Surgery

Note

Overexpression of human epidermal growth factor receptor 2 in canine primary lung cancer

Authors

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Running head: HER2 Expression in Canine Lung Tumor
Abstract

Human epidermal growth factor receptor 2 (HER2) overexpression has been reported
in various human cancers. HER2-targeted therapies showed clinical responses in humans with
HER2-positive tumors. The incidence of canine primary lung cancer (cPLC) is increasing, but
there are no effective systemic therapies for dogs with late-stage cPLC. HER2-targeted therapy
could be an option for cPLC, but HER2 expression in cPLC remains unknown. We evaluated
HER2 expression in cPLC. Immunohistochemical analysis revealed that 3 samples (19%)
scored 3+; 8 (50%), 2+; 5 (31%); and 1+ and 0 (0%), 0. Of the cPLC tissues, 69% were HER2
positive (scored ≥2+). These data would lead to further evaluation of the role of HER2 in cPLC
as a mechanism of malignancy and therapeutic target.

Keywords

Canis familiaris, lung cancer, oncogene protein human epidermal growth factor receptor 2
Human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase and one of the members of the human epidermal growth factor receptor family [2]. HER2 amplification and protein overexpression have been reported in various human cancers, including breast, gastric, and lung cancers [5, 11, 14, 19]. HER2 has been shown as both a diagnostic marker and therapeutic target in these tumors [5]. HER2-targeted therapies, such as lapatinib and trastuzumab, have been developed and showed clinical responses in humans with HER2-positive cancers [1, 8, 22].

In human medicine, lung cancer is the leading cause of cancer-related death worldwide [11]. Approximately 85% of human lung cancers are non-small cell lung cancers (NSCLCs), which consisted of three distinct histologic subtypes: squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma [15]. HER2 was overexpressed in a proportion of patients with NSCLC and related to tumorigenesis of NSCLC [13]. HER2 protein overexpression is observed in 6–35% of patients with NSCLC, and HER2 gene amplification is noted in 10–20% of such patients [13]. Subsets of patients with high HER2 immunohistochemical (IHC) expression (score of 3+) were found to have a good response to HER2-targeted antibody, trastuzumab [7, 9]. Moreover, several emerging approaches to target HER2-positive NSCLC have been established, and clinical trials are ongoing to evaluate the efficacy of new HER2-targeted therapies, such as trastuzumab emtansine and margetuximab [11].

Canine primary lung cancer (cPLC) is relatively uncommon in dogs compared to humans, with an estimated incidence of 1.5–4.2 cases per 10,000 dogs [3, 4]. However, its incidence is increasingly reported because of improved diagnostic procedures, increased pet
longevity, and environmental changes [17]. Surgical excision is a more likely effective therapy
for dogs, in which surgical remission could be achieved, and the median survival time is 330
days in these cases but 28 days in dogs that could not be rendered free of visible disease [16].
Although systemic therapy is required for dogs with late-stage cPLC, chemotherapy has been
largely unrewarding in the gross disease setting [21]. Treatment with molecular- and antigen-
targeted therapies could be beneficial, but such targeted therapies have yet to be thoroughly
examined in dogs with cPLC [21].

As with human cancer, HER2 overexpression has been reported in dogs with canine
mammary gland carcinoma, transitional cell carcinoma, and apocrine gland anal sac
adenocarcinoma [6, 18, 24], and HER2-targeted therapies, such as lapatinib and HER2-
targeting recombinant *Listeria* vaccines, have shown an anti-tumor effect on canine HER2-
positive cancers [12, 18]. HER2 could be a therapeutic target and diagnostic marker for HER2-
positive cPLC, but HER2 expression in cPLC is still unclear. This study aimed to evaluate
HER2 mRNA and protein expression in cPLC tissues.

To evaluate mRNA expression of *HER2*, fresh cPLC (n=6) and normal lung (n=4)
tissues were obtained from dogs with cPLC and healthy beagles, respectively. The cPLC tissues
were obtained from dogs with cPLC at the Veterinary Medical Center of Tokyo between April
2017 and December 2018. To investigate the IHC expression of HER2, paraffin-embedded
tissues of cPLC (n=16) were analyzed. The tissues were surgically removed between January
2011 and December 2016. The tissues samples were diagnosed by veterinary pathologists
certified by the Japanese College of Veterinary Pathologists at the Department of Veterinary
Pathology at the University of Tokyo. All patients underwent clinical staging by thoracic
radiography and computed tomography.

Permission for the collection and use of resected tissue for this study was obtained from the owners. Healthy dog tissue was obtained from healthy beagles euthanized for other experiments. All procedures were approved by the Animal Care and Use Committee at the University of Tokyo (approval number, P17-074).

The total RNA was extracted and reverse transcribed using a transcriptase (ReverTra Ace, Toyobo, Osaka, Japan). Real-time polymerase chain reaction (PCR) was performed using a premix reagent (THUNDERBIRD SYBR qPCR Mix, Toyobo), specific primers for HER2 (forward primer, CAGCCCTGGTCACCTACAA; reverse primer, CCACATCCGTAGACAGGTAG), and a real-time PCR system (StepOnePlus, Thermo Fisher Scientific, Waltham, MA, USA). mRNA expression was analyzed using the standard curve method, and expression value was normalized using gapdh (forward primer, TGACACCCACTCTTCCACCTTC; reverse primer, CGGTTGCTGTAGCCAAATTCA) as an internal control.

The cPLC tissues were retrospectively evaluated by IHC analysis. All samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and cut into 4-µm-thick serial sections. Paraffin-embedded tumor sections were dewaxed and rehydrated in xylene and graded ethanol. Then, antigen retrieval was performed using Dako Target Retrieval Solution, pH 9.0 (Agilent Technologies, Santa Clara, CA, USA), by microwaving for 15 min at 750 W. After washing with Tris-buffered saline containing 0.1% Tween 20 (TBS-t), endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. Then, specimens were washed with TBS-t and incubated in 8% skimmed milk for 1 hr at 20–28°C to reduce
nonspecific binding before overnight incubation with primary antibodies, which included mouse IgG1 anti-HER2 monoclonal antibodies (clone, CB-11, Leica Biosystems, Wetzlar, Germany) diluted 1:40, at 4°C in a humidified chamber. A negative control was incubated with purified mouse IgG1, κ isotype antibody (clone, MG1-45, BioLegend, San Diego, CA, USA), under identical conditions. After washing with TBS-t, sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Envision+ System-HRP Labeled Polymer; K4001; Agilent Technologies) for 30 min at 20–28°C. Thereafter, the sections were washed with TBS-t, incubated with 3,3’ diaminobenzidine (Dojindo Laboratories, Rockville, MD, USA) solution for 3 min, and counterstained with Mayer’s hematoxylin. In the negative control, sections were subjected to the same procedures. The results were quantified based on the recent guidelines proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [23].

The statistical method and software used were Fisher’s exact test (R Development Core Team, 2018) and the beeswarm R package (version 0.2.3, 2016). A P-value<0.05 was considered statistically significant.

mRNA expression levels of HER2 were evaluated in cPLC and normal lung tissues. Variation in the mRNA expression of HER2 in cPLC tissues was higher than that in normal tissues and high HER2 mRNA expression was more frequently observed in cPLC tissues compared to normal lung tissues (Fig. 1). Of 6 cPLC samples, 5 expressed higher HER2 mRNA expression than any normal samples (p<0.05).

In the IHC analysis, 16 cPLC tissue samples were collected from 16 dogs (5 castrated male, 3 intact male, 7 spayed female, and 1 intact female dogs) of different breeds (5
Chihuahuas, 4 Miniature Dachshunds, 2 Shih Tzu, 1 Beagle, 1 English Pointer, 1 Saluki, and 1 Toy Poodle). The mean age was 12.3 ± 1.3 years (population standard deviation), ranging from 10.2 to 15.1 years. Three tumors were located on right cranial, 3 on the right middle, 2 on the right caudal, 1 on the left cranial, and 7 on the left caudal area (Table 1).

HER2-positive cPLC tissues demonstrated strong and broad staining of the circumferential membrane of cPLC cells and negative or weakly positive staining on peri-tumor normal cells and normal lung tissues via IHC staining (Fig. 2). We analyzed the HER2 expression score based on the ASCO/CAP guidelines (Table 2) to evaluate the staining pattern. Representative staining patterns of each score are shown in Fig. 2. HER2 expression score analysis demonstrated that 3 samples (19%) scored 3+; 8 samples (50%), 2+; 5 samples (31%), 1+; and 0 sample (0%), 0 (Table 3). In total, 69% of cPLC tissues were HER2 positive (scored ≥2+). There was no relationship between HER2 expression scores and clinical characteristics (Table 1).

In the current study, high HER2 mRNA expression was frequently found in cPLC tissues and circumferential membrane staining of canine HER2 in cPLC. HER2 expression was strongly detected in 69% of cPLC tissues, while HER2 was negative in normal lung tissues. Because most cases were resectable early-stage diseases and the number of patients was limited, we could not observe a correlation between HER2 expression and clinical characteristics, such as TNM classification, clinical stage, and histologic subtype. Further studies are required to investigate the association between HER2 expression and malignancies in cPLC.

Our results demonstrated that approximately 70% of cPLC overexpressed HER2. Recently, Lorch et al. reported that 38% of canine pulmonary adenocarcinoma possessed
somatic \textit{HER2} point mutation mainly in hotspot V659E transmembrane domain and constitutively activated \textit{HER2} downstream signaling [10]. Importantly, they demonstrated that no cases having \textit{HER2} point mutation showed overexpression of \textit{HER2} mRNA and protein, as similar to typical finding that \textit{HER2} amplification/overexpression and gene mutations are mutually exclusive [10]. They also showed one out of \textit{HER2} wild type four tissues overexpressed \textit{HER2} protein [10]. The discrepancy in frequency of \textit{HER2} protein overexpression between the reports and our results could be because of the difference in clones of antibody, populations and/or scoring systems. Overall, through our and Lorch \textit{et al.} findings, majority of cPAC possessed \textit{HER2} abnormality including overexpression and gene mutations, which would be beneficial findings for diagnosis and development of a novel therapy.

An increasing number of \textit{HER2}-targeted therapies are developed in human medicine and some drugs, such as lapatinib, afatinib, and trastuzumab, showed clinical benefit for patients with NSCLC [11]. Since cPLC share clinic pathologic features with NSCLC [10] and canine HER2 has 92\% amino acid homology with human \textit{HER2} [20], \textit{HER2}-targeted therapies, which showed anti-tumor effect against NSCLC, could exhibit clinical benefit for cPLC. In fact, one of the \textit{HER2}-targeted therapies, neratinib, have shown an anti-tumor effect on \textit{HER2}^{V659E} cPLC cell line which displayed constitutive activation of \textit{HER2} downstream signaling \textit{in vitro} [10]. Moreover, other types of \textit{HER2}-targeted therapies, such as trastuzumab and \textit{HER2}-targeting recombinant \textit{Listeria} vaccines, have shown potential anti-tumor effect against cPLC cell lines and dogs with osteosarcoma [10,12]. Because these therapies are independent on \textit{HER2} downstream signaling, they might be potential novel therapies for dogs with \textit{HER2}-positive cPLC. Above all, there are several therapeutic options targeting \textit{HER2} in cPLC, and
our preliminary data would lead to further preclinical and clinical evaluation
of these HER2 targeted therapies in cPLC.
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Figure Legends

Fig. 1. Human epidermal growth factor receptor 2 (HER2) mRNA expression in canine primary lung cancer (cPLC) (n=6) and normal lung tissues (n=4). Of 6 cPLC samples, 5 expressed higher HER2 mRNA than any normal samples ($p<0.05$).

Fig. 2. Representative staining pattern of human epidermal growth factor receptor 2 (HER2) in canine primary lung cancer (cPLC) (A) and normal lung tissue (C). Circumferential membrane staining is complete and intense in cPLC. (A) hematoxylin and eosin staining of cPLC and normal tissues. (B, D) Scale bar, 200 µm.

Fig. S1. Human epidermal growth factor receptor 2 (HER2) scoring of canine primary lung cancer (cPLC) based on the American Society of Clinical Oncology/College of American Pathologists guidelines. Representative images of the staining pattern of HER2 in cPLC. Scale bar, 100 µm.
Table 1. Characteristics of dogs and the corresponding human epidermal growth factor receptor 2 expression

| No. | Breed*<sup>a</sup> | Age (years) | Sex<sup>b</sup> | Location       | TNM classification | Classification         | IHC<sup>c</sup> score |
|-----|-----------------|-------------|----------------|-----------------|--------------------|------------------------|------------------------|
| 1   | CH              | 13.2        | MC             | Right cranial   | T1 N0 M0           | Adenocarcinoma         | 1+                     |
| 2   | CH              | 11.5        | FS             | Right middle    | T1 N0 M0           | Adenocarcinoma         | 1+                     |
| 3   | TP              | 11.3        | MC             | Left caudal     | T1 N1 M0           | Adenocarcinoma         | 1+                     |
| 4   | SAL             | 11.8        | M              | Left caudal     | T1 N1 M0           | Adenocarcinoma         | 1+                     |
| 5   | SHI             | 10.2        | FS             | Left caudal     | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 6   | CH              | 12.3        | MC             | Left caudal     | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 7   | MLT             | 13.6        | MC             | Left caudal     | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 8   | SHI             | 11.8        | MC             | Left cranial    | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 9   | MD              | 11.2        | FS             | Right middle    | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 10  | CH              | 11.2        | M              | Right cranial   | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 11  | CH              | 14.2        | F              | Right cranial   | T1 N0 M0           | Adenocarcinoma         | 2+                     |
| 12  | MD              | 15.1        | M              | Right middle    | T1 N0 M0           | Adenocarcinoma         | 3+                     |
| 13  | MD              | 12.3        | FS             | Left caudal     | T2 N0 M1           | Adenocarcinoma         | 3+                     |
| 14  | BG              | 13.0        | FS             | Right caudal    | T1 N0 M0           | Alveolar carcinoma     | 3+                     |
| 15  | EP              | 12.8        | FS             | Right caudal    | T1 N0 M0           | Bronchial cell carcinoma | 2+                 |
| 16  | MD              | 12.3        | FS             | Left caudal     | T1 N0 M0           | Squamous cell carcinoma | 1+                 |

<sup>a</sup> CH, Chihuahua; TP, Toy Poodle; SAL, Saluki; SHI, Shih Tzu; MLT, Maltese; MD, Miniature Dachshund; BG, Beagle; EP, English Pointer

<sup>b</sup> M, male; MC, male castrated; F, female; FS, female spayed

<sup>c</sup> IHC, immunohistochemistry
Table 2. Human epidermal growth factor receptor 2 scoring methods proposed by the American Society of Clinical Oncology/College of American Pathologists

| Score | Description |
|-------|-------------|
| 3+    | Circumferential membrane staining that is complete, intense, and in >10% of tumor cells |
| 2+    | Circumferential membrane staining that is incomplete and/or weak/moderate and in >10% of tumor cells or Complete and circumferential membrane staining that is intense and in <10% of tumor cells |
| 1+    | Incomplete membrane staining that is faint/barely perceptible and in >10% of tumor cells |
| 0     | No staining observed or Membrane staining that is incomplete and is faint/barely perceptible and in <10% of tumor cells |
Table 3. Human epidermal growth factor receptor 2 scores in canine primary lung cancer tissue samples

| Score | Negative | Positive |
|-------|---------|----------|
| 0     | 0 (0%)  | 2+ 0 (0%) |
| 1+    | 5 (31%) | 3+ 0 (0%) |

Number (%)
Fig. 1

![Box plot showing HER2/GAPDH levels in cPLC and Normal Lung tissues.](image-url)

- The y-axis represents HER2/GAPDH levels, ranging from 0 to 4.
- The x-axis categorizes samples into cPLC and Normal Lung.
- The box plot shows the distribution of HER2/GAPDH levels with median, quartiles, and outliers.
Fig. 2

| cPLC   | HER2  | Normal lung | HE   |
|--------|-------|-------------|------|
| A      |       |             |      |
| B      |       |             |      |
| C      |       |             |      |
| D      |       |             |      |
Fig. S1

HER2 score

0  1+  2+  3+

[Images of tissue sections with HER2 scores]