Calprotectin and β-Catenin Expression in Canine Hepatoid Gland Tumors and Correlation with Macrophage Infiltration

Claudia Rifici †, Giada Giambrone †, Stefania Di Giorgio, Ettore Napoli ©, Gabriele Marino ©, Giuseppe Mazzullo © and Alessandra Sfacteria *

Department of Veterinary Sciences, University of Messina, Via G. Palatucci, 98168 Messina, Italy; claudia.rifici@unime.it (C.R.); giada.giambrone@studenti.unime.it (G.G.); stefania.digiorgio@unime.it (S.D.G.); ettore.napoli@unime.it (E.N.); gabriele.marino@unime.it (G.M.); giuseppe.mazzullo@unime.it (G.M.)* Correspondence: alessandra.sfacteria@unime.it † These authors contributed equally to this work.

Abstract: β-catenin is deregulated in cancer malignancies and drives the epithelial-to-mesenchymal transition (EMT). Calprotectin plays antioxidant activities, modulates inflammation and immune responses, and influences cell migration and invasion. Calprotectin can contribute to the progression of various types of cancer. Macrophages expressing calprotectin (MAC387) have been related to M1 polarization and promote EMT. In this study, β-catenin and calprotectin expression in canine hepatoid gland tumors and its relationship with MAC387-positive macrophages is reported. B-catenin was membranous and strong in hyperplasia and adenomas, moderate or weak in well-differentiated carcinomas, and absent in less-well-differentiated carcinomas. In cells with squamous differentiation, β-catenin was weak or absent. In benign and malignant lesions, MAC/387 positivity was found in both macrophages and clusters of cells with squamous differentiation arranged in whorls centered on ductal-like spaces. These clusters were more voluminous in carcinomas, sometimes with a center of lamellar keratin (horny pearls) and were surrounded by neoplastic hepatoid cells variably positive to calprotectin. The number of calprotectin-positive macrophages progressively increased in the stroma of carcinomas. These findings suggest that hepatoid glands are a useful model for studying the different roles of β-catenin and calprotectin in the tumor milieu and their involvement in tumor differentiation and EMT.

Keywords: hepatoid gland; calprotectin; β-catenin; macrophages; immunohistochemistry; dog

1. Introduction

Hepatoid or circumanal glands are modified sebaceous glands located primarily in the perianal skin. The lobules of these glands consist of mature hepatoid cells and peripheral basal reserve cells. Hepatoid gland tumors, classified as adenoma, epithelioma, and carcinoma, are the most common type of skin neoplasia in dogs and have a marked sex predilection in male dogs [1]. Aged non-castrated male dogs have a higher risk of hepatoid cell adenomas, implying androgen dependency, whereas perianal gland carcinomas occur in castrated or intact males, implying no hormonal dependency [2–4]. Adenomas are encapsulated masses composed of polyhedral cells, resembling hepatocytes, arranged as cords, islands, and trabeculae. Hepatoid gland epitheliomas have low-grade malignancy and are characterized by a proliferation of reserve cells, with fewer hepatoid cells. Carcinomas of the perianal gland are composed of trabeculae and cords of hepatoid cells or a mixture of reserve cells and hepatoid cells. Well-differentiated hepatoid gland carcinomas have a histological architecture and morphology like those of adenomas, except for the infiltrative growth at the tumor margins. Small foci of well-differentiated carcinoma may occur within adenomas, which may represent carcinoma in situ. Hepatoid gland carcinoma rarely metastasizes; therefore, these tumors may provide insight into the understanding of the mechanism of cancer pathogenesis [5,6].
β-catenin, a 92 K-Da glycoprotein responsible for cell-to-cell and extracellular matrix adhesion, binds directly to the intracytoplasmic domain of E-cadherin and to the network of cytoskeleton actinic microfilaments. E-cadherin–catenin complexes regulate the functional integrity of the epithelium by mediating specific intercellular adhesion. β-catenin plays a critical role in the Wnt signaling pathway, which is involved in various cellular processes such as cell growth, differentiation, embryonic development, tissue homeostasis, and stem cell maintenance [7]. A high frequency of mutations in members of the cellular adhesion complexes in some human tumors suggests that these molecules may play a role in neoplastic development. β-catenin mutations inhibit the degradation of β-catenin proteins that accumulate in the cytoplasm and enter the nucleus where they bind to the Tcf/Lef (T-cell factor/lymphoid enhancer factor) family of transcription factors and activate the transcription of some genes involved in proliferation, such as those encoding for c-myc3 and cyclin D1 [8–10].

Calprotectin is a protein that plays a crucial role in various cellular processes. In tumors, calprotectin exhibits unique functions and is involved in various aspects of tumorigenesis and disease progression. Calprotectin, also known as S100A8/S100A9, belongs to the S100 protein family, known for its role in cellular processes, such as signal transduction, inflammation, and immune response. Calprotectin, which is composed of two subunits, S100A8 (MRP8) and S100A9 (MRP14), encoded by separate genes, is a key protein in inflammation and is used as a reliable and useful marker. Granulocytes, monocytes, macrophages, and ileal tissue eosinophils all express calprotectin. Peripheral blood monocytes carry the antigen extra- and intracellularly, neutrophils only carry it intracellularly [11]. The S100A8/A9 complex has antibacterial, antifungal, immunomodulating and antiproliferative effects. Recently infiltrating monocytes/macrophages express MRP14 [12]. The antibody MAC387 specifically recognizes MRP14 and, to a lesser extent, the MRP8/MRP14 heterocomplex [13]. The ability of MAC387 to recognize MRP14 allows it to target these cells during early acute inflammation. Moreover, calprotectin acts as an antioxidant and plays a role in tumor cell survival and stress-responsive mechanisms. It can protect cells from oxidative stress and apoptosis, contributing to their resistance to various stressors. This function is important for maintaining normal cell function and preventing premature cell death [14]. Calprotectin can also be expressed by macrophages, which can infiltrate neoplastic lesions. Tumor-associated macrophages (TAMs) exert both pro-tumor and anti-tumor activities. One of the mechanisms by which macrophages contribute to tumor progression is by releasing pro-inflammatory molecules, including calprotectin. Studies have shown that calprotectin released by macrophages can promote the epithelial-to-mesenchymal transition (EMT), a process associated with increased tumor cell motility and invasiveness [15]. On the other hand, intracellular calprotectin has also been shown to induce DNA damage and activate DNA damage response pathways in head and neck squamous cell carcinoma (HNSCC) cells. This activation of DNA damage signaling pathways further contributes to G2/M cell cycle arrest and the suppression of cell proliferation [16]. Furthermore, calprotectin can modulate the Wnt/β-catenin pathway, which is crucial for the maintenance of cell differentiation and tissue homeostasis in various organs, and the alteration of alteration is responsible for the EMT and cancer progression [17]. Further investigation into the interplay between calprotectin and β-catenin may provide valuable insights into the development and progression of cancers.

2. Materials and Methods

Thirty samples of hepatoid glands were retrieved from the archives of the Unit of Pathology of the Department of Veterinary Sciences of Messina. All the samples were from male dogs, of different breeds, with a median age of 9 y.o. They included 10 hyper-
plasias/adenomas, and 20 carcinomas subclassified, according to Mauldin et al. [18], as well differentiated and poorly differentiated (Figure 1).

Figure 1. Canine hepatoid glands. Normal glands (a, 10×), hyperplasia/adenoma (b, 10×), well-differentiated carcinoma (c, 5×) and poorly differentiated carcinoma (d, 5×). Hematoxylin&Eosin stain.

Absence of skin inflammation and ulceration was a sample inclusion criterium. For immunohistochemical staining, five-micrometer-thick sections were deparaffinized in xylene and rehydrated in graded alcohols. Slides were steamed in 0.01 mol/L sodium citrate buffer, at pH6, in a microwave oven for 15 min. Endogenous peroxidase activity was quenched by 0.3% hydrogen peroxide in methanol. Nonspecific reactions were blocked via incubation with 2.5% BSA (bovine serum albumin) for 30 min. Slides were then incubated over night at 4 °C with the primary antibody (Ab). A list of primary antibodies are reported in Table 1. Slides were then incubated for 30 min at room temperature with a secondary antibody, as shown in Table 1, developed using an avidin-peroxidase complex (BioSpa, Milan, Italy), revealed with DAB (Diaminobenzidine) or Vector Nova Red (Vector Laboratories Inc., Newark, USA), and counterstained with hematoxylin.

Table 1. Primary and secondary antibody used for HIC.

<table>
<thead>
<tr>
<th>Antibody I</th>
<th>Clone</th>
<th>Type of Antibody I</th>
<th>Dilution</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>H-102</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology Inc., Dallas, TX, USA</td>
</tr>
<tr>
<td>Macrophage marker</td>
<td>MAC387</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology Inc., Dallas, TX, USA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody II</th>
<th>Antibody type</th>
<th>Dilution</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit IgG-B</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>BioSpa, Milan, Italy</td>
</tr>
<tr>
<td>Goat anti-mouse IgG-B</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>BioSpa, Milan, Italy</td>
</tr>
</tbody>
</table>

For each sample, negative controls were performed by omission of primary Ab or substitution with normal immunoglobulins from the same species of primary Abs. Immunohistochemical stains were interpreted by assessing membranous, cytoplasmic, and nuclear immunoreactivity. A semi-quantitative scoring system was applied to study the intensity of β-catenin staining, which was evaluated as follows: negative (0), weak (1), moderate (2), and strong (3).

MAC387-positive TAM localization was assessed as intratumoral or stromal following the guidelines given by Salgado et al. [19] for the evaluation of tumor-infiltrating lymphocytes (TILs) in breast tumors. Briefly, intratumoral TAMs immediately abutted the neoplastic epithelium, while stromal TAMs were dispersed in the tumor stroma and not directly in contact with neoplastic cells. Starting from the most representative area, in terms
of macrophage density, the cells were counted in 10 fields at 40x each for the intratumoral and stromal compartment.

Statistical Analysis

Intratumoral and stromal TAMs were counted, and statistical analysis was carried out through Jamovi computer software (version 2.4.14—https://www.jamovi.org/, accessed on 15 February 2024). The correlation between the intratumoral and stromal localization was performed with a Mann–Whitney U test for adenomas and the Welch test for carcinomas. The correlation between the number of TAMs and tumor type was determined with the one-way ANOVA test, followed by the Tukey and Games–Howell post hoc tests for intratumoral and stromal TAMs, respectively. Statistical significance was based on a 5% (0.05) significance level.

3. Results

3.1. β-Catenin

B-catenin expression was membranous in all examined samples, but staining intensity differed. Epithelial cells from normal hepatic glands were strongly labeled. B-catenin immunoreactivity was similarly strong in hyperplasia and adenomas, moderate or weak in well-differentiated carcinomas, and often absent in less differentiated carcinomas. Hyperplasia/adenoma showed a very strong dark brown membranous stain that appeared to be distributed homogeneously in all the cells of the sample. Cytoplasm was slightly stained and dusty in appearance (Figure 2a). In carcinomas, membrane positivity showed a weaker stain than benign lesions with a wide variability inside the single neoplastic lesion and between different samples of carcinomas. Membrane positivity was easily detectable if the hepatoid cell was still well differentiated; on the contrary, low or no intensity was associated with a less differentiated or anaplastic cellular pattern. Sometimes, these different patterns of staining were detectable in the same microscopic field (Figure 2b). In poorly differentiated lesions, characterized by the increased proliferation of basal cells and the altered morphology of hepatoid cells, membrane positivity for β-catenin was very weak and fragmented; in the above cases, cytoplasmic staining was always weak or absent (Figure 2c,d).

Figure 2. Immunohistochemistry for β-catenin in hepatoid gland tumors. (a) Adenoma: strong dark brown membranous stain; (b,c) well-differentiated carcinomas: moderate or scarce membrane staining; (d) less-well-differentiated carcinomas: rare or absent membrane staining. Note the different stain patterns in the same microscopic field (b) from the well-differentiated (left) to the less differentiated cells (right). IHC, 20 x.

Reserve cells from normal hepatoid glands, nodular hyperplasia, adenomas, and carcinomas were not stained for β-catenin. In cells with squamous differentiation in both
benign and malignant lesions, β-catenin was detectable only in the early stages of whorls formation, becoming weak or absent in clusters of squamous differentiated cells. These squamous cells had scattered and unconstant positivity for β-catenin but showed diffuse positivity for the anti-cytokeratin AE1/AE3 antibody, and negativity for the anti-Vimentin antibody (Supplementary Materials, Figure S1) (Figure 3a).

Figure 3. β-catenin and calprotectin (MAC-387). β-catenin is weak or absent in squamous-differentiated cells (a, asterisk); macrophages and clusters of cells with minimal atypia, oval to elongated, loosely arranged and separated by thin cytoplasmic bridges or characterized by a large cytoplasm of homogeneous appearance, translucent and containing a fading or absent nucleus (squamous metaplasia) arranged in whorls centered on ductal-like spaces (b, arrow). Clusters of squamous cells in carcinomas, surrounded by neoplastic hepatoid cells variably positive to calprotectin (c,d, arrow). IHC, 10×.

3.2. Calprotectin

In both benign and malignant lesions, MAC/387 antibody positivity was found in both macrophages (Figure 3b) and clusters of cells with minimal atypia, oval to elongated, loosely arranged and separated by thin cytoplasmic bridges (Figure 3c) or characterized by a large cytoplasm of homogeneous appearance, translucent and containing a fading or absent nucleus (squamous metaplasia) arranged in whorls centered on ductal-like spaces. In the benign lesions, the hepatoid cells were negative. These clusters were more voluminous in carcinomas, sometimes with a center of lamellar keratin (horny pearls), and were surrounded by neoplastic hepatoid cells variably positive to calprotectin (Figure 3d).

3.3. MAC387-Positive TAMs

Numerous macrophages were positive for immunohistochemical detection with MAC387 (calprotectin) antibody. The statistical analysis of the results was hindered by the relatively low number of samples in each group. Anyway, the number and distribution varied in adenomas, and in well-differentiated and undifferentiated carcinomas, as shown in Table 2.
### Table 2. Comparison between intratumoral and stromal macrophages for tumor types and the p-value for each statistical test used.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Intratumoral Macrophages</th>
<th>Stromal Macrophages</th>
<th>Test</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia/adenoma</td>
<td>46 ± 8</td>
<td>137 ± 8</td>
<td>Mann–Whitney U</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Well-differentiated carcinoma</td>
<td>14 ± 8</td>
<td>277 ± 85</td>
<td>Welch</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>17 ± 11</td>
<td>281 ± 91</td>
<td>Welch</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The difference between intratumoral and stromal macrophages was statistically significant for both adenomas and carcinomas \((p < 0.001)\). Indeed, stromal localization was represented more, as shown in Figure 4.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Bar chart showing the different distributions of macrophages for adenomas, and well-differentiated and undifferentiated carcinomas.

The one-way ANOVA test showed statistical significance for the comparison of intratumoral macrophages with tumor type \((p < 0.001)\). The post hoc Tukey test showed significant results for the comparisons between adenomas and carcinomas. Analog statistical significance resulted in the comparison of stromal macrophages with tumor type \((p < 0.001)\). The post hoc Games–Howell test showed significant results for the comparison between adenomas and carcinomas.

### 4. Discussion

\(β\)-catenin-mediated signals are necessary for the normal development of skin and glands not only during embryogenesis but also in adult tissues. Mutations of \(β\)-catenin-mediated intracellular signals are responsible for tumorigenesis [20].

Our results showed that canine perianal normal and neoplastic glands are positive for \(β\)-catenin. Considering the strong membrane positivity in benign lesions and its decreased expression, as long as the neoplasm increases in malignancy, let us suggest that the \(β\)-catenin is dysregulated even in hepatoid glands. The lack of nuclear staining in our dataset of tumors could be related to tumor differentiation and could explain the reported scarce incidence of poorly differentiated tumors and metastatization despite the malignancy. Moreover, the observations of \(β\)-catenin membranous staining could be of...
potential diagnostic value, especially in distinguishing adenomas from well-differentiated carcinomas or areas of carcinomas intermingled with adenoma-like ones.

In veterinary medicine, the immunohistochemical expression of β-catenin has also been described in canine pilomatricoma [21], gastro-intestinal tumors [22–24], mammary gland tumors [25–28], and cutaneous melanotic tumors [29]. In neoplastic cells, a loss of membrane positivity, increase in cytoplasmic positivity, and nuclear staining are the typical immunohistochemical patterns of invasive and metastatic acquisition of the tumor.

A role for β-catenin in the modulation of the immune microenvironment has been postulated. In addition to its involvement in the initiation and the development of many tumors, it was shown that canonical Wnt/β-catenin signaling was activated during monocyte-to-macrophage differentiation and that it regulated the polarization of macrophages to the M2 phenotype in hepatocellular carcinoma [30].

The MAC387 antibody we used efficiently identified TAMs in the hepatoid tumor sections. Tumor-associated macrophages are classified as an M1 proinflammatory phenotype (classically activated) or M2 anti-inflammatory phenotype (alternatively activated) based on the stimuli they receive in the microenvironment. The MAC387 antibody recognizes the molecule L1 or calprotectin (S100A8/A9), an intracytoplasmic antigen expressed by granulocytes, monocytes, and macrophages in tissues. The epitope recognized appears to be well conserved and is predicted to have crossreactivity with a wide variety of species, including canines. MAC387 macrophages, such as those identified in this work, are suggested to be of the M1 type, linked to tissue damage and anti-tumor effects [31].

To date, there are no reports in the literature about the presence and role of macrophages in hepatoid gland tumors; the data here reported showed an increase in calprotectin (MAC387)-positive macrophages, in the invasive front of neoplastic lesions [19], compared with their number in intratumoral areas. The comparison of intratumoral and stromal macrophages showed statistical significance in all samples, with a greater amount of calprotectin-positive TAMs in the stromal area. Additionally, the comparisons of intratumoral and stromal TAMs with tumor type were significant. From the post hoc tests, adenomas presented higher intratumoral macrophages than both well-differentiated and undifferentiated carcinomas did. Concurrently, the amount of stromal TAMs was greater in carcinomas than adenomas.

Calprotectin is a multifaceted protein that plays a significant role in tumor cells and normal cells. It exhibits antioxidant activities, modulates inflammation and immune responses, and influences cell migration and invasion [14]. Modifications in the expression and function of calprotectin are implicated in various types of cancer and contribute to disease progression.

In the cells of the hepatoid glands, the expression of calprotectin, as shown in this work, could indicate a normal adaptive response to hormonal stimuli, or oxidative stress, to which the glands are supposed to be exposed during the progression of neoplasia. Its increased expression in cells with squamous differentiation could serve as a stimulus for the infiltration of M1 macrophages, and a better inflammatory anti-neoplastic response may be one of the possible explanations for the lower metastatic activity of carcinomas of hepatoid glands. Calprotectin can scavenge reactive oxygen species (ROS), protecting tumor cells from oxidative stress. Moreover, S100A8/A9 appears to be directly associated with the level of squamous differentiation and keratinization in HNSCC, where calprotectin appears to suppress cell cycle progression, growth, and migratory invasion [16,17,32].

The role of calprotectin varies depending on the specific context and cancer type. The biological effects of S100A8/A9 depend on the extra- or intracellular localization of the protein complex. Likely released by infiltrating polymorphonuclear leukocytes, macrophages, and epithelial cells, extracellular S100A8/A9 in the tumor microenvironment is associated with inflammation-induced tumor progression and may serve as a prognostic marker in some types of cancer [14]. For example, S100A8/A9 is often abnormally elevated in tumors originating in tissues that are negative for the protein complex, whereas intracellular levels decrease in tumors such as HNSCC that originate from tissues with constitutive
expression [16,33]. Intracellular functions of S100A8/A9 in squamous epithelial cells may orchestrate gene networks critical in cellular development and squamous epithelial cell differentiation. Hepatoid gland carcinomas express both androgen and estrogen receptors [34], and, as well known to occur in the prostate, squamous metaplasia could be related to the hormonal status of the subject and calprotectin, the molecule responsible for this epithelial substitution.

5. Conclusions

The results demonstrate that β-catenin is a valid marker for evaluating less-well-differentiated neoplastic histotypes or even small clusters of malignant cells inside well-differentiated hepatoid tumors. Also, the interaction between β-catenin and calprotectin could be a therapeutic strategy for inhibiting hepatoid gland tumor progression and metastasis. Further research is needed to explore the mechanisms underlying this interaction and to identify potential therapeutic targets for intervention.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pets1010006/s1, Figure S1: Hepatoid gland carcinoma. Squamous cells, arranged in whorls centered on ductal-like spaces, showed diffuse positivity for the anti-cytokeratin AE1/AE3 antibody (a, 20×) and negativity for the anti-Vimentin antibody (b, 20×). IHC.

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