IMMUNOPHENOTYPE OF THE CANINE MAMMARY TUMORS REGARDING CD44 AND CD24 IMMUNOMARKERS

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Summary
The cancer stem cell theory indicates that cancers develop from a subset of malignant cells that have stem cell characteristics (i.e., CD44+/CD24-). The aim of the study was to investigate the expression of CD44 and CD24 biomarkers in diverse canine mammary tumors. The material was represented by canine mammary tumors that were presented to the Laboratory of Pathologic Anatomy from the Faculty of Veterinary Medicine Cluj-Napoca, Romania. The tissue samples (i.e., from the mammary neoplasms) were fixed in 10% neutral buffered formalin and processed by paraffin technique. Immunohistochemical staining methods for CD44 (Santa Cruz) and CD24 (Santa Cruz) were performed on formalin-fixed, paraffin-embedded tissue sections. The labeled Streptavidin Biotin method (LSAB+system-HRP, Dako) and Rat ABC Staining System (Santa Cruz) was used for immunomarkers detection. The CD44 and CD24 positive cells were counted in 5 high-power microscopic fields (400x). Eleven canine mammary tumors (i.e., simple and complex mammary tumors) presented in diverse canine breeds were assessed. In our paper, canine mammary tumors had CD44+/CD24+ (i.e., 63.60%) or CD44-/CD24+ (i.e., 36.36%) immunophenotypes. Nevertheless, no CD44+/CD24- (revealing cancer stem cells) mammary tumors were noticed in our report. However, statistically significant correlations were established between the CD44 and CD24 evaluated biomarkers.

Key words: mammary cancer, dog, stem cells, CD44, CD24.

Introduction
Mammary cancers represent one of the main causes of death in female dogs, and women. One of the hallmarks of this disease is its extensive local tumor invasion and systemic spreading. Currently, in female dogs, the surgery is the treatment modality of choice in locally limited disease. Nevertheless, in advanced stage of the disease, most chemotherapeutic approaches failed to significantly improve the outcome (Baba and Cătoi, 2007).

Attempts to better understand the molecular foundation for mammary cancer have focused on studying gene and protein expression profiles of patient samples (Lawson et al., 2009). The cancer stem cell (CSC) theory indicates that cancers develop from a subset of malignant cells that have stem cell characteristics.

However, some recent studies suggested the development from CSC of a variety of malignancies, including brain, liver, colon, prostate, pancreatic and breast cancer (Shmelkov et al., 2005; Lawson et al., 2009; Ischenko et al., 2010). These CSC possess characteristics of both stem cells and cancer cells (i.e., properties of self-renewal, asymmetric cell division, resistance to apoptosis, independent growth, tumorigenicity and metastatic potential) (Miyamoto et al., 2003; Al-Hajj and Clarke, 2004; Leach, 2005). The purpose of this study was to investigate the expression of a specific set of markers (i.e., CD44 and CD24, which are specific for mammary CSC), in diverse canine mammary tumors.
Material and methods

The material was represented by canine mammary tumors that were presented to the Laboratory of Pathologic Anatomy from the Faculty of Veterinary Medicine Cluj-Napoca, Romania. The tissue samples (i.e., from the mammary neoplasms) were fixed in 10% neutral buffered formalin. For histopathologic study, the tissue samples were subsequently embedded in paraffin wax, sectioned at 4 µm thicknesses and stained with hematoxylin and eosin. The diagnosis criterion of lipid-rich carcinoma of the mammary gland was based on WHO (World Health Organization) classification in dogs and cats (Misdorp et al., 1999).

Immunohistochemical staining methods for CD44 (IM7 cod sc-18849, SANTA CRUZ) and CD24 (C-20 cod sc-7034, SANTA CRUZ), were performed on formalin-fixed, paraffin–embedded tissue sections (4-µm-thickness). Steam antigen retrieval in citrate buffer (pH 6.0) was used. Commercially available antibodies tested were diluted according to the manufacturers’ instructions. The labeled Streptavidin Biotin method (LSAB+system-HRP, K0679, Dako) and Rat ABC Staining System (sc-2019, SANTA CRUZ) was used for immunomarkers detection. CD44 and CD24 positive cells were counted in 5 high-power microscopic fields (i.e., 400x). The tumors having values lower than 3% for both biomarkers were considered as negative tumors. The proportion of positive neoplastic cells (i.e., percentage) was calculated. Positive and negative control slides were included in each experiment. Tissue analysis was performed using an Olympus system for image acquisition and analysis, respectively an Olympus BX51 microscope equipped with Olympus Cell B software. The data obtained was interpreted statistically using TTest (p<0.05).

Results and discussions

In our study were utilized 11 canine mammary tumors (i.e., simple and complex mammary carcinomas, squamous cell mammary carcinoma and mammary fibroadenoma) presented in diverse canine breeds (Table 1). Canine mammary tumors originated from different breeds, such as: Stray dog (4 subjects), Caniche (2 subjects), Caucasian dog, Cocker, German shepherd, Bichon, and Basset-Hound (1 subject each). Tumor size varied from 5 to 20 cm.

Statistically significant correlations were established between the two assessed immunomarkers (i.e., CD44 and CD24) (p<0.0001). Membrane and cytoplasm staining for both biomarkers was in general moderate to intense in mammary neoplastic cells. However, CD44+ immunolabeling was restricted to a reduced number of cells comparing to CD24 immunomarker (Figs. 1 and 2). According to this, the percentage of CD44+ cells in examined mammary tumors was smaller (i.e., obtained values range from 0 to 14.1%) comparing to CD24. Oppositely, numerous CD24+ cells were noticed in all examined canine mammary tumors (obtained values range from 9 to 81.6%). As for CD44 immunomarker, several negative tumors were encountered (e.g., cases 3, 5, 6 and 11) for this marker, while no negative tumors were noticed for CD24. The following tumor types, regarding CD44/CD24 immunophenotype, were obtained: CD44+/CD24+ (63.60%) and CD44-/CD24+ (36.36%).

Another important suggestion in our study is the lack of CD44+/CD24- tumors that indicates the presence of CSC. In our paper we did not noticed malign tumors that have the CSC immunophenotype suggested in numerous reports. Dissimilar results were obtained in our paper (involving canine mammary tumors) comparing to some other studies involving breast mammary neoplasms (Lawson et al., 2009; Ischenko et al., 2010).

Lately, confirmation for the presence of CSC in different tumors was suggested. These cells have been named CSC since they have the capacity to self-renew and produce differentiated cells
without stem-cell properties (Lee et al., 2008). If the condition is correct, the CSC theory may provide a new understanding of the carcinogenesis and new strategies for cancer prevention and therapy (Li et al., 2007; Trosko, 2009).

The CSC hypothesis is a deeply different model of tumorigenesis composed of two separate (but dependent on each other) components; (a) the tumors derive from tissue stem and/or progenitor cells through the dysregulation of the normal process of self-renewal (Al-Hajj and Clarke, 2004). (b) The CSC are defined as cancer cells that have the capacity to divide into new malignant stem cells and daughter cells that differentiate and create the heterogeneous tumor cell mass (Ischenko et al., 2010).

The first confirmation of a cancer stem cell origin for breast cancer was reported by Al-Hajj et al. (2003). Using cells derived from primary breast tumours, they confirmed that a subpopulation inside the samples could create palpable tumours in diabetic combined immunodeficient/immunocompromised mice. All of the tumourigenic cells expressed CD44, alone or in conjunction with ESA (epithelial specific antigen; EpCAM) but did not express CD24 (Al-Hajj et al., 2003). In our paper, none of the examined canine mammary tumors had the CSC immunophenotype suggested in various reports (i.e., CD44+/CD24-). This study offered some insights regarding the two biomarkers of the CSC (CD44+/CD24-) in canine mammary tumors. However, the new tumorigenesis hypothesis from CSC has to be assessed in numerous spontaneous and experimental tumor types. It is clear that there is still much that is unknown about the variety and heterogeneity of the different CSC populations (Lawson et al., 2009).

Conclusions

The presence of CSC in mammary tumors could have prognostic significance and multiple practical applications. In our paper, canine mammary tumors had CD44+/CD24+ (i.e., 63.60%) or CD44-/CD24+ (i.e., 36.36%) immunophenotypes. Nevertheless, no CD44+/CD24- mammary tumors were noticed in our report. However, statistically significant correlations were established between the CD44 and CD24 evaluated biomarkers.

Acknowledgements

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### Table 1. Details regarding the examined tumors.

<table>
<thead>
<tr>
<th>No.</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Castration</th>
<th>Tumor size (cm)</th>
<th>Tumor type</th>
<th>Metastasis</th>
<th>CD44 (%)</th>
<th>CD24 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cocker</td>
<td>10</td>
<td>No</td>
<td>17/12</td>
<td>Complex carcinoma</td>
<td>No</td>
<td>9.5</td>
<td>81.6</td>
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<tr>
<td>2</td>
<td>German Shepherd</td>
<td>9</td>
<td>No</td>
<td>10/20</td>
<td>Complex carcinoma</td>
<td>No</td>
<td>3.2</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Caniche</td>
<td>10</td>
<td>No</td>
<td>5/4.5</td>
<td>Carcinoma in benign mixed tumor (BMT)</td>
<td>No</td>
<td>0</td>
<td>41.2</td>
</tr>
<tr>
<td>4</td>
<td>Stray dog</td>
<td>10</td>
<td>No</td>
<td>15/15</td>
<td>Complex carcinoma</td>
<td>No</td>
<td>7.1</td>
<td>16.9</td>
</tr>
<tr>
<td>5</td>
<td>Stray dog</td>
<td>5</td>
<td>No</td>
<td>6/5.6</td>
<td>Carcinoma in BMT</td>
<td>No</td>
<td>2</td>
<td>29.5</td>
</tr>
<tr>
<td>6</td>
<td>Stray dog</td>
<td>14</td>
<td>No</td>
<td>10/9.4</td>
<td>Anaplastic carcinoma</td>
<td>No</td>
<td>2.8</td>
<td>46.6</td>
</tr>
<tr>
<td>7</td>
<td>Caniche</td>
<td>9</td>
<td>No</td>
<td>8/7.5</td>
<td>Carcinoma in BMT</td>
<td>Yes</td>
<td>14.1</td>
<td>30.1</td>
</tr>
<tr>
<td>8</td>
<td>Caucasian dog</td>
<td>10</td>
<td>No</td>
<td>7.5/7</td>
<td>Squamous cell mammary carcinoma</td>
<td>No</td>
<td>4.5</td>
<td>25.5</td>
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<tr>
<td>9</td>
<td>Stray dog</td>
<td>13</td>
<td>Yes</td>
<td>5/4.8</td>
<td>In situ ductal and lobular carcinoma</td>
<td>No</td>
<td>3.3</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>Bichon</td>
<td>11</td>
<td>No</td>
<td>7.5/4</td>
<td>Mammary fibroadenoma</td>
<td>No</td>
<td>9.4</td>
<td>10.2</td>
</tr>
<tr>
<td>11</td>
<td>Basset-Hound</td>
<td>6</td>
<td>No</td>
<td>6/5.5</td>
<td>Simple carcinoma</td>
<td>No</td>
<td>0</td>
<td>55.5</td>
</tr>
</tbody>
</table>

**Fig. 1.** Solid mammary carcinoma – CD44+ tumoral cells; immunohistochemistry, 400x.

**Fig. 2.** Simple mammary carcinoma – CD24+ tumoral cells (discrete membranary reaction); immunohistochemistry, 400x.