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Unveiling HER2 immunoexpression in canine hepatoid gland neoplasms: clinicopathological and morphological associations

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ABSTRACT

Canine hepatoid gland neoplasms (HGNs) are significant clinical concerns due to their high prevalence and diverse biological behaviour. Human epidermal growth factor receptor 2 (HER2), a tyrosine kinase receptor implicated in various aspects of tumorigenesis, has been extensively studied in human and animal neoplasms but remains unexplored in HGNs. This study aimed to assess HER2 immunoexpression in canine HGNs and its association with clinicopathological and morphological features. A total of 61 formalin-fixed paraffin-embedded samples, including normal hepatoid glands ($n = 10$), hepatoid gland adenomas (HGAs, $n = 20$), hepatoid gland epitheliomas (HGEs, $n = 16$), and hepatoid gland carcinomas (HGCs, $n = 15$), were analysed using immunohistochemistry. HER2 expression was scored based on percentage positivity and staining intensity. HER2-positive expression was detected in 50% of HGEs (score 2 +) and 73.3% of HGCs, with 36.4% of cases scoring 3 + . In contrast, all HGAs and normal hepatoid tissues were HER2-immunonegative. Statistical analysis revealed significant differences in HER2 expression among normal and neoplastic hepatoid glands ($p < 0.001$). Only in HGCs, HER2 expression was significantly associated with tissue invasion ($p = 0.007$), mitotic count ($p = 0.033$), and nuclear pleomorphism ($p = 0.007$). These findings suggest that HER2 may play a role in the progression of malignant HGNs, particularly HGCs. This preliminary study highlights the potential of HER2 as a diagnostic marker and emphasizes the need for further investigation into its prognostic value and role in HER2-targeted therapy for canine HGCs.

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1. Introduction

Among various tumour markers, human epidermal growth factor receptor 2 (HER2) has been widely investigated as a valuable biomarker for its prognostic and predictive indicators in cancer patients worldwide [1,2]. HER2, also known as c-erbB-2 or HER2/neu, belongs to a family of tyrosine kinase receptor that plays a crucial role in the pathogenesis of cancers. It controls cell growth, proliferation, and differentiation through multiple signal transduction pathways [3]. Oncogenic activation of HER2 typically arises from gene amplification, leading to overexpression of the protein on the cell membrane, which triggers signal transduction controlling the cell cycle [4]. HER2 overexpression has been investigated in several human cancers, including breast, gastric, colorectal, ovarian, and lung cancers [5–9]. The majority of HER2 research in human has focused on breast cancer, demonstrating that HER2 overexpression and gene amplification are associated with an aggressive tumour phenotype, poor clinical outcomes, higher recurrence rates, and worse disease-related survival [10,11]. Furthermore, its prognostic value is crucial for staging breast cancer patients who could benefit from HER2-targeted therapies like trastuzumab, which has been shown to enhance overall survival [11]. This highlights the importance of HER2 as both a prognostic marker and a therapeutic target.

In veterinary oncology, there is increasing interest in HER2 overexpression across various tumour types, including mammary gland tumours, lung carcinomas, transitional cell carcinomas, anal sac carcinomas, thyroid carcinomas, and gastrointestinal tract carcinomas [12–17]. However, reports on HER2 overexpression in dogs have been inconsistent, and its prognostic significance in mammary carcinomas remains unclear. Some studies have indicated that HER2 overexpression in canine malignant mammary tumours is associated with nuclear pleomorphism, mitotic count, and histological grade, suggesting a potential role in tumorigenesis [18,19]. In contrast, other studies have found no

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significant prognostic relevance of HER2 overexpression in mammary carcinoma [12,20]. Although clinical trials of HER2-targeted therapies, such as trastuzumab, have not been conducted in canine mammary carcinomas, previous research has demonstrated that trastuzumab can inhibit the growth of canine carcinoma cell lines [21]. This finding suggests significant potential for developing future therapeutic strategies for canine cancers.

Canine hepatoid gland neoplasms (HGNs) are common tumours that originate from modified sebaceous glands around the anus. They are clinically significant due to their high prevalence and variable biological behaviour. These neoplasms constitute 25% of all cutaneous epithelial tumours [22]. Their biological behaviour varies from benign to malignant. HGNs are classified into three main types: adenomas, epitheliomas, and adenocarcinomas. Benign hepatoid gland adenomas (HGAs) account for 60% of all tumours in the perianal region, and their progression is androgenic hormone-dependent [23]. Therefore, surgical removal along with castration is the treatment of choice [24]. Hepatoid gland epitheliomas (HGEs), low-grade malignancies constitute 30% of perianal tumours. Clinically, they exhibit local invasiveness and ulceration, hence increasing the risk of infection [25]. Effective treatments include wide surgical excision, laser ablation, or cryotherapy for invasive lesions [26]. Hepatoid gland carcinomas (HGCs) are the most aggressive type of HGNs, exhibiting invasive and metastatic characteristics. They represent 3–21% of perianal tumours [23]. Metastasis rates are approximately 15–22% of cases, with spread to lymph nodes, abdominal organs, and lungs, leading to severe systemic illness and paraneoplastic complications [24,27]. Recommended treatments include extensive excision followed by chemotherapy, with or without radiation [28]. However, non-resectable tumours with distant metastasis exhibit low radiation responsiveness, which has little impact on disease-free intervals or survival times [24,28]. These limitations highlight the urgent need to develop effective treatments.

To our knowledge, no studies have investigated HER2 expression in canine hepatoid gland tissues. This study aimed to (1) assess HER2 immunoexpression in canine HGNs, comparing expression among normal hepatoid glands, HGAs, HGEs, and HGCs, and (2) evaluate the association between HER2 expression and clinicopathological features, along with morphological aspects in canine HGNs. This preliminary study could contribute to improved prognostic markers and facilitate the development of HER2-targeted therapy for canine HGNs.

2. Materials and methods

2.1. Ethical approval

The research protocols were approved by the animal ethics committee of the Faculty of Veterinary Medicine, Chiang Mai University (Ref. No. R14/2563), under the guidelines for the Care and Use of Experimental Animals, National Research Council of Thailand.

2.2. Sample collection

Fifty-one formalin-fixed paraffin-embedded (FFPE) samples of canine HGNs, collected from January 2019 to December 2021, were retrieved from the archives of the Small Animal Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, and the Vet and Vitro Central Laboratory, Bangkok, Thailand. Individual dog data, including age, breed, sex, and neutering or spaying status, were recorded along with clinicopathological features, consisting of tumour location and size (maximum length), presence of skin ulceration and necrosis, tissue invasion, and lymphatic metastasis. Additionally, 10 normal hepatoid gland tissue samples were obtained post-mortem from dogs aged 6 to 12 years at the Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Chiang Mai University, to assess HER2 expression in normal tissues.

2.3. Histopathological examination

To confirm the primary diagnosis, all FFPE tissue samples were cut into 4 μm thickness, deparaffinized, rehydrated, and stained with haematoxylin and eosin. The histological type of each sample was categorized based on the World Health Organization Classification of Epithelial Tumors in Domestic Animals [29] by two board-certified veterinary pathologists from the College of Veterinary Specialties of Thailand. Additionally, peripheral tissue invasion, lymph node metastasis, nuclear pleomorphism, and mitotic count were evaluated as detailed in the following sections.

2.3.1. Assessment of peripheral tissue invasion and regional lymph node metastasis

Peripheral tissue invasion was defined as the growth of primary tumour tissue into the adjacent connective tissue and/or muscle, while regional lymph node metastasis referred to the spread of cancer cells from the primary tumour to nearby lymph nodes. These characteristics were assessed in the HGN samples and were histologically classified as either present or absent. The presence of at least one area of infiltrating neoplastic cells in the surrounding connective tissue or

cancer cells within the excised regional lymph nodes was considered indicative of invasion or metastasis, respectively.

2.3.2. Assessment of nuclear pleomorphism

Nuclear pleomorphism was assessed in the most pleomorphic regions of the tumour sample by examining 10 high-power fields (HPF) (400 × magnifications). The degree of nuclear pleomorphism was subjectively classified into three scores: mild, moderate, and marked. Mild pleomorphism was defined by small tumour nuclei with minimal size increase compared to normal hepatoid cells. These nuclei exhibited regular nuclear membrane, uniform nuclear chromatin, and less than 5% of tumour cells had nuclei twice the size of adjacent tumour cells. Moderate pleomorphism was characterized by intermediate variation in nuclear size and shape, with 5–10% of tumour cells exhibiting nuclei at least twice the size of adjacent tumour cells. Marked pleomorphism was identified by significant variation in nuclear size and shape, including vesicular nuclei, irregular nuclear membrane, and often prominent nucleoli. This category occasionally included very large and bizarre nuclear forms, with more than 10% of tumour cells displaying nuclei at least twice the size of adjacent tumour cells [18,30].

2.3.3. Assessment of mitotic count

The quantification of mitotic figures was performed following the method described by Meuten *et al* [31]. Briefly, mitotic figures were counted as the cumulative number of mitoses in 10 consecutive HPF at 400 × magnifications within areas of highest proliferative activity. Only tumour cells with clear mitotic features were included, defined by the following criteria: (1) absence of nuclear membrane, (2) clearly visible hairy projections of nuclear material (condensed chromosome), and (3) nuclear material in identifiable stages – clotted (prophase), in-plane (metaphase/anaphase), or separate clots (telophase). Areas with extensive necrosis, apoptotic or pyknotic cells, and ambiguous cells with hyperchromatic nuclei were excluded. Mitotic counts of each HGN cases were recorded as absolute numbers of mitotic figures and categorized into two groups: Group 1; a total of < 8 mitotic figures, and Group 2; ≥ 8 mitotic figures [30].

2.4. HER2 immunohistochemistry

Immunohistochemical staining was performed using a polymer-based detection system (MedaView^{TM} One-step Polymer-horseradish peroxidase (HRP) labelled Detection System; Medaysis, Livermore, CA, USA) following the manufacturer's instructions. FFPE tissue blocks were cut into 4-µm-thick sections, mounted on coated slides, then deparaffinized and rehydrated in xylene and graded ethanol, respectively. Antigen retrieval was performed by immersing the sections in Epitope Retrieval Solution (pH 9.0; Novocastra, Leica, Newcastle Upon Tyne, UK) and boiling them in a 750W microwave for 10 minutes. To block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in methanol (MedaView^{TM}, Medaysis, Livermore, CA, USA) for 10 minutes at room temperature, followed by washing with phosphate-buffered saline (PBS, pH 7.2). Nonspecific reactions were blocked by treating with Novolink 0.4% Casein in PBS at 37°C for 30 minutes. The tissue sections were then incubated with the primary antibody (1:100 in dilution), rabbit polyclonal anti-human c-erbB-2 oncoprotein (HER2/neu) antibody (A0485, DAKO), at 4°C overnight in a humidified chamber. Thereafter, the sections were rinsed with PBS and incubated with Polymer HRP anti-mouse and anti-rabbit antibodies (MedaView^{TM}, Medaysis, Livermore, CA, USA) for 20 minutes at room temperature. Antigen-antibody reactions were visualized by applying 3,3′-diaminobenzidine tetrahydrochloride solution (MedaView^{TM}, Medaysis, Livermore, CA, USA) for 3 minutes. Slides were counterstained with Mayer's haematoxylin, dehydrated in graded ethanol, and mounted using a permanent mounting medium. Canine urothelial carcinoma tissue was used as a positive and negative control [32]. For negative controls, the primary antibody was replaced with PBS.

2.5. Immunohistochemical evaluation

HER2 immunoreactivity was evaluated following the 2023 guidelines for human breast cancer proposed by the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) [33]. The staining pattern of HER2 in normal hepatoid gland tissues and HGNs were scored as follows: 0 = no staining or incomplete, faint/barely perceptible membrane staining detected in <10% of tumour cells, 1 + = incomplete and faint membrane staining observed in >10% of tumour cells, 2 + = weak to moderate complete membrane staining detected in > 10% of tumour cells, and 3 + = intense and complete circumferential membrane staining observed in >10% of tumour cells. Scores of 0 and 1 + were classified as negative, while scores of 2 + and 3 + were considered positive for HER2 expression.

2.6. Statistical analysis

Descriptive statistics were used to describe the characteristics of each canine HGN type, including demographic data (age, sex, and breed), tumour size and location, skin ulceration, necrosis, tissue invasion, lymphatic metastasis, nuclear pleomorphism, and mitotic count. The differences in HER2 expression

scores among normal hepatoid glands and HGNs were analysed using the Kruskal – Wallis test and *post hoc* multiple comparisons using Dunn's test with Bonferroni adjustment. Fisher's exact test was used to analyse the association of clinicopathological and morphological features with HER2 expression in HGNs. Variables associated with HER2 expression ($p < 0.05$) were further analysed using a conventional or exact logistic regression analysis when appropriate. All statistical analyses were performed using the STATA statistical software release 16.1 (STATA Corp., College Station, TX, USA). A $p < 0.05$ was considered statistically significant.

3. Results

Tissue samples of canine HGNs were obtained from 51 dogs of different breeds and sexes. The breeds included Mixed-breed ($n = 14$), Shih Tzu ($n = 9$), Poodle ($n = 6$), Siberian husky ($n = 6$), Beagle ($n = 3$), Golden Retriever ($n = 3$), Labrador Retriever ($n = 2$), Miniature pincher ($n = 2$), Chi Hua Hua ($n = 2$), Jack Russel ($n = 1$), Dachshund ($n = 1$), Thairidge back ($n = 1$), and Bang Kaew ($n = 1$). The mean age was 9.67 ± 2.67 years (range: 4–15 years). Most of the HGNs were located in the perianal region ($n = 41$, 80.4%). The mean tumour diameter was 3.90 ± 2.04 cm (range: 1.5–10 cm). Additional clinicopathological and morphological data for different canine HGN types are summarized in Table 1. For comparison, normal hepatoid gland tissues were collected post-mortem from 10 dogs of different breeds, including Mixed-breed ($n = 4$), Poodle ($n = 3$), Shih Tzu ($n = 1$), Beagle ($n = 1$), and Golden Retriever ($n = 1$). These dogs included intact males ($n = 2$), castrated males ($n = 2$), intact females ($n = 2$), and spayed females ($n = 4$), with a mean age of 8.2 ± 2.2 years (range: 6–12 years).

3.1. HER2 expression in normal hepatoid gland tissues and HGNs

Histopathological observations of normal and neoplastic hepatoid glands are shown in Figures 1A, C, E, and G. The tumours were categorized into 20 HGAs, 16 HGEs, and 15 HGCs. HER2 immunoexpression scores for each sample are summarized in Table 2. Significant differences in HER2 expression were observed among normal and neoplastic hepatoid glands ($p < 0.001$). All normal hepatoid glands were

Table 1. Clinicopathological and morphological features of canine hepatoid gland adenomas (HGAs), hepatoid gland epitheliomas (HGEs), and hepatoid gland carcinomas (HGCs).

		Histological Diagnosis		
Clinicopathological and morphological features	Number of samples	HGAs ($n = 20$) (%)	HGEs ($n = 16$) (%)	HGCs ($n = 15$) (%)
Age (years)				
<10	26	14 (53.9)	7 (26.9)	5 (19.2)
≥10	25	6 (24.0)	9 (36.0)	10 (40.0)
Sex				
Intact males	32	17 (53.1)	9 (28.1)	6 (18.8)
Castrated males	11	0 (0.0)	5 (45.5)	6 (54.5)
Intact females	2	1 (50.0)	1 (50.0)	0 (0.0)
Spayed females	6	2 (33.3)	1 (16.7)	3 (50.0)
Tumor size (cm)				
<3	17	12 (70.6)	5 (29.4)	0 (0.0)
3–5	24	8 (33.3)	10 (41.7)	6 (25.0)
>5	10	0 (0.0)	1 (10.0)	9 (90.0)
Location				
Perianal	41	17 (41.4)	15 (36.6)	9 (22.0)
Tail based	7	2 (28.6)	1 (14.3)	4 (57.1)
Prepuce	2	1 (50.0)	0 (0.0)	1 (50.0)
Perivulva	1	0 (0.0)	0 (0.0)	1 (100.0)
Ulceration				
Present	24	5 (20.8)	9 (37.5)	10 (41.7)
Absent	27	15 (55.6)	7 (25.9)	5 (18.5)
Necrosis				
Present	21	2 (9.5)	7 (33.3)	12 (57.1)
Absent	30	18 (60.0)	9 (30.0)	3 (10.0)
Tissue invasion				
Present	10	0 (0.0)	0 (0.0)	10 (100.0)
Absent	41	20 (48.8)	16 (39.0)	5 (12.2)
Lymphatic metastasis				
Present	7	0 (0.0)	0 (0.0)	7 (100.0)
Absent	37	17 (46.0)	13 (35.1)	7 (18.9)
N/A	7	3 (42.9)	3 (42.9)	1 (14.3)
Nuclear pleomorphism				
Mild	35	20 (57.1)	12 (34.3)	3 (8.6)
Moderate	9	0 (0.0)	4 (4.44)	5 (55.6)
Marked	7	0 (0.0)	0 (0.0)	7 (100.0)
Mitotic count				
<8	38	20 (52.7)	14 (36.8)	4 (10.5)
≥8	13	0 (0.0)	2 (15.4)	11 (84.6)

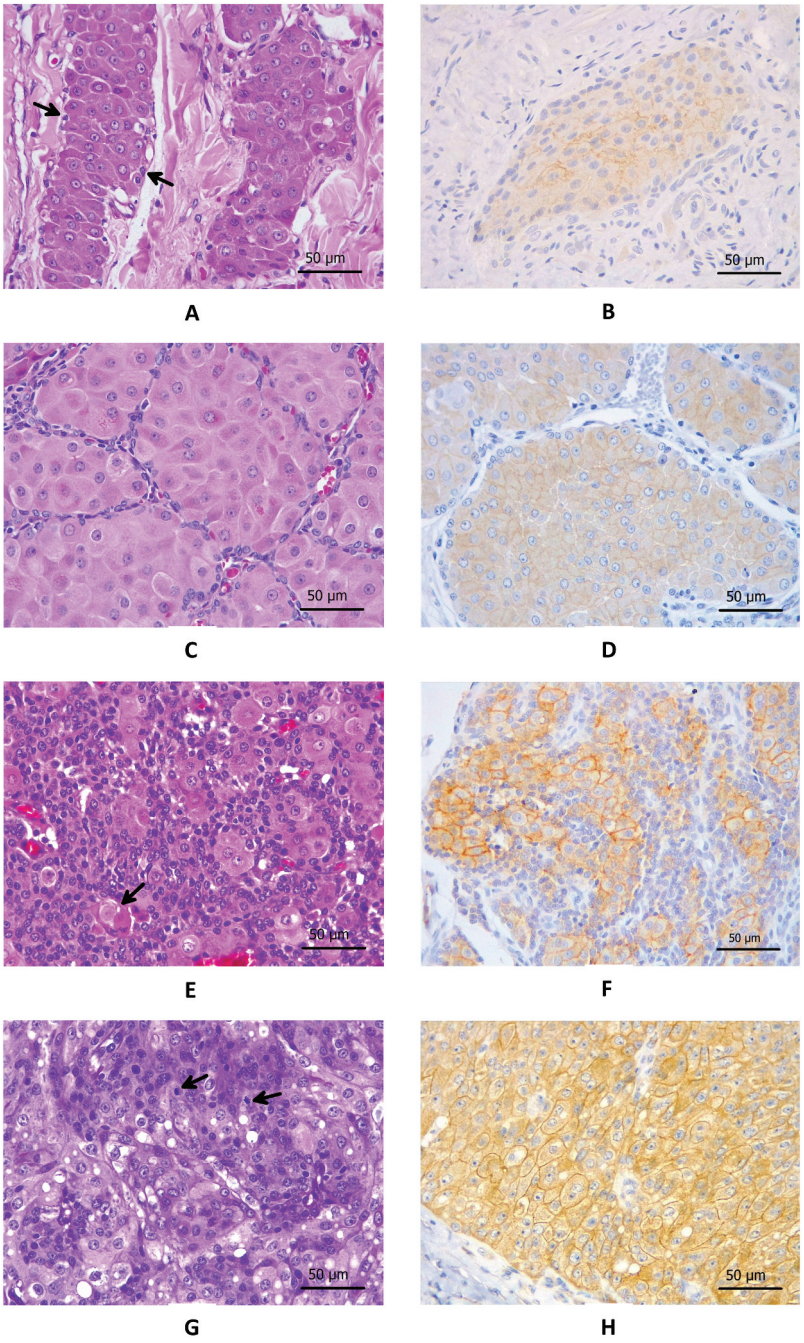

Figure 1. Histopathological and immunohistochemical staining patterns of human epidermal growth factor receptor 2 (HER2) in normal canine hepatoid gland and hepatoid gland neoplasms. (A) Normal hepatoid glands; the glands composed of uniform polyhedral cells with abundant eosinophilic cytoplasm (mature hepatoid cells), arranged in trabeculae and rimmed by a single peripheral layer of basaloid reserve cells with hyperchromatic nuclei and scant eosinophilic cytoplasm (arrows). Each glandular unit is separated by fibrovascular stroma. (B) Mature hepatoid cells showed no immunoreactivity, with only a few of cells displaying faint incomplete membranous immunoreactivity (score 0). (C) Hepatoid gland adenoma; well-differentiated mature hepatoid cells with centrally located nuclei and abundant finely granular eosinophilic cytoplasm, arranged in islands and anastomosing trabeculae which separated by fine fibrovascular stroma. (D) Weak and incomplete membranous immunoreactivity was observed in well-differentiated mature hepatoid cells (score 1 +). (E) Hepatoid gland epithelioma; marked proliferation of basaloid reserve cells with fewer well-differentiated mature hepatoid cells showing abundant eosinophilic cytoplasm. Occasional squamous metaplasia was observed (arrow). (F) Neoplastic well-differentiated hepatoid cells presented weak to moderate complete membranous immunoreactivity (score 2 +), whereas basaloid reserve cells were immunonegative. (G) Hepatoid gland carcinoma; irregular arrangement of malignant hepatoid cells, with marked cellular atypia and nuclear pleomorphism. Malignant hepatoid cells showed small to large, round to oval nuclei with prominent nucleoli and moderate to abundant eosinophilic and vacuolated cytoplasm. Mitotic figures were frequently observed (arrows). (H) Intense and complete circumferential membranous immunoreactivity was detected in malignant hepatoid cells (score 3 +). (A, C, E, G); Haematoxylin and Eosin staining. (B, D, F, H); HER2 immunohistochemistry using DAB. Magnifications, 400 × .

Table 2. HER2 expression scores in normal and neoplastic hepatoid glands.

		Negative (%)		Positive (%)		
Histology diagnosis	Number of samples	0	1 +	2 +	3 +	*p*-value
Normal	10	7 (70.0)	3 (30.0)	0 (0.0)	0 (0.0)	<0.001
HGA	20	7 (35.0)	13 (65.0)	0 (0.0)	0 (0.0)	
HGE	16	2 (12.5)	6 (37.5)	8 (50.0)	0 (0.0)	
HGC	15	1 (6.6)	3 (20.0)	7 (46.7)	4 (26.7)	

HER2-negative, with 7 out of 10 (70%) samples scoring 0 (Figure 1B). In HGAs, all samples were immunonegative, with 13 of 20 (65%) scoring 1 + , indicating faint and incomplete membranous immunolabeling in more than 10% of well-differentiated mature hepatoid cells (Figure 1D). Among HGEs, 8 of 16 (50%) cases exhibited HER2-positive with a score of 2 + , showing weak to moderate complete circumferential membrane staining in more than 10% of neoplastic well-differentiated mature hepatoid cells (Figure 1F). For HGCs, HER2-positive was observed in 11 of 15 (73.3%) cases, with 4 of these 11 (36.4%) scoring 3 + , indicating strong and complete circumferential membrane staining in more than 10% of malignant hepatoid cells (Figure 1H). Notably, 3 of the 4 cases (75%) with a score of 3 + exhibited anaplastic features with intense positive immunolabeling.

3.2. Association of clinicopathological and morphological features with HER2 expression in HGNs

The statistical evaluation of HER2 expression based on demographic data (age and sex), tumour size and location, skin ulceration, necrosis, tissue invasion, lymphatic metastasis, mitotic count, and nuclear pleomorphism revealed no significant associations in either HGAs or HGEs (Tables 3 and 4). Conversely, HER2 expression in HGCs was significantly associated with the presence of tissue invasion ($p = 0.004$), mitotic count ($p = 0.033$) and nuclear pleomorphism ($p = 0.007$) (Table 5). Our study revealed that HGCs with local invasion were 23.97 times more likely to be HER2-positive than those without local invasion (95% CI: 2.25- Infinity). HGCs exhibiting moderate and marked nuclear pleomorphism were 6.89 and 23.72

Table 3. Associations of clinicopathological and morphological features with HER2 expression in hepatoid gland adenomas ($n = 20$).

		HER2 expression		
Clinicopathological and morphological features	Number of samples	Negative (%)	Positive (%)	*p*-value
Age (years)				N/A
<10	14	14 (100.0)	0 (0.0)	
≥10	6	6 (100.0)	0 (0.0)	
Sex				N/A
Intact males	17	17 (100.0)	0 (0.0)	
Castrated males	0	0 (0.0)	0 (0.0)	
Intact females	1	1 (100.0)	0 (0.0)	
Spayed females	2	2 (100.0)	0 (0.0)	
Tumor size (cm)				N/A
<3	12	12 (100.0)	0 (0.0)	
3–5	8	8 (100.0)	0 (0.0)	
>5	0	0 (0.0)	0 (0.0)	
Location				N/A
Perianal	17	17 (100.0)	0 (0.0)	
Tail based	2	2 (100.0)	0 (0.0)	
Prepuce	1	1 (100.0)	0 (0.0)	
Perivulva	0	0 (0.0)	0 (0.0)	
Ulceration				N/A
Present	5	5 (100.0)	0 (0.0)	
Absent	15	15 (100.0)	0 (0.0)	
Necrosis				N/A
Present	2	2 (100.0)	0 (0.0)	
Absent	18	18 (100.0)	0 (0.0)	
Tissue invasion				N/A
Present	0	0 (0.0)	0 (0.0)	
Absent	20	20 (100.0)	0 (0.0)	
Lymphatic metastasis				N/A
Present	0	0 (0.0)	0 (0.0)	
Absent	17	17 (100.0)	0 (0.0)	
N/A	3	3 (100.0)	0 (0.0)	
Nuclear pleomorphism				N/A
Mild	20	20 (100.0)	0 (0.0)	
Moderate	0	0 (0.0)	0 (0.0)	
Marked	0	0 (0.0)	0 (0.0)	
Mitotic count				N/A
<8	20	20 (100.0)	0 (0.0)	
≥8	0	0 (0.0)	0 (0.0)	

N/A, Not applicable.

Table 4. Associations of clinicopathological and morphological features with HER2 expression in hepatoid gland epitheliomas ($n = 16$).

Clinicopathological and morphological features	Number of samples	HER2 expression		*p*-value
		Negative (%)	Positive (%)	
Age (years)				≈ 1.000
<10	7	4 (57.1)	3 (42.9)	
≥10	9	4 (44.4)	5 (55.6)	
Sex				≈ 1.000
Intact males	9	4 (44.4)	5 (55.6)	
Castrated males	5	3 (60.0)	2 (40.0)	
Intact females	1	0 (0.0)	1 (100.0)	
Spayed females	1	1 (100.0)	0 (0.0)	
Tumor size (cm)				0.282
<3	5	4 (80.0)	1 (20.0)	
3–5	10	4 (40.0)	6 (60.0)	
>5	1	0 (0.0)	1 (100.0)	
Location				≈ 1.000
Perianal	15	7 (46.7)	8 (53.3)	
Tail based	1	1 (100.0)	0 (0.0)	
Prepuce	0	0 (0.0)	0 (0.0)	
Perivulva	0	0 (0.0)	0 (0.0)	
Ulceration				≈ 1.000
Present	9	4 (44.4)	5 (55.6)	
Absent	7	4 (57.1)	3 (42.9)	
Necrosis				0.315
Present	7	2 (28.6)	5 (71.4)	
Absent	9	6 (66.7)	3 (33.3)	
Tissue invasion				N/A
Present	0	0 (0.0)	0 (0.0)	
Absent	16	8 (50.0)	8 (50.0)	
Lymphatic metastasis				≈ 1.000
Present	0	0 (0.0)	0 (0.0)	
Absent	13	6 (46.2)	7 (53.8)	
N/A	3	2 (66.7)	1 (33.3)	
Nuclear pleomorphism				0.569
Mild	12	7 (58.3)	5 (41.7)	
Moderate	4	1 (25.0)	3 (75.0)	
Marked	0	0 (0.0)	0 (0.0)	
Mitotic count				0.467
<8	14	8 (57.1)	6 (42.9)	
≥8	2	0 (0.0)	2 (100.0)	

N/A, Not applicable.

times more likely to be HER2-positive than those with mild nuclear pleomorphism (95% CI: 0.53-Infinity and 95% CI: 1.75-Infinity, respectively). Furthermore, HGCs with a mitotic count ≥8 were 30 times more likely to be HER2-positive than those with a mitotic count <8 (95%CI: 1.41–638.15) (Table 6).

4. Discussion

HER2, the most commonly targeted oncogene in human cancers, serves as a useful prognostic and predictive biomarker. The relevance of HER2 in veterinary oncology is gaining attention, and its expression may provide insights into tumour behaviour and potential therapeutic targets. This study is the first to document the immunoexpression of HER2 in normal and neoplastic canine hepatoid gland tissues, along with their clinicopathological and morphological features. Our findings revealed a significant difference in HER2 expression among normal and neoplastic hepatoid glands ($p < 0.001$). Specifically, HER2-positive expression was observed in 73.3% of HGCs and 50% of HGEs, while all HGAs and normal hepatoid tissues were immunonegative. These results align with previous studies reporting positive HER2 expression in canine transitional cell carcinomas of the urinary bladder and malignant mammary tumours in both canine and feline, whereas non-neoplastic urothelium, benign mammary tumours, and normal mammary glands were negative [14,18,34]. Tsuboi *et al.* [32] reported that the percentage of HER2-positive cases and HER2 scores were higher in canine urothelial carcinoma than in polypoid cystitis, with normal bladder tissues exhibiting negative expression. Based on these findings, we speculate that HER2 overexpression in HGCs may play a role in the malignant transformation of hepatoid gland tissues. Our results are further supported by several studies linking HER2 overexpression or gene amplification with aggressive forms of human cancers, particularly breast, gastric, and ovarian cancers [35–37]. Overexpression of HER2 leads to increased homodimerization and heterodimerization, initiating a strong pro-tumorigenic signalling cascade [38]. These interactions activate downstream signalling pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt pathway, which is crucial for cell survival and growth, and the mitogen-activated protein kinases (Ras/Raf/MEK/MAPK) pathway, which primarily regulates cell proliferation and differentiation [39]. The overactivation of these HER2 signalling pathways

Table 5. Associations of clinicopathological and morphological features with HER2 expression in hepatoid gland carcinomas (n = 15).

Clinicopathological and morphological features	Number of samples	HER2 expression		p-value
		Negative (%)	Positive (%)	
Age (years)				≈ 1.000
<10	5	1 (20.0)	4 (80.0)	
≥10	10	3 (30.0)	7 (70.0)	
Sex				0.097
Intact males	6	2 (33.3)	4 (66.7)	
Castrated males	6	0 (0.0)	6 (100.0)	
Intact females	0	0 (0.0)	0 (0.0)	
Spayed females	3	2 (66.7)	1 (33.3)	
Tumor size (cm)				0.235
<3	0	0 (0.0)	0 (0.0)	
3–5	6	3 (50.0)	3 (50.0)	
>5	9	1 (11.1)	8 (88.9)	
Location				0.596
Perianal	9	2 (22.2)	7 (77.8)	
Tail based	4	1 (25.0)	3 (75.0)	
Prepuce	1	0 (0.0)	1 (100.0)	
Perivulva	1	1 (100.0)	0 (0.0)	
Ulceration				≈ 1.000
Present	10	3 (30.0)	7 (70.0)	
Absent	5	1 (20.0)	4 (80.0)	
Necrosis				≈ 1.000
Present	12	3 (25.0)	9 (75.0)	
Absent	3	1 (33.3)	2 (66.7)	
Tissue invasion				**0.004**
Present	10	0 (0.0)	10 (100.0)	
Absent	5	4 (80.0)	1 (20.0)	
Lymphatic metastasis				0.103
Present	7	0 (0.0)	7 (100.0)	
Absent	7	4 (57.1)	3 (42.9)	
N/A	1	0 (0.0)	1 (100.0)	
Nuclear pleomorphism				**0.007**
Mild	3	3 (100.0)	0 (0.0)	
Moderate	5	1 (20.0)	4 (80.0)	
Marked	7	0 (0.0)	0 (0.0)	
Mitotic count				**0.033**
<8	4	3 (75.0)	1 (25.0)	
≥8	11	1 (9.1)	10 (90.9)	

N/A, Not applicable.

Table 6. The exact logistic regression analysis of the association between clinicopathological and morphological features of hepatoid gland carcinomas and HER2- positive expression.

Clinicopathological and morphological features	HER2-positive expression (%)	Median OR	95% CI	p-value
Tissue invasion				
Absent	1/5 (20.0)	Reference		
Present	10/10 (100.0)	23.97	2.25-Inf.	**0.007**
Nuclear pleomorphism				
Mild	0/3 (0.0)	Reference		
Moderate	4/5 (80.0)	6.89	0.53-Inf.	0.143
Marked	7/7 (100.0)	23.72	1.75-Inf.	**0.017**
Mitotic count				
<8	1/4 (25.0)	Reference		
≥8	10/11 (90.9)	30.00	1.41–638.15	**0.029**

OR, Odds ratios; CI, Confidence interval.

drives oncogenic transformation, characterized by enhanced cell proliferation, resistance to apoptosis, angiogenesis, and metastasis [3,40].

Notably, 3 of 15 HGC cases exhibited anaplastic features characterized by lack of cellular differentiation, all of which demonstrated intense circumferential membrane staining with a score of 3 + . This finding may be related to the prominent Golgi apparatus in anaplastic cells. The Golgi apparatus plays a critical role in cellular functions and homoeostasis, including protein glycosylation, membrane trafficking, and secretion [41]. In anaplastic tumours, dysfunction in the Golgi apparatus is often observed, leading to aberrant glycosylation, secretion, and altered protein trafficking [42,43]. These alterations may result in enhanced signalling pathways that promote HER2 overexpression and its downstream effects, contributing to tumorigenic processes [44,45]. Interestingly, the intense HER2 immunolabelling observed in anaplastic HGCs aligns with our previous study, which noted the highest immunohistochemical scores of Cyclooxygenase-2 (COX-2) expression in anaplastic HGCs [46]. This suggests that both HER2 and COX-2 may play critical

roles in the tumorigenesis of anaplastic tumours, a relationship that warrants further investigation. In support of this hypothesis, Millanta *et al.* [47] demonstrated a significant correlation between increased COX-2 expression and HER2 overexpression (p = 0.013) as well as tumour dedifferentiation (p = 0.03) in canine invasive mammary carcinomas. These results suggest that COX-2 may contribute to mediating HER2-induced mammary tumours.

As 8 out of 16 (50%) HGE cases were detected as HER2-positive with a score of 2 + , this suggests a possible role of HER2 in the progression of low-grade malignant tumours. We speculate that these findings may be attributable to the association between HER2 and other tumour factors, such as vascular endothelial growth factor (VEGF), which are known to promote tumour progression. This hypothesis is supported by several studies in both humans and animals, demonstrating that HER2-positive tumours, such as mammary gland and gastric cancers, often exhibit elevated VEGF levels, which enhances tumour vascularization and growth [48–50]. Furthermore, a previous study on VEGF levels in canine HGAs and HGEs before and after tamoxifen treatment found significantly higher VEGF levels in HGEs at diagnosis, with levels decreasing during remission within one month of treatment. However, VEGF levels markedly increased during tumour recurrence within six months post-treatment. These findings suggest that VEGF may serve as a prognostic marker for tumour progression and recurrence [26]. Therefore, we hypothesize that HER2 and VEGF co-expression may contribute to the progression of HGNs, a possible association that requires further study.

In this study, we demonstrate that there was no association between clinicopathological and morphological variables and HER2 expression in HGAs and HGEs. However, HER2-positive immunoexpression in HGCs was significantly associated with tissue invasion (p = 0.004), nuclear pleomorphism (p = 0.007) and mitotic count (p = 0.033). Our findings align with previous studies highlighting a correlation between HER2 overexpression and aggressive tumour behaviours, including higher histological grade, marked nuclear pleomorphism, increased mitotic activity, and poor survival outcomes [18,19,51,52]. Furthermore, the pathological features of increased malignancy, such as tumour invasion, were associated with HER2 expression, demonstrated by significant overexpression of HER2 mRNA and Epidermal growth factor receptor (EGFR) in invasive matrix-producing mammary gland carcinoma compared to in situ carcinoma [53]. In our study, the exact logistic regression analysis revealed that HGCs with local invasion were 23.97 times more likely to be HER2-positive than those without local invasion (95%CI: 2.25-Inf). This result supported previous studies suggesting that HER2 facilitates tumour invasiveness by activating signalling pathways and promoting extracellular matrix degradation, primarily through the upregulation of matrix metalloproteinases (MMPs) [54,55]. Additionally, a study by Luo *et al.* revealed that HER2 can down-regulate the expression of E-cadherin and up-regulate the expression of Twist, promoting the invasion and migration of human gastric cancer cells [56]. According to nuclear pleomorphism and mitotic count, the higher values of these two morphological aspects reflect abnormal cell growth, rapid proliferation, and poor differentiation, which are considered indicators of aggressive malignant tumours and negative prognostic factors [18,57]. In this study, HGCs exhibiting moderate and marked nuclear pleomorphism were 6.89 (95% CI: 0.53-Infinity) and 23.72 times (95% CI: 1.75-Infinity) more likely to be HER2-positive than those with mild nuclear pleomorphism. Additionally, HGCs with a mitotic count ≥8 were 30 times (95%CI: 1.41–638.15) more likely to be HER2-positive than those with a mitotic count <8. These results suggest that HER2 expression could be involved in the regulation of cell cycle progression in canine HGCs. A previous study supports our findings, indicating that HER2 positivity is associated with higher-grade features in canine mammary carcinomas, such as marked nuclear pleomorphism and elevated mitotic counts [18]. Similarly, in a human study, the copy number of the c-erbB-2 gene was strongly correlated with histological grade, number of mitotic figures, and degree of nuclear atypia, highlighting the role of HER2 in tumorigenesis in breast carcinomas [58]. Furthermore, previous studies reported a correlation between HER2 positivity, higher grade of nuclear atypia, and increased mitotic activity in gastric adenocarcinoma, linking these morphological abnormalities to worse prognosis and a greater potential for metastasis [59,60].

According to our findings, HER2-positive expression was predominantly detected in malignant HGNs. In HGCs, HER2 expression was significantly associated with tissue invasion, nuclear pleomorphism and mitotic count. Additionally, the overexpression of HER2 was observed in all HGC cases with lymphatic metastasis. These results are consistent with findings in several human and animal cancers [18,19,58,60]. Thus, it seems plausible that HER2 may play a crucial role in tumour progression, and HER2-targeted therapy could potentially be useful as an additional treatment for canine HGCs. However, the exact role of HER2 in canine hepatoid gland tumorigenesis remains unclear and requires further investigation with a larger sample size. Regarding the prognostic value, HER2 gene amplification and protein overexpression in human breast cancer are well-

established predictors of shorter disease-free intervals and overall survival times [61,62], indicating that HER2 is an independent adverse prognostic factor. In the veterinary literature, the prognostic implications of HER2 are varied. Some studies have shown that HER2 overexpression correlates significantly with shorter survival times and negative clinicopathological parameters in invasive mammary carcinomas [51,63]. Conversely, other studies have reported no significant association between HER2 expression and overall survival or a worse prognosis [12,64]. These controversial findings highlight the necessity for further studies to investigate the prognostic value of HER2 expression in relation to disease-free intervals and overall survival outcomes in canine HGCs.

While our findings provide valuable insights into HER2 overexpression in canine HGCs, there are certain factors to consider. Due to the retrospective nature of the study, not all information was available for each sample. In particular, lymphatic assessment was incomplete for some cases, and data on previous medications, such as NSAIDs, especially COX-2 inhibitors, were limited. As some previous studies have reported that COX-2 inhibitors may reduce HER2 protein levels, which could influence HER2 expression [65,66]. However, the specific impact of COX-2 inhibitors on HER2 expression in the tissue samples analysed remains unclear. Additionally, the lack of follow-up data limits our ability to evaluate the prognostic implications of HER2 in this tumour. Therefore, further studies should investigate HER2 expression as a prognostic indicator and explore the therapeutic potential of HER2-targeted treatments in canine HGCs. These new insights will be important in cancer treatment and prognosis.

5. Conclusion

Our study demonstrated that HER2-positive immunoexpression was predominantly detected in canine HGCs. This is the first report to show that HER2 overexpression is associated with certain clinicopathological and morphological features indicative of increased malignancy in HGCs. These findings suggest that HER2 may play a role in tumorigenesis and could potentially influence treatment decisions. Our preliminary study highlights the need for further investigation into the prognostic value of HER2 expression and the therapeutic potential of HER2-targeted treatments in canine HGCs.

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Disclosure statement

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