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Expression of MCM3, Ki-67 and PCNA in Canine Mammary Tumors

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Abstract

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Mammary tumors are one of the most prevalent neoplasms in female dogs. Histologic grade of canine mammary carcinoma has been assessed to predict prognosis, and recently several molecular prognostic factors are being investigated to permit a more precise prognosis. In this study, the expression of minichromosome maintenance protein 3 (MCM3), Ki-67 and proliferative cell nuclear antigen (PCNA) was analyzed in 68 mammary tissues and correlated with histologic grade. Labeling index of MCM3, Ki-67 and PCNA in malignant mammary lesions was significantly higher than that in benign mammary lesions. Among the malignant tumors, more pronounced expression of the markers was observed in mammary ductal carcinomas than adenocarcinomas. Labeling index of three markers showed positive association with histologic grade, highest in G3 malignant mammary tumors and lowest in G1 tumors. Among the markers, expression of MCM3 showed higher

coefficients with histologic grade than that of Ki-67 and PCNA. Based on the positive correlation result between histologic grade and labeling index of markers, MCM3, Ki-67 and PCNA can be useful as additional prognostic factors. Moreover, MCM3 may be a superior proliferation marker than Ki-67 and PCNA.

Key words : canine mammary tumors, cell proliferative marker, histologic grade, immunohistochemistry, MCM3

CONTENTS

Ⅰ. Introduction

Mammary tumors are one of the most prevalent type of neoplasms in female dogs and approximately half of the tumors are malignant [14, 27]. This malignancy represents one of the principal causes of death owing to neoplastic disease. In most cases of canine mammary tumors, masses are unnoticed by owners of dogs at the preliminary stages because they induce no specific complaints, and most of the dogs are clinically healthy when they are initially presented [26]. In view of above, an early diagnosis and accurate evaluation of prognosis are immensely important. An accurate evaluation of histologic grade of canine mammary carcinoma represents an important element of histopathological diagnosis since it allows prediction of the prognosis [26, 27]. In addition, attempts are made to take advantage of additional cell proliferative markers which would permit a more precise prognosis [33].

In both human and veterinary medicine, several proliferative biomarkers have been used for various precancerous and cancerous conditions. The most widely used conventional proliferation markers include Ki-67 and proliferative cell nuclear antigen (PCNA) [13]. Ki-67 is expressed in the nuclei of cells in the G_1, S, G_2, and M phases of the cell cycle but not in the G_0 phase [8]. However, the precise function in cell proliferation still remains unclear although report suggests that it may be required for ribosome synthesis during cell proliferation [4, 16]. Moreover, Ki-67 only provides limited information on cell cycle state [24, 31]. PCNA is known to act as an auxiliary factor for DNA polymerase δ, and is involved in DNA repair mechanisms as well as replication [22, 29]. Furthermore, PCNA may also be up-regulated in nonproliferating cells [28]. In this respect, the relationship

between PCNA expression to proliferation has been controversial.

The minichromosome maintenance (MCM) proteins are essential for the initiation and elongation of DNA replication [30]. MCM proteins are relatively constant and stable throughout the cell cycle but rapidly disappear after entry into the G_0 phase and terminal differentiation stage in tissues [20]. This makes MCMs specific markers of cell proliferation. The MCM proteins comprise 8 proteins, MCM2 to MCM9 [17]. The function of MCM8, which associates with chromatin at the onset of S-phase, is distinct from MCM2-7 and that of MCM9 is unknown. Although MCM2-7 are functionally and structurally related [6], recent findings suggest that MCM3 cooperates with cyclin-dependent kinases to exclude MCM2-7 from the nucleus to prevent inappropriate rereplication [15]. Anti-MCM antibodies have been investigated as a prognostic factor in various human neoplasms [7, 10, 25, 32], however only few in veterinary medicine [2, 12, 21]. Recent studies have shown that immunohistochemical staining for MCM proteins is associated with histologic grade in various neoplasms [7, 21, 32].

The aims of this study were to examine the distribution pattern of cell proliferative markers including MCM3, Ki-67 and PCNA in canine mammary tumors and also to correlate the frequency of expression of the markers with histologic grade.

Ⅱ. Materials and Methods

1. Tissue samples

This study was performed on formalin-fixed 65 canine mammary masses classified as 22 adenomas, 20 adenocarcinomas and 23 ductal carcinomas (Table 1), referred to Laboratory of Veterinary Pathology, College of Veterinary Medicine, Jeju National University from 2010 to 2012. Diagnosis had been performed on the basis of clinical and histopathologic findings. Three normal canine mammary glands were used as control. Age of the female dogs with mammary tumor ranged from 3 to 19 years (Mean 9.8 ± 3.57 years). The dogs included various breeds.

Table 1. Number of canine mammary samples examined

Histopathologic diagnosis		Number of cases
Malignant	Ductal carcinoma	23
	Adenocarcinoma	20
Benign	Adenoma	22
Normal	Negative control	3

2. Histopathologic examination

The samples were routinely processed for histopathologic examination and sections were stained with hematoxylin and eosin (H&E). The histologic grade (G) of mammary carcinoma was determined using guidelines suggested by Clemente *et al*. [5]. The evaluation of the histologic grade included three parameters scored on a scale from 1 to 3 points: tubule formation, nuclear pleomorphism and mitotic index (Table 2). The sum of the points enabled the distinction of 3 malignancy grades: 3-5 points (G1), 6-7 points (G2), 8-9 points (G3) (Table 3).

Table 2. Histologic grading system of canine mammary carcinoma

Score	Tubule formation	Nuclear pleomorphism	Mitoses in 10 HPF*
1	> 75%	Mild	0-9
2	10-75%	Moderate	10-19
3	< 10%	Marked	> 19

* : High power field (x400)

Table 3. Summary of histologic malignant grade for canine mammary carcinoma

Grade of malignancy	G1	G2	G3
Total score	3 to 5	6 to 7	8 to 9

3. Immunohistochemistry

Immunohistochemical staining for MCM3, Ki-67 and PCNA was performed as follows: formalin-fixed paraffin embedded sections were cut 2 to 3 μm thick, mounted on silane-coated slides (Muto Pure Chemicals, Japan), deparaffinized in xylene, rehydrated in graded ethanol solutions and then washed in distilled water. Endogenous peroxidase activity was blocked with 10% H_2O_2 in phosphate buffered saline (PBS, pH 7.2) for 10 minutes. After being washed in PBS, antigen retrieval was performed with EDTA (10 mM, pH 9.0) for MCM3 and citrate (10 mM, pH 6.0) for Ki-67 and PCNA at 100℃ for 1 hour. The sections were then allowed to cool and rinsed in PBS. The primary antibodies to MCM3, Ki-67 and PCNA were applied in a humidified chamber at 37℃ for 1 hour (Table 4). Following the primary incubation, the sections were washed in PBS and incubated with EnVision^{TM}/HRP, Rabbit/Mouse (EVN) reagent (Dako, Denmark) at 37℃ for 45 minutes. After a further wash in PBS, the color reaction was developed with 3, 3´-diamino-benzidine tetrahydrochloride (Dako, Denmark) and counterstained with Mayer hematoxylin (Sigma, USA). Positive and negative controls were included in all reactions.

Table 4. Type, dilution and source of antibodies for immunohistochemistry

Antibody	Type/Clone	Dilution	Source
MCM3	Monoclonal mouse/101	1 : 50	Dako, Denmark
Ki-67	Monoclonal mouse/MIB-1	1 : 50	Dako, Denmark
PCNA	Monoclonal mouse/PC10	1 : 200	Dako, Denmark

4. Quantification of immunolabeling

Frequency of expressions of MCM3, Ki-67 and PCNA in neoplastic mammary epithelium was determined by calculating a labeling index for each marker. Labeling index was calculated using method suggested by Wojnar *et al.* with some modification [32]. Five fields which had the highest number of positive tumor cells were selected in every section and a labeling index was evaluated scoring the brown-labeled cells nuclei under x400 magnification (Olympus BX51, Japan). The expression of the biomarkers was appraised semiquantitatively by evaluating the mean percentage of positive cells (Table 5).

Table 5. Criteria for labeling index in canine mammary tumors

Immuno-histochemical labeling	No reaction	Weak	Moderate	Strong	Intensive
	0-3%	4-25%	26-50%	51-75%	> 75%
Labeling index	0	1	2	3	4

5. Statistical analysis

The statistical analysis was conducted with the SPSS software (SPSS *ver.* 21.0). Association between labeling indices of histopathologic diagnosis were assessed using Kruskall-Wallis test. Mann-Whitney test was used for post hoc comparison between each histopathologic diagnosis and for comparison between ductal carcinoma and adenocarcinoma. Using Spearman's correlation analysis, linear relationship between the labeling index of each marker and histologic grade was assessed. P value under 0.05 was considered to be statistically significant.

Ⅲ. Results

1. Histopathologic features of canine mammary tumors

Mammary ductal carcinomas were composed of neoplastic cells that surround slitlike lumina with multiple epithelial layers. Most neoplastic cells were arranged in tubules or cords that often lined by a double epithelial cell layers resembled normal mammary ducts. Neoplastic cells showed marked cellular and nuclear pleomorphism (Table 6). These neoplastic cells had round to oval nuclei with prominent nucleoli and scant basophilic cytoplasm (Fig. 1). Mitotic figures, strong invasive tendency and neoplastic cell emboli in lymphatic and blood vessels were frequently found (Fig. 2). Mammary adenocarcinomas were composed of one type of cell and showed invasive tendency to surrounding tissues (Fig. 3). These tumors showed various arrangement of neoplastic cells such as tubulopapillary (Fig. 4A), solid (Fig. 4B), comedo (Fig. 4C) and cribriform (Fig. 4D) type. These types of cell arrangement were also found in ductal carcinoma, but comedo type and cribriform type were much more frequently observed in ductal carcinomas than in adenocarcinomas. Adenomas had well-demarcated noninfiltrative lesions composed of uniform sized neoplastic mammary glands with mostly single cell layer of epithelium.

Table 6. Histologic comparison between canine mammary ductal carcinomas and adenocarcinomas

Histologic features	Ductal carcinomas	Adenocarcinomas
Epithelial distribution	More than double layer	Single layer
Staining characteristic	Basophilic	Eosinophilic
Pleomorphism	Generally high	Various
Nucleoli	Prominent	Various
Tumor cell emboli	Frequent	Various

2. Histologic grade of mammary tumors

Between those malignant mammary tumors, overall histologic grade of ductal carcinomas showed higher tendency than that of adenocarcinomas. The histologic grade of ductal carcinoma cases included only G3 and G2, but low grade G1 in adenocarcinoma occupied 6 (30%) out of 20 cases (Table 7).

Table 7. The result of histologic grade in canine malignant mammary tumors

Histopathologic diagnosis	Number of cases (%)			Total
	G1	G2	G3	
Ductal carcinoma	0 (0.0)	15 (65.2)	8 (34.8)	23
Adenocarcinoma	6 (30.0)	8 (40.0)	6 (30.0)	20

3. Expression pattern of cell proliferative markers

MCM3, Ki-67 and PCNA were expressed in the nuclei of tumor cells in both lobules and ducts. In general, Ki-67 showed lower positivity than MCM3 and PCNA, and 7 cases including 4 cases of adenocarcinoma and 3 cases of adenoma did not express Ki-67 antigens. PCNA showed higher positivity than MCM3, and tended to show nonspecific binding to normal tissues. Difference of expression pattern among each proliferative marker is demonstrated in Figure 5.

4. Comparison between histopathologic diagnosis and labeling index

Labeling index of MCM3, Ki-67 and PCNA in malignant mammary lesions was significantly higher than those in benign mammary lesions ($P<0.001$, $P=0.005$, $P=0.004$) (Table 8). Among the malignant tumors, more pronounced expression of MCM3 and Ki-67 was observed in ductal carcinomas than adenocarcinomas ($P=0.019$, $P=0.024$). However for PCNA, the difference between ductal carcinoma and adenocarcinoma proved to be insignificant ($P=0.227$).

Table 8. Mean labeling index for three markers in canine mammary samples

Histopathologic diagnosis		Labeling index (Mean ± SD)		
		MCM3	Ki-67	PCNA
Malignant	Ductal carcinoma (n=23)	3.0 ± 0.69	1.7 ± 0.79	3.7 ± 0.48
	Adenocarcinoma (n=20)	2.4 ± 0.79	1.2 ± 0.79	3.4 ± 0.44
Benign	Adenoma (n=22)	1.7 ± 0.81	0.9 ± 0.42	3.1 ± 0.63
Normal	Negative control (n=3)	0	0	0.3 ± 0.47

5. Comparison between histologic grade of malignant mammary tumors and labeling index

The labeling index of three markers showed significant positive correlation with the histologic grade, highest in G3 malignant mammary tumors and lowest in G1 tumors (Table 9). Among the markers, the highest correlation coefficient with histologic grade was found in MCM3 (r=0.608; $P<0.001$), followed by Ki-67 (r=0.496; $P<0.001$) and PCNA (r=0.420; $P=0.001$).

Table 9. Results of comparison between histologic grade of malignant mammary tumors and labeling index

Histologic grade	Labeling index (Mean ± SD)		
	MCM3	Ki-67	PCNA
G3 (n=14)	3.0 ± 0.76	1.7 ± 0.80	3.8 ± 0.41
G2 (n=23)	2.7 ± 0.76	1.5 ± 0.83	3.4 ± 0.50
G1 (n=6)	2.0 ± 0.58	0.8 ± 0.69	3.3 ± 0.94

Ⅳ. Discussion

The mammary gland is one of the most common sites of tumor development in dogs and cats [18, 19]. To predict prognosis of diseases, unlike feline mammary tumors in which size is the most important prognostic factors, combination of grading systems, size, invasiveness and the overall health of the patient should be considered in canine mammary tumors [19]. Several histopathologic grade and clinical features of canine mammary gland tumors have been widely studied from a prognostic standpoint and recently several molecular prognostic factors are being investigated to permit a more precise prognosis [13, 26, 27]. This study was conducted to examine the expression of MCM3, Ki-67 and PCNA, which might be useful as additional prognostic factors in canine mammary tumors.

Many studies have proved that MCM proteins can be reliable markers for proliferative or malignant cells in human medicine [7, 10, 25, 32]. MCM proteins, which include the group of eight proteins (MCM2 to 9), are responsible for the start and maintenance of replication [17]. MCM2-7 proteins has similar biochemical functions and are equally important for chromosome replication [6], but MCM8 has distinct function from MCM2-7 and the function of MCM9 is unknown [17]. MCM2-7 might act as DNA helicases and are dissociated from chromatin after replication in order that they restrict DNA synthesis to only once per cell cycle and regulate DNA elongation [30]. Recent findings suggest that MCM3 cooperates with cyclin-dependent kinases to exclude MCM2-7 from the nucleus to prevent inappropriate rereplication [15]. These features make MCM3 protein more specific indicator of cell proliferation.

Although only a few studies have investigated MCM protein expression in

veterinary medicine to date, these studies also have shown that MCM proteins could be sensitive proliferation markers in various tumors. Berlato *et al.* [2] and Nowak *et al.* [21] reported that MCM proteins are sensitive and useful markers of proliferation in cutaneous mast cell tumors, mammary adenocarcinomas and soft tissue fibrosarcomas. Ishino *et al.* [12] evaluated the distribution pattern and frequency of MCM7 and Ki-67 expression in canine pituitary corticotroph adenomas and concluded that MCM7 may be superior to Ki-67 as a proliferation marker.

In the present study, the distribution pattern of cell proliferative biomarkers including MCM3, Ki-67 and PCNA and the expression of these markers as related to the histologic grade were analyzed in 68 canine mammary tissues. Immunohistochemical staining was conducted and labeling index of each marker was calculated. Labeling index of the markers in malignant mammary tumors was significantly higher than that in benign mammary tumors. In a similar way, Reena *et al.* [23] documented MCM2 expression in malignant breast lesions was significantly higher than that in benign breast lesions. Our results showed that between the malignant neoplasms, more pronounced expression of MCM3 and Ki-67 in ductal carcinomas as compared to adenocarcinomas was observed. In addition, ductal carcinomas had higher grade of malignancy than adenocarcinoma. Histopathologically, neoplastic cells of ductal carcinoma showed marked nuclear and cellular pleomorphism, and mitosis and emboli in blood or lymphatic vessels were frequently observed. However mammary adenocarcinomas showed variable features of malignancy according to histopathologic tumor type. Based on the histopathologic characteristics and immunohistochemical results, mammary tumors arose from mammary ductal epithelium may have higher malignant tendency than those arose from secretory lobular epithelium.

There was a significant correlation between histologic grade of canine malignant mammary tumors and immunoreactivity for each marker. The

higher the grade was, the more positively staining cells were observed in most malignant mammary tumors. Among the markers, statistically MCM3 showed higher coefficients with the grade of malignancy than Ki-67 and PCNA.

The expression of Ki-67 in histologic grade of malignant mammary tumors showed similar pattern as MCM3. However, overall positivity of Ki-67 was generally lower than immuno-labeling of MCM3. Although Ki-67 has been widely used as a cell proliferation marker, it has frequently proven to be of limited diagnostic and prognostic value [24, 31]. Ki-67 presents in the nuclei of cells in the G_1, S, G_2 and M phases of the cell cycle, but not in G_0 and early G_1 phase [1]. Also the expression of Ki-67 is affected by external factor such as nutrient deprivation, which could subsequently underestimate the number of cells in the cycle. In comparison to MCM proteins, Ki-67 showed reduced sensitivity and specificity in several human cancers [11]. This is in accordance with our observations, which showed lower immunopositivity for Ki-67 compared to MCM3.

PCNA is a nuclear protein which is necessary for DNA synthesis in eukaryotes [22, 29]. However, PCNA has a major limitation as a proliferation marker because of its redundant role in DNA repair. In the previous studies, PCNA showed lower sensitivity for tumor cells than MCM proteins [3, 9]. Our results showed the labeling index of PCNA was correlated with the histologic grade, however the statistical significance was lower than MCM3 and Ki-67. Moreover, PCNA tended to show nonspecific binding which suggests that PCNA might have a chance of detecting normal cells as well as cancer cells. In view of the above, MCM3 protein might be a better candidate marker of cell proliferation than Ki-67 and PCNA in canine mammary tumors.

In summary, we have proved that MCM3, Ki-67 and PCNA are reliable markers for proliferating cells in canine mammary tumors. These cell

proliferative markers can be useful as additional prognostic factors for improving estimation of prognosis and guiding therapeutic decisions. Moreover, among these markers, MCM-3 may be a superior proliferation marker than Ki-67 and PCNA.

Ⅴ. Conclusion

We conducted histopathologic and immunohistochemical examinations using cell proliferative markers MCM3, Ki-67 and PCNA on 68 canine mammary tissues and the following results were obtained.

1. Labeling index of MCM3, Ki-67 and PCNA in malignant mammary tumors was significantly higher than that in benign mammary tumors. Among the malignant tumors, more pronounced expression of the markers was observed in mammary ductal carcinomas than adenocarcinomas.

2. There was a significant correlation between the grade of malignancy and immunoreactivity for each marker.

3. Statistically expression of MCM3 showed higher coefficients with the grade of malignancy than Ki-67 and PCNA.

To sum up, MCM3, Ki-67 and PCNA could be useful as additional prognostic factors for improving estimation of prognosis and guiding therapeutic decisions in canine mammary tumors. In addition, among these markers MCM3 may be a superior proliferation marker than Ki-67 and PCNA.

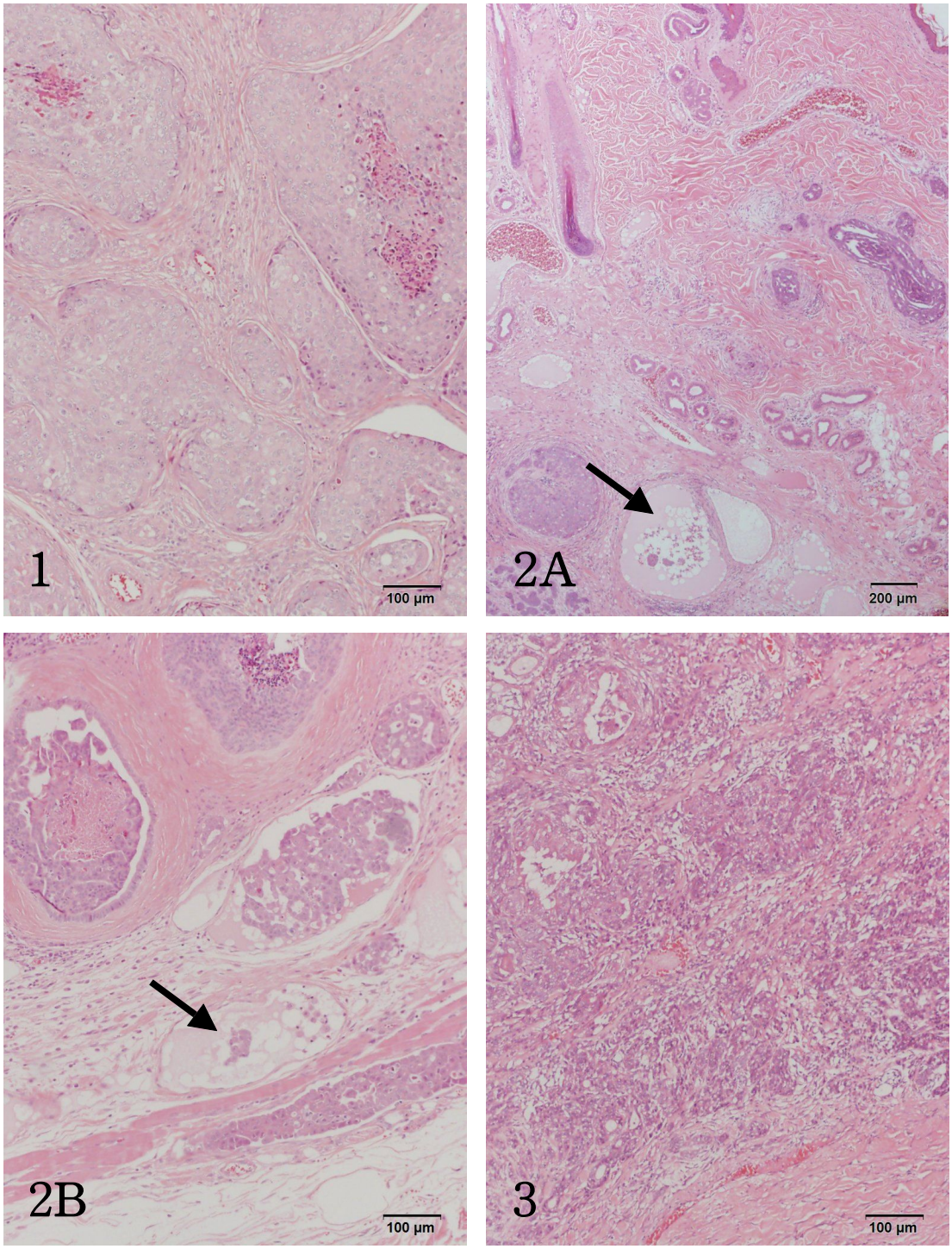
Legends for Figures

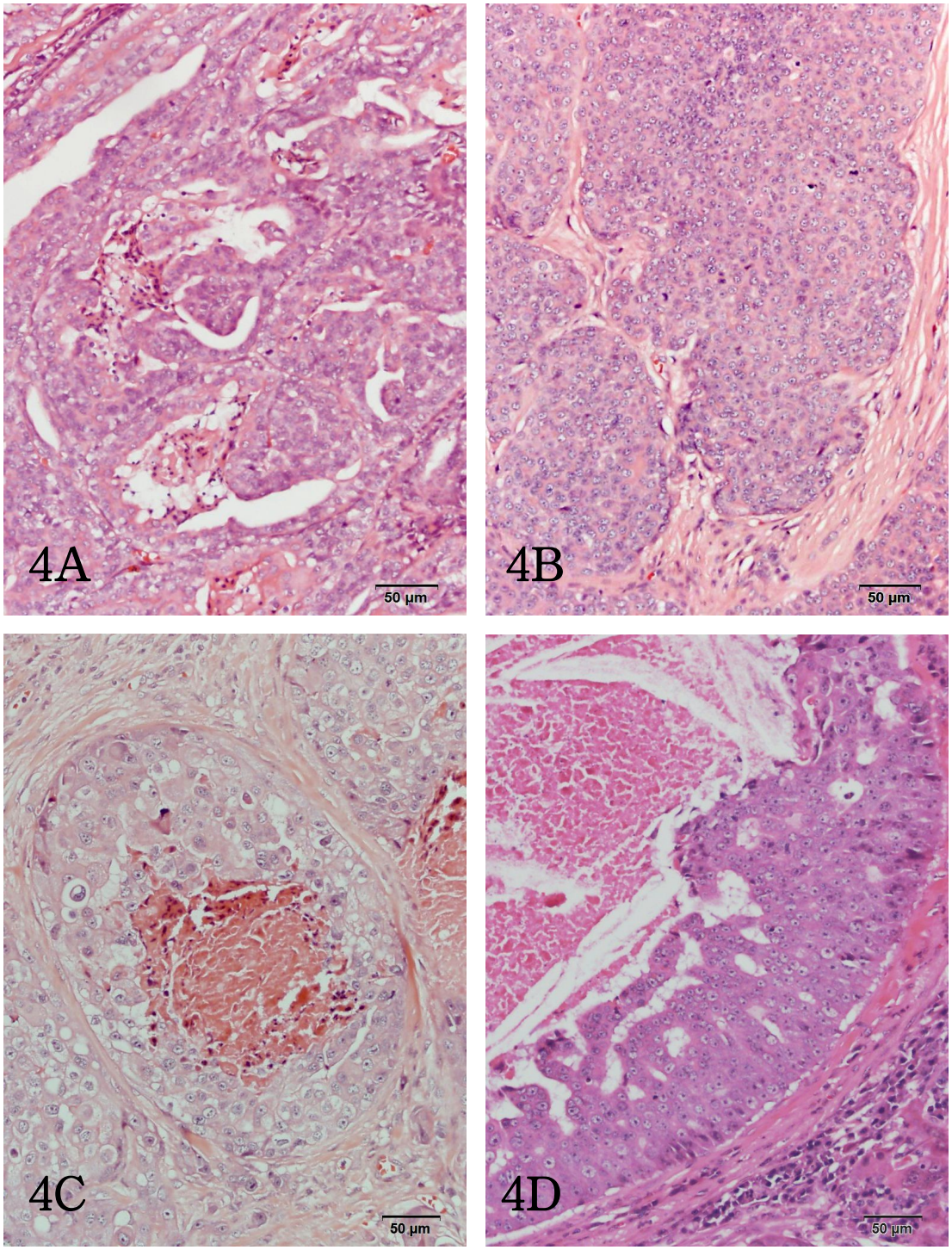
Fig. 1. Mammary ductal carcinoma. The neoplastic ducts were lined by a bi- or multi-layered epithelium and neoplastic cells had prominent nucleoli and basophilic cytoplasm, H&E, x100.

Fig. 2. Tumor emboli (arrows) in superficial (A, H&E, x40) and deep (B, H&E, x100) lymphatic plexus in ductal carcinoma.

Fig. 3. Mammary adenocarcinoma. The tubules were lined by single- to multi-layered epithelium and showed invasive tendency to adjacent tissues, H&E, x100.

Fig. 4. Neoplastic cell arrangement in mammary ductal carcinoma and adenocarcinoma. A: Tubulopapillary type had neoplastic tubules arranged in a sessile or pedunculated papillary fashion. B: Solid type had neoplastic cells arranged in solid sheets or masses without lumina. C: Comedo type had necrotic areas within the center of the neoplastic cell aggregates. D: Cribriform type had neoplastic epithelial cells formed a sievelike arrangement, H&E, x200.





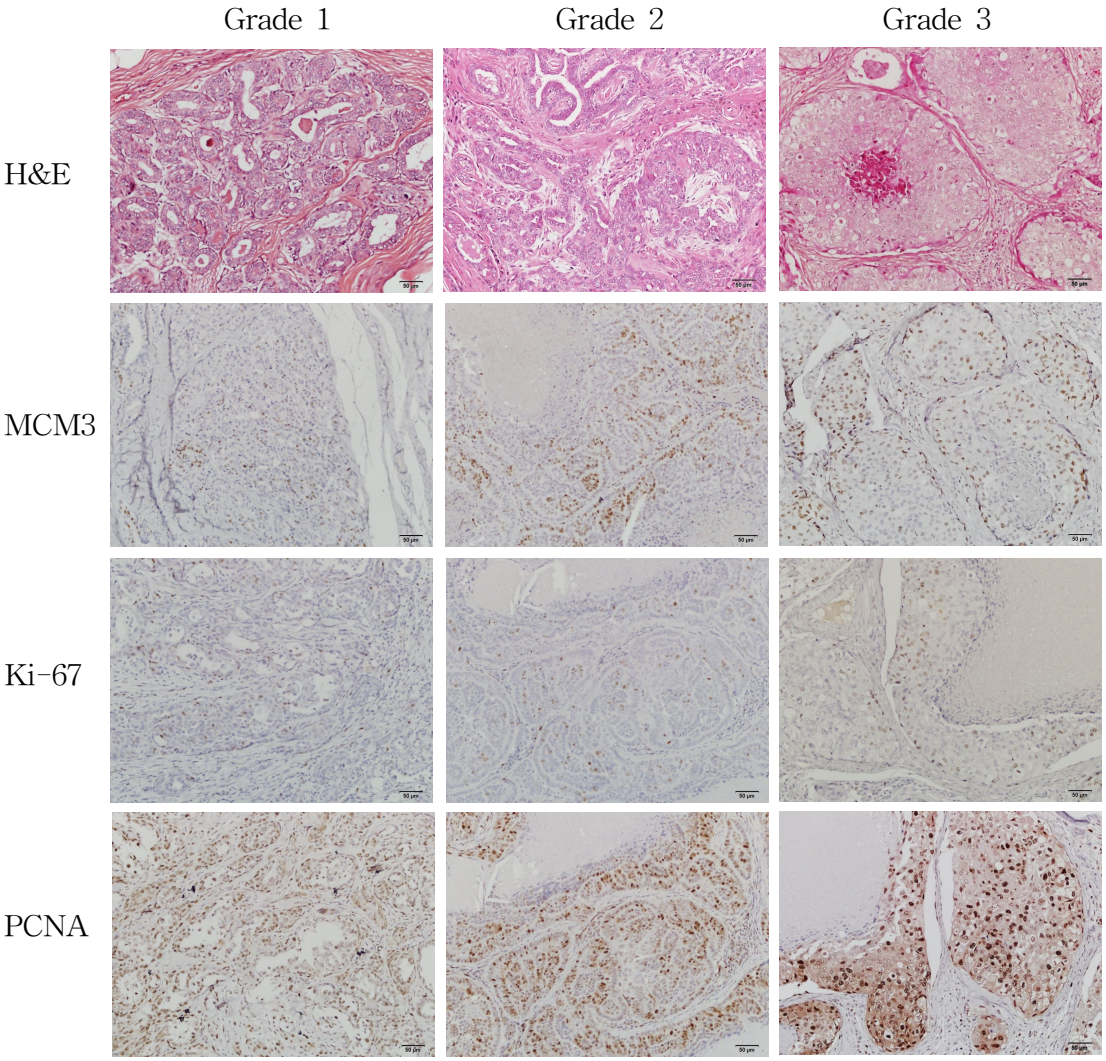


Fig. 5. Histopathologic features (H&E) and expression of MCM3, Ki-67 and PCNA (immunohistochemical staining) in each grade of mammary tumors. The higher the grade was, the more immunopositive cells in neoplastic tissues were observed, x200.

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초록

개 유선종양에서 MCM3, Ki-67 및 PCNA의 발현

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개에서 유선종양은 흔히 발생되는 종양 중 하나이다. 종양의 악성도를 정확히 평가하는 것은 질병의 예후를 예측할 수 있기 때문에 병리조직학적으로 매우 중요한 요소이다. 최근에는 보다 정확한 예후평가를 위해 minichromosome maintenance (MCM) protein, Ki-67, proliferative cell nuclear antigen (PCNA)과 같은 세포증식성 마커들이 사용되고 있다. 본 실험의 목적은 개의 유선 종양에서 MCM3, Ki-67, PCNA와 같은 세포증식성 마커들의 발현 여부를 조사하고, 항원의 발현도와 조직학적 악성도 간의 상관관계를 분석하고자 하였다. 총 68건의 유선조직에 대하여 MCM3, Ki-67, PCNA 항체를 이용하여 면역조직화학염색을 실시하였으며, 각각의 항원 발현과 조직학적 종양의 악성도를 평가하여 상관성을 비교 분석하였다. 세포증식성 마커들의 발현은 선과 도관의 종양화된 상피세포의 핵에서 관찰되었으며, 악성 유선종양에서 항원의 발현도가 양성 유선종양에 비하여 현저히 높았다. 또한 악성 유선종양 중에서는 선유래암종에 비해 도관유래암종에서 항원의 발현도와 조직학적 악성도가 높았다. 조직학적인 악성도 평가 결과와 항원의 발현도를 비교했을 때, 세 항체 모두에서 조직학적인 악성도가

높아질수록 항원의 발현도가 증가하는 것이 관찰되었고 통계학적으로 높은 상관관계를 나타냈다. 세 항체 중에서는 MCM3의 통계학적인 유의성이 Ki-67과 PCNA보다 높았다. 이러한 결과를 토대로 MCM3, KI-67, PCNA 모두 개의 유선종양의 예후를 판정하는데 보조적인 역할을 할 수 있을 것으로 사료되며, 마커들 중에는 MCM3가 Ki-67과 PCNA에 비해 우수한 세포증식성 마커로 판단된다.

중심어 : 개 유선종양, 면역조직화학염색, 세포증식성 마커, 조직학적 악성도, MCM3