Flow Cytometric DNA Ploidy Analysis of Feline Mammary Tumors

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ABSTRACT

Flow cytometric DNA analysis was performed on biopsies from 9 nonmalignant and 111 malignant (primary and metastatic) feline mammary lesions. In our series, 46.3% of the primary mammary carcinomas appeared to be aneuploid, whereas all but one benign breast lesion were diploid. The degree of aneuploidy in carcinomas was low, with a relatively high number of primary tumors (12 of 82) displaying hypodiploidy. Aneuploidy was not found to be correlated with any specific histological tumor type, vascular invasion, tumor size, or histological malignancy grade or with the separate components thereof.

Comparison of the ploidy in primary and metastatic tumors from the same cases revealed a remarkable stability, both in time and location of appearance of the metastases.

It is concluded that with respect to DNA ploidy feline mammary carcinoma has more in common with canine mammary carcinoma than with human mammary carcinoma. Further prospective studies are necessary to clarify the implications of aneuploidy in feline mammary carcinoma for tumor behavior and prognosis.

INTRODUCTION

FCM analysis of DNA ploidy is frequently used to detect the presence of genetic abnormalities in tumor cells (reviewed in Refs. 1 and 2). In human breast cancer, DNA aneuploidy is found in a high percentage (50–80%) of cases (1, 2) and appears to be associated with unfavorable prognostic factors such as steroid receptor negativity (3-6). In addition, DNA ploidy has been reported to have an independent effect on prognosis in some subsets of patients (3). In humans, comparisons of the DNA content of primary and metastatic sites of breast cancer in the same patients have revealed a remarkable stability (7-9). This, in combination with the low incidence of intratumoral variation in ploidy, has led to the hypothesis that human mammary cancer is of monoclonal origin (2). Although a positive correlation between ploidy and various clinicopathological parameters associated with an unfavorable prognosis was reported in several studies (3-6, 9-15), the results are still controversial and no firm conclusions can be drawn.

Feline mammary cancer is an attractive model for diagnostic and therapeutic studies since it shares many features with human breast cancer, including histopathological appearance, pattern of metastasis, and poor prognosis (16, 17). As in women with breast cancer, tumor size, the condition of regional lymph nodes at time of surgery, and mitotic index are related to prognosis (18). Nuclear DNA analysis of feline mammary tumors has been reported only once (16). In that study, using image cytometry, similar types of DNA histograms were found as in human breast cancer, albeit at different frequencies. However, no attempt was made to correlate the DNA content with clinicopathological parameters.

The aim of this study was 3-fold: (a) to determine the degree and frequency of aneuploidy in malignant and nonmalignant primary feline mammary tumors, (b) to compare the DNA ploidy in primary malignant tumors with local recurrent tumors and metastases, and (c) to establish a possible relation between DNA ploidy and some clinicopathological characteristics. For this purpose, DNA content was measured with FCM in 122 feline benign and malignant mammary lesions.

MATERIALS AND METHODS

Animals. During the years 1986–1989, mammary tumor specimens from 88 cats (87 females, 1 male) were collected at surgery or autopsy for histopathological examination and determination of their nuclear DNA content using FCM.

Mastectomy was the only form of treatment in 50 cats, whereas in 13 cats chemotherapy was given after a biopsy was taken (doxorubicin, 30 mg/m², i.v., in a 2-week cycle for 10 weeks). Thirty-one cats were euthanized at the owners' request because of advanced mammary cancer; 6 of these animals had also had biopsies at an earlier stage of the disease. We found 6 cases where there was simultaneous occurrence of cancers in two mammary glands. Histological examination revealed the same histological morphology in 3 cases and different morphologies in the remaining 3. The latter 3 were considered carcinoma duplex. In total, 11 nonmalignant tissues (2 nonaffected mammary glands and 9 benign tumors) from 10 cats and 111 malignant tumor specimens (82 primary tumors and 29 metastases) derived from 79 cats were collected. In 1 cat both a benign and a malignant mammary tumor were collected. The diagnosis and histopathological characterization of all primary tumors are listed in Table 1.

All cases were evaluable for ploidy analysis. The diameter of 65 primary tumors was known. The diameter was measured on the unfixed specimen and the largest size was used for determination of the T category (T1, <2 cm; T2, 2–5 cm; T3, >5 cm). The mean age of the cats with carcinomas was 10 years and of the benign tumor group, 7.5 years.

Since in a large number of cases histological data concerning the involvement of the regional lymph nodes were not available, no pathological staging of the tumors was possible. Since no standardized protocol for clinical staging was followed by the various clinicians, no correlations were made between the ploidy on the one hand and disease-free interval and survival on the other hand.

Sample Preparation. Immediately after surgery or autopsy, the sample was placed in tissue culture medium at 4°C and was transported to our laboratory within 1 hr. After removing fat and necrotic tissue, for most tumors at least one part was snap-frozen in liquid nitrogen and stored at −70°C; an adjacent part was fixed in phosphate-buffered 10% formalin for histopathological examination.

Histological Examination. The histological examination was done by one of the authors (W. M.) according to the World Health Organization classification for tumors in domestic animals (19), on paraffin-embedded hematoxylin- and eosin-stained sections, without prior knowledge of the FCM results. The classification was slightly extended by introduction of a distinct cribriform tumor type. The histological examination included diagnosis of the lesions (carcinoma versus benign), typing of the carcinomas, and estimation of the relative amounts of vital and necrotic tumor tissue and of normal tissue. Also the absence or presence of vascular invasion and the histological malignancy grade and the components thereof, i.e., the mitotic index, the differentiation grade, and the nuclear grade, were determined as described before (20).

Flow Cytometric Analysis of DNA Content. Frozen samples were...
thawed at room temperature and cut into small pieces. Suspensions of single nuclei were prepared with the detergent-trypsin procedure described by Vindelev et al. (21) and were stained with propidium iodide (Sigma Chemical Co., St. Louis, MO). From paraffin-embedded blocks of formalin-fixed specimens, 35-µm sections were cut and deparaffinized in xylol. Cell suspensions were obtained by treating the deparaffinized sections with 0.5% pepsin (Sigma) in saline for 30 min, according to the procedure described by Hedley et al. (22).

The cells were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Boehringer, Mannheim, FRG). Stained samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA). Filtered demineralized water was used as sheath fluid. For excitation of propidium iodide fluorescence, LP 515 and SP 560 filters were used in combination with a 560-nm chromatic beam splitter. Emission was measured with an LP 590 barrier filter. For excitation of 4′,6-diamidino-2-phenylindole dihydrochloride fluorescence, a UG 365 filter was used in combination with a 400-nm chromatic beam splitter. Emission was measured using a 435-nm barrier filter.

Nuclear suspensions of frozen material were analyzed in the presence of chicken RBC or normal cat WBC as external standards and, in some cases, with both standards separately. The DNA content of feline cells was found to be 2.28 ± 0.08 (mean ± SE) times that of reference chicken RBC. In samples derived from paraffin-embedded tissues, normal nonneoplastic cells, when present in sufficient quantities (>10%) as determined by histological examination, served as an internal control. As a rule, at least 10,000 cells were measured in each assay.

Calculation of DNA Index. The DNA index was defined as the ratio of the modal channel number of the G0i peak of the tumor cell population to that of the G0i peak of normal diploid cells.

Tumors with a distinct G0i population with DI ≠ 1.0 were classified as aneuploid. When more than one aneuploid cell population was present, the tumor was classified as multiploid. Tumors showing a large G2-M peak (>20% of measured cells) in addition to a diploid G0i peak were classified as tetraploid when a separate G2-M peak at twice the channel number was present too. Tetraploid tumors were considered aneuploid.

Statistical Analysis. The data were analyzed with the trend test and the Fisher exact test (two-sided). The level of significance was set at P = 0.05.

RESULTS

All tumor specimens tested contained more than 15% tumor cells, which is above the sensitivity limit for the detection of aneuploidy by the flow cytometer (23). The use of frozen specimens (n = 104) for the determination of ploidy yielded, in general, satisfactory results. In contrast, paraffin-embedded samples (n = 40) gave insufficient results (coefficient of variation > 8.5) in 8 cases, and these 8 tissues were excluded from further consideration. The ploidy of four paraffin-embedded tumors could only be designated as "peridiploid"; i.e., these tumors were further considered as if they were diploid. In 14 tumors, the ploidy was determined in both frozen and paraffin-embedded tissues; in 3 cases no comparison was possible, due to insufficient results for the paraffin-embedded tissues, and in the remaining 11 cases concordant DNA index values were obtained in 10 samples. The one exception (K 138) was classified as aneuploid (DI = 0.89) using frozen material and diploid using paraffin-embedded material; the former was considered decisive. Examples of different DNA profiles are demonstrated in Fig. 1.

The coefficient of variation for the G0i peak of the tumor cells was 2.91 (1.6-5.1) in frozen samples (n = 83) and 5.01 (2.6-8.4) in paraffin-embedded material (n = 20).

DNA Ploidy in Primary Lesions. Flow cytometric analysis of normal mammary glands (n = 2) displayed diploid G0i peaks. Of the 9 nonmalignant mammary tumors (6 fibroadenomas, 2 borderline-type tumors, and 1 hyperplasia without atypia), 8 were diploid. The benign aneuploid tumor (hyperplasia; DI = 1.15) was found in a cat (K198) which simultaneously had a multiploid malignant tumor (DI = 0.74; 0.78; 0.89). DNA FCM results of 82 primary malignant tumors are presented in two ways: first, the overall incidence of diploid, single aneuploid, and multiploid tumors (Table 2) and second, the frequency distribution of individual cell stemlines found in this series (Fig. 2). A clustering of stemlines is found in the diploid/near-diploid region, 51.9% of the stemlines being diploid, 42% being near-diploid [15.9 hypodiploid (DI < 1.0), 26.1% hyperdiploid (1.0 < DI ≤ 1.4)], and only 6.82% being (hypo-)tetraploid (DI > 1.4).

Multiploid, defined as the presence in a tumor of two or more aneuploid stemlines, was observed in 6 carcinomas. In 3

Table 1 Ploidy in 91 feline primary mammary tumors in relation to type of histology

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of tumors</th>
<th>Diploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Borderline tumor</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Tubular carcinoma</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Cribriform carcinoma</td>
<td>31</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Solid carcinoma</td>
<td>12</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Carcinoma displaying multiple differentiations</td>
<td>25</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2 Ploidy distribution in 82 primary feline mammary carcinomas

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>No. of cancers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>44</td>
<td>53.7</td>
</tr>
<tr>
<td>Single aneuploid</td>
<td>32</td>
<td>39.0</td>
</tr>
<tr>
<td>Multiploid</td>
<td>6</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1. Representative DNA profiles of feline mammary carcinomas, redrawn from original recorder tracings (frozen samples). The DNA index (x-axis) is plotted against the relative number of cells (y-axis). A, tubular carcinoma; B, partly infiltrating and in situ carcinoma of solid type; C, infiltrating carcinoma of solid type; D, cribriform carcinoma. CRBC, chicken RBC.
cases the second aneuploid peak had a DI value twice the value of the first aneuploid peak, indicating the possibility that the second stemline was derived via polyploidization.

Comparison of the Ploidy in Primary and Local Recurrent/Metastatic Tumors. To determine whether progression of feline mammary tumors is associated with a change in the degree of ploidy, we investigated the DNA content in local recurrent and metastatic lesions. Samples \( n = 29 \) from primary and metastatic tumors of the same animal were obtained in 17 cats; the animals either underwent serial biopsies during the disease or had biopsies from multiple sites at one time. In 25 of the 29 metastases, the same stemlines as in the primary tumor were found (Table 3). In 1 cat (K 160) the primary tumor was found to be hyperdiploid (DI = 1.15), whereas the pulmonary metastasis was scored as peridiploid. In cats K 205, K 190, and K 352, an extra stemline was found in one of the metastatic lesions in addition to the stemlines also found in the primary tumor. In 1 case (K 193), 2 primary mammary carcinomas of different histological types with different DNA indexes (DI = 0.87 and 1.0) were found; only the diploid stemline was found in both metastases.

Relation of DNA Index and Clinicopathological Parameters. In our series of malignant tumors, no correlation was found between the ploidy and histological type of the tumors (Table 1). Likewise, ploidy status and tumor diameter, mitotic index, and grades of differentiation and histological malignancy were not statistically associated (Table 4). A low nuclear grade tended to be associated with diploidy, but the comparison did not reach statistical significance \( P = 0.08; \) Table 4). Although a difference in vascular invasion was found between diploid and aneuploid tumors, the latter being more invasive, this difference was not significant \( P = 0.08; \) Table 4).

**DISCUSSION**

In the present study, DNA aneuploidy was determined in histologically defined malignant and nonmalignant mammary lesions in the cat. In our series, 46.3% of the carcinomas appeared to be aneuploid, whereas all but one of the benign mammary lesions were diploid. The one exception, being a lobular hyperplasia without atypia, was detected in a cat also bearing a second multiploid malignant mammary tumor. In humans, the presence of hyperplasia with or without epithelial atypia appears to be an important risk factor for subsequent development of breast cancer (24). Whether this is also true for feline mammary tumors remains to be tested. The occurrence of aneuploid benign tumors in animals also developing malignant tumors was also observed by Rutteman et al. (25) in canine mammary tumors.

The overall frequency of 46.3% aneuploid primary carcinomas is lower than the 61% aneuploidy reported by Prop et al. (16) in his series of 44 feline mammary tumors analyzed by image cytometry. However, an accurate comparison between the two studies is difficult, because both the methodology and tumor material used and the criteria for aneuploidy were different in the two studies. The aneuploidy prevalence observed in this study is low compared to the percentages of aneuploidy usually reported in humans (1, 3, 5, 6, 9, 10, 15, 26) and dogs (25, 28).

In the present series of feline mammary carcinomas, a clustering of stemlines in the (near-) diploid range (DI = 0.55–1.5)
was observed, with a remarkably high number of tumors (n = 12, 14.6%) displaying hypodiploidy. In human breast cancer, most investigators report a typical bimodal distribution of stemlines (3, 5, 10, 11, 15), which reflects two possible mechanisms of karyotypic evolution. As pointed out by Ewers et al. (10) and confirmed recently by Shackney et al. (27) in a computer simulation model of genetic evolution in human solid tumors, the generation of (hyp-)tetrapoloid stemlines (DI > 1.4) may have occurred via the sequence of polyploidization of (near-) diploid stemlines and chromosomal loss. The evolution of low aneuploid tumors may have occurred by a small loss or gain of single chromosomes. Polyploidization could double the dose of growth-promoting genes activated by structural chromosomal abnormalities. This may confer a growth advantage to cells that have undergone polyploidization if the growth-promoting effect is dose dependent (27). Our results suggest that, although polyploidization occurs in feline mammary carcinomas, it probably is not an important mechanism by which feline mammary tumor cells obtain a selective growth advantage. In view of the common finding of hypodiploid stemlines in feline and canine mammary carcinomas (25, 28), it is interesting to determine whether loss of suppressor genes in both tumor systems plays a role in the activation of growth-promoting genes. The presence of hypodiploid stemlines in human breast cancer is a rare event and has been observed in 0 to 8% of the cases (3, 6, 10, 14, 29–31).

In canine mammary carcinomas (25, 28), the distribution of DI values is very similar to those found in feline mammary carcinomas. Hypodiploidy was nearly as common in the dog as in the cat (respectively, 20.6% and 14.6%). It was speculated that in the dog the distribution over a greater number of chromosomes (n = 78) of genes coding for vital functions might allow chromosomal loss to a greater extent (25). However, this hypothesis cannot hold for feline mammary tumors, since this species harbors only 38 chromosomes.

Multiploidy was observed in only 6 cases (7.3%) and seems to be a rather rare event in feline mammary cancer. However, we have to interpret these results with caution, since multiploidy may be underestimated if defined by the required presence of two aneuploid stemlines. We cannot exclude the possibility that within aneuploid tumors diploid tumor cells were present, since FCM analysis is not suited to discriminate normal from neoplastic cells within a diploid peak. In this respect it is important to stress that in our series 53.7% of feline mammary carcinomas contained diploid stemlines. In human breast cancer, multiploidy was observed in 8–17% (3, 10, 14, 29, 31) and in canine mammary cancer in 5–13% of the cases (25, 28, 32).

By comparing the DNA content of primary and metastatic tumors derived from the same animals, it was found that the DNA index of primary cancers remained constant in 25 of the 29 analyzed metastases, irrespective of time and location of occurrence. In only 3 cases an additional stemline was found in a metastatic lesion which was not present in the primary tumor. The results, which are in line with those obtained in human breast cancer (8, 9, 26, 33), could indicate that progression of feline mammary carcinomas in general is not associated with a change in ploidy. This does not necessarily conflict with the clonal evolution theory of Nowell (34), since at clinical presentation feline mammary carcinomas might already represent an ultimate stage of progression. More important, subtle chromosomal changes not leading to a change in ploidy might be more significant than gross chromosomal abnormalities during progression of feline mammary carcinomas. The high incidence of metastatic lesions containing only diploid stemlines supports this notion. Karyotypic analysis of feline mammary carcinomas should give more insight into the specific chromosomal changes involved in this disease.

We did not find a significant correlation between the ploidy and the histological malignancy grade or the separate components thereof, i.e., differentiation grade, nuclear grade, and mitotic index. However, we did observe a nonsignificant trend towards an association of aneuploidy and an increased nuclear grade.

In the human situation, the relationship between the ploidy and the histological malignancy grade is still controversial (5, 9, 11–13, 15, 30). Most investigators do report on a positive correlation between the ploidy and nuclear grade (4, 9, 14).

Similar to our study, Rutteman et al. (25) failed to find a correlation between the ploidy and the histological malignancy grade or its components in canine mammary tumors. The lack of a significant correlation between the ploidy and nuclear grade in both systems possibly is related to the phenomenon that many aneuploid mammary tumors of cat and dog are characterized by only a small loss or gain of DNA, which need not have an impact on nuclear volume or staining characteristics.

Although in our series of carcinomas neither the size nor the vascular invasion was significantly correlated with the ploidy, our results did indicate a trend towards aneuploid tumors being more invasive. In an earlier study by Weyer and Hart (18), the size of the primary tumor was reported to be an important prognostic factor, whereas vascular invasion was only correlated with prognosis via metastases in the regional lymph nodes. The presence of vascular invasion in the primary tumor could reflect the condition of the regional lymph nodes, as shown by McDivitt et al. (30). Proper clinical staging of feline mammary carcinoma patients should give more insight into a possible relationship between the ploidy and lymph node involvement.

In humans, there is no agreement between the reports on the relationship between ploidy and diameter (3–6, 10–12, 29, 30) or between the reports on ploidy and the involvement of the regional lymph nodes (3, 5, 6, 9–11, 14, 29, 30).

Comparison between feline, canine, and human mammary carcinomas with regard to the DNA content indicates that there is more similarity between feline and canine mammary carcinomas in this respect than between human and feline mammary carcinomas. Despite the observed difference in aneuploidy evolution in cats and humans, we still consider feline mammary carcinomas to be valuable models for studies of therapeutic intervention (35, 36).

Further prospective follow-up studies should establish whether the presence of aneuploid stemlines in feline mammary tumors has any implications for tumor behavior and prognosis.

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