NOTE Pathology

Relationship between Major Histocompatibility Complex Class I Expression and Prognosis in Canine Mammary Gland Tumors

Toshiyuki TANAKA1), Terumasa SHIMADA2), Hideo AKIYOSHI3), Junichiro SHIMIZU1), Cao ZHENG1), Li YIJUN1), Keiichiro MIE1), Akiyoshi HAYASHI3), Mitsuru KUWAMURA3), Fumio HOSHI4) and Fumihito OHASHI1)*

1)Laboratory of Veterinary Surgery, Department of Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinku-Oraikita, Izumisano, Osaka 598–8531, Japan
2)Veterinary Medical Center, Department of Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinku-Oraikita, Izumisano, Osaka 598–8531, Japan
3)Laboratory of Veterinary Pathology, Department of Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinku-Oraikita, Izumisano, Osaka 598–8531, Japan
4)Department of Small Animal Internal Medicine, School of Veterinary Medicine, Kitasato University, 23–35–1 Higashi, Towada, Aomori 034–8628, Japan

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ABSTRACT. The aim of this study was to evaluate MHC class I expression and prognosis using tumor tissues surgically removed from 9 dogs with mammary gland carcinomas and from 13 dogs with complex carcinomas. We assessed MHC class I expression and its correlation with tumor size, B2M expression, infiltration of lymphocytes, histological grade and prognosis. Hematoxylin and eosin-stained sections were histologically graded using the Elston and Ellis grading method. MHC class I expression on tumor cells was evaluated using the avidin-biotin peroxidase complex method. Loss of MHC class I expression from canine mammary gland carcinomas was significantly correlated with poor prognosis (P<0.05). Loss of MHC class I expression showed no association with poor prognosis in canine mammary gland complex carcinomas, because the data were not balanced. Only 1 of 13 (7.6%) canine mammary gland complex carcinomas showed loss of MHC class I expression. All 13 of these dogs showed good prognosis. Thus, the low frequency of MHC class I expression loss from canine mammary gland complex carcinomas may be associated with good prognosis. Taken together, these results suggest that loss of MHC class I expression may be associated with poor prognosis in canine mammary gland carcinomas.

KEYWORDS: beta2-microglobulin, canine, mammary gland carcinoma, MHC class I.

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Major histocompatibility complex (MHC) class I antigens are composed of both variable chain MHC class I proteins and beta2-microglobulin (B2M) [18], an invariant chain essential for the structural stability and optimal function of these proteins [16]. In humans, MHC class I antigens are expressed on the surface of most nucleated cells [12]. Loss or downregulation of MHC class I expression has been reported in many types of cancer, including breast cancer [1, 2, 18]. The frequency of MHC class I expression loss and/or downregulation in breast cancer ranges from 37 to 88% [1, 18]. This loss or downregulation of MHC class I expression has been associated with disease progression and/or poor clinical outcome [2]. Cells with loss of these antigen proteins from the cell surface may escape recognition by CD8+ T lymphocytes [18]. Loss of MHC class I expression in breast cancer is caused by loss of B2M expression and/or function [4]. In dogs, mammary gland tumors are the second most commonly occurring neoplasms [13]. These tumors show different growth patterns and biological behavior, depending on whether the tumors are simple or complex carcinomas [9, 11]. Analysis of potentially important prognostic factors in canine mammary gland tumors has been the focus of many studies. Prognostic factors in dogs with these tumors include tumor size, histological type, evidence of metastasis at the time of diagnosis, clinical stage and histological grade [3, 8, 17]. Although canine and human mammary gland tumors have similar features, including histological appearance and biological behavior [20], no study to date has evaluated the relationship between loss or downregulation of MHC class I expression and prognosis in dogs with these tumors. This study therefore assessed MHC class I expression and its correlations with B2M expression, tumor size, infiltration of lymphocytes, histological grade and prognosis in dogs with mammary gland carcinoma and complex carcinomas.

This observational study involved 22 female dogs with mammary gland tumors, including 9 with mammary gland carcinomas and 13 with mammary gland complex carcinomas, admitted to the Veterinary Clinical Center of Osaka Prefecture University from June 2009 to October 2010. Histopathological findings were used to classify the tumors according to the criteria of a recently validated system [10]. Median dog age was 12 years (range 8 to 14 years). None of the dogs had metastases. Clinical data collected for all dogs included age at diagnosis, tumor size, treatment, relapse and...
survival. Dogs were followed-up for at least 2 years after surgical excision of the mammary gland tumors with assessment of no relapse or survival, rather than the disease-free interval. Since different types of treatment and deaths due to unrelated causes or euthanasia were considered confounders of survival time as an end point [6], dogs that died from unrelated causes or were euthanized were excluded. All dogs were treated by surgery only. Tumor tissue was removed and subjected to histopathological and immunohistochemical analysis. The study protocol was approved by the animal ethics review committee of Osaka Prefecture University.

Hematoxylin and eosin (HE)-stained sections were histologically graded using the Elston and Ellis grading method as described previously [8, 19]. Grading was based on (1) gland tubules and acini formation, (2) pleomorphism of tumor cell nuclei and (3) mitotic counts with each feature scored from 1 to 3 points. Tumors with 3–5, 6–7 and 8–9 points were considered as having histological grades 1 (well differentiated), 2 (moderately differentiated) and 3 (poorly differentiated), respectively (Fig. 1). Infiltration of lymphocytes was evaluated using HE-stained sections semiquantitatively with − indicating no infiltration and + indicating mild, moderate or diffuse infiltration.

MHC class I expression on tumor cells was assayed using mouse anti-dog MHC class I monoclonal antibody (VMRD, Inc., Pullman, WA, U.S.A., diluted 1:200) and a commercial streptavidin-biotin kit (LSAB+ Kit/HRP; Dako North America, Inc., Carpinteria, CA, U.S.A.). Briefly, sections, 1 cm in diameter, were embedded in optimal cutting temperature (OCT) embedding medium (Tissue Mount; Chiba Medical Co., Ltd., Saitama, Japan) and cut, air dried at room temperature for 15 min and again washed. The sections were incubated with streptavidin conjugated to horseradish peroxidase (HRP) at room temperature for 30 min, washed, incubated with streptavidin conjugated to horseradish peroxidase (HRP) at room temperature for 30 min, washed in PBS for 5 min and developed for 5 min using a 3,3′-diaminobenzidine chromogen (DAB) H2O2 solution. Color development was stopped by diluting in distilled deionized H2O, and the sections were counterstained with hematoxylin. Normal mammary gland or non-tumor lymph nodes of canine tissue were used as positive controls. MHC class I expression score was measured as described previously [18]. MHC class I-expressing tumor cells were analyzed in 20 different fields of each tumor, and the values reported represent the means of the area calculated. MHC class I expression was regarded as negative, if immunoreactivity was observed in <10% of tumor cells. MHC class I expression was regarded as positive, if >10% of tumor cells were stained (Fig. 2).

B2M expression on tumor cells was assessed similarly using rabbit anti-dog B2M serum (10 µg/ml, diluted 1:500), the kind gift of Dr F. Hoshi (Kitasato University School of Veterinary Medicine, Towada, Japan) [14, 15] and a commercial streptavidin-biotin kit (LSAB+ Kit/HRP; Dako). To assay B2M expression, formalin-fixed paraffin-embedded sections of surgical specimens were deparaffinized and boiled for 15 min in a microwave oven for antigen retrieval. The sections were incubated with 3% H2O2 at room temperature for 10 min, washed with PBS for 5 min and incubated with 5% skimmed milk in PBS at room temperature for 60 min. The sections were subsequently incubated with optimally diluted rabbit anti-dog B2M serum at room temperature for 30 min, washed, incubated with biotinylated anti-mouse IgG (Dako) at room temperature for 30 min, washed, incubated with streptavidin conjugated to horseradish peroxidase (HRP) at room temperature for 30 min, washed in PBS for 5 min and developed for 5 min using a 3,3′-diaminobenzidine chromogen (DAB) H2O2 solution. Color development was stopped by diluting in distilled deionized H2O, and the sections were counterstained with hematoxylin. Normal mammary gland or non-tumor lymph nodes of dogs were used as positive controls. B2M expression score was measured as above (Fig. 2).

All statistical analyses were performed using R software (version 2.12.1). Continuous variables were assessed using Fisher’s exact test with statistical significance set at P<0.05. To assist in determining between-group differences, effect size statistics were calculated for each dependent variable. An effect size (Cramer’s coefficient of association) of 0.5
was defined as a meaningful between-group difference. MHC class I expression score was categorized as negative or positive; tumor size was classified as >3 cm or <3 cm; B2M expression was classified as negative or positive; infiltration of lymphocytes was classified as negative or positive; histological grade was classified as 1, 2 or 3; and disease-free interval was classified as >24 months or <24 months.

In 9 dogs with mammary gland carcinomas, the histological grade was 1 in 1 dogs (11%), 2 in 3 dogs (33%) and 3 in 5 dogs (56%). Immunohistochemical analysis of MHC class I expression in mammary gland carcinomas showed that 5 dogs had a positive score and 4 dogs had a negative score. Regarding MHC class I expression, immunoreactivity was observed in the membrane and cytoplasm. Tumors from 4 of the 9 dogs (44.4%) had lost MHC class I expression (Table 1). Of the 5 dogs with positive scores for MHC class I expression, 4 (80%) had tumors >3 cm in size, and 1 (20%) had a tumor <3 cm in size. Of the 4 dogs with negative scores for MHC class I expression, 3 (75%) and 1 (25%) had tumors >3 cm and <3 cm in size, respectively. Fisher’s exact test showed no significant association between MHC class I expression and tumor size (Table 1). Of the 5 tumors positive for MHC class I expression, 3 (60%) were positive, and 2 (40%) were negative for B2M expression. Regarding B2M expression, immunoreactivity was observed in the membrane and cytoplasm. All 4 tumors negative for MHC class I expression were also negative for B2M expression. Fisher’s exact test revealed no significant association between MHC class I and B2M expression (Table 1). However, the effect size statistic was large (0.63), suggesting that loss of MHC class I expression was significantly associated with loss of B2M expression (Table 1). Of the 5 tumors positive for MHC class I expression, 3 (60%) were positive, and 2 (40%) were negative for infiltration of lymphocytes. Of the 4 dogs with negative scores for MHC class I expression, 0 (0%) and 4 (100%) were positive and negative for infiltration of lymphocytes, respectively. Fisher’s exact test revealed no significant association between MHC class I and infiltration of lymphocytes (Table 1). However, the effect size statistic was large (0.63), suggesting that loss of MHC class I expression was significantly associated with infiltration of lymphocytes (Table 1). Of the 5 tumors positive for MHC class I expression, 1 (20%), 3 (60%) and 1 (20%) were of histological grades 1, 2, and 3, respectively, whereas the tumors of all 4 dogs negative for MHC class I expression were of histological grade 3. Fisher’s exact test showed that MHC class I expression was not significantly associated with histological grade (Table 1). Staining intensity had no association with histological grade. All 5 dogs positive for MHC class I expression survived without relapse or shortened survival for 2 years after surgical treatment. In contrast, all 4 dogs negative for MHC class I expression relapsed or died during the 2 years after surgical treatment (median survival, 3.5 months; range, 1 to 6). Fisher’s exact test showed a significant relationship between loss of MHC class I expression and shorter event-
In the 13 dogs with mammary gland complex carcinomas, the histological grades was 1 in 7 dogs (54%), 2 in 4 dogs (31%) and 3 in 2 dogs (15%). Immunohistochemical analysis of the 13 mammary gland complex carcinomas showed that 12 (92.4%) were positive and 1 (7.6%) was negative for MHC class I expression. Regarding MHC class I expression, immunoreactivity of luminal epithelial cells was observed in the membrane and cytoplasm. Immunoreactivity of myoepithelial cells was the same as that of luminal epithelial cells. Of the 12 tumors positive for MHC class I expression, 6 (50%) each had tumors >3 cm and <3 cm in size, although the tumor negative for MHC class I expression was >3 cm in size. Fisher’s exact test showed that the association between MHC class I expression and tumor size was not statistically significant (Table 1). Immunohistochemical analysis of the 12 tumors positive for MHC class I expression showed that 11 (92%) were positive for B2M expression and 1 (8%) was negative. Regarding B2M expression, immunoreactivity of luminal epithelial cells was observed in the membrane and cytoplasm. Immunoreactivity of myoepithelial cells was the same as that of luminal epithelial cells. The tumor negative for MHC class I expression was positive for B2M expression. Fisher’s exact test and the small effect size statistic (0.53) suggested that loss of MHC class I expression was significantly associated with loss of B2M expression (Table 1). Of the 12 tumors positive for MHC class I expression, 6 (50%), 4 (33%) and 2 (17%) were of histological grades 1, 2 and 3, respectively. The tumor negative for MHC class I expression was of histological grade 1. Fisher’s exact test showed no significant association between MHC class I expression and histological grade (Table 1). Staining intensity had no association with histological grade. All 12 dogs with tumors positive for MHC class I expression and the 1 dog with a tumor negative for MHC class I expression survived without relapse for 2 years after surgical treatment. Fisher’s exact test showed no significant association between loss of MHC class I expression and the event-free interval (Table 1). Previously reported prognostic factors in dogs with mammary gland tumors included tumor size, histological type, evidence of metastasis at the time of diagnosis, clinical stage and histological grading using the Elston and Ellis grading method [3, 8, 17]. This study showed that loss of MHC class I expression was associated with loss of B2M expression and prognosis. In human cancers, abnormalities in MHC class I expression can be caused by alterations in the MHC class I processing machinery, including TAP1, TAP2, tapasin, LMP2, LMP7 and B2M [2]. In human breast cancer, loss of MHC class I expression is caused by loss of B2M expression and/or function [4]. Tumor cells that do not express MHC class I proteins on their surfaces can escape recognition by CD8+ T lymphocytes [18]. Thus, loss or downregulation of MHC class I expression has been associated with disease progres-

| Tumor size | Positive n (%) | Negative n (%) | P  | Effect size |
|------------|----------------|----------------|----|-------------|
| >3 cm      | 4 (80)         | 3 (75)         | 0.72|             |
| <3 cm      | 1 (20)         | 1 (25)         | 0.05|             |

| B2M expression | Positive | Negative | P  | Effect size |
|----------------|----------|----------|----|-------------|
| Positive       | 3 (60)   | 0 (0)    | 0.12|             |
| Negative       | 2 (40)   | 4 (100)  | 0.63|             |

| Infiltration of lymphocytes | Positive | Negative | P  | Effect size |
|-----------------------------|----------|----------|----|-------------|
| Positive                    | 3 (60)   | 0 (0)    | 0.12|             |
| Negative                    | 2 (40)   | 4 (100)  | 0.63|             |

| Histological grade | Positive | Negative | P  | Effect size |
|---------------------|----------|----------|----|-------------|
| 1                   | 1 (20)   | 0 (0)    | 0.09|             |
| 2                   | 3 (60)   | 0 (0)    | 0.8 |             |
| 3                   | 1 (20)   | 4 (100)  |     |             |

| Event-free interval | Positive | Negative | P  | Effect size |
|---------------------|----------|----------|----|-------------|
| >24 months          | 5 (100)  | 0 (0)    | 0.008|            |
| <24 months          | 0 (0)    | 4 (100)  | 1 |             |

P was calculated using Fisher’s exact test with statistical significance set at P<0.05. An effect size of 0.5 was identified as a meaningful difference between groups.
Carcinoma were correlated. Moreover, infiltration by CD8-positive expression of MHC class I and infiltration of lymphocytes, invading surrounding tissues and/or starts to metastasize to regional lymph nodes or distant organs [7]. None of the dogs in our study showed any evidence of metastasis. Therefore, loss of MHC class I expression may be independent of tumor size.

This study is limited by the small sample size. In future studies, a larger number of mammary gland tumors should be evaluated with regard to loss of MHC class I expression as an independent prognostic factor by multivariate analysis, and the mechanism of MHC class I expression loss should also be explored. In conclusion, our findings suggested that loss of MHC class I expression may be a factor associated with poor prognosis in dogs with mammary gland carcinoma.

REFERENCES

1. Aptsiauri, N., Cabrera, T., Garcia-Lora, A., Lopez-Nevo, M. A., Ruiz-Cabello, F. and Garrido, F. 2007. MHC class I antigens and immune surveillance in transformed cells. *Int. Rev. Cytol.* 256: 139–189. [Medline] [CrossRef]

2. Chang, C. C., Campoli, M. and Ferrone, S. 2003. HLA class I defects in malignant lesions: what have we learned? *Keio J. Med.* 52: 220–229. [Medline] [CrossRef]

3. Chang, S. C., Chang, C. C., Chang, T. J. and Wong, M. L. 2005. Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998–2002). *J. Am. Vet. Med. Assoc.* 227: 1625–1629. [Medline] [CrossRef]

4. Chen, H. L., Gabriolovich, D., Virmanni, A., Ratmani, I., Girgis, K. R., Nadaf-Rahrov, S., Fernandez-Viña, M. and Carbone, D. P. 1996. Structural and functional analysis of β2 microglobulin abnormalities in human lung and breast cancer. *Int. J. Cancer* 67: 756–763. [Medline]

5. Estrella-Lima, A., Araújo, M. S., Costa-Neto, J. M., Teixeira-Carvalho, A., Barron-Melo, S. M., Cardoso, S. V., Martins-Filho, O. A., Serakides, R. and Cassali, G. D. 2010. Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates. *BMC Cancer* 10: 256. [Medline] [CrossRef]

6. Gilbertson, S. R., Kurzman, I. D., Zachrau, R. E., Hurvitz, A. I. and Black, M. M. 1983. Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs. *Vet. Pathol.* 20: 127–142. [Medline]

7. Hsieh, C. H., Hsu, Y. J., Chang, C. C., Liu, H. C., Chuang, K. L., Chuang, C. K., Pang, S. T., Hasumi, K., Ferrone, S. and Liao, S. K. 2009. Total HLA class I loss in a sarcomatoid renal carcinoma cell line caused by the coexistence of distinct mutations in the two encoding beta2-microglobulin genes. *Cancer Immunol. Immunother.* 58: 395–408. [Medline] [CrossRef]

8. Karayannopoulou, M., Kaldymidou, E., Constantinidis, T. C. and Dessiris, A. 2005. Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *J. Comp. Pathol.* 133: 246–252. [Medline] [CrossRef]

9. Misdorp, W. and Hart, A. A. 1976. Prognostic factors in canine mammary carcinoma. *J. Natl. Cancer Inst.* 56: 779–786. [Medline]

10. Misdorp, W., Else, R. W., Hellmén, E. and Lipscumb, T. P. 1999. Histological classification of mammary tumors of the dog and the cat. pp. 11–29. In: Armed Forces Institute of Pathology and the American Registry of Pathology and the World Health Organization Collaborating Center for Worldwide Reference on Comparative Oncology, vol. 7, Washington, D.C.

11. Misdorp, W., Cotchin, E., Hampe, J. F., Jabara, A. G. and Von Sondersleben, J. 1973. Canine malignant mammary tumors. 3. Special types of carcinomas, malignant mixed tumors. *Vet. Pathol.* 10: 241–256. [Medline] [CrossRef]

12. Morrison, L. A., Lukacher, A. E., Braciale, V. L., Fan, D. P. and Braciale, T. J. 1986. Differences in antigen presentation to MHC class I and II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163: 903–921. [CrossRef]

13. Moulton, J. E. 1990. Tumor of the mammary gland. pp. 518–552. In: Tumors in Domestic Animals. 3rd ed. (Moulton, J. E. ed.), Univ. California Press, Berkeley.

14. Nakajima, Y., Hoshi, F., Higuchi, S. and Kawamura, S. 1999. The complete amino acid sequence of dog beta2-microglobulin. *J. Vet. Med. Sci.* 61: 517–521. [Medline] [CrossRef]

15. Nakajima, Y., Hoshi, F., Higuchi, S. and Kawamura, S. 2001. Determination of canine beta2-microglobulin in plasma and urine.
by enzyme-linked immunosorbent assay. J. Vet. Med. Sci. 63: 343–345. [Medline] [CrossRef]

16. Pedersen, L. O., Hansen, A. S., Olsen, A. C., Gerwien, J., Nissen, M. H. and Buus, S. 1994. The interaction between beta 2-microglobulin (beta 2m) and purified class-I major histocompatibility (MHC) antigen. Scand. J. Immunol. 39: 64–72. [Medline] [CrossRef]

17. Philibert, J. C., Snyder, P. W., Glickman, N., Glickman, L. T., Knapp, D. W. and Waters, D. J. 2003. Influence of host factors on survival in dogs with malignant mammary gland tumors. J. Vet. Intern. Med. 17: 102–106. [Medline] [CrossRef]

18. Redondo, M., Garcia, J., Villar, E., Rodrigo, I., Perea-Milla, E., Serrano, A. and Morell, M. 2003. Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. Hum. Pathol. 34: 1283–1289. [Medline] [CrossRef]

19. Saleh, F. and Abdeen, S. 2007. Pathobiological features of breast tumours in the State of Kuwait: a comprehensive analysis. J. Carcinog. 6: 12. [Medline] [CrossRef]

20. Uva, P., Aurisicchio, L., Watters, J., Loboda, A., Kulkarni, A., Castle, J., Palombo, F., Viti, V., Mesiti, G., Zappulli, V., Marconato, L., Abramo, F., Ciliberto, G., Lahm, A., La Monica, N. and de Rinaldis, E. 2009. Comparative expression pathway analysis of human and canine mammary tumors. BMC Genomics 10: 135. [Medline] [CrossRef]