Altered Expression of Claudin-1 Is Related With Malignancy in Canine Thyroid Tumors

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**Abstract.** Background/Aim: Claudins (CLDNs) are crucial structural and functional components of tight junctions playing an important role in maintaining cell polarity, controlling paracellular diffusion and regulating cell growth and differentiation of epithelial cells. CLDNs are differentially expressed in neoplastic lesions compared to the corresponding healthy tissues; therefore, they are thought to play a role in tumorigenesis and tumor progression. Reduced expression of CLDN-1 has been observed in several types of human cancers, including thyroid tumors. There are no reports of CLDN-1 immunoexpression in normal and neoplastic canine thyroid tissues. Material and Methods: CLDN-1 immunoexpression was investigated in normal canine thyroid gland (n=2), benign (n=1) and malignant (n=10) tumors, as well as neoplastic emboli (n=6). Results: CLDN-1 was constitutively expressed in normal canine follicular epithelium. Ninety percent of the malignant canine thyroid lesions showed absence or reduced CLDN-1 expression compared to that of normal thyroid gland. Additionally, a cytoplasmic subcellular location of CLDN-1 was recorded in the malignant epithelial cells and neoplastic emboli. Conclusion: These findings link altered expression of CLDN-1 to neoplastic transformation and suggest that CLDN-1 expression is associated with malignant canine thyroid tumors and their vascular invasion.

Tight junctions (TJs) are the most apical components of the junctional complex in epithelial and endothelial cells. The two major functions of TJs in epithelial cell layers are regulation of paracellular permeability, known by barrier or gate function, and maintenance of cell polarity, known by fence function. Because of their ability to recruit signaling proteins, TJs are considered to be involved in the regulation of proliferation, differentiation and other cellular functions (1, 2).

Claudins (CLDNs) (from the Latin “claudere”- to close) are the critical sealing proteins of TJs. In mammals, the claudin protein family is composed of 27 members that are highly tissue-specific, while their several combinations can explain the observed physiologic differences in the sealing properties of TJs in the different epithelia (1). CLDNs help preserve cell polarity and paracellular flux of normal epithelial tissue (2), thus deregulation of these molecules has been shown to contribute to tumor progression by affecting cell proliferation, invasion and metastasis (2, 3).

Based on the degree of similarity determined by the analysis of whole-length sequences of CLDNs, Claudin-1 (CLDN-1) is considered a classic claudin with stronger sequence homology in mammals (1, 2). CLDN-1 has been associated with the pathogenesis of different types of human tumors (2) since changes in their structure may lead to abnormal influx of nutrients and other signaling proteins that could induce special types of cellular differentiation (4).

In humans, reduced expression of CLDN-1 has been observed in undifferentiated thyroid carcinomas (5), high-grade prostatic adenocarcinomas (6), stage II colonic cancer (7) and correlates with shorter overall survival in lung adenocarcinomas (8). On the other hand, increased expression of CLDN-1 has been associated with papillary and squamous morphology (9, 10) and to an invasive and metastatic phenotype in thyroid and colon cancer (3, 10). It is reasonable to speculate that down-regulation, as well as up-regulation of CLDN-1, may contribute to development and progression of cancer and that the role of this specific protein may vary considerably depending on the tissue involved and according to the histotyloge type and grade of the neoplasm (2).

To the best of our knowledge, CLDN-1 has not been studied in canine thyroid tissues. Therefore, the main
purpose of the present study was to investigate the immunoexpression of CLDN-1 in normal and neoplastic canine thyroid gland.

Materials and Methods

Thirteen canine thyroid specimens, obtained either during surgery or necropsy examination, were selected from the archives of the Laboratory of Veterinary Pathology, ICBA-UP (Portugal). Tissues were fixed in 10% buffered formalin and paraffin-embedded. Serial consecutive 2 μm-thick sections were made, one for routine histological diagnosis and the others for the immunohistochemical study. Sections were independently examined by three pathologists. Thyroid tumors were classified according to the criteria proposed by the World Health Organization (11) (Table I).

For the immunohistochemical study, antigen retrieval was performed on dewaxed sections by immersion in citrate buffer (10 mM, pH 6.0) in a pressure cooker for 3 min. The Novolink™ Max-Polymer detection system (Novoceastra, Newcastle, UK) was used for visualization according to the manufacturer’s instructions. Slides were incubated overnight at 4°C with the ready-to-use rabbit polyclonal anti-CLDN-1 (359A-17; Cell Marque, CA, USA) and the monoclonal antibody Ki-67 (MIB-1) (Dako, Glostrup, Denmark), diluted 1:50. Negative controls consisted in replacing the primary antibody by another of the same immunoglobulin isotype. Positive controls consisted in sections of human breast tissue known to express anti-CLDN-1 and samples of canine lymphoma presenting high Ki-67 proliferative index (PI).

Claudin-1 immunoreactivity was assessed according to the distribution and intensity of labeled cells. The distribution was registered as the percentage of positive cells of the total neoplastic population, as follow: 0, <5%; 1, 6%-20%; 2, 21%-40%; 3, 41%-60%; 4, 61-80%; or 5, 81-100%. The labeling intensity was scored as: 0, negative; 1, weak; 2, moderate; 3, strong. The subcellular location was classified as membranous and/or cytoplasmic. A semi-quantitative estimation of CLDN-1 expression was performed using a composite score obtained by multiplying the values of the distribution by the intensity (score, 0-15). This method allows a comparison of the architectural arrangement adopted by neoplastic cells, with a reduction in the intensity and number of positive cells in the most solid areas of the tumors (Figure 1c). When present, CLDN-1 labeling was recorded in the membranous and/or cytoplasmic (Figure 1d). In the remaining malignant neoplasm, CLDN-1 expression was similar to canine normal thyroid gland (Table I). One out of the 2 thyroid malignant mixed tumors presented null CLDN-1 score in the mesenchymal neoplastic cells (chondroid and osseous areas). The other case showed lower score and CLDN-1 labeling was visualized at the cytoplasmic level (Table I).

All malignant tumors with vascular invasion demonstrated lower CLDN-1 score in neoplastic epithelial cells when compared with normal canine thyroid gland. CLDN-1 score values in neoplastic emboli were also lower. In all emboli lesions, the cytoplasm pattern was prevalent (Figure 1e and f). Four cases of neoplastic emboli showed a higher score compared with the respective primary thyroid lesion, while the remaining two cases exhibited CLDN-1 expression similar to that recorded in the primary tumor site (Table I). The Ki-67 PI of normal canine thyroid gland was minimal (<1%). The average PI value of the malignant neoplasms was 38.0% (standard deviation (SD)±16.4%). With the exception of a follicular carcinoma, the highest PI values obtained belonged to the malignant lesions with recognized vascular invasion (Table I). However, a clear relationship between Ki-67 PI and CLDN-1 score expression in the epithelial cells of the malignant lesions was not found.

Discussion

Thyroid tumors are relatively uncommon in dogs. Approximately 88% of all canine thyroid tumors are malignant and follicular carcinoma remains the most
common histological subtype (13). Despite the small size of our sample, the majority of canine thyroid neoplasms included in our study were malignant, although the compact cellular carcinoma was the most encountered subtype. Reinforcing previous data, herein the animals affected were mainly females of adult or old age (13). A considerable number of Boxers was also observed in our sample (13). This finding might be explained by the popularity of these specific breed in our geographical localization rather than a racial predisposition.

In normal canine thyroid gland, follicular epithelial cells presented strong and lateral membranous CLDN-1 expression. However, some human studies have described a complete absence of this protein expression in normal thyroid gland (14). The expression pattern of CLDNs is highly tissue-specific and most tissues express multiple CLDNs (2). Thus, CLDN-1 can be constitutively expressed in canine normal thyroid tissues. Additionally, Jakab et al. (2008) detected a strong CLDN-1 immunoexpression in normal canine mammary gland. The localization of CLDN-1

Figure 1. (a) Normal thyroid gland. The follicular epithelial cells show strong membranous lateral CLDN-1 expression. Note the minimal nuclear expression of Ki-67 in epithelial cells. IHC; ×400 for both inserts. (b) Follicular adenoma. Neoplastic epithelial cells show strong to moderate membranous expression of CLDN-1. IHC; ×200. (c) Malignant mixed tumor. Decreased expression of CLDN-1 in neoplastic cells. Note the weak cytoplasm CLDN-1 staining in malignant epithelial cells. Ki-67 expression in the majority of neoplastic cells IHC; ×400, 200x for each insert. (d) Compact-cellular carcinoma. Note the strong membranous expression of CLDN-1 in malignant neoplastic cells. IHC; 400x. (e and f) Moderate cytoplasmic staining of CLDN-1 in neoplastic emboli. IHC; ×400 and ×200, respectively. IHC, immunohistochemistry; CLDN, claudin.
along the lateral membrane of normal canine thyroid epithelium may indicate that CLDN-1 was present not only in the apical areas, which contained TJs structures, but also in extrajunctional areas, as previously reported in mammary epithelium of dogs (15). Although the significance of extrajunctional expression of claudins remains unclear, studies suggest that this expression, outside the classical apical TJs areas, might serve as pool(s) for junctional claudins and alternatively play a role in cell–cell and cell–matrix adhesion or signaling (16).

The single benign tumor of our study showed a slight decrease of CLDN-1 score in the epithelial neoplastic population; however, in 90% of the malignant cases, a considerable reduction or absence in CLDN-1 score was observed when compared to normal canine thyroid tissue. Interestingly, such loss was more evident in less differentiated areas where neoplastic cells adopted a solid arrangement. The differences in CLDN-1 expression between benign and malignant tumors are in agreement with the study of Tzelepi et al. (2008), which described a reduction of CLDN-1 expression from follicular adenomas to carcinomas, specifically in the poorly-differentiated and undifferentiated types of human thyroid carcinomas (5). Furthermore, previous studies demonstrated that loss or reduced expression of CLDNs occur in several types of human (2) and canine cancers (17). In fact, decreased expression of CLDN proteins in cancer is consistent with the generally accepted idea that tumorigenesis is accompanied by a disruption of TJs, a process that can lead to the loss of cohesion, invasiveness and lack of differentiation observed in cancer cells (2).

Similarly to what has been described in human cancers (3, 18), a concurrent cytoplasmic subcellular location of CLDN-1 was found in 50% of the canine malignant thyroid tumors. Among these, 80% showed signs suggestive of poor prognosis, such as vascular invasion and high PI. Dhawan et al. (2005) reported that the de-localization of CLDN-1 from TJs to the cytoplasm occurred in tumors with unfavorable behavior (3). Bezdekova et al. (2012) also concluded that, in human colorectal cancer, subcellular alteration of CLDN-1 from its typical membranous position may be due to structural changes in TJs that will lead to profound modifications in cell morphology and disturbances in paracellular permeability, thus resulting in an abnormal flux of nutrients and signaling proteins, which is considered critical for tumor growth and survival. The mechanisms underlying the cytoplasmic internalization of CLDNs in cancer are under investigation although post-translational modifications, such as phosphorylation (19), mutations (3) and promoter gene hypermethylations (20) have been mentioned as possible alternatives. Furthermore, the cytoplasmic location of CLDN-1 is associated with induction of "epithelial-mesenchymal transition" (EMT), a mechanism that may favor the spread of carcinomas (18). During this process, epithelial cells dedifferentiate towards a mesenchymal phenotype by losing cell-cell adhesion structures, altering their polarity, reorganizing their cytoskeleton and becoming isolated and motile and, therefore, facilitating tumor progression and metastasis (21).

In our study, tumors with vascular invasion presented the lowest CLDN-1 score values among the epithelial neoplastic population. In turn, the neoplastic emboli exhibited an equal or
higher CLDN-1 scores compared to the epithelial neoplastic cells of the primary lesion. Previous data suggest a correlation between decreased CLDN-1 expression and cancer invasion and metastasis (4, 7). This finding was discussed by Chao et al. (2009) who reported that increased expression of CLDN-1 suppressed the cancer cell migration, invasion and metastasis, concluding that CLDN-1 is a cancer invasion and metastasis suppressor. Additionally, re-expression of CLDN-7 and CLDN-2 in metastatic lesions was demonstrated in previous human and feline mammary tumor studies (12, 22). Park et al. (2007) suggested that the re-expression of adhesion proteins enables the circulating tumor cells to resettle at the metastatic sites facilitating the intercellular adhesion, fundamental for metastatic tumor progression (22).

Additionally, the Ki-67 PI of canine thyroid tumors was assessed. We demonstrated that all neoplastic lesions expressed Ki-67 regardless of the histological type involved, as previously demonstrated by Brown and colleagues (23). Among the thyroid lesions, higher Ki-67 PI was also associated with a more malignant phenotype, which may reinforce the potential of this protein in determining its cellular activity and predicting the behavior of canine thyroid lesions.

Increasing evidence suggests that: (i) CLDN-1 regulates cell polarity and paracellular permeability of epithelial cells in a tissue-specific manner, thus representing a key factor in maintaining the structural and functional integrity of normal tissues; (ii) altered expression of this molecule may represent an acquired feature that can lead to loss of cohesion, detachment and invasion of cancer cells; (iii) the circulating tumor cells, to resettle in metastatic sites, tend to re-express adhesion molecules. These reported facts may explain why CLDN-1 is strongly expressed in normal epithelial cells of canine thyroid gland, is lost or expressed in an abnormal subcellular location in malignant canine thyroid lesions and further over-expressed in neoplastic emboli compared to the corresponding primary lesion.

The present work constitutes the first investigation regarding CLDN-1 expression in normal and neoplastic canine thyroid tissues. The present study documented a membranous lateral expression of CLDN-1 in normal canine thyroid gland suggesting that this molecule may have a physiological role in this tissue. Furthermore, we described a possible relation between the decrease of CLDN-1 expression and the neoplastic transformation of the canine thyroid epithelial cells. Additionally, altered expression of CLDN-1 in canine thyroid malignant tumors, characterized either by reduced expression and cytoplasmic location, may constitute a mechanism for tumor progression.

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