RESEARCH ARTICLE

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

Ahad Muhammadnejad1*, Elahe Keyhani2, Pejman Mortazavi1, Farkhondeh Behjati2, Iraj Sohrabi Haghdooost1

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 (Philibert 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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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. Paraflin 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 HIC staining with the CD34 marker; therefore, the phrase VI 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 (Antufermo et al., 2007).

CD34 IHC test: In this method, 4 hot spot regions at 100x magnification were selected, then microvessel were counted at 400x magnification (0.17 mm²) 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 400x 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

<table>
<thead>
<tr>
<th>Grade</th>
<th>HER-2+ (n)</th>
<th>HER-2- (n)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2</td>
<td>10</td>
<td>0.043</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>12</td>
<td></td>
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<tr>
<td>III</td>
<td>4</td>
<td>1</td>
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<td>Stage</td>
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<td>I</td>
<td>2</td>
<td>11</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
<td>4</td>
<td>1</td>
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</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Tumor size:</td>
<td></td>
<td></td>
<td>0.466</td>
</tr>
<tr>
<td>T1</td>
<td>5</td>
<td>11</td>
<td></td>
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<tr>
<td>T2</td>
<td>5</td>
<td>11</td>
<td></td>
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<tr>
<td>T3</td>
<td>2</td>
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<tr>
<td>Margin:</td>
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<td></td>
<td>0.016</td>
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<tr>
<td>Involve</td>
<td>10</td>
<td>6</td>
<td></td>
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<tr>
<td>Free</td>
<td>2</td>
<td>17</td>
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<tr>
<td>LVI:</td>
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<td></td>
<td>0.797</td>
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<tr>
<td>Positive</td>
<td>5</td>
<td>10</td>
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<tr>
<td>Negative</td>
<td>7</td>
<td>13</td>
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<tr>
<td>Ki-67:</td>
<td></td>
<td></td>
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<tr>
<td>Low</td>
<td>0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>7</td>
<td></td>
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<tr>
<td>High</td>
<td>8</td>
<td>3</td>
<td></td>
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<tr>
<td>MVD-CD34:</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>3</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>1</td>
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Discussion

CMGTs have recently received great attention since they are the most important tumor 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 cyclooxygenase-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., 2012).
In this study, 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 G0 (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 (Kebe., 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 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-2 and Ki-67 low and luminal B was divided into 2 groups of (ER and/or PR, HER-2, Ki-67 high) and (ER and/or PR, HER-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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References


Young RJ, Reed MW (2012). Anti-angiogenic therapy: concept to clinic. Microcirculation, 19, 115-25