Novel Putative Fragile Sites Observed in Feline Fibroblasts Treated with Aphidicolin and Fluorodeoxyuridine

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ABSTRACT. Fragile sites are non-randomly distributed chromosomal breaks and gaps observed in the cells cultivated under certain conditions. Feline fragile sites were analyzed using skin fibroblast strains after the treatments with aphidicolin and fluorodeoxyuridine in combination with caffeine. Three aphidicolin-induced fragile sites (A1q21, C2q13 and E1p21) as well as a folate-sensitive site (B1q14) were observed in all the 3 fibroblast strains tested for each treatment group. The loci in A1q21 and B1q14 are very close to that reported previously for peripheral blood lymphocytes and lung cells. Two chromosomal break points in C2q13 and E1p21 seem to be new fragile sites. Fifteen candidates for feline fragile sites were also assigned their locations in feline chromosomes. Both the incidence and distribution of feline fragile sites in skin fibroblasts seem to be different at least in part from those in lymphocytes. — KEY WORDS: aphidicolin, chromosomal break, feline, fluorodeoxyuridine, fragile site.

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Fragile sites are non-random chromosomal breaks and gaps observed in the cells under the folate-deficient condition [25] as well as those treated with particular mutagen, carcinogen and clastogens [31]. A great number of papers has been reported the fragile sites in human chromosomes, and 116 sites have already been authorized and assigned their location to specific chromosomal bands until 1993 [24]. For domestic animals, however, only limited numbers of information are available [17–21, 23, 28]. So far, only two papers concerning feline fragile sites have been published. Stone *et al*. are the first to report 3 feline fragile sites commonly observed in blood lymphocytes of 3 domestic cats [21]. Using lung cell culture from a new born kitten, Rønne has detected 22 putative fragile sites[20].

Fragile sites have been milestones in the studies on the human diseases with chromosomal instability [25]. Though the significance of fragile sites in the development of cancer is still to be examined, the evidences of chomosomal instability in normal cells derived from cancer patients have been accumulated [2, 8, 14, 27]. It is important to assign the chromosomal break point frequently observed in a particular type of tumor in order to identify a specific gene which is responsible for the onset of the disease. Recently, the close correlations between specified fragile sites and particular neoplasms have been reported [9, 13, 26]. It is possible that more accurate information will reveal the direct correlation between fragile sites and the break points in chromosomes. These information would be also very helpful in the diagnosis and treatment of malignant tumors in domestic animals, especially in companion animals. Therefore, the determination of the precise locations of fragile sites are an urgent proposal in order to estimate their significance in the tumorigenesis in domestic animals.

Fragile sites in human chromosomes were studied most in peripheral blood lymphocytes. For the study on feline fragile sites, however, lymphocytes are less suited, because the culture often suffer a heavy loss of lectin-activated cells by aggregation with platelets. In human, the fragile sites in skin fibroblasts treated with aphidicolin differ in both frequency and distribution as compared with those observed in lymphocytes [10, 11]. In the present study, we have investigated novel fragile sites induced in feline skin fibroblasts, because the banding pattern of their chromosomes have been well-characterized [12].

MATERIALS AND METHODS

Feline fibroblast strains: Small patches of skin were excised from foot pads of four healthy female domestic cats under the sterile condition. The skin fragments were minced with scissors in the Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS(-)), and cell suspension obtained was inoculated into 25 cm^2 plastic culture flasks containing Eagle's minimum essential medium (Nissui) supplemented with MEM sodium pyruvate solution (GIBCO), nonessential amino acid solution (GIBCO), and 10% inactivated fetal bovine serum (Hyclone). The flasks were incubated in the fumidified atmosphere containing 5% CO_2 at 37°C and medium was replenished periodically until the growth of the fibroblasts reached confluence.

Drug treatment: Single-cell suspension was prepared from a confluent culture of fibroblasts by trypsinization and inoculated into a plastic dish containing 10 m*l* of the growth medium. The dish was then incubated under the condition mentioned above for 2 days. Drug treatment was carried out by the procedure described by Yunis and Soreng with slight modifications [30]. Briefly, the log-phase culture of fibroblast strains FFB2, FFB3 and FFB4 were treated with

25 ng/m*l* fluorodeoxyuridine (FUdR) for 18 hr. Caffeine was, then, added to the culture at 2.2 mM and the treatment continued for addtional 6 hr. Alternatively, the exponentially growing cells from strains FFB1, FFB3 and FFB4 was treated with 50 ng/m*l* aphidicolin (Wako Pure Chemical Co.) for 24 hr. For chromosome analysis, colcemid was added at 0.05 mg/m*l* 30 min prior to the termination of the drug treatments.

Analysis of break points in feline chromosomes: The drug-treated cells were harvested by trypsinization and resuspended in hypotonic solution (0.075 M KCl). After the hypotonic treatment for 10 min at room temperature, the cells were fixed by addition of ice-cold acetic acid-methanol (1:3) solution. The fixation was completed by changing the fixative for several times. Cell suspension in the fixative were dropped onto slides and air-dried [6, 7]. The slides thus prepared were stained with quinacrine mustard-Hoechist 33258 solution [29].

The chromosome preparation was examined using a fluoromicroscope (Nikon Optiphoto). Well-spread metaphases were photographed and analyzed after Q-band karyotyping. Chromosomal breaks and gaps were identified their localization in the chromosomes [3, 12]. Mostly, more than 50 metaphase cells were analyzed for each strains.

RESULTS

Figure 1 shows a typical Q-banded metaphase cell treated with aphidicolin. Thirty three intact chromosomes and 5 chromosomes containing isochromatid breaks/gaps were observed. Analysis of karyotypes revealed that the breaks/gaps are present in chromosomes A1p, C2q (both C2 chromosomes are affected), E1p and E3p.

Figure 2 summarizes the locations of breaks or gaps observed in chromosomes of the 260 feline fibroblasts examined. The result includes all the 86 break points induced by both aphidicolin and FdUrd-caffeine. Of these locations, no. 1, no. 8 and no. 27 correspond with the fragile sites observed by Stone and Stephens [23] using concanavalin A-activated lymphocytes. Neither gap nor break was detected at no. 1 in FFB1 and FFB3 cells treated with aphidicolin, while 3.7% and 2.0% of FFB4 cells had the gaps and breaks at this position after the treatment with FUdR+caffeine and aphidicolin, respectively. The gaps and breaks at the locus no. 8 were observed in 0, 2.63 and 2.0% of FFB1, FFB3 and FFB4 cells, respectively, after aphidicolin treatment. At position no. 27, however, the drug induced gaps and breaks only in FFB1 at very low frequency (1.0%). These results may indicate that the incidence and distribution of aphidicolin-induced chromosomal breaks and gaps in feline fibroblasts are different from those in lymphocytes.

Tables 1 and 2 show the percentages of the breaks and gaps at the locations commonly observed in all three strains for each treatment group. Five loci were apparent after treatment with apidicolin (Table 1) and FUdR+caffeine induced 3 other breaks/gaps sites (Table 2), although the percentage of each site differ greatly. In total, the incidence of 3 aphidicolin sites (A1q21, C2q13 and E1p21) and 1 FUdR+caffeine site (B1q14) are shown to be higher than 4% and it may be reasonable to accept as fragile sites of domestic cats according to the criteria employed by Yunis and Soreng [30]. As loci no. 78 in E3p12, no. 86 in Xq23, no. 30 in B2q12 and no. 36 in B3q22 are observed in all the strains examined even at the overall incidence lower than 4%, they should be treated as possible candidates of feline fragile sites. The gaps and breaks at no.74 in E1p21 were also detected at high yield in FFB2 (7.0%) and FFB4 (14.8%) treated with FUdR+caffeine. The locus 9 in A1q21 is close to A1q21–22 at which Stone *et al*. identified an aphidicolin-induced fragile site [21]. Considering the difference in staining methods for regional assignment of fragile sites, it is possible that aphidicolin induces a fragile site at the same locus (A1q21–22) in both types of feline cells. The fragile site in B1q14 seems to be same as that observed by Rønne [20]. So far, loci in bands C2q13 and E1p21 should be counted as new fragile sites in fibroblasts.

Additional candidates of feline fragile sites are listed in Table 3. Thirteen sites were observed at the frequency higher than 4% in at least one fibroblast strain. Of these sites, loci no. 3 and no. 69 showed high incidence of aphidicolin-induced gaps and breaks in FFB1 and FFB4.

DISCUSSION

Large number of fragile sites have been reported for human chromosomes [24], while the studies on those in domestic animals are limited [17–21, 23, 28]. For domestic cat, only 2 reports have been published. Stone *et al*. have firstly reported 3 fragile sites commonly observed in feline lymphocytes from 3 healthy donnors [21]. Rønne has identified 22 fragile sites in methotrexate/bromodeoxyuridine-treated lung cells from a normal newborn kitten [20].

In the present study, we detected 3 aphidicolin-induced putative fragile sites in all the 3 feline fibroblast strains examined. At least 2 of these are different from those identified previously [20, 21]. It is important to determine the locations of fragile sites in the cells from different tissues to rationalize the chromosomal break points in tumors of domestic animal. As suggested by Murano *et al*. for human cells [10, 11], the distribution and frequency of aphidicolin-induced fragile sites also seems to be different between lymphocytes and fibroblasts of domestic cats. Stone *et al*. have shown that there is canine breed specific variation in the frequency of aphidicolin-inducible fragile sites [22]. Because a similar variation in domestic cats with different genetic background is possible, more detailed studies on the feline chromosomal stability is required for understanding of feline fragile sites.

All the fibroblast strains used in this study were established from unrelated domestic cats. Because all the human fragile sites are not always expressed in all individuals, the loci listed in Table 3 may also be a potential

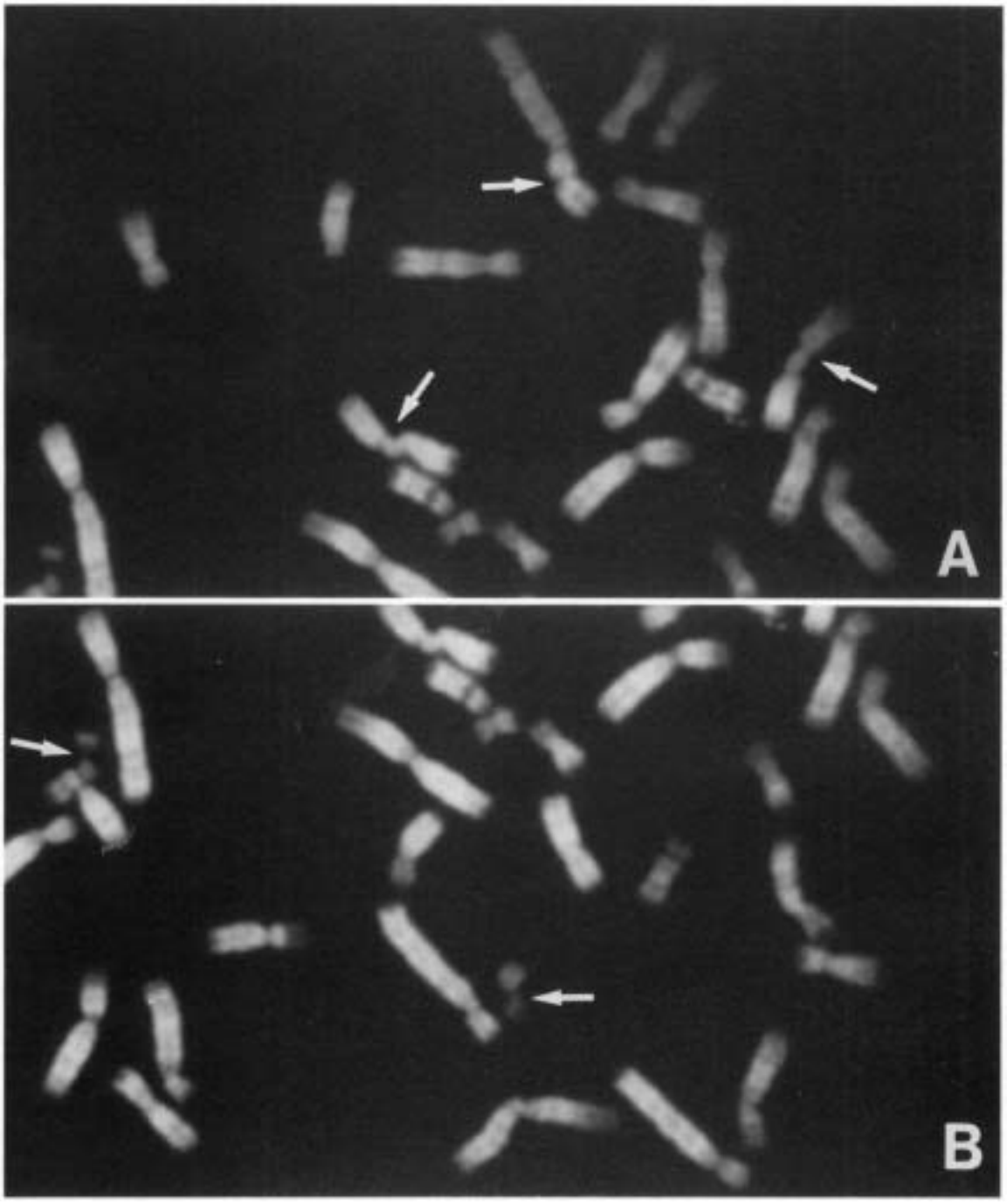

Fig. 1. (A) A part of Q-banded metaphase spread of a feline fibroblast treated with aphidicolin. Three chromosomal breaks/gaps in A1p and C2q (both C2 chromosomes) are indicated by arrows. (B) The other part of same metaphase shown in (A) includes 2 breaks/gaps in E1p and E3p (arrows).

candidates for feline fragile sites. Interestingly, 7 sites of these were observed at relatively high frequency by Rønne [20]. It is widely accepted the incidence of folate-sensitive fragile sites is greatly dependent on the intracellular pool size of both folate and nucleotides[4, 5, 16]. Because the variation of these quantities among a variety of tissues is highly probable, the incidence of fragile sites may vary significantly among the different types of cells employed.

At present, only 26 feline fragile sites have been identified including 2 novel sites observed. Though the information on the fragile sites in domestic cats are very limited, it is probable that the low incidence of feline fragile sites, as

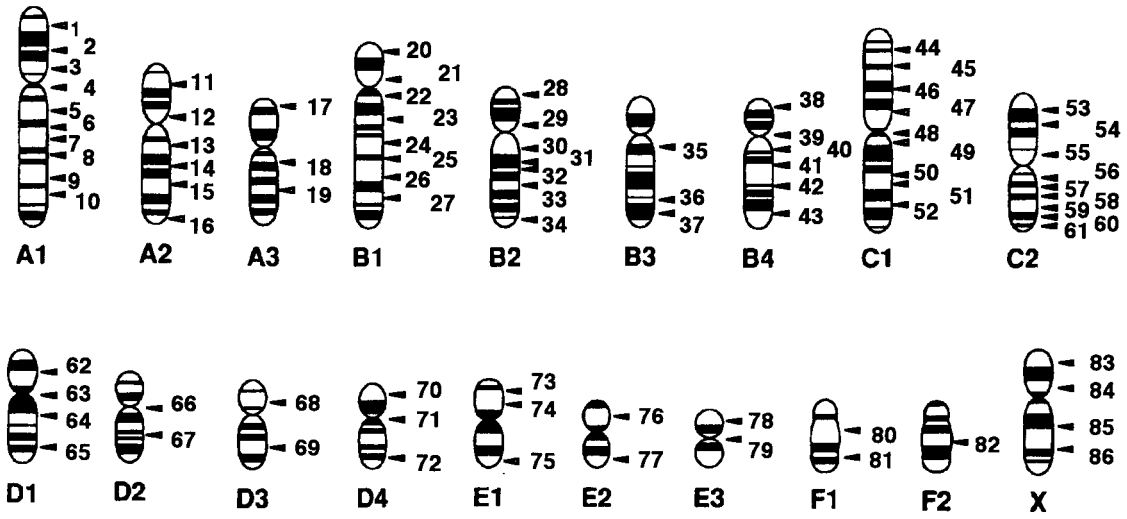

Fig. 2. The integrated map of chromosomal location of breaks and gaps induced by both aphidicolin and fluorodeoxyuridine plus caffeine in feline fibroblasts. Eighty-six different loci are identified (arrowheads).

Table 1. Percentage and chromosomal locations of gaps and breaks commonly induced in aphidicolin-treated feline fibroblast strains

Position	Location	FFB1	FFB3	FFB4	Net
9	A1q21	2.80	2.63	8.00	4.64
56	C2q13	9.00	7.89	16.00	11.23
74	E1p21	3.00	7.89	12.00	7.61
78	E3p12	4.00	2.63	2.00	2.90
86	Xq23	1.00	2.63	2.00	1.81

Table 2. Percentage and chromosomal locations of gaps and breaks commonly observed in feline fibroblast strains treated with fluorodeoxyuridin+caffeine

Position	Location	FFB2	FFB3	FFB4	Net
23	B1q14	1.00	10.20	7.41	5.95
30	B2q12	1.50	2.04	7.41	2.98
36	B3q22	1.00	2.04	3.70	1.98

Table 3. Percentage and chromosomal locations of breaks/gaps which were induced by aphidicolin (APC) and fluorodeoxyuridine/caffeine (FdU) at the incidence higher than 4% in at least one strain

Position	Location	FFB2FdU	FFB3FdU	FFB4FdU	FFB1APC	FFB3APC	FFB4APC
3	A1p14	1.00	-	-	13.00	-	6.00
11	A2p21	-	2.04	3.70	-	-	6.00
13	A2q13	-	4.08	-	14.00	-	-
15	A2q23	1.00	4.08	-	3.00	-	8.00
24	B1q23	-	10.20	-	-	-	-
26	B1q25	1.00	2.04	-	1.00	-	8.00
28	B2p13	-	-	-	3.00	-	8.00
33	B2q31	-	4.08	-	-	2.63	-
50	C1q22	1.00	4.08	-	-	-	4.00
55	C2p11	-	-	7.40	-	-	-
69	D3q14	-	-	3.70	13.00	-	8.00
70	D4p14	-	-	-	5.00	-	-
80	F1q13	6.50	2.04	-	-	-	-

compared with that in human, may have resulted from the selective elimination of inferiors in establishing the breeds. Alternatively, it is possible that the cellular sensitivity to fluorodeoxyuridine plus caffeine and aphidicolin may different among mammalian species. Conventionally, same procedure and drug concentrations as those for the detection of human fragile sites have been employed to study those in domestic animals. Precise dose-induction relationship of fragile sites for each mammalian spieces, therefore, should be established.

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