Overexpression of HER-2 via immunohistochemistry in canine urinary bladder transitional cell carcinoma (TCC) - A marker of malignancy and possible therapeutic target

Running title

HER-2 in canine urinary bladder transitional cell carcinoma

Francesca Millanta[1], Joseph Impellizeri[2], Leo McSherry[3], Guido Rocchigiani[1], Luigi Aurisicchio[4], George Lubas[1]

[1] Department of Veterinary Sciences, University of Pisa (Italy)

[2] Veterinary Oncology Services, PLLC (New York, NY, USA)

[3] Antech Diagnostic (Irvine, CA, USA)

[4] Evvivax (Rome, Italy)

Corresponding author: Joseph Impellizeri; oncologyvet@yahoo.com; www.petcancerinformation.com

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ABSTRACT

Transitional cell carcinoma (TCC) is the most commonly diagnosed neoplasm in the urinary bladder. Distant metastases to the regional lymph nodes, lungs, abdominal organs, or bones are noted up to 50% of dogs at death. Surgical excision is often not practicable as TCC typically involve the trigone of the bladder and/or occur multifocally throughout the bladder with field cancerization. Therapeutic approaches are very challenging and the requirement to evaluate alternative therapeutic protocols that may prolong survival times in dogs bearing these tumours is compelling. We assessed the immunohistochemical expression of HER-2 in 23 cases of canine TCCs of the urinary bladder and compare it to non-neoplastic urothelium in order to evaluate a rationale for targeted therapies and gene-based vaccines. HER-2 positivity was recorded in 13/23 (56%) neoplastic lesions. The receptor was significantly overexpressed in neoplastic than in non-neoplastic samples (P=0.015). According to our preliminary results it would be of interest to further evaluate the role of HER-2 in canine TCCs as a marker of malignancy and a therapeutic target for cancer vaccine and antibodies. Moreover, the significantly different overexpression of HER-2 in TCCs than in non-neoplastic urothelium further supports to investigate its role in the progression toward malignancy of non-neoplastic lesions.

The c-erbB2 gene encodes the second member of the epidermal growth factor receptor (EGFR) family, namely Her-2/neu. It is a defective transmembrane tyrosine kinase receptor, which is involved in the control of epithelial cells growth and differentiation. The overexpression of this protein is considered as a poor prognostic factor in many human carcinomas (breast, ovary) and the amplification in patients with lymph-node metastases is more common. [1] The incidence of overexpression of HER-2/neu in bladder cancer has been reported to have a wide variation ranging from 2% to 71% of cancers tested . [2,3] In urothelial tumors of the urinary bladder, HER-2/neu expression has been reported for over 10 years, and yet, there is no clear correlation between prognosis and recurrence rate. [4] In transitional cell carcinomas (TCCs) of the bladder it is reported that HER-2/neu is overexpressed with a greater frequency in higher grades (40%) and stages (38%) than in lower grades (0%) and stages (8%). [3,5] Currently, the only approved treatments of locally advanced or metastatic disease are cisplatin-based chemotherapy combinations, but recently targeted therapies have yielded promising results alone or combined with traditional chemotherapy. Among these, the anti-HER-2 antibody trastuzumab has been proposed as a target for therapeutic intervention in human breast cancer. [6]Two compounds have been registered for HER-2-positive breast cancer treatment: trastuzumab (Herceptin™), a humanised antibody directed against the HER-2 extracellular domain [7], and lapatinib, a small molecule acting as a dual EGFR and HER-2 tyrosine kinase inhibitor. [8] Although both drugs improve progression-free survival, many patients' tumours will exhibit primary resistance, or develop secondary resistance, to anti-HER-2 therapies. The recent significant improvement of survival gained with pertuzumab (Perjeta™, a dimerisation blocking antibody) or trastuzumab emtansine (T-DM1, a cytotoxic drug vectored by trastuzumab binding) further validate the relevance of the target and opened the way for new strategies. [9] In addition, different vaccine platforms targeting HER-2 have been evaluated clinically including mainly peptide-based, protein-based and dendritic cells (DC)-based vaccines . However, in most cases such approaches results in poor immunogenicity and therapeutic outcomes. [10]

In dogs, TCC is the most commonly diagnosed neoplasm in the urinary bladder. [11] Distant metastases to the regional lymph nodes, lungs, abdominal organs, or bones are noted in up to 50% of dogs at death. Surgical excision is not often feasible as TCC typically involves the trigone of the bladder and/or occur multifocally throughout the bladder with field cancerization. [12] Medical treatment is also challenging: chemotherapy with either mitoxantrone or carboplatin in combination with piroxicam, along with surgical excision is recommended, but response rates are often low. [13, 14, 15, 16] A recent pilot study using a combination of toceranib and vinblastine did not result in improved response rates. [17] Among comparative medicine studies HER-2-positive mammary tumors showed marked high similarities between human and canine antigen expression. [18, 19] In this study, we aimed to evaluate the immunohistochemical expression of HER-2 in canine TCCs of the urinary bladder and to compare it with non-neoplastic urothelium.

Twenty-three cases of canine TCCs from the urinary bladder were retrospectively analyzed. Tissue specimens were collected from dogs (13 females and 10 males) of different breeds (8 mixed breed, 3 Labrador Retriever, 2 West Highland white terrier, 2 Toy poodle, 2 Wheaton Terrier, 1 each of Akita Inu, Old English Sheepdog, Wheaton Terrier, Beagle, Dachshund, and Pug). The mean age was 10.9 years ±2.5 (population Standard Deviation), range: 6-15 years. The tissue samples were submitted to a veterinary diagnostic lab (1) and evaluated by a single pathologist affiliated to (2) and consisted of 20 invasive TCCs and 3 "in situ" papillary TCCs. The control population consisted of 5 cases of non-neoplastic canine urothelium retrieved from the archives of (3). Specimens were from dogs (one each for Cocker Spaniel, Dachshund, Beagle, Pug, and Mixed Breed) of age ranging from 7 to 12 years. Additional slides from each case were retrospectively evaluated by immunohistochemistry for HER-2 expression at (3).

Briefly, 5 µm-thick sections, mounted on coated slides (Superfrost plus, Menzel-Glaser, Germany) and dried at 37° C for 24h, were dewaxed and rehydrated in distilled water through a series of graded alcohols. To unmask the antigen, the slides were microwaved in 10 mM citrate buffer, pH 6, for 5

minutes at 750 W (three times). Endogenous peroxidases were blocked with 0.5% hydrogen peroxide for 30 minutes followed by 3 washes in 0.05% Tween Tris-buffered saline (TBS-tween) solution at pH 7.6. The polyclonal anti- HER2/neu antibody (Dako, Glostrup, Denmark, ref A0485, dilution 1 in 250 TBS, incubation at room temperature) was applied for 1 hour, followed by rinsing in TBS/Tween. Tissues were then incubated at room temperature for 30 min with anti-rabbit Envision®+ System-HRP (Dako). After washing again with TBS/Tween, the peroxidase reaction was developed for 10 min with 3,3-diaminobenzidine (DAB) (Impact DAB®, Vector Laboratories, Burlingame, CA), rinsed with deionized water and lightly counterstained with Mayer's haematoxylin. Sections from a human breast and a canine mammary carcinoma, known to react with HER-2 antibody, were used as a positive control, while negative controls were performed omitting the primary antibody and replacing the primary monoclonal antibody with a subtype matched unrelated primary antibody. The IHC protocol adopted had already been described in canine mammary tissues [20] and results were quantified according to recent guidelines proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). Only tumors scoring 3+, with a strong, complete, homogeneous membrane labeling (chicken-wire pattern) in >30% of cells, were considered positive (HER-2 overexpressing) . [21] Chi-square tests were used to assess associations between categorical variables. Statistical significance was based on a 5% significance level.

In control cases 4 out of 5 (80%) showed a weak cytoplasmic immunoreactivity and focal areas without any immunoreactivity. One case showed a nuclear immunolabelling predominantly evident at the basal cell layer. All the cases were considered as negative (score 0). In spite of that, in neoplastic specimens, a membranous immunolabelling, of intensity varying from weak, to moderate to strong was observed. In detail, two of the three *"in situ"* papillary TCCs showed a strong and complete membranous immunolabelling in more than 30% neoplastic cells and scored 3+ (67%), while one scored 1+. Of the 20 invasive TCCs, 11 (55%) scored 3+, 6 (30%) scored 2+, and 3 (15%) scored 1+. The results are summarized in Table 1. HER-2 positivity was recorded in 13/23 (56%) neoplastic lesions. HER-2 was significantly overexpressed in neoplastic than in non-neoplastic samples

(*P=0.015)*, while no significant differences were observed between *"in situ"* and invasive TCCs (*P*=0.2). HER-2 overexpression was not significantly associated to sex, age or breed.

Effective treatments for TCC in dogs are still lacking as the mainstay continues to be systemic medical therapy, which usually consists of chemotherapy, non-selective and selective cyclooxygenase inhibitors, and combinations of these. [12]Research is however setting the stage for further progress against the cancer. Because of close similarities between dogs and humans with invasive TCC, promising research in dogs is expected to translate into humans to improve the outlook in both species. [21] In humans targeted therapy with novel drugs directed at specific molecular pathways opens promising new avenues to improve patient outcome and trastuzumab and cytotoxic agents conjugated to trastuzumab such as T-DM1 have shown promising results for patients with advanced bladder cancer. [23, 24]

TCC is associated with high risk of metastatic disease and therefore represents an ideal model to evaluate cancer immunotherapy. New immunotherapies, such as gene-based vaccines have the great potential ability to elicit potent immune responses against a target antigen. Engineered viral vectors, for instance, recently gained attention for the treatment of infectious diseases, such as Ebola [25] and Zika [26]. This property is particularly relevant for cancer patients, because tumours can rapidly induce immune-suppression in the host, compromising the response to the vaccine via a variety of complex mechanisms. Recently, ADXS-cHER2, an HER2-directed cancer immunotherapy for the treatment of canine osteosarcoma based on the vector *Listeria spp* has suggested therapeutic effects in dogs with osteosarcoma that had minimal residual disease following standard of care (amputation and follow-up chemotherapy). [27] Similarly, a genetic vaccine based on a combination of Adenovirus and DNA electroporation has been shown to be immunogenic in dogs [28] and some authors (LA, JAI, GL) are currently testing its antitumor efficacy in solid tumors overexpressing HER2. This idea is in line with a growing alliance of scientists involved in cancer research and drug development which recognizes the inclusion of dogs with cancer in a comparative and integrated translational drug development path as a possible means to markedly accelerate cancer drug discovery. [29]

For this purpose, we tried to assess the expression of HER-2 in a subset of transitional cell carcinomas and in non-neoplastic canine urothelium in order to evaluate if this receptor is overexpressed in this type of tumors. To the authors' knowledge, this is the first study that evaluates this expression and according to our data, the percentage of HER-2 overexpressing canine TCCs is similar to that reported in previous human studies. [4]HER-2 overexpression is widely documented in primary human tumors as a prognostic indicator [30, 31] and, more recently, a HER-2 homologue with 92% amino acid identity has been described in canine mammary tumors, which shares similar biological implications with human breast cancer and can be immunologically targeted by anti-HER-2 antibodies. [19]According to our preliminary results it would be of interest to further evaluate the role of HER-2 in canine TCCs as a marker of malignancy and as a therapeutic target for cancer vaccine and antibodies. Moreover, the significantly different overexpression of HER-2 in TCCs than in non-neoplastic urothelium further supports justification to investigate its role in the progression toward malignancy from non-neoplastic lesions.

Footnotes

[1] Antech Diagnostics, Lake Success, NY

[2] Dr. Dave Gamble, DVM

[3] Department of Veterinary Sciences of the University of Pisa

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Figure legends

Fig 1. Immunohistochemical expression of HER-2 in canine urinary bladder. 1a: hyperplastic urothelium in a chronic cystitis: a weak and focal cytoplasmic immunoreactivity (20x magnification, streptavidin-biotin peroxidase method, haematoxylin counterstain). 1b: a strong and membranous immunolabelling in a transitional cell carcinoma scoring 3+ and recorded as overexpressing HER-2 (40x magnification, streptavidin-biotin peroxidase method, haematoxylin counterstain)

HER-2 overexpression		N cases (%)		*P* Value
Lesion	N cases	Yes	No	
TCC	23	13 (56%)	10 (44%)	0.015
Non neoplastic	5	0	5 (100%)	

Tab.1. HER-2 overexpression in canine transitional cell carcinomas (TCC) and in non-neoplastic samples of the urinary bladder