RESEARCH ARTICLE

Overexpression of HER-2/neu in Malignant Mammary Tumors; Translation of Clinicopathological Features from Dog to Human

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Abstract

Background: Canine mammary gland tumors (*CMGTs*) are the most common tumor found in bitches. Changes in HER-2/neu genes in human breast cancer (*HBC*) lead to decrease in disease-free survival (*DFS*) and overall survival rate (*OSR*). Previous studies have demonstrated that the biological behavior of malignant mammary gland tumors (*MMGTs*) is similar to that of *HBC*. The present study aimed at evaluating the relationship between overexpression of HER-2/neu and clinicopathological features in *MMGTs* to represent a model of prognostic factors for *HBC*. Materials and Method: The clinicopathological data of 35 *MMGTs* were obtained. Immunohistochemical staining with HER-2, Ki-67 and CD34 markers was conducted with sections from paraffin-embedded blocks. According to standard protocols, histological type, grade, margin status, lymphovascular invasion (LVI), HER-2/neu score, proliferation rate and microvessel density (*MVD*) of tumors were determined and the association of HER-2/neu overexpression with these parameters was assessed statistically. Results: The *IHC* results showed that 12 (34.3%) cases were HER-2/neu positive. Statistical analyses indicated a significant relationship between HER-2 positivity and tumor grade (p=0.043), which also was demonstrated with cancer stage (p=0.035), tumor margin involvement (p=0.016), proliferation index (p=0.001) and *MVD* (p=0.001); however, there was no statistical relationship between *LVI* and tumor size. Overexpression of the HER-2/neu gene in *MMGTs* results in similar biological behavior as that of *HBC*; as a result, these tumors have can be considered to have important similarities in clinicopathological characteristics. Conclusions: *MMGTs* can be regarded as an *HBC* animal model. Further studies in this field would result in new treatments that could be beneficial for both dogs and humans.

Keywords: Canine mammary gland tumors - human breast cancer - HER-2/neu gene

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Introduction

Canine mammary gland tumors (*CMGTs*) are the most common tumor found in bitches; based on published statistics, 34-93% of these tumors are malignant (Akhdar et al., 2011). Currently, surgery is the primary and most cost-effective treatment for *CMGTS*; however, due to local recurrence and early metastases in malignant mammary gland tumors (*MMGTs*), the post-surgery overall survival rate (*OSR*) is low. Studies have shown that within the first 2 years after surgery, recurrence risk of invasive tumors is 13 times higher than that of non-invasive tumors (Simon et al., 2006; Lorenza et al., 2010). On the other hand, recurrence times are different in *MMGTs*, as some tumors with unfavorable histopathology are associated with later relapse. Similar to human breast cancer (*HBC*), several prognostic factors are associated with the development of *MMGTs* (Philbert et al., 2003).

Changes in HER-2/neu genes in *HBC* have received great attention over the past 15 years, and numerous *HBC* oncology studies focused on the diagnosis and treatment of individuals carrying this gene. In humans, this gene is located on chromosome 17, while the HER-2/neu gene (derived from the name of the human gene) is located on chromosome 1q13.1 in canines (Hus et al., 2009). Overexpression and amplification of this gene has been shown by immunohistochemistry (IHC) and in situ hybridization (*ISH*) methods, respectively. During the mutation time of this gene, intracellular signaling cascade of epidermal growth factor receptor is hyperactivated; as a result, tumor cells grow more quickly and their doubling time decreases. In addition, chemo-resistance occurs among cancer patients (Akhdar et al., 2011; Ryska et al., 2011). The relationship between changes of HER-2/neu genes and tumor grade, tumor proliferation, lymphovascular invasion (*LVI*) and rate of tumour angiogenesis has been studied in *HBC*, and these changes have been demonstrated to result in poor

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prognosis (Schoppmann et al., 2010; Chen et al, 2011; Park et al., 2012). Previous studies have demonstrated that the biological behavior of MMGT is similar to that of *HBC*, thus it is regarded as the *HBC* animal model (Queiroga et al., 2011). The present study aimed at evaluating the relationship between overexpression HER-2/neu in *MMGTs* and tumor grade, proliferation, *LVI* and rate of tumor angiogenesis, in order to provide a model of prognostic factors in *CMGTs* and to further test the validity of this *HBC* model.

Materials and Methods

This study was a retrospective observational study that was blinded in all of the pathological diagnostic stages. The data of 41 bitches with initial MMGT history but with no previous treatment record were collected from the beginning of January 2009 to May 2012 from several small animal clinics and hospitals in Tehran. Surgery methods varied from nodulectomy to complete chain (unilateral) resection. Only those cases with histology test results of malignant epithelial neoplasms (*MENs*) or malignant epithelial neoplasms– special types were investigated. The age of the animals ranged between 4 and 12 years. All clinical data, including ovariohysterectomy (*OHE*) records, the number of involved breasts, the presence or absence of local invasion, lymph node involvement or recognizable metastases, type of mammary tumor removal surgery technique, tumor size and primary Histopathological results were collected from the files of the animals; in some cases, their owners were contacted to provide the missing data. Paraffin blocks were transferred to the pathology laboratory and stained slides with hematoxylin and eosin (*H&E*) staining were prepared after a second sectioning. All of the sections were twice examined by the pathologist. Improper fixation and suspicion of the diagnosis of intraepithelial lesions (*IELs*) or malignant *MENs* resulted in the exclusion of 4 and 2 samples, respectively. Accordingly, 35 animals were included in the study. Histologic classification and tumor grading were performed based on the protocol proposed by Goldschmidt et al. (2011).

For the *H&E* staining, observations of tumor cells in blood vessels were reported as vascular invasion (*VI*) positive. In addition, *VI* was also considered in the second IHC staining with the CD34 marker; therefore, the phrase *LVI* was used in the final report (Uzzan et al., 2004; Geovanni et al., 2009).

Since several blocks were available for each tumor, the margin between the tumor border and its healthy edge was accurately examined, and the observations of tumor cells were recorded as a positive margin in the seemingly healthy edge.

Clinical cancer staging (TNM) was carried out according to the protocol recommended by Owen 1980 (Angélica et al., 2011).

Five-micron thick blocks were provided to the IHC laboratory and were stained with HER-2 (Dako, Colone: mAb), Ki-67 (Dako: MIB-1) and CD34 (Dako, OBQEnd 10) antibodies by using the following method. First, the sections were maintained at 37°C for 24 h; then, they were incubated for 15 min at 60°C inside a microwave. Deparaffinization and rehydration stages were passed in a xylene and ethanol solution series, and a methanol solution containing hydrogen peroxidase was used as a blocking agent. The sections were incubated for 10 min in the phosphate buffered saline (*PBS*) container for antigen retrieval. After incubating the tissues with primary and secondary antibodies, ready made solutions of diaminobenzidine (*DAB*) and hematoxylin were used to reveal staining.

The results of IHC were interpreted by using a light microscope according to the following semi-quantitative method.

HER-2/neu IHC test: According to the American Society of Clinical Oncology/College of American Pathologists (*ASCO/CAP*) guidelines (2007) in which only Score +3 was considered positive (Antuofermo et al., 2007).

CD34 IHC test: In this method, 4 hot spot regions at 100× magnification were selected, then microvessel were counted at 400× magnification (0.17 mm^2) and the mean count of each slide was recorded. The results were reported as low microvessel density (*MVD*; less than 20), medium *MVD* (20-40) and high *MVD* (>40) (Dhakal et al., 2009).

Ki-67 IHC test: Ten fields were randomly selected and 100 epithelial cells were counted at 400× magnification. Rates of nuclear immunoreactivity were stated as percentages <10%, 10-25% and <25%, which corresponded to low, medium and high, respectively (Jones et al., 2009).

Statistical analysis

Statistical significance of differences was analyzed by Chi-square test using BioState® 2008. A 'p value' of less than 0.05 was statistically regarded as significant.

Results

The mean of age of the dogs included in the present study was 8±0.4 years; in terms of the involvement of mammary glands (*MGs*), 24 (68.6%) had only 1 MG involved, 9 (25.7%) had 2 *MGs* involved and 2 (5.7%) had all 3 *MGs* involved. With regard to tumor distribution, 19 (54.3%) tumors occurred in the left *MGs* and the 4 left *MGs* had the most involvement in 16 (45.7%) animals. In addition, 82.5% of tumors occurred in the abdominal *MGs*. 74.3% of the dogs had records of *OHE*, although sufficient data were unavailable regarding *OHE* before and after puberty. The surgery methods applied in this study for MMGT treatment were lumpectomy (37.2%), mammectomy (25.7%), regional mastectomy (34.3%) and unilateral resection (2.8%), respectively. In terms of tumor size, T1=45.7%, T2=45.7% and T3=8.6%, which indicated that more than 90% of the tumors were up to 5 cm. Clinical staging results showed that stage II tumors, that had a frequency of 51.4%, were the most common clinical stage in the present study. In histopathological terms, tumor types included simple carcinoma in 57.2%, mixed-type carcinoma in 11.5%, complex carcinoma in 11.5%, mucinous carcinoma in 5.7%, spindle cell carcinoma in 5.7% and micropapillary invasive carcinomas, anaplastic carcinoma, inflammatory carcinoma and ductal carcinoma

Table 1. Clinico-pathological Results of *MMGTs* in the Present Study

		HER-2$^+$ (n)	HER-2$^-$ (n)	P
Grade:	I	2	10	p=0.043
	II	6	12	
	III	4	1	
Stage:	I	2	11	p=0.035
	II	6	11	
	III	4	1	
	IV	0	0	
Tumor size:	T1	5	11	p=0.466
	T2	5	11	
	T3	2	1	
Margin:	Involve	10	6	p=0.016
	Free	2	17	
LVI:	Positive	5	10	p=0.797
	Negative	7	13	
Ki-67:	Low	0	13	p=0.001
	Moderate	4	7	
	High	8	3	
MVD-CD34:	Low	0	7	p=0.001
	Moderate	3	15	
	High	9	1	

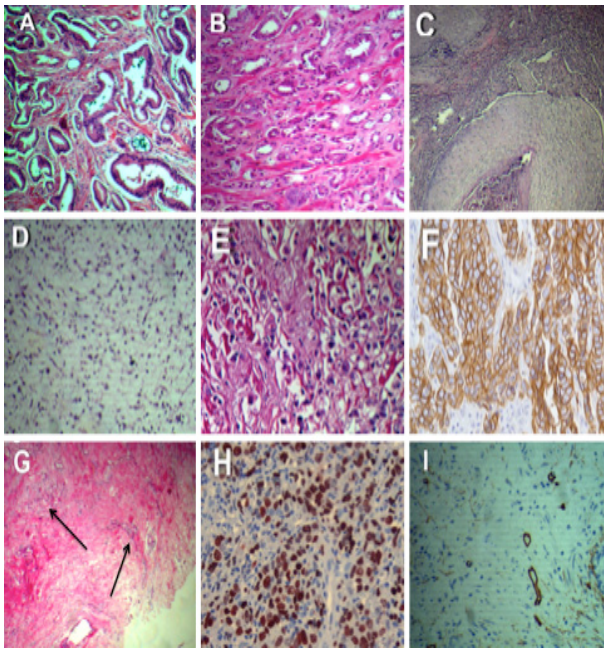

Figure 1. Microscopic Views of Malignant Mammary Tumors. (A) *H&E* staining of simple carcinoma (original magnification, ×20); (B) *H&E* staining of complex carcinoma (original magnification, ×20); (C) *H&E* staining of mix carcinoma (original magnification, ×10); (D) *H&E* staining of mucinous carcinoma (original magnification, ×10); (E) *H&E* staining of anaplastic carcinoma (original magnification, ×40); (F) *IHC* staining with Her-2 antibody. This micrograph illuminates a score 3+ feature (original magnification, ×40); (G) the arrows indicate the infiltration of tumoral cells (*H&E* staining; original magnification, ×4); (H) *IHC* staining with Ki-67 antibody. Immunoreactive nuclei indicate the high proliferation (original magnification, ×40); (I) *IHC* staining with CD34 antibody. Immunoreactive microvessels have been illustrated in this micrograph (original magnification, ×20)

occurred in 2.8%of. In summary, malignant epithelial neoplasms and malignant epithelial neoplasms – special types occurred in 88.6% and 11.4% respectively. In this study, grade II tumors had a frequency of 51.4%, which was the highest rate. Approximately 45.7% of the tumors had margin involvement, and a positive *LVI* was reported in 42.9% of tumors (Figure 1).

The IHC results showed that 12 (34.3%) cases were HER-2/neu positive, and 37.2%, 31.4% and 31.4% of cases were Ki-67 low, moderate and high, respectively; while, in the *MVD*-CD34 group, 20%, 51.4% and 28.6% of cases were low, moderate and high, respectively. In terms of histopathology, 10 (83.3%) HER-2-positive cases had simple carcinoma, while others had micropapillary invasive carcinoma and ductal carcinoma.

Statistical analyses indicated a significant relationship between HER-2 positivity and tumour grade (p=0.043), which also was demonstrated with cancer stage (p=0.035), tumor margin involvement (p=0.016), proliferation coefficient (p=0.001) and tumor *MVD* (p=0.001); however, there was no statistical relationship between *LVI* and tumor size (Table 1).

Discussion

CMGTs have recently received great attention since they are the most important tumour observed in bitches and they exhibit similar histological, biological and epidemiological behaviours as those of *HBC*. Research findings of the recent decade have demonstrated several similarities between *HBC* and *CMGTs* in terms of incidence and risk factors, histologic features, clinical course and molecular markers (Queiroga et al., 2011). Many studies have demonstrated that the relevant molecular biomarkers and their relationship with prognosis in *HBC* were also involved in *CMGTs*, and both types of tumors have similar biological behaviors. Consequently, most biomarkers used in *HBC* have received attention in *CMGTs* and studies involving these canine tumors have had similar results to those involving *HBC*. Although in most cases the role of CMGT biomarkers has imitated those of the *HBC* model, results demonstrating the role of genes related to cycloxygenase-2 (*COX-2*) and *COX-2* inhibitors were first made in *CMGTs* and have now been used in studies of *HBC* (Klopfleisch et al., 2011).

Since the introduction of the expensive medication Trastuzumab (*INN: trade name Herceptin®*) in *HBC* for the treatment of changes in HER-2/neu genes, many studies have been conducted regarding the performance of this gene in *HBC* and its effect on the prognosis of disease. Numerous findings in human studies have shown that overexpression and amplification of this gene in *HBC* results in the reduction of *DFS* and *OSR* (Tortora., 2011). Initially, due to technical weaknesses and lack of uniform diagnostic protocols, the rate of false positives was high. However, since technical and diagnostic protocols were optimized and an in situ technique was used besides IHC, variation rates of this gene in *HBC* reached 15-20% (Vogel, 2012).

There are not enough studies regarding variation frequencies of the HER-2/neu gene in *CMGTs* to decisively state the percentage mean; however, this limited number of studies indicated that the mutation frequency is approximately equal to that of *HBC* (Nieto et al.,

2007; Angélica et al., 2011; Chu et al., 2011; Klopfleisch et al., 2011). In some studies in which positive HER-2 was reported to be high in *CMGTs*, benign and in situ tumors were also examined and +2 cases were regarded as positives (Antuofermo et al., 2007). Nevertheless, according to the *ASCO/CAP* guidelines (2007), in humans, cases of ductal carcinoma in situ (DCIS) should not be considered HER-2-positive; on the other hand, a score of +2 must be re-evaluated by fluorescence in situ hybridization (*FISH*) (Ejlertsen et al., 2009). In the present study, the rate of positive HER-2 cases among *MMGTs* was 34.3%, which was consistent with similar studies.

In this report, the mean age of dogs with *MMGTs* was 8±0.04 years. Metzeger et al. showed that, from biological point of view, the age of 8 year-old dogs is equal to that of 48-51 years in humans (Metzger et al., 2005; Queiroga et al., 2011). Investigations have demonstrated that the peak incidence of *CMGTs* occurs in dogs aged approximately 7-10 years, which is equal to 44-56 years in humans and is roughly equal to the peak of *HBC* incidence (Jamal et al., 2007; Baquet et al., 2008). In terms of the involved anatomic region as well as the histopathological results, this study was in line with other similar studies (Karyannopoulo et al., 2005; Andrade et al., 2010). Any change in the effect of the HER-2/neu gene in *HBC* is related to tumor grading since, by activating epidermal growth factor receptor 2, both tumor cell proliferation pathways and the cellular growth cycle are activated (Ramadan et al., 2011). The result of activating the cellular growth cycle is the emergence of several mitotic figures in tumor cells, and several anisocytoses and anisokaryoses are observed. Since, in tumor grading, parameters of mitotic count and pleomormphic cells along with tubular formation are considered, the effect of mutations in HER-2/neu on tumor grade can be justified. As shown in Table 1, the relationship between HER-2 and tumor grade is significant.

Ki-67 is a protein that is detected in all growth stages of the cell cycle, with the exception of G_0 (Khoruzhenko et al., 2010). Tumors with a fast growth cycle have a high emergence percentage of this protein. In the variation time of HER-2/neu in *HBC*, the proliferation index quickly increases. High Ki-67 in these patients indicates the activation of cancer cells, which results in rapid invasion and metastases (Miglietta et al., 2009). In the present study, this phenomenon also occurred, which was consistent with similar studies.

Tumor margin is important in determining prognosis. In breast-conserving surgeries (*BCS*), margin involvement indicates local recurrence risk (Dunne et al., 2009). In many cases of *HBC*, the relationship between variations in the HER-2 gene and margin involvement has been demonstrated; it has been recently shown that, in DCIS patients with HER-2 positivity and high Ki-67 expression, local recurrence risk increases after *BCS*. In this study, 45.7% of tumors exhibited margin involvement and had a significant relationship with HER-2 positivity. Since there was a positive relationship between HER-2/neu and Ki-67 in this study, it is reasonable to expect the increase of local recurrence risk in HER-2-positive *MMGTs*, according to the study by Racovitch et al. (2012) however, prospective studies should be conducted in this regard.

In recent years, angiogenesis has become widely studied in various tumors. Based on the theory proposed by Folkman (1971) tumors are unable to grow and invade unless angiogenesis occurs. Several papers have supported the relationship between HER-2/neu positivity and increased angiogenesis in *HBC* (Vamesu., 2007; Tortora., 2011). It is believed that HER-2 mutations result in increased metabolic activity of tumor cells; thus, hypoxia-inducible factor 1- alpha (*HIF1-α*) is induced. Consequently, tumor angiogenesis is initiated and endothelial cells rapidly increase in the tumor location (Kebel., 2007). Currently, *MVD* evaluation is a cost-effective method for measuring angiogenesis in tumors. Meta-analyses have demonstrated a relationship between *MVD* and *OSR*, which increases relapse risk in *HBC* (Uzzan et al., 2004; Nieto et al., 2007). In the present study, the relationship between HER-2 and *MVD* was strongly significant and showed that angiogenesis parameters in *MMGTs* were also similar to the *HBC* pattern.

Clinical staging is an important method for determining appropriate clinical procedures in human tumors. Due to limitations in resources and costs, lymph node imaging is not performed in veterinary oncology; only in cases of diagnosed lymphadenopathy are regional lymph nodes are removed. Surgeons prefer to resect lymph nodes in regional mastectomy and unilateral resection surgeries. On the other hand, due to the issues related to cost in veterinary medicine, metastases are examined by abdominal and chest X-ray as well as abdominal sonography, and other diagnostic methods are rarely used. In light of these issues, it is recommended to use clinical staging instead of histological staging (Azizun et al., 2008). In the present study, due to the comparison with *HBC*, we attempted to collect as accurate of clinical data as possible. In this study, the relationship between HER-2 and clinical staging was significant, and the results of clinical staging were consistent with other histological prognostic findings. This relationship was appropriately stated for *HBC* as well (Burestein and Winer, 2009; Aksu et al., 2011).

Tumor size is an important and valuable prognostic factor in *HBC* and *MMGTs*. In the present study, in contrast to the viewpoints of some authors, there was no relationship between HER-2 and tumor size. In *HBC*, several factors such as the existence or lack of hormonal receptors, various types of epidermal growth receptors and tumor suppressing genes affect tumor size (Gama et al., 2008; Goldherish et al., 2011). Some studies have demonstrated a direct relationship between HER-2/neu expression and tumor size in *HBC*, while others reject such a relationship. In the *St. Gallen consensus* (2011), breast cancer was revised in terms of subtypes as follows (Gama et al., 2008). In terms of clinicopathological definition, luminal A was divided into ER and/or PR^+, $HER\text{-}2^-$ and Ki-67 low and luminal B was divided into 2 groups of (ER and/or PR^+, $HER\text{-}2^-$, Ki-67 high) and (ER^+ and/or PR^+, $HER\text{-}2^+$, Ki-67 any). In *MMGTs*, similar subtypes to those of humans have been reported (Sassi et al., 2010; Cintra et al., 2012). showed that certain subtypes [Erb-B2 overexpression and basal-like (Triple negative)] were

associated with larger tumor size relative to that of other subtypes (Baqaria et al., 2012). In the present study, data regarding existence or lack of hormonal receptors were unavailable; as a result, data regarding their molecular subtypes were not available. According to Baqaria et al. (2012) and Citra et al. (2012) and by referring to the biological similarity theory between *HBC* and *MMGTs*, it can be deduced that tumor groups of basal-like subtypes also had larger tumor sizes in this study. Although subtype Erb-B2 overexpression is also associated with larger tumor size, it is possible that a portion of HER-2-positive tumors in this study were placed in luminal B (HER-2^+) subtype, as ER and PR were not considered. Consequently, using this hypothesis, lack of a relationship between tumor size and HER-2-positivity is justified. Of course, it is clear that complementary studies are needed to prove this theory.

LVI is routinely evaluated in *HBC* in pathological terms. However, its interpretation is sometimes difficult. Previous studies have shown that, in the patients with positive *LVI*, prognosis is abated and overall survival is decreased (Mohammed et al., 2007; Ragage et al., 2010). In this study, although *LVI* was positive in 42.9% of all *MMGTs*, there was no statistical relationship between HER-2 positivity and *LVI*. In a prospective study, Ejlertsen et al; concluded that the risk of a second recurrence was high in *HBCs* with positive *LVI*, while the HER-2/neu gene likely had no effect on transmission of low-risk status toward high-risk (Ejlertsen et al., 2009). However, other studies describe an altogether different view. Although all authors agree that positive *LVI* increases local recurrence risk or metastases, it seems that its parameters have not been well understood; thus, it has been shown that any disorder involving gene pathways related to molecular adhesion and matrix metalloproteinases (*MMPs*) results in the increase of premature *LVI* risk (Dicken et al., 2006).

The results of this study showed that the biological pattern of changes HER-2/neu expression was almost identical between *HBC* and *MMGTs*; as a result, similar clinicopathological properties existed between them. Some authors have obtained a comparable result in similar studies; however, Hus et al. (2009) believed that the biological behavior of the HER-2/neu gene in *MMGTs* was completely different from that of *HBC* (Hus et al., 2009).

It can be deduced from the results of this research that *MMGTs* can be regarded as an *HBC* model; likewise, similar studies evidently have described this issue (Andrade et al., 2010; Hasiwa et al., 2011; Queiroga et al., 2011). Ethical issues are the point that should be considered in modeling studies. Three principles of reduction, refinement and replacement should be considered in modeling diseases in animals (Workman et al., 2010; Pinho et al., 2012). Cancer xenograft models performed in athymic nude mice are accepted as the best laboratory models of cancers. It is clear that *CMGTs* could not be taken as a laboratory model of *HBC* based on these 3 ethical principles; only after the new treatment has successfully passed all *in vitro* and *in vivo* tests and the treatment is shown to be beneficial for dogs as well, could *CMGTs* be used as pre-clinical animal models.

In conclusion, overexpression of the HER-2/neu gene in *MMGTs* results in similar biological behavior as that of *HBC*; as a result, these tumors have similar clinicopathological characteristics. Therefore, *MMGTs* can be regarded as an *HBC* animal model. Further studies in this field would result in new treatments that could be beneficial for both dogs and humans.

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