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Metastasis prognostic factors and cancer stem cell-related transcription factors associated with metastasis induction in canine metastatic mammary gland tumors

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ABSTRACT

Background: Canine mammary gland tumor (MGT) is the most common cancer in aged female dogs. Although it's important to identify reliable metastasis or prognostic factors by evaluating related to cell division, adhesion, and cancer stem cell-related transcription factor (TF) in metastasis-induced canine MGT, but there are limited studies.
Objectives: We aimed to identify metastasis prognostic factors and cancer stem cell-TFs in canine MGTs.
Methods: Age-matched female dogs diagnosed with MGT only were classified into metastatic and non-metastatic groups by histopathological staining of MGT tissues. The mRNA levels of cancer prognostic metastasis molecular factors (*E-cadherin*, *ICAM-1*, *PRR14*, *VEGF, HPRT1*, *RPL4* and *hnRNP H*) and cancer stem cell-related TFs (*Oct4*, *Sox2*, and *Nanog*) were compared between metastatic and non-metastatic canine MGT tissues using qRT-PCR analysis.
Results: The mRNA levels of *ICAM-1*, *PRR14*, *VEGF*, *hnRNP H*, *Oct4*, *Sox2*, and *Nanog* in metastatic MGT group were significantly higher than those in non-metastatic MGT group. However, mRNA level of *RPL4* was significantly lower in metastatic MGT group. Loss of *E-cadherin* and *HPRT1* was observed in the metastatic MGT group but it was not significant.
Conclusions: Consistent expression patterns of all metastasis-related factors showing elevation in *ICAM-1*, *PRR14*, *VEGF*, *hnRNP H*, *Oct4*, *Sox2*, and *Nanog,* but decreases in *RPL4* levels occurred in canine MGT tissues, which was associated with metastasis. Thus, these cancer prognostic metastasis factors and TFs of cancer stem cells, except for *E-cadherin* and *HPRT1*, can be used as reliable metastasis factors for canine MGT and therapeutic strategy.

Keywords: Canine; mammary gland tumor; metastasis; prognostic molecular markers; stem cell markers

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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INTRODUCTION

In dogs with cancer, evaluation and prediction of metastasis of mammary gland tumor (MGT) is important because metastasis is the main cause of death. Complex carcinoma is the most common type of malignant MGT, which has a high probability of metastasis and associated with a poor prognosis [1]. While a variety of potential metastatic markers in animal oncology is of great significance, it is also important not to misdiagnose false positives and avoid overaggressive treatment of older dogs with cancer to improve prognosis. Therefore, it is necessary to compare specifically determined patterns of well-known metastasis markers in a particular type of cancer, such as MGT, to suggest high accuracy and robustness of prognosis. Tumor cell-cell adhesion, cell division, angiogenesis, and cancer stem cell-related mechanisms play roles in cancer metastasis. Therefore, it is important to comparatively evaluate the expression levels of the associated molecules, such as E-cadherin, intercellular adhesion molecule-1 (*ICAM-1*), proline rich protein 14 (*PRR14*), vascular endothelial growth factor (*VEGF*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L4 (*RPL4*), and heterogeneous nuclear ribonucleoprotein H (*hnRNP H*) between metastatic and non-metastatic MGT tissues in canines.

We believe that these cancer prognostic molecular markers, as well as cancer stem cell markers, could provide basis for a metastatic prognosis, but there are limited studies on canine cancer. Currently, targeting cancer stem cells (CSCs), in which only a small subset of tumor cells are capable of initiating and sustaining tumor growth, is actively used for the development of new cancer therapies [2]. The high stemness of CSCs in cancer makes it possible to consider aggressive prognosis due to rapid proliferation capacity and mutational possibility [3]. In particular, stemness-related transcription factors (TFs) are highly expressed in embryonic stem cells and in some types of adult stem cell populations, and can be detected in tumor samples, suggesting the presence of cancer stem cells [4,5]. Currently, approximately 25 TFs have been reported to be related to stemness. Of these, *Oct4*, *Sox2*, and *Nanog* comprise a core regulatory network for embryonic stem cell maintenance and self-renewal. A retrospective study that included a cohort of human cancer patients correlated TF expression with survival outcomes, which may also be useful for assessing patient prognosis [6]. Thus, these TFs may also play a role as prognostic markers, especially in metastatic cancer cells. This information can be used to explain the correlation or co-detect the expression level of these stemness-related TFs and prognostic molecular markers in certain types of canine cancer to evaluate metastatic prognosis for establishing therapeutic strategies. In addition, the expression level of these TFs are clearly related to the age of the patient; hence, all patients need to be age-matched.

In the present study, we aimed to compare and evaluate these candidate molecules including cancer metastasis prognostic factors and stem-related TFs of CSCs in metastatic and non-metastatic canine MGTs. Furthermore, we aimed to investigate whether these factors were effective in predicting metastasis in age-matched canine MGT.

MATERIALS AND METHODS

Diagnosis and sampling of metastatic and non-metastatic MGT dogs

Six intact age-matched female dogs diagnosed with MGT (13–14 years old) by clinical and physiological diagnosis and radical surgery at Gyeongsang National University Animal Medical Center were selected for this study. All dogs in the study group were evaluated by the veterinary

with respect to physical (age and breed) and clinical (metastasis) characteristics. They had no previous history of diagnosis or treatment for other cancers and were only diagnosed with MGT. For radiographic examination, lateral radiographic images were obtained preoperatively. X-ray tubes were used to acquire BFSS images. BFSS images were obtained using an E7239X Rotanode Toshiba X-ray tube (Toshiba Electron Tubes and Devices Company, Tokyo, Japan) containing a nominal focal spot size 2.0 mm. All radiographic images were obtained using a Konica computed radiography system (Regius model 190, KONICA, Japan).

Tumor fragments from dogs with MGT with or without metastasis were collected, and the collected tumor fragments were divided into two sections with one large section and three small sections for histopathological and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Histopathological staining

For histopathological analysis, one large section was fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin wax. Histological sections (4 μm) were stained with hematoxylin and eosin (H&E) using standard histological procedures for histopathology. The other three small sections were immersed in RNAlater (Invitrogen Life Technologies, Carlsbad, CA, USA) as an RNA stabilizing solution for qPCR analysis.

RNA isolation and qRT-PCR

The expression of metastasis-related genes was analyzed in tissues using qRT-PCR. Total RNA was extracted from both the tissues in metastatic and non-metastatic MGT groups using the easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Korea). The quantity and quality of RNA were measured using an OPTIZEN NANO Q spectrophotometer (Mecasys, Korea), with an A_{260}/A_{280} ratio of 1.8 ± 0.2, indicating the purity of preparation. Complementary DNA (cDNA) was synthesized with 500 ng of RNA using HiSenScript™ RH(-) RT PreMix Kit (iNtRON Biotechnology), and the reaction was conducted at 42°C for 1 h. qRT-PCR was conducted on a Rotor Gene Q cycler (Qiagen, Germany) using RealMOD™ Green AP 5x qPCR mix (iNtRON Biotechnology), supplemented with 50 ng cDNA and specific primer sets.

The PCR reaction cycle was as follows: initial activation at 95°C for 12 min, followed by 40 cycles of PCR at 95°C for 15 s, 60°C for 25 s, and 72°C for 25 s. Melting curves, amplification curves, and cycle threshold values (Ct values) were analyzed using the Rotor-Gene Q Series Software (Qiagen). All PCR products were confirmed by electrophoresis using 1.5% agarose gel with 0.1 mg/mL ethidium bromide for nonspecific amplification. The Ct values were normalized to *GAPDH* expression level and all samples were analyzed in triplicates; the primer sequences used for qRT-PCR are shown in **Table 1**.

Statistical analysis

All experimental data were analyzed using an independent t-test available in GraphPad Prism. Results are shown as mean ± SE of the mean. Statistical significance was considered at $p < 0.05$, $p < 0.01$, and $p < 0.0001$.

RESULTS

In the radiographic evaluation of the non-metastatic MGT group, a round soft tissue opacity mass was observed in the caudal abdomen on lateral abdominal radiography scans (**Fig. 1A**,

Table 1. Primer sequences used for qRT-PCR

Gene	Primer sequence (5′-3′)	GenBank accession number
E-Cadherin	F: CTGATGCTGAGGATAACTGAGG R: TACAAACTGTGACCTAAGGATCG	NM_001287125.2
ICAM-1	F: AACTGTAGTACCTCATGCAACC R: CACATCAGTCAGTTCAAAGAGC	L31625.1
PRR14	F: AAAGCAACTTAAAGAAGCATCCC R: ACTGTTTCAAGGAAATTCCAAAGG	XM_005636489.4
VEGF	F: CGAGTACATCTTCAAGCCATCC R: GTGATGTTGAACTCCTCAGTGG	AF133250.1
HPRT1	F: GACTGAAGAGCTACTGTAATGACC R: TCTTTGGATTATGCTCCTTGACC	NM_001003357.2
RPL4	F: AATGAGAAACCGTCGTCGTATCC R: GGAGCAAGTTTCAGAATGTTCAGC	NM_001252409.1
hnRNP H	F: GGTGCTTATGGTGGAGGTTATGG R: ACAATGTCCTGTTGTGCTCTGG	XM_538576.2
Oct4	F: F: AGTGAGAGGCAACCTGGAGA R: GATACTGGTGCCCCTGAGAA	XM538830.1
Sox2	F: AGTCTCCAAGCGACGAAAAA R: CCACGTTTGCAACTGTCCTA	DR105272
Nanog	F: GGTAAAACTCCCACCCACCT R: TTTCTGCCACCTCTTGCTTT	XM543828.2
GAPDH	F: GGAGAAAGCTGCCAAATATG R: ACTGTTGAAGTCACAGGAGA	XM_038444404.1

F, forward; R, reverse.

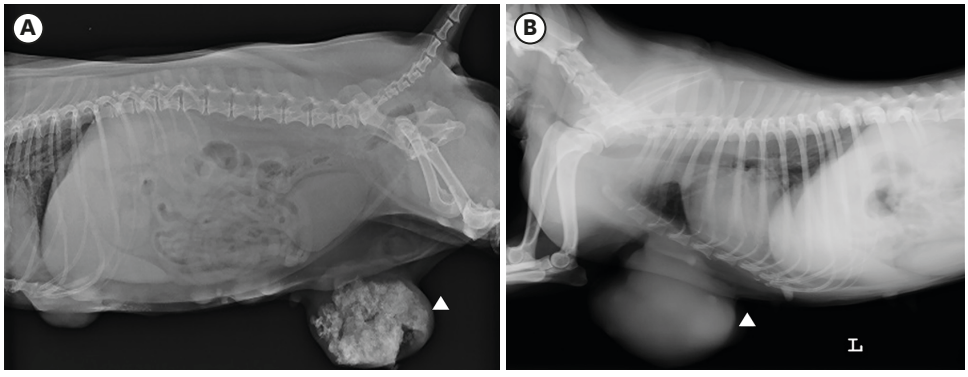

Fig. 1. Representative radiographic images of female dogs with MGT. All radiography scans in non-metastatic MGT (A) and metastatic MGT groups (B) show protruded soft tissue mass in the abdominal or chest regions in lateral view. MGT, mammary gland tumor.

white arrowhead), which protruded out from the chest in the metastatic MGT group (**Fig. 1B**, white arrowhead). In the radiographic images analyzed, we could not find any other tumors or abnormalities except for MGT in all dogs.

All age-matched female dogs aged 13–14 years were classified into non-metastatic and metastatic groups by histopathological diagnosis. The tumors of dogs were successfully removed by radical surgery, and the isolated tumor fragments were used for histopathological staining. In all MGT groups, the parameters employed for histological classification were determined according to the Canine Mammary Neoplasms Histological Classification, modified methods from Misdorp et al. [7,8]. The non-metastatic MGT group displayed myxoid changes in the mammary glands (**Fig. 2A**). Proliferating cells were epithelial cell and showed mitotic figures that indicated carcinoma arising in a mixed tumor (**Fig. 2B**). Neoplastic growth and anisokaryosis were observed in the malignant mammary mixed tumors (**Fig. 2C and D**). In the metastatic MGT

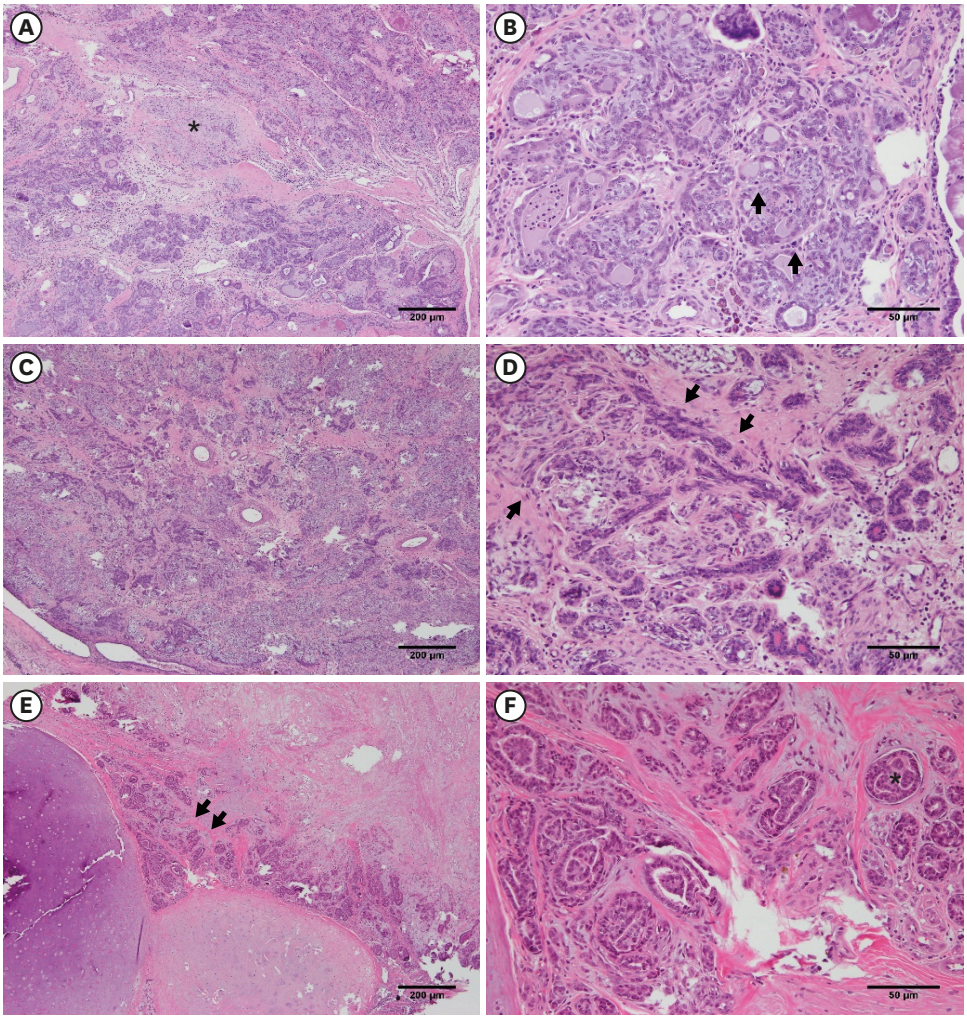

Fig. 2. Histological analysis of metastatic and non-metastatic MGT tissues. (A) Myxoid change (asterisk) in mammary gland. (B) Proliferating cells and mitotic figures (arrows) in non-metastatic MGT group. (C) and (D) show neoplastic growth (arrows) and anisokaryosis in non-metastatic malignant mammary mixed tumor. (E) and (F) display neoplastic growth (arrows) and tumor cell invasions in lymph duct (asterisk) that indicate metastatic malignant mammary mixed tumor. (A), (C), and (E); magnification 40× and scale bar, 200 μm; (B), (D), and (F); magnification 200× and scale bar, 50 μm.
MGT, mammary gland tumor.

group, there was neoplastic growth in the mammary gland and tumor cell invasion in the near lymph duct, indicating a malignant mammary mixed tumor (**Fig. 2E and F**). In two dogs in the non-metastatic MGT group, tumors were classified as mammary adenocarcinoma and carcinoma arising from a complex tumor, respectively. The other two dogs in the metastatic MGT group were classified as having malignant mammary mixed tumors, and metastasis was confirmed in the lymph ducts.

The mRNA level in each sample was analyzed by normalizing the Ct value using *GAPDH* as a reference gene. All samples were analyzed in triplicates and normalized using the average of Ct values in the control group. For the analysis depicted in **Figs. 3A** and **4A**, the relative mRNA level in the control group is shown as "1.0," and the represented values in the metastatic MGT group indicate the rates of fold change in the control group. In **Figs. 3B** and **4B**, each amplification product is shown by agarose gel electrophoresis that supported

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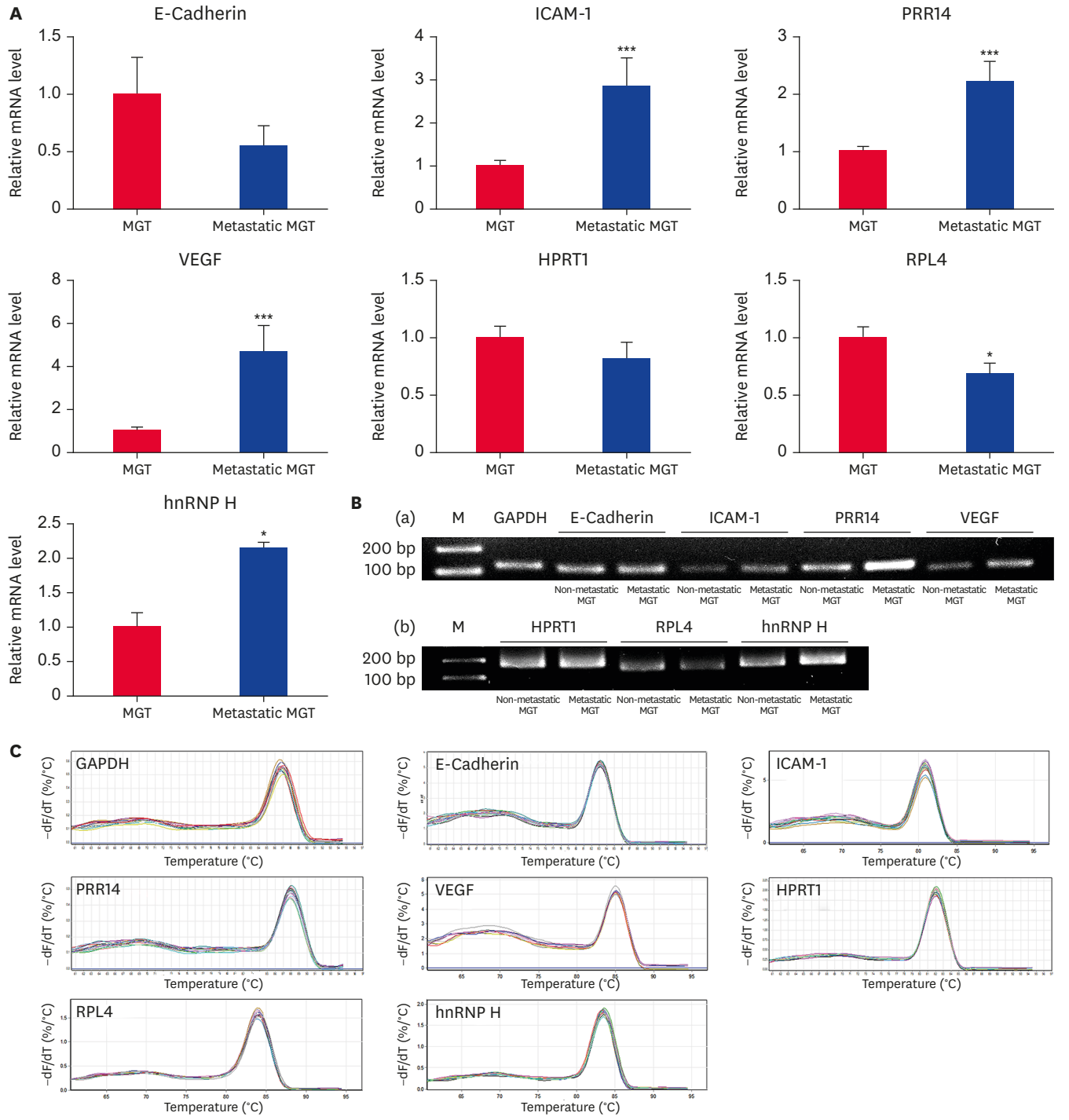

Fig. 3. Analysis of the expression levels of metastasis prognostic factors in MGT tissues. (A) qRT-PCR is used to assess the mRNA levels of metastasis prognostic factors. The asterisks indicate significant differences, $^{*}p < 0.05$, and $^{***}p < 0.0001$. The graphs present mean ± SD. (B) Ethidium bromide-stained 1.5% agarose gel electrophoresis shows qRT-PCR amplified products. (a) Lane M: 100 bp DNA ladder (100–200 bp); lane 1: GAPDH; lane 2, 3: E-Cadherin (non-metastatic MGT, metastatic MGT, respectively); lane 4, 5: ICAM-1 (non-metastatic MGT, metastatic MGT, respectively); lane 6, 7: PRR14 (non-metastatic MGT, metastatic MGT, respectively); lane 8, 9: VEGF (non-metastatic MGT, metastatic MGT, respectively). (b) Lane M: 100bp DNA ladder (100–200 bp); lane 1, 2: HPRT1 (non-metastatic MGT, metastatic MGT, respectively); lane 3, 4: RPL4 (non-metastatic MGT, metastatic MGT, respectively); lane 5, 6: hnRNP H (non-metastatic MGT, metastatic MGT, respectively). (C) Melting curves generated by qRT-PCR analyzed products. For each sub-graph, temperature is displayed in the x axis and the derivative reporter signal is displayed in the y axis.
MGT, mammary gland tumor; qRT-PCR, quantitative real-time polymerase chain reaction.

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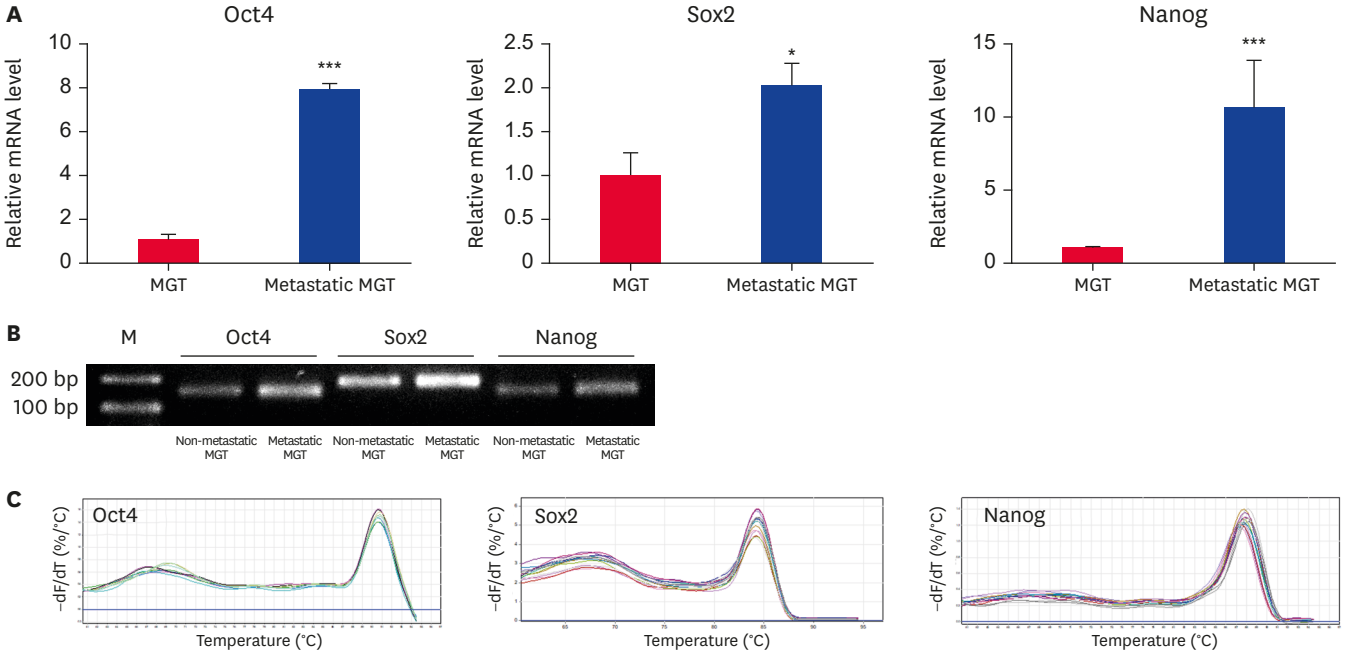

Fig. 4. Analysis of the expression levels of stemness-related transcription factors in MGT tissues. (A) qRT-PCR is used to assess the mRNA levels of stemness-related transcription factors. The asterisks indicate significant differences, $^{*}p < 0.05$, and $^{***}p < 0.0001$. The graphs present mean ± SD. (B) Ethidium bromide-stained 1.5% agarose gel electrophoresis of qRT-PCR amplified products. Lane M: 100bp DNA ladder (100–200 bp); lane 1, 2: Oct4 (non-metastatic MGT, metastatic MGT, respectively); lane 3, 4: Sox2 (non-metastatic MGT, metastatic MGT, respectively); lane 5, 6: Nanog (non-metastatic MGT, metastatic MGT, respectively). (C) Melting curves generated by qRT-PCR analyzed products. For each sub-graph, the temperature is displayed in the x axis, the derivative reporter signal is displayed in the y axis.
MGT, mammary gland tumor; qRT-PCR, quantitative real-time polymerase chain reaction.

the analysis presented in **Fig. 3A**. The corresponding melting curves for all of genes showing single peaks are presented in **Figs. 3C** and **4C**.

The mRNA levels of cancer prognostic metastasis molecular factors (*E-cadherin*, *ICAM-1*, *PRR14*, *VEGF*, *HPRT1*, *RPL4* and *hnRNP H*) and stemness-related TFs (*Oct4*, *Sox2*, and *Nanog*) were comparatively evaluated in cancer fragments of metastatic and non-metastatic MGT dogs using qRT-PCR analysis (**Figs. 3** and **4**). As cancer prognostic metastasis molecular markers, mRNA levels of *ICAM-1*, *PRR14*, *VEGF* and *hnRNP H* were significantly higher in metastatic MGT group than in non-metastatic MGT group ($p < 0.0001$ and $p < 0.05$). In contrast, mRNA level of *RPL4* was significantly lower ($p < 0.05$) in metastatic MGT group than in non-metastatic MGT group. However, there was no significant difference between these at the mRNA level of *E-cadherin* and *HPRT1*. The mRNA levels of *Oct4*, *Sox2*, and *Nanog* as stemness-related transcriptional factors were significantly higher in metastatic MGT group than in non-metastatic MGT group ($p < 0.0001$ and $p < 0.05$).

DISCUSSION

Cancer cells exhibit uncontrolled cell division, and over-activation of positive regulators that are related to cell division which can lead to malignancy. These metastatic cancer cells can spread from where metastasis begins to distant parts of the body. The acquisition of metastasis requires several fundamental steps, including loss of cell-cell adhesion, motility, and the ability to digest through the basement membrane to enter the circulation. EMT is thought to be the

underlying mechanism for the development of metastatic potential, and this process is related to cancer stem cells. Hence, we considered cell division, cell-cell adhesion, and cancer stem cell-related factors to identify candidates for metastasis prognostic factors in canine MGT.

In canine oncology, MGT is the most common cancer in aged female dogs. Therefore, these prognostic biomarkers can be used to discriminate grade tumors based on aggressiveness and metastatic risk, and further predict the prognosis and disease-free survival rates after surgical excision or chemotherapy of the tumors in older dogs with cancer. While extensive screening using various biomarkers can help predict aggressiveness and metastatic risk, it can be impossible to identify low-risk cancer patients among older dogs, and they often have poor prognosis due to over-treatment. Therefore, it is necessary to compare specifically determined patterns in which well-known metastasis markers in certain cancers have already been defined to improve accuracy and robustness. In the actual metastatic MGT, we compared expression patterns that depended on well-known metastatic factors, such as *E-cadherin*, *ICAM-1*, *PRR14*, *VEGF*, *HPRT1*, *RPL4*, *hnRNP H* and cancer stem cell-related TFs, including *Oct4*, *Sox2*, and *Nanog*, to suggest high accuracy and robustness of prognosis.

In the present study, we considered the age-dependent effect and all female dogs were matched according to similar age of approximately 13–14 years old. To understand the specific metastatic factors in dogs with MGT, the only dogs selected for the metastatic MGT group had lymph duct metastasis, while no tumors were found in other sites or organs.

In most studies, the analysis of metastatic factors in cancer tissues is usually performed using immunohistochemical staining. However, we evaluated mRNA levels of well-known cancer prognostic metastasis molecular factors, such as *ICAM-1*, *PRR14*, and *VEGF*, showing that their levels higher in the metastatic MGT group than in the non-metastatic MGT group using *q*RT-PCR analysis (**Fig. 3**). As an adhesion molecule, *E-cadherin* maintains intercellular contacts that confine cells to the primary tumor site. The expression level of *E-cadherin* is known to have a negative correlation with the metastatic potential of the tumor [9] and critically regulate the invasion and progression of human breast cancer and canine MGT [10]. We observed the loss of *E-cadherin* in canine metastatic MGT tissues (**Fig. 4**). The loss of *E-cadherin* expression, which mediates cell-cell junctions to maintain the morphology of cells and tissue architecture, is associated with tumor metastasis. In normal canine mammary glands, *E-cadherin* shows a distinct pattern of expression in epithelial cells [11,12], but immunohistochemical analysis of *E-cadherin* in canine MGT showed reduced membranous expression in malignant neoplasia in the first report [11]. The reduction in E-cadherin expression was associated with malignancy, with all benign tumors analyzed exhibiting strong intercellular immunostaining; however, results obtained from undifferentiated cases were conflicting in canine MGT [13]. If the tumor cells have the ability to overcome cell-cell adhesion, they enter the circulation and invade the surrounding tissue to establish new metastatic colonies. Despite the prolific studies in the human setting on implications of cell adhesion in cancer, there are still few publications on this subject available in canine species. Therefore, the specific role of cadherin-mediated cell adhesion in canine MGT has not yet been fully elucidated [11-14]. In the present study, *E-cadherin* was identified as a sensitive prognostic metastasis factor in canine MGT because its level was reduced in cancer tissues of MGT, in which lymph ducts metastasis was induced.

ICAM-1 is also known to play an important role in cancer [15] but there is an argument on the regulatory relationship between *ICAM-1* in canine MGT and human breast cancer.

It has been reported that the downregulation of *ICAM-1* attenuated metastatic ability or overexpression in human breast cancer [16]. Even these arguments have not been reported in the understanding of the role of MGT in the progression of canine MGT. In this study, we observed that mRNA levels of *ICAM-1*, another important molecule involved in tumor cell adhesion, were increased in MGT tissues with metastasis to lymph ducts. Thus, the risk of metastasis induction can be predicted by the elevation of ICAM-1 in canine MGT.

In this study, the mRNA levels of *PRR14* and *VEGF* have been shown to be increased in MGT tissues that have metastasized to lymph ducts. Cancer cell invasion is known to occur primarily in the G1/G0 cell cycle arrest state [17]; therefore, some genes such as *PRR14* indicating specific cell cycle states can be used to predict the metastatic potential of cancer. However, it is not clear whether *PRR14* induces metastasis or progression to malignant tumors in canine MGT. The overexpression of *PRR14* in cancer leads to deregulation of the DNA damage pathway [18] which has been reported in various cancers, including lung and breast cancers in humans [19]. Furthermore, *PRR14* overexpression was associated with the possibility of metastasis of colon cancer. Although observation of the promotion of metastasis by *PRR14* in canine MGTs is rare, we observed a higher mRNA level of *PRR14* in the metastatic MGT than in the non-metastatic MGT.

As the most potent inducer of angiogenesis, *VEGF* is required for tumor metastasis and invasion, and the prognostic value of VEGF has been reported in canine malignant tumors [20]. In canine and feline mammary carcinomas, the level of circulating VEGF was significantly increased compared to that in healthy animal dogs.

HPRT1, *RPL4*, and *hnRNP H* are well-known factors as housekeeping genes and they showed specific patterns of expression in particular cancers and association with prognosis of cancers. However, these candidate factors have not been reported in canine cancer, to the best of our knowledge. In this study, we compared mRNA expression levels of these factors between non-metastatic and metastatic MGT tissues (**Fig. 3**). *HPRT1* recycles nucleotides for cell division and cell viability through purine salvage pathway [21], and higher expression of *HPRT1* in breast tumors suggests worse clinical prognosis [22]. However, we observed no significant difference in mRNA level of *HPRT1* between metastatic and non-metastatic MGT tissues. It may suggest that the patterns of *HPRT1* expression level in canine MGT tissue could different from that in human breast cancer tissue. Ribosomal proteins (RPs) are associated with ribosome assembly and protein translation for cell growth and cell survival. In particular, *RPL4* plays important role in regulating cell cycle transition; however, over-expression of this protein is involved in cell proliferation in human non-small cell lung cancer and gastric cancer [23]. In contrast, *RPL4* was under-expressed as shown by its mRNA levels in HER2-positive breast cancer with brain metastasis [24], suggesting that different types of cancer have specific expression of *RPL4*. In this study, *RPL4* was downregulated in metastatic MGT. It demonstrates that human breast cancer and canine MGT shows similarity with regard to mRNA level of *RPL4*. Furthermore, decreased *RPL4* indicates poor prognosis including metastasis. *hnRNP*s have been suggested as novel prognostic biomarkers in human cancer studies that involved angiogenesis, extracellular matrix, and cell invasion in many cancers [25]. In this study, mRNA expression of *hnRNP H* was significantly increased in metastatic MGT compared to non-metastatic MGT tissues. We would suggest that *hnRNP H* and *RPL4* could be used as novel candidates for predicting the ability to metastasize in canine MGT, but *HPRT1* is not involved in this process.

Embryonic specific TFs are abnormally expressed in human tumors [4,5], suggesting the presence of CSCs. A retrospective study on patient cohorts correlating TF expression with the survival outcome in specific tumor types suggested that the levels of TF expression may also be useful for assessing the prognosis in human patients [6]. However, information on the association between TF and canine cancer is limited. In canine mast cell tumors, immunohistochemical expression of Oct4 was not an accurate prognostic indicator, and no significant differences were found between the histopathological grades of *Oct4* expression in various tumors [26]. On the other hand, expression of *Oct4* and *Sox2* [27], or *Nanog*, *Oct4,* and *STAT3* [28] have been identified in CSCs of canine osteosarcoma; and these TFs can be used for their distinctive sensitivity to anticancer agents as a reliable experimental model to assay drug efficacy. Detection of mRNA expression levels of these TFs through immunohistochemistry staining and *q*RT-PCR analysis can aid in tumor diagnosis, classification, and selection of suitable therapeutic strategies. In particular, *Oct4*, *Sox2*, and *Nanog* as core stemness-related TFs, indicated advanced disease stages in various cancers, including breast and lung cancers [29], HER2+ breast cancer patients [30]. In canine neoplasms, positive expression of Oct4 was reported by immunohistochemistry; and the proportion of *Oct4* positive cells and the intensity of immune-reactivity varied both within and between tumor types [31]. All of these TFs in CSCs can be meaningful prognostic indicators of metastatic potential in primary cancer. *Oct4*, *Sox2*, and *Nanog* expression was increased with lymph node metastasis in renal carcinoma, non-small cell lung cancer, and breast cancer patients, respectively [32-34]. We also observed that the mRNA levels of *Oct4*, *Sox2*, and *Nanog* were higher in the metastatic MGT than in the non-metastatic MGT. The expression level of *Oct4* is associated with clinical and histopathological prognostic indicators of cancer and can be considered as a prognostic cancer factor [35]. Expression of *Sox2* expression has been correlated with poor prognosis in squamous cell carcinoma [36], gastric carcinoma [37], small cell lung cancer [38], and ovarian carcinoma [39]. The other TFs, *Nanog*, have also been associated with poor prognosis in various cancers, including breast [40], colorectal [41], gastric [42], ovarian [43], and liver cancers [44]. Thus, elevation of *Oct4*, *Sox2*, and *Nanog* in canine MGT tissues can be used as a strong evidence for the induction of metastasis due to primary cancer in the present study.

According to these results, *ICAM-1*, *PRR14*, *VEGF*, *RPL4* and *hnRNP H* are the cancer prognostic metastasis factors, and *Oct4*, *Sox2*, and *Nanog* are the TFs of CSCs that can be used as reliable factors for metastasis of canine MGT. Although each of these individual factors also have sufficient significance as a prognostic factor, a combined expression pattern of multiple factors may predict metastasis as a more reliable indicator.

In conclusion, we suggest that identical expression patterns showing higher levels in all metastasis-related factors *ICAM-1*, *PRR14*, *VEGF*, *hnRNP H*, *Oct4, Sox2*, and *Nanog*, but decreased *RPL4* levels, and not significant loss in *E-cadherin* and *HPRT1* occurred in the MGT issues with metastasis. Accordingly, the evaluation of expression levels of individual factors can be used as prognostic factors, but the combination of multiple factors to evaluate their expression pattern is a more reliable metastatic prognostic marker in canine MGT. Furthermore, interfering with the expression of these factors could act as suppressors of canine MGT metastasis.

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