from G to A). All the lymphoma cell lines used in this study had a genetic mutation of *p53*, but the 3201 lymphoma cell line had a substitution of the amino-acid sequence.

Expression of mdm2 mRNA in feline lymphoma cell lines: In the 30 cycle amplification, as shown in Fig. 4A, bands

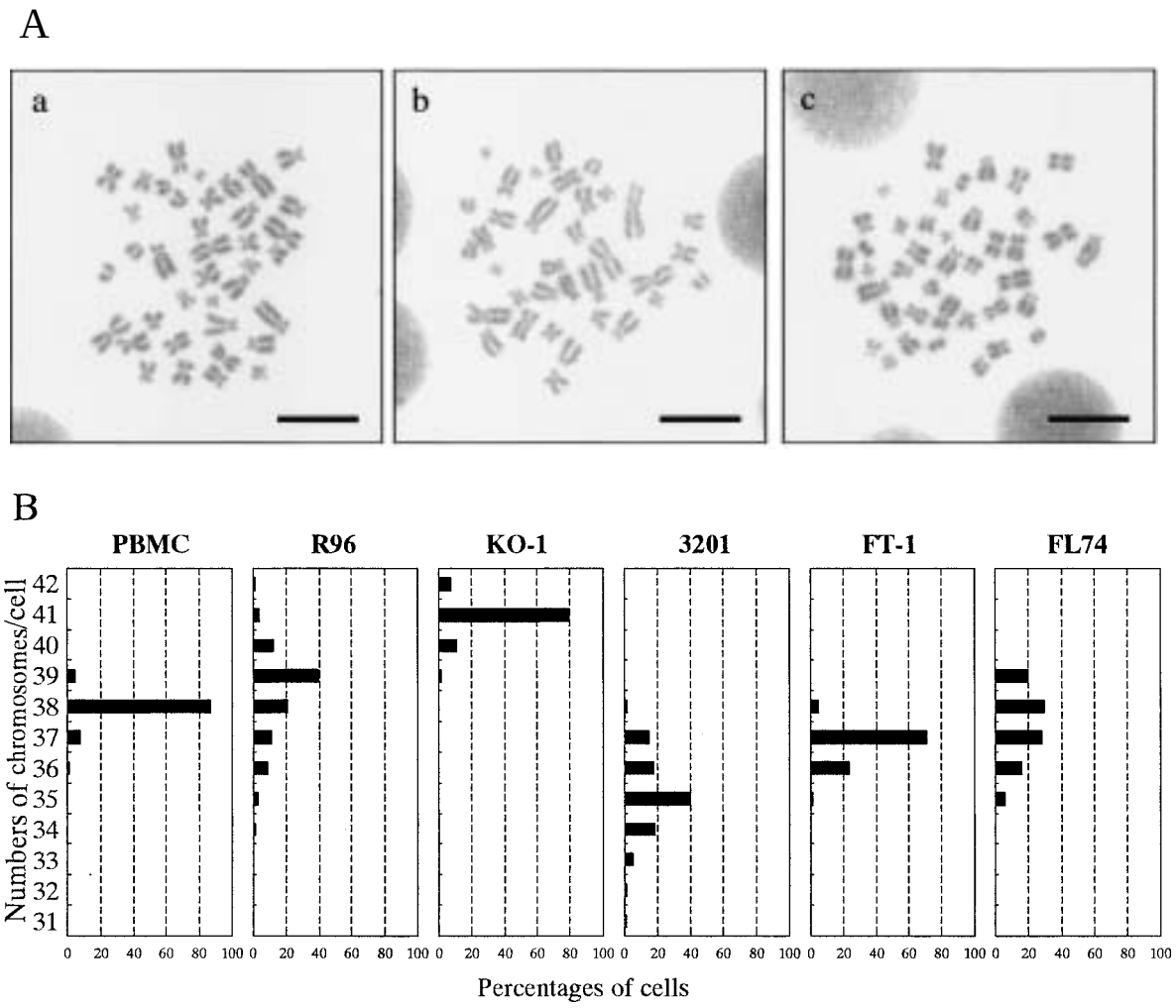

Fig. 2. Chromosomal instability in cat lymphoma cell lines. (A) Representative metaphase spreads from R96 cells. Metaphase spreads were prepared from R96 cells, stained with Giemsa, and examined under a light microscope. Panel a shows a typical euploid spread (2N=38). Panel b shows a typical hypoploid spread (2N=35). Panel c shows a typical hyperploid spread (2N=40). Bars=5 μm. (B) Distribution of chromosomes in clinically healthy cat PBMC and five lymphoma cell lines. Metaphase cells, enriched by treatmentof colcemid for 2 hr, were harvested and metaphase chromosome spreads were prepared. The Giemsa-stained chromosomes were directly analyzed under a light microscope. At least 300 metaphase spreads were examined for the control PBMC and each cell line.

Table 1. Mutations of *p53* cDNA in 5 lymphoma cell lines

Cell lines	Mutations	
	Nucleotides	Amino acids
R96	TAC→TAT	Tyr^{155}→Tyr (silent)
KO-1	GAA[a)]→GAG	Glu^{35}→Glu (silent)
	GAA	
3201	TGC→CGC	Cys^{235}→Ala
FT-1	TAC→TAT	Tyr^{155}→Tyr (silent)
FL74	GAA[a)]→GAG	Glu^{35}→ Glu (silent)
	GAA	
	TAC→TAT	Tyr^{155}→Tyr (silent)
	AAG→AAA	Lys^{350}→Lys (silent)

a) Heterozygous.

derived from *mdm2* mRNA were clearly detected in the control cells isolated from a healthy cat and in all lymphoma cell lines. There were no differences in the depth of bands among the control cells and the lymphoma cell lines. In the amplification of 20 cycles, very faint bands derived from *mdm2* mRNA were detected in both the control and the five lymphoma cell lines (Fig. 4B). There were also no differences in the depth of bands among the control and the lymphoma cell lines. The data suggest that none of the lymphoma cell lines used in this study were accompanied by MDM2 overexpression, which could contribute to inactivation of P53.

DISCUSSION

Chromosomal instability is frequently observed in a variety of human cancers and has been shown to correlate with the acquisition of malignant phenotypes [2]. Centrosome amplification, and the resulting increase in the frequency of aberrant mitoses, is a major cause of chromosomal instability in human cancers [1, 19, 29]. Centrosome amplifications were observed in the R96, 3201 and FL74 lymphoma cell lines (Fig. 1B & Table 2). To the best of our knowledge, this is the first report of centrosome amplification in cat tumors. Moreover, chromosome instabilities were also observed in the R96, 3201 and FL74 lymphoma cell lines (Fig. 2B & Table 2). In contrast, the KO-1 and FT-1 cell lines showed no difference in the magnitude of centrosome amplification or chromosomal instability as compared to PBMC isolated from a clinically healthy cat (Table 2). Statistical analysis revealed that a strong correlation exists between centrosome amplification and chromosomal instability in feline lymphoma cells. In order to perform metaphase spreads, the method for counting the chromosome-number per cell, there is a need to culture tumor cells in order to increase number of the mitotic cells. In contrast, immunostaining for centrosomes is easy to perform on a routine clinical basis as compared to the metaphase spreads. The strong correlation between centrosome amplification and chromosomal instability indicates that centrosome immunostaining may be an alternative method for the exam-

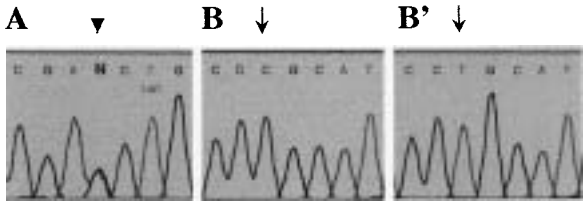

Fig. 3. Analysis of *p53* gene sequences in feline lymphoma cell lines. A. Heterozygous sequences of *p53* gene of R96 cell line. Waves of adenine and guanine are observed at nt. 591 (an arrow head), showing that mutation occurs in one allele. B. Point mutation of *p53* gene of 3201 cell line. A point mutation is indicated by an arrow (B) compaired with the wild-type sequence (B').

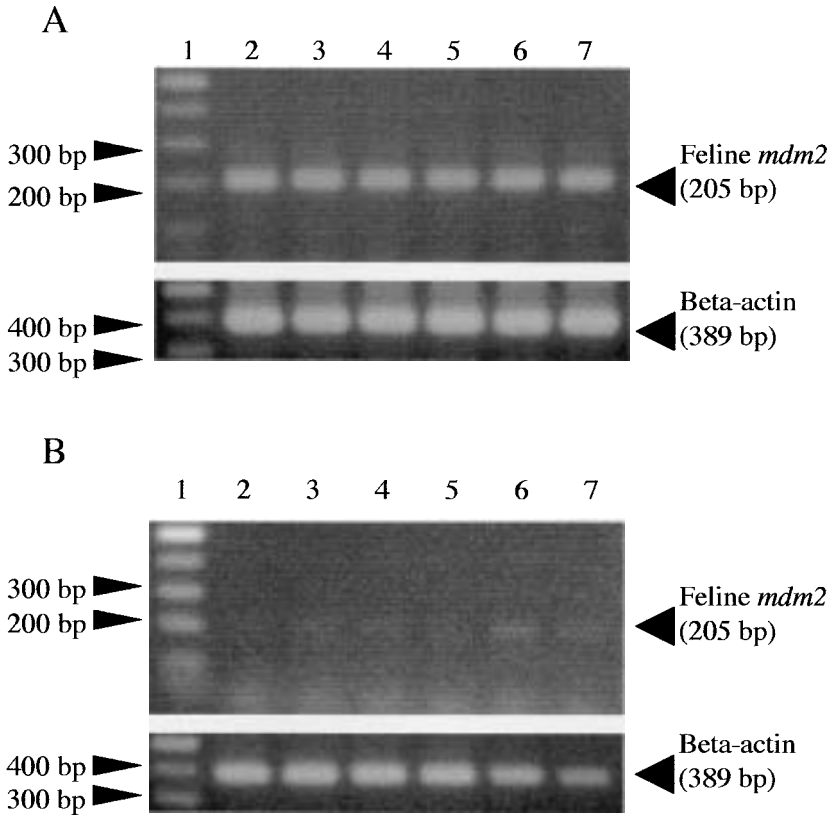

Fig. 4. RT-PCR analysis for *mdm2* mRNA. Reverse transcription of 1 μg RNA samples extracted from control PBMC and five lymphoma cell lines was performed. PCR amplifications were performed by 30 cycles (Panel A) and 20 cycles (Panel B) of denaturation (1 min 94°C), annealing (1 min 54°C) and polymerization (1 min 72°C), after an initial hot start (9 min 94°C). Feline β-actin cDNA was also amplified for each amplification cycle as a control. Lane number 1–7 indicates DNA ladder, PBMC, R96, KO-1, 3201, FT-1 and FL74, respectively.

Table 2. Centrosome hyperamplification and *p53* mutation in feline lymphoma cell lines

Cell line	Centrosome	Chromosome (mean ± SD)	*p53* *	*mdm2*	FeLV antigen
R96	++	38.33 ± 1.41	–	–	+
KO-1	–	40.98 ± 0.43	–	–	–
3201	++	34.94 ± 1.24	+	–	–
FT-1	–	36.75 ± 0.56	–	–	+
FL74	+	37.50 ± 1.09	–	–	+

* Mutations with amino-acid substitutions.

ination of chromosomal instability, which is correlated with malignant phenotypes [2].

In this study, we examined the correlation between centrosome amplification, p53 mutation and inactivation of P53 caused by *mdm2* overexpression. RT-PCR analysis revealed the absence of *mdm2* mRNA overexpression in all the cell lines (see below). In the 3201 cell line, mutational inactivation of *p53*, and centrosome amplification as well as chromosomal instability, was detected. This indicates that inactivation of P53 was caused by gene mutations which resulted in centrosome amplification in the cell lines (Table 2). These observations suggest that the aberration of P53 function also lead to centrosome amplification and chromosomal instability in cat tumors as in human and dog tumors. In contrast, no change was observed in the predicted amino-acid sequence of *p53* cDNA in the KO-1 and FT-1 cell lines, and no difference in the magnitude of centrosome amplification or chromosomal instability as compared to PBMC isolated from a clinically healthy cat was observed (Table 2). As for the 3201, KO-1 and FT-1 cell lines, the observations were consistent with previous studies showing that centrosome amplification and the resulting chromosomal instability are associated with the loss of functional P53 [7, 8, 41].

Interestingly, the newly established R96 cell line and FL74 cell line had no amino-acid substitution of P53, but showed significant centrosome amplification and obvious chromosomal instability (Table 2). The results of RT-PCR for *mdm2* also showed that expression levels of mdm2 mRNA in the cell lines were not any different from PBMC isolated from a healthy cat and other lymphoma cell lines. Therefore it can be concluded that overexpression of *mdm2* did not occur in the R96 and FL74 cells. No expression of MDM2 protein was detected in the R96 and FL74 cells, the other cell lines or the control cells as determined by immunostaining for feline MDM2 using polyclonal antibody against the C-terminal of human MDM2 which is very similar to the amino-acid sequence of feline *mdm2* (data not shown). Eventually, noticeable centrosome amplification and chromosomal instability were observed in the R96 and FL74 cells, although both mutational inactivation of *p53* and inactivation of P53, mediated by MDM2 overexpression did not occur. There are two possibilities to explain this phenomenon: (1) unknown multiple factors may be involved in centrosome homeostasis apart from P53 functions, or (2) unknown mechanisms related with P53 functions are in play, such as inactivation of downstream targets of P53 other than MDM2, or the inactivation of P53 by P53 associated proteins which inhibit P53 function. As for the second possibility, it has been revealed that some viral proteins such as SV 40 large T antigen, adenoviral E1B and human papilloma viral E6, bind to P53 and inactivate the functional P53 [23, 31, 43]. Because the R96 and FL74 cells produce FeLV, it is possible that the viral proteins may bind to P53 and inactivate the functional P53. Clonal integration of proviral FeLV at the loci of proto-oncogenes and common integration regions such as *c-myc*, *flvi-2 (bmi-1)*, *fit-1* and *pim-1* have been shown to be associated with the development of cat lymphomas [25, 37]. The *myc* gene is frequently activated by FeLV(32%) either by transduction or by proviral insertion [37]. However, the activation of the *myc* gene alone is not sufficient in inducing the tumors, strongly suggesting a multistep process of FeLV leukemogenesis [27]. It has been seen that the aberration of *p53* occurs less frequently in spontaneous cat lymphomas as compared to tumors in humans and dogs [26]. These observations may suggest that the viral protein produced by FeLV binds to P53 and is associated with tumorigenesis in cat lymphomas. It seems that FT-1 cells retaining the wild type of P53 and showing normal centrosome behavior inspite of FeLV infection may be contradictory to this hypothesis. However, the FT-1 cell line was established more than 20 years ago [24], and thus, chromosomal instability may have been reduced during the long term cell culturing process. The hypothesis is currently under investigation in our laboratory.

In this study, we found mutations of *p53* with and without amino-acid substitutions in all the five feline lymphoma cell lines examined. It is known that the aberration of *p53* occurs less frequently in spontaneous cat lymphoma [26]. These findings strongly support the possibility of *p53* mutations, which were derived from the long term cell culturing. Actually, the aberrations of *p53* in the lymphoma cell lines were rather similar in each cell line (Table 1). The data suggest that further analyses are necessary to examine the correlation between the aberration of P53 and centrosome amplification using spontaneous cat lymphomas.

In conclusion, through this study it can be seen that centrosome amplification occurs in cat lymphoma cell lines and is strongly correlated with chromosomal instability. It was also revealed that centrosome immunostaining may be an alternative method for the examination of chromosomal instability, which in turn is correlated with malignant phenotypes. Furthermore, in this study it was observed that

centrosome amplification occurred despite none of p53 mutations involving amino-acid substitution or inactivation of P53 mediated by MDM2 overexpression. It is of great importance to further elucidate the molecular linkage between P53 functions, FeLV proteins and other proteins, which might be involved in centrosome homeostasis, using spontaneous cat lymphomas.

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