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Research article

Investigation of serum interleukin-8 level and clinicopathological parameters in canine mammary gland tumors

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Abstract

This study aimed to evaluate serum interleukin-8 (IL-8) levels in female dogs with mammary gland tumors (MGTs) and the correlation of these levels with clinicopathological parameters. Serum IL-8 levels from 25 female dogs with MGTs and 10 healthy intact female dogs were measured using canine IL-8 ELISA test kit. The animal age varied from 6 to 16 years (median=10.5 years), with 36% (n=9) dogs under 10 years, and 64% (n=16) over 10 years. Thirty-two percentage of dogs (n=8) with MGTs presented with a tumor up to 6 months, 36% (n=9) between 6 months and 12 months and 32% (n=8) for greater than 12 months. Stage II (56%, n=14) tumors were the most classification, followed by stage I (16%, n=4), stage III (16%, n=4), and stage V (12%, n=3). Among these tumor tissues, 3 samples (12%) were hyperplasia, 9 samples (36%) were benign mixed tumor and 13 samples (52%) were malignant tumor. The mean serum IL-8 levels (± SE) in tumor-bearing dogs (868.42 ± 142.99 pg/ml) were significantly higher than in the control group (299.10±129.52 pg/ml) (P=0.025). Although serum IL-8 levels tended to be higher in bigger tumor size, those tended to be lower in malignant tumors. However, there was no correlation with duration of tumor presentation prior to surgery, clinical staging, and histopathological diagnosis. In conclusion, serum IL-8 levels were higher in canine MGTs when comparing to healthy dogs. Further larger sample size investigations are required to clearly define its potential as predictive biomarker.

Keywords: Canine, Interleukin-8, Mammary gland, Serum, Tumor

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INTRODUCTION

Mammary gland tumor (MGT) is the most common tumor in female dogs and approximately one-half are malignant tumors (Antuofermo et. al., 2007; Brodey et al., 1983). It has been reported that canine MGT was presented in 13.4% of all tumors in dogs and in 41.7% of all tumors in intact female dogs (Sorenmo, 2003). Previous study revealed an increasing risk of 0.5, 8 and 26% depending on whether an ovariohysterectomy is performed before the 1st, 2nd or 3rd estrus cycle, respectively; however, if the bitches were spayed later, the risk of developing malignant tumors was the same as for an intact bitch (Schneider et al., 1970). Human and canine MGT have many similarities regarding biological behavior and metastasis (Mac Ewen et al., 1990). Therefore, canine MGT is an ideal translational model for comparative studies in relation to human breast cancer prognosis and treatment (Moe et al., 2001).

Clinical staging is used to classify MGT in dogs through examination of the tumor size, regional lymph node involvement and distant metastasis. The treatment of choice is surgery. Approximately 50% of dogs developed a new tumor in the ipsilateral remaining glands after a regional mastectomy had been performed (Stratmann et al., 2008). However, several dogs with MGT may need the combination treatments of surgery, chemotherapy, and radiotherapy. Several disease features that correlate a worse prognosis for canine malignant mammary tumors are masses greater than 3 cm, invasive masses, ulcerated masses, longer than 6 months of duration, enlarged lymph nodes with neoplastic cells, poor differentiated or anaplastic carcinomas, inflammatory carcinoma, lack of estrogen receptors and invasive into the vascular and lymphatic system (Chang et al., 2005; Chavey et al., 2007). Metastasis is the most malignant behavior of cancers. It occurs when tumor cells spread from primary tumor sites to secondary sites, including regional lymph nodes and distant organs, with many molecular pathways. Currently, peripheral blood or serum biomarker as minimally invasive diagnostic tool is interesting in small animal medicine.

Cytokines are low molecular weight small proteins secreted by cells and tissues associated with the inflammatory process, immune reaction and tumorigenesis which regulate autocrine and paracrine cell functions (Waugh and Wilson, 2008). Cytokines can control tumor growth by regulating cells in response to a range of stresses (Derin et al., 2007). The review of cytokines in canine tumorigenesis as the translational model has been reported in lymphoma, melanoma, osteosarcoma and MGT (Irac et al., 2019). In human cancer patients, high level of systemic cytokine can be resulted by interaction between tumors and microenvironment and cytokine production by tumor itself (Salvatore et al., 2017).

Serum interleukin-8 (IL-8) levels are considered an important poor predictive biomarker in human breast cancer patients (Benoy et al., 2004) and canine mammary carcinoma (Gelaleti et al., 2012). IL-8 was overexpressed in malignant breast cancer cells that showed a higher transcriptional activity of the IL-8 gene promotor (Freund et al., 2004). In contrast, serum IL-8 levels measured in various of tumor-bearing dogs did not correlated with tumor type, tissue origin and histological features (Haas et al., 2015). However, the potential role as the tumor marker in dogs with MGT is scarce and unknown. The objectives of this study were to evaluate the serum IL-8 levels in dogs with MGT and the correlation of these levels with the clinicopathological parameters: duration of tumor presentation prior to surgery, clinical staging, and histopathological diagnosis.

MATERIALS and METHODS

Sample collection

This study obtained ethical approval from the Kasetsart University Committee on Animal Use and Care (reference number ACKU60-VET-041). Peripheral blood samples and tumor tissues were collected from 25 female dogs with MGTs that had undergone surgical resection at the Soft Tissue Surgery Unit of the Kasetsart Veterinary Teaching Hospital (Bang Khaen campus, Bangkok, Thailand). Serum controls were obtained from 10 healthy intact female dogs outside the estrus period, with no tumor and surgical history, without inflammation, aged 8-12 years and with normal blood profiles. Female dogs in the study group were examined for physical and pathological characteristics. Duration of tumor presentation prior to surgery was recorded. Clinical staging according to TMN system (tumor size, metastasis and lymph node involvement) established by the WHO for canine MGTs (Cassali et al., 2010), which recommended primary tumor size (T) – T0: no evidence of tumor; T1: <3 cm; T2: between 3 and 5 cm; T3: >5 cm; lymph node involvement (N) – N0: no apparent involvement; N1: unilateral involvement; N2: bilateral involvement; and distant metastasis (M) – M0: no evident metastasis; M1: distant metastasis including non-regional lymph nodes. Staging included 5 classifications – Stage 1 (T1N0M0); Stage 2 (T2N0M0); Stage 3 (T3N0M0); Stage 4 (T1-3N1M0); Stage 5 (T1-3N0-1M0) was assigned as I, II, III, IV or V according to the extension and prognostic establishment. The existence of metastasis at the time of surgery was described. Determination of the survival time and disease-free interval were also followed up.

Sample processing

Blood samples were collected (3 ml) into plain serum tubes (without anticoagulant) and allowed to clot at room temperature. The serum was separated by centrifugation (1000×g, 25 min) and immediately cryopreserved at -80°C until used for IL-8 evaluation. The tumor tissues of canine MGT were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Four-µm thick sections were stained with hematoxylin and eosin for pathological examination. The tumor tissues were histopathologically classified into three types: hyperplasia, benign mixed tumor, and malignant tumor.

Quantification of serum IL-8 levels

Serum IL-8 levels were evaluated using a sandwich enzyme immunoassay technique with canine CXCL/IL-8 Quantikine ELISA test kit (R&D Systems Inc., USA) according to the manufacturer's instructions. The antibody used in this study was a mouse monoclonal antibody specific canine IL-8. The assays were carried out in duplicate. The optical density (OD) was measured at 450 nm in a microplate reader (Thermo Scientific, USA).

Statistical analysis

The data were descriptively analyzed to determine normality. The clinicopathological features and serum IL-8 levels were separated in each group and analyzed using the Mann-Whitney U test or the Kruskal-Wallis one-way

ANOVA test. A P-value of <0.05 was considered statistically significant. All values were expressed as the mean ± standard error (SE). Data analysis was carried out using the NCSS 2007 software package (Kaysville, UT, USA).

RESULT

The data were collected from 25 female dogs presenting MGTs. The evaluated clinicopathological parameters were duration of tumor presentation prior to surgery, clinical staging, and histopathological diagnosis. The correlation between some clinicopathological parameters and serum IL-8 levels were analyzed using the Kruskal-Wallis One-way ANOVA test. The data analysis showed that serum IL-8 levels were significantly elevated in canine mammary tumor patients compared with the control. The average serum IL-8 level (±SE) of the canine MGT group was 868.42±142.99 pg/ml. The average serum IL-8 level of the normal group was 299.10±129.52 pg/ml. Differences between the two sample groups were analyzed using the Mann-Whitney U test (P=0.025; Table 1 and Figure 1a). The serum IL-8 level tended to be higher in dogs having the duration of tumor presentation prior to surgery for less than 6 months, in dogs with clinical stage III and in dogs with a histopathological type of hyperplasia. However, the IL-8 levels were not significantly different in time course (P=0.328) (Figure 1b), clinical stage (p=0.943) (Figure 1c) or histopathological type (P = 0.37) (Figure 1d).

Table 1 Correlation between serum IL-8 levels (mean±SE) and clinicopathological parameters

Clinicopathological parameter	N	IL-8 (pg/ml)
Samples		
Normal group	10	299.10±129.52
Mammary tumor group	25	868.42±142.99
p-value		0.025
Duration		
<6 months	8 (32%)	1028.54±300.28
6-12 months	9 (36%)	693.44±286.23
>12 months	8 (32%)	574.10±149.72
p-value		0.328
Clinical stage		
I	4 (16%)	560.63±224.03
II	14 (56%)	772.72±189.48
III	4 (16%)	1101.32±645.74
V	3 (12%)	532.11±169.99
p-value		0.943
Histopathological diagnosis		
Normal	4	299.10±129.52
Hyperplasia	3 (12%)	1300.12±827.51
Benign Mixed Tumor	9 (36%)	629.99±138.39
Malignant Tumor	13 (52%)	730.14±205.230
p-value		0.37

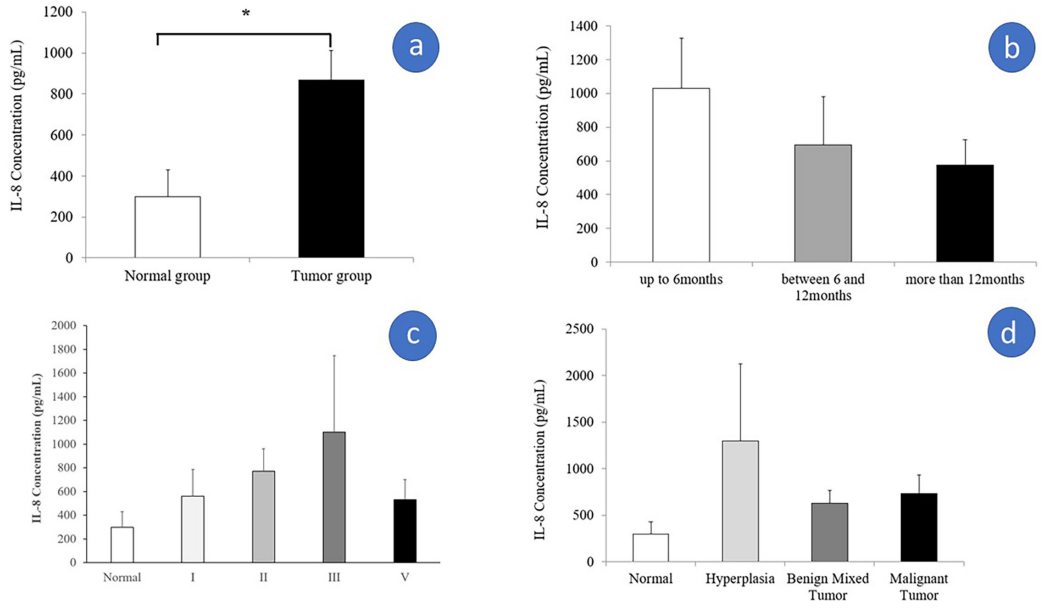

Figure 1 Serum IL-8 levels in female dogs with mammary gland tumors and controls (a), with mammary gland tumors – correlation between time courses (b), with mammary gland tumors – correlation between clinical stages (c) and with mammary gland tumors – correlation between histopathological types (d) (* = $P < 0.05$).

The animal age of tumor group varied from 6 to 16 years (median = 10.5 years), with 36% (n=9) dogs under 10 years 64% (n=16) over 10 years. There was greater incidence in intact female dogs (80%, n=20) than in spayed dogs (20%, n=5). There were no significant differences among the duration of tumor presentation times. The data from the sample group showed that 32% (n=8) of dogs presented with a tumor up to 6 months, 36% (n=9) between 6 months and 12 months and 32% (n=8) for greater than 12 months. Most of the tumor-bearing dogs (76%, n=19) presented multiple gland involvement compared to single gland involvement (12%, n=3). Of the sampled dogs, 4 (16%) had a tumor less than 3cm, 16 dogs (64%) had a tumor between 3cm and 5cm and 5 dogs (20%) had a tumor bigger than 5cm. The results of applying clinical staging based on the TNM system were: stage I, 16% (n=4); stage II, 56% (n=14); stage III, 16% (n=4); stage IV, 0% (n=0); and stage V, 12% (n=3). Most clinical stage II dogs were characterized by a maximum tumor size diameter between 3cm and 5cm without lymph node involvement and distant metastasis. In the 3 dogs (10%) that presented clinical stage V there was evidence of local tumor recurrence after surgical treatment. Moreover, these dogs also had distant metastasis at the time of diagnosis, with 2 dogs presenting lung metastasis and 1 dog presenting splenic metastasis. In the tumor group, 15 dogs (60%) had a survival time greater than 12 months in the follow up period, while 6 dogs (24%) had a survival time between 6 months and 12 months and 4 dogs (16%) had a survival time of less than 6 months.

Another important diagnostic procedure for identifying tumor type was histopathology. The histopathological classification was performed by a pathologist at the Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. Total canine MGT specimens from the

25 patients were classified according to principle cell types (hyperplasia, benign mixed tumor, and malignant tumor). Among the 25 primary tumor tissues, 3 samples (12%) were hyperplasia, 9 samples (36%) were benign mixed tumor consisting of 5 samples (55.55%) of adenoma and 4 samples (44.44%) of cystadenoma. Thirteen samples (52%) were malignant tumors consisting of 8 samples (61.54%) of adenocarcinoma and 5 samples (38.46%) of complex carcinoma.

DISCUSSSION

To consider serum IL-8 levels as a poor prognostic tumor marker of canine MGT progression. This study determined the serum IL-8 levels in female dogs with MGT and the correlation of the levels with clinicopathological parameters: duration of tumor presentation prior to surgery, clinical staging and histopathological diagnosis.

Based on this study, the mean serum IL-8 levels (±SE) in tumor presenting dogs (868.42±142.99 pg/ml) were higher than in the control group (299.10±129.52 pg/ml) significantly (P=0.025). According to clinicopathological data from the 25 patients, canine MGT was more common in female dogs older than 10 years old and approximately 50% of these dogs were histologically diagnosed malignants. This result was similar to previous studies in human breast cancer (Benoy et al., 2004) and canine mammary carcinoma (Gelaleti et al., 2012) though the number of cases in this study was too small. Although serum IL-8 levels were significantly higher in female dogs with time course of tumors more than 18 months (Gelaleti et al., 2012), we found no significant differences among the duration of tumor presentation prior to surgery for less than 6 months, between 6 months and 12 months and greater than 12 months. However, this collected data by history taking from the owners may be too difficult to know precisely.

For clinical staging, most tumors were in clinical stage II characterized by a maximum tumor size diameter between 3 cm and 5 cm without both lymph node involvement and distant metastasis. However, 3 dogs had distant metastasis (to the lung and spleen) in clinical stage V at the time of diagnosis. The survival rate was 60% in canine MGT dogs had a survival time longer than 12 months. Serum IL-8 levels tended to be higher in clinical stage III with increasing tumor size, being consistent with the previous study (Haas et al., 2015). IL-8 is reported to regulate the tumor growth and survival through angiogenesis, autocrine and paracrine mechanisms (Raman et al., 2007). IL-8 may function for promoting angiogenesis, proliferation, migration, infiltrating neutrophils, and tumor-associated macrophages within the tumor microenvironment (Waugh and Wilson, 2008). Moreover, the large tumors usually have many tumor cells and inflammatory environment which can result high serum IL-8 levels (Raman et al., 2007). Based on our results, although the concentration of IL-8 in each clinical stage were not significantly difference. The highest concentration was found in clinical stage III that described average tumor diameter more than 5cm. It might be related to tumor size and initial stage of distant metastasis. This result demonstrated that high concentration of serum IL-8 could be associated with early period of tumor formation. Moreover, large tumor size could be expressed high levels of serum IL-8 also. Another study showed that IL-8 expression as prognostic factor correlated inflammatory and

angiogenic factors with tumor malignancy in canine MGT (Perez et al., 2000).

Based on histological diagnosis in this study, serum IL-8 levels tended to be increased in hyperplasia but decreased in malignant tumors. In the present study, hematological parameters and blood chemistry profile of all dogs were normal. The overexpression of IL-8 in hyperplasia may depend on a proper stimulus that might not be given with increasing malignancy (Baggiolini et al., 1989). Another reason is that the malignant tumors related local IL-8 overexpression leads to a blockage of its systemic production through a negative feedback mechanism (Haas et al., 2015). The function of systemic and local IL-8 may be different; therefore, both should be compared in future studies. Several previous studies have been controversial. On the one hand, IL-8 may play a protective role because its overexpression associated with higher survival rate without metastasis (Derin et al., 2007; Zuccari et al., 2008). IL-8 overexpression may have a positive role in controlling tumor growth after chemotherapy (Lee et al., 2004). On the other hand, increased serum IL-8 level was associated with poor prognosis, tumor progression, metastasis, recurrence and shorter survival rate in human breast cancers (Benoy et al., 2004; Yao et al., 2006; Angelo and Kurzrock, 2007), melanoma, gastric, ovary and prostatic cancer (Xie, 2001) and canine MGT (Gelaleti et al., 2012). Benoy et.al (2004) showed that a high level of serum IL-8 was associated to a worse clinical course, a higher tumor load, the liver or lymph node metastasis, recurrence, and shorter survival rate in human breast cancer. More than 60% of patients with metastatic breast cancer had increased serum IL-8 levels. Survival rate was significantly shorter for human breast cancer patients with IL-8 levels above 17.2 pg/ml. Expression of IL-8 correlated with disease progression in human melanoma. IL-8 levels were higher in gastric cancer surgical tissues than in normal mucosa and correlated with vascularization. Patients with ovarian cancers showed significantly higher levels of IL-8 when compared to patients with benign gynecological disorders. A high level of serum IL-8 in prostate cancer correlated with increasing stage of tumor. Gelaleti et al., (2012) have revealed that 80% of the dogs with MGTs which died during follow-up had elevated IL-8 levels. The IL-8 concentration was significantly and positively correlated with new recurrence and metastasis.

However, the univariate analysis showed no significant difference in tumor dogs for each tumor duration (P=0.328), clinical stage (P=0.943) and histopathological tumor type (P=0.37). These results may be due to the relatively small number of samples and the fact that only serum IL-8 levels at the time of diagnosis were investigated. Moreover, the limitations of present study include we did not measure the serum IL-8 levels expressed in various times after surgery during follow-up. Therefore, we could not know the changing levels in the individual stage of tumor progression and their role in tumor recurrence and related processes. Further research is required to clarify the relationship between IL-8 levels and canine mammary tumors, including before and post-surgical treatment with more time series.

CONCLUSION

This study showed that the serum IL-8 levels in dogs presenting MGTs were significantly higher than in normal dogs. Although there were no significant differences among the clinicopathological parameters, these results tended to support that serum IL-8 can be used as a non-invasive prognostic serum marker for MGT progression. However, the results did not conclusively demonstrate that IL-8 levels were associated with the severity of MGTs. On the other hand, some studies found significantly high serum IL-8 levels in the malignancy group. The current results may have been due the small size of the sample group that limited the evaluation of significant differences for parameters. Further study might clarify whether IL-8 has strong enough potential to be used as both a prognostic marker and a severity indicator.

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CONFLICT of INTEREST

There is no conflict of interest.

AUTHOR CONTRIBUTION

Natapat Monkong performed experiments, analyzed data, and co-wrote the paper. Chaiyakorn Thitiyanaporn analyzed statistical data and co-wrote the paper. Nutawan Niyatiwatchanchai performed experiments. Tassanee Jaroensong designed, performed experiments, analyzed data, and co-wrote the paper.

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