Immunohistochemical Detection of Mdm2 and p53 in Feline Mammary Gland Tumors

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ABSTRACT. The objective of this study was to evaluate nuclear reactivity of Mdm2 and p53 proteins by immunohistochemical means in feline mammary gland tumors; 12 adenomas which included 6 adenomatous lesions obtained from the tissue adjacent to adenocarcinomas, and 22 adenocarcinomas. Seven adenomas and 18 adenocarcinomas showed moderate or marked Mdm2 reactivity. 16 adenomas and 14 adenocarcinomas showed moderate to marked p53 reactivity, but 9 adenomas showed none. Discordant Mdm2 overexpression was found in 5 adenomas and 3 adenocarcinomas, although co-overexpression of Mdm2 and p53 was found in 15 adenocarcinomas. These results suggest that nuclear overexpression of Mdm2 is present in the tumors of early stage without p53 overexpression and related to feline mammary gland tumorigenesis. Nuclear overexpression of p53 is more frequent in adenocarcinomas, but not in adenomas.

KEY WORDS: feline mammary tumor, Mdm2, p53.

MATERIALS AND METHODS

Tumors: The samples were collected from feline mammary tumors removed surgically during a 5-year period at Yamaguchi University. The samples consisted of formalin-fixed, paraffin wax-embedded tissue from 28 tumors (6 adenomas and 22 adenocarcinomas). In addition, six samples of adenomatous lesions were obtained from the tissue adjacent to adenocarcinomas. Sections from each sample were stained with hematoxylin and eosin, and histological diagnosis was carried according to the World Health Organization classification for feline mammary gland tumors [20]. The adenomas were observed all in females of mixed breeds (5) and Chinchilla (1) aging 6 to 14.5 years and classified as intermediate and 3 poorly differentiated types arising also in females of mixed breeds (14), Siamese (5), Persian (1), Chinchilla (1), Russian blue (1) aging 9 to 16 years (average 11.5 years).

Immunohistochemistry and scoring: Serial 5 µm sections were mounted on amino-silane-coated slides. The sections were deparaffinized with xylene, rehydrated through graded ethanol, and microwave-pretreated in 10 mM citrate buffer, pH 6.0 for 6 min. All the following steps were conducted at 25°C. Endogenous peroxidase and avidin/biotin (Nichirei, Tokyo, Japan) were quenched by immersion in 3% hydrogen peroxide and endogenous avidin/biotin blocking solution, respectively. The sections were incubated with normal rabbit or goat serum for 10 min. Anti-Mdm2 mouse mono-
clonal antibody (Santa Cruz Biotechnology, Santa Cruz, U.S.A.) and rabbit anti-p53 polyclonal antibody FL-393 (Santa Cruz Biotechnology) were applied for 120 and 30 min, respectively. The primary antisera were diluted at 1:75, and a biotin-streptavidin immunoperoxidase method was used. Biotinylated rabbit anti-mouse IgG,A,M antibody (Nichirei) and goat anti-rabbit IgG antibody (Nichrei) were applied as secondary antibodies for 20 min before treatment with peroxidase-conjugated streptavidin (Nichirei). After treatment with the substrate chromogen (3-amino-9-ethylcarbazol; AEC), the sections were counterstained with hematoxylin. Non-immune mouse or rabbit sera, as sources of irrelevant primary antibodies, were used as negative controls. To quantify the reaction, the positive cells in 100 fields at ×400 magnification were counted, averaged, and expressed as percentages. The extent of the nuclear reactivity was classified as either absent, mild (1 to 10% of tumor cells), moderate (11 to 50%) or marked (51 to 100%).

**Statistical Analysis:** The correlation between Mdm2 and p53 expression and histological stage was analyzed by Fisher’s exact test. A P-value less than 0.05 was considered statistically significant.

**RESULTS**

The results of immunohistochemical nuclear reactivity of Mdm2 and p53 are summarized in Table 1.

**Analysis of Mdm2 protein:** Five of the 12 adenomas (included adenomatous lesions obtained from the tissue adjacent to adenocarcinomas) showed marked Mdm2 reactivity (57, 64, 67, 80 and 82% of tumor cells, respectively; Fig. 1a), and 2 showed moderate reactivity (13 and 42% of tumor cells); 5 showed mild (3, 4, 5 and 8% of tumor cells) or none. Twelve of the 22 adenocarcinomas showed marked Mdm2 reactivity (ranged from 53 to 90% of tumor cells; Figs. 1c and 1e), and 6 showed moderate reactivity (ranged from 60 to 84% of tumor cells) than in the former (moderate: 38 and 40% of tumor cells or none). Increased Mdm2 reactivity was also significantly higher in the latter (ranged from 60 to 84% of tumor cells) than in the former (moderate: 38 and 40% of tumor cells or none).

**Analysis of p53 protein:** Only three of the 12 adenomas showed marked or moderate p53 reactivity (38, 40 and 81% of tumor cells), and nine showed none (Fig. 1b). Nuclear reactivity of p53 was significantly low in adenomas compared with that of Mdm2. In contrast, twelve of the 22 adenocarcinomas showed marked p53 reactivity (ranged from 52 to 90% of tumor cells; Fig. 1d), and 4 showed moderate reactivity (14, 29, 47 and 49% of tumor cells); 6 showed mild (8% of tumor cells) or none (Fig. 1f). In four of the 6 cases with both adenomatous and carcinomatous lesions, p53 reactivity was also significantly higher in the latter (ranged from 60 to 84% of tumor cells) than in the former (moderate: 38 and 40% of tumor cells or none). Increased p53 reactivity was statistically significant in adenocarcinomas compared with adenomas (P<0.01). No correlation between p53 nuclear reactivity and histological features was observed within adenocarcinomas.

To investigate a correlation between expression of Mdm2 and p53, moderate to marked reactivity for Mdm2 and p53 were regarded as overexpression. Of the 12 adenomas, 5 had discordant overexpression (Mdm2 positive and p53 negative), but 2 had co-overexpression of Mdm2 and p53, and 1 had discordant p53 overexpression. Discordant Mdm2 overexpression was also observed in 3 adenocarcinomas, although co-overexpression of Mdm2 and p53 was observed in 15 adenocarcinomas. No correlation between co-overexpression of Mdm2 and p53 and histological features was observed within adenocarcinomas.

**DISCUSSION**

High levels of Mdm2 nuclear expression were observed in feline mammary adenomas and adenocarcinomas. Seven of the 12 adenomas and 18 of the 22 adenocarcinomas showed moderate or marked Mdm2 reactivity. In four of 6 cases with adenomatous and adenocarcinomatous lesions, Mdm2 reactivity was higher in the latter than in the former. Increased Mdm2 reactivity was observed in adenocarcinomas compared with adenomas, but not statistically significant (p<0.1). The results suggest that nuclear overexpression of Mdm2 is related to feline mammary gland tumorigenesis and present in tumors of early stage. Recent studies focused on Mdm2 expression in human epithelial neoplasms. A similar result has been reported in colorectal adenocarcinoma, Mdm2 expression was detected in both adenomas and adenocarcinomas and associated with negative p53 expression [1]. Our recent study has suggested that increased Mdm2 expression is an early event in canine cir-

### Table 1. Nuclear immunoreactivity of Mdm2 and p53 proteins in feline mammary gland tumors

<table>
<thead>
<tr>
<th>Histological type</th>
<th>No. Cats</th>
<th>Mdm2</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenomas</td>
<td>12</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>22</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

a) – = absent; + = mild (1–10%); ++ = moderate (11–50%); +++ = marked (51–100%).
b) Included six cases with both adenomatous and adenocarcinomatous lesions.
Fig. 1. Immunohistochemistry for Mdm2 and p53 in feline mammary gland tumors. (a) Adenoma (lobular type) shows marked Mdm2 nuclear reactivity. Mixed breed, aged 10 years. (b) Adenoma (lobular type) shows marked p53 nuclear reactivity. The same case as that in Fig. 1a. (c) Adenocarcinoma (well differentiated type) shows marked Mdm2 nuclear reactivity. Mixed breed, aged 10 years. (d) Adenocarcinoma (well differentiated type) shows marked p53 nuclear reactivity. The same case as that in Fig. 1c. (e) Adenocarcinoma (intermediate type) shows marked Mdm2 nuclear reactivity. Mixed breed, aged 12 years. (f) Adenocarcinoma (intermediate type) shows no detectable p53 nuclear reactivity. The same case as that in Fig. 1e. Biotin-streptavidin-immunoperoxidase method/AEC. Counter-stained with hematoxylin. Magnification: × 200.
cumanal gland tumorigenesis, and its expression decreased in adenocarcinomas [24]. On the other hand, overexpression of Mdm2 has been found more frequently in human carcinomas of higher grade and associated with tumor invasiveness and aggressiveness in the breast [10, 18], liver [26], kidney [11], prostate [16], and lung [2]. Further studies are needed to clear the functional significance of Mdm2 expression in feline epithelial cell tumorigenesis.

The study showed that the level of p53 nuclear reactivity was significantly low in adenomas compared with adenocarcinomas and similar to that of Mdm2 in adenocarcinomas. The results suggest that p53 nuclear overexpression is frequent in adenocarcinomas, but not in adenomas. However, no correlation between p53 nuclear reactivity and histological features was observed within adenocarcinomas. Wild type p53 protein is rapidly eliminated by virtue of its short half-life, which is considered to preclude immunohistochemical detection. In contrast, an extended half-life of mutated p53 protein causes the protein to accumulate in the nucleus of the affected cells, enabling it to be detected immunohistochemically [8, 14]. Thus, immunohistochemical detection of p53 protein indicates that a mutation is present in p53 gene. Recently, p53 gene mutation and nuclear accumulation have been reported in mammary gland tumors in cats [22] and dogs [15, 23], and associated with aggressive tumor behavior. On the other hand, it has been reported that p53 gene mutation is an early event in human breast cancer [5].

p53 protein and Mdm2 are functionally closely related, and their expression is controlled by an autoregulatory feedback loop [21, 30]. Expression of Mdm2 is consequent upon p53 activation. Concomitant overexpression of Mdm2 and p53 has been observed in human epithelial neoplasms including those of the breast [3, 4], prostate [16], stomach [13], liver [7], and bladder [29] and associated with high tumor grade and tumor invasiveness and aggressiveness. A reciprocal relationship between the presence of a p53 mutation and Mdm2 gene amplification was demonstrated in canine soft-tissue sarcomas [25]. By contrast, Mdm2 overexpression has been shown to be associated with negative p53 expression [1]. Mdm2 overexpression was present in feline mammary gland adenomas in the absence of p53 overexpression. Discordant overexpression (Mdm2 positive and p53 negative) was found in three adenocarcinomas, although co-overexpression of Mdm2 and p53 was found in 15 adenocarcinomas. The results suggest that p53 overexpression is frequent in adenocarcinomas and may be present after Mdm2 overexpression. Expression of Mdm2 may be not consequent upon p53 activation during feline mammary gland tumorigenesis. Our recent study suggested that discordant Mdm2 overexpression in the absence of p53 overexpression was frequent in canine circumanal gland adenocarcinomas [24].

In conclusion, nuclear overexpression of Mdm2 is present in the tumors of early stage without p53 overexpression and related to feline mammary gland tumorigenesis. Nuclear overexpression of p53 is more frequent in adenocarcinomas, but not in adenomas.

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