IMMUNOHISTOCHEMICAL EXPRESSION OF CD 44, CD24 AND ESA IN CANINE MAMMARY TUMOURS

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Abstract: Identification of Cancer stem cell lineage subpopulations of tumor cells within the tumor mass offer to identify targets for therapeutic interventions and a better understanding of carcinogenesis. The main objective of this study was to evaluate the prognostic value of cancer stem cell lineage markers i.e., CD44, CD24 and Epithelial specific antigen (ESA) in canine mammary tumours using triple immunohistochemistry. A total of 40 surgically excised tissue samples of canine mammary tumours were obtained during the eight month study period. These tumors were classified according to WHO classification of mammary tumors of dog (2002), as Carcinoma – tubular, Carcinoma – Papillary cystic, Squamous cell carcinoma and Carcinosarcoma with tumor grades I & II respectively. Of 40 samples, 24 selected cases were subjected to triple immunohistochemistry using antibodies against CD 44, CD 24 and ESA. Two cases were positive for cancer stem cell phenotype i.e., CD44+/CD24−/low/ESA+ showing colocalized expression of CD44, epithelial specific antigen (ESA) with membranous staining and absence or isolated staining for CD24, which correlated with both the cases showing distant metastases and reduced average survival period of 122 days, indicating poor survivability and distant metastasis. CD44+/CD24−/ESA+ expression was seen in carcinosarcoma (Grade I) indicating an epithelial mesenchymal transition and could favor metastasis. triple immunohistochemical expression of cancer stem cell phenotype i.e., CD44+/CD24+/ESA+ favored distant metastasis with poor prognosis and could be used as a prognostic indicator in canine mammary tumors as evident from this study.

Key words: Canine mammary tumor, CD 44, CD24, ESA

INTRODUCTION

Tumours of the mammary glands are one of the common neoplasms encountered in female dogs and ranked second compared to skin neoplasms [1]. The incidence of mammary carcinoma in canines is three times that documented in humans and shares several important epidemiological, morphological, clinico-pathological and biochemical features compared to humans [2]. Close similarity that exist between cancer genes of dog and human cancer tissue especially of human tumor initiating cells (CD44+/CD24−) makes the dogs as an excellent tumor models [2-6]. A population of cancer stem cells (CSC) that have the exclusive ability to extensively proliferate and form new tumors can be identified based on marker expression especially, CD44+/CD24−/low/ESA+ which have more than 50 fold tumourigenic potential and as few as 200 cells with this phenotype were able to form tumours in SCID mice consistently, whereas other lineages failed to form tumors [7]. CSC population with the phenotype of CD44+/CD24−/low
correlated with metastatic potential signifying canines as genuine models for studying Human breast cancer [8]. Recent data suggest that canine mammary tumours possessed the ability to generate tumourspheres having stem cell property [9]. Insights into the CSC marker expression in canine mammary tumours would help greatly by enhancement of current perspectives in breast cancer research and to use canines as suitable animal model for studying breast cancer. Thus, the primary objective of this study was to evaluate the prognostic value through co localized expression of cancer stem cell phenotype CD44+/CD24low/ESA+ in mammary tumours of dogs.

MATERIALS AND METHODS

The current study was conducted at Department of Veterinary Pathology, Madras Veterinary College, Chennai, for a period of 14 months from 2008-2009.

Tissues: 40 surgically excised tissue samples were collected from female dogs aged between 2-17 yrs (mean age: 9.45 yrs) of various pure and mixed breeds. Three representative tissue samples (different locations of tumor) from each case were collected and fixed in neutral buffered formalin for 48 hrs and processed to get sections of 3-4µm thickness. The tumours were classified histologically and graded according to the standered criteria [10,11] respectively.

24 selected cases were subjected to triple immunohistochemistry based on the area free of necrosis, hemorrhage, inflammatory cells and certain areas showing malignant cells with mitotic figures and invasiveness were considered.

Triple immunohistochemistry of CD 44, CD 24 and ESA using polymer detection system: Tissue sections were subjected to triple immunostaining using the following antibodies: mouse monoclonal CD44 (Clone: 156-3C11), CD24 (clone: SN3b) and epithelial specific antigen (ESA) (clone: VU-1D9), M/s. Lab Vision, USA. The secondary antibodies included goat anti-mouse HRP polymer and goat anti-mouse alkaline phosphatase polymer (Lab Vision, USA). Chromogens used were DAB, Fast Red (Labvision, USA). Additional chromogens such as vector blue and vector vip (Vector laboratories, USA) were also used. Various chromogen combination was used in tandem to arrive at the best combination of chromogens, where the least background noise was achieved. Denaturing solution was obtained from M/s Biocare, USA, was used to remove the primary antibody complex before proceeding into second and third immunostaining procedure in order to prevent cross reaction, leaving the colored reaction product intact. Antigen retrieval was standardized, where all three antigens were retrieved optimally using 10mM of Tris-EDTA, pH 9.0 using a microwave oven (Kenstar # 9925) at 1. Hi-power (> 1000 W) for 5 min - 1 change, 2. 800W for 5min - 1 change 3. 500W for 25 min - 1 change

Following boiling, the slide rack were brought to room temperature for 20 minutes and washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by treating with hydrogen peroxide 3 per cent in methanol for 10 min. Non-specific staining was eliminated by incubating the sections ultra V block. The sections were incubated with the ready to use (RTU) mouse monoclonal CD 44 primary antibody for 60 min at room temperature, in a humid chamber.

They were then incubated with HRP polymer based secondary antibody for 30 min at room temperature (RT). The color was developed at room temperature with a freshly prepared solution of DAB (3,3’ diamine benzidine) for the first staining sequence [12]. This was followed by applying denaturing solution (Biocare # DNS001) for 3 min at RT. Sections were washed thrice thoroughly with phosphate-buffered saline. Second staining sequence included Incubation with mouse monoclonal (1:100) CD24 for 6 hr at RT. Incubated with HRP polymer based secondary antibody for 30 min at RT followed with chromogen Vector VIP in 50 mM Tris HCl pH 7.6 for 10 min. Followed by denaturing solution (Biocare) for 3 minutes at RT was applied to remove the earlier applied immune complexes. Slides were washed with TBS-T before using AP polymer secondary antibodies. The third immunostaining sequence involved incubation with mouse ESA primary antibody (RTU) overnight at room temperature at RT in an incubator followed by alkaline phosphatase polymer based secondary antibody was incubated for 30 min at RT. Vector blue in 50 mM Tris HC1 pH 8.2 for 15 min was added as the third chromogen. Sections were counterstained with 1:10 dilution of haematoxylin. The slides were dried at 37°C for 1h in an incubator and mounted with Vectamount for permanent mounting.

The criterion for CD24 negative / low staining was based on absence or partial staining of CD24 molecule without interference with positive signals for CD44
& ESA. All immunohistochemistry process was accompanied with human breast ductal adenocarcinoma as positive controls. Negative controls included slides without primary antibodies.

RESULTS

Immunohistochemistry: The results of immunohistochemistry are presented in Table 1. All 24 samples were subjected to triple immunohistochemistry.

CD44: Out of 24 cases 15 cases were positive for CD44. The staining pattern was mostly cytoplasmic and membranous involving the neoplastic epithelial cells (Fig 1). Myoepithelial cells showed variable, moderate immunoreactivity in most cases.

CD24: Out of 24 cases subjected to triple immunohistochemistry, 18 cases were positive for CD24. The staining pattern observed in neoplastic epithelial cells were apically accentuated membranous staining (Fig. 2) and a few cases showed cytoplasmic staining.

ESA: Out of 24 cases, ESA was positive in 11 cases and the staining pattern was membranous in the basolateral surface (Fig. 3) and rest of the cases showed mainly cytoplasmic staining.

CD44/CD24/ESA - Stem cell lineage marker: Of the 24 cases CD24 was positive along with CD44 as colocalized targets in six cases with the phenotype i.e., CD44+/CD24+/ESA-. ESA expression was also seen along with CD24 in five cases showing co-localized pattern of reaction with the phenotype CD44+/CD24+/ESA+ (Fig. 4).

Out of 24 cases subjected to triple immunohistochemistry two cases of carcinosarcoma were positive for cancer stem cell lineage markers i.e., CD44+/CD24+/low/ESA showing colocalized expression of CD44 and ESA with membranous staining (Fig.5). Desquamated intact cell showed strong positive reaction to CD44 and ESA (Figs. 6-8).

DISCUSSION

CD44: Out of 24 cases, 15 (62.50%) cases were positive for CD44 expression. Of these 15 cases, two had thoracic metastasis and out of these two cases, one had lymph node metastases. Other cases which had thoracic or lymph node metastases did not express CD44. The role of CD44 in human breast cancer cannot be confidently used as a reliable prognostic indicator [13] due to the variation in its ligand, hyaluronan. Hence, when evaluating the role of CD44, epithelial stromal interactions as well as ligand and isotype expression profiles should be considered [14].

All the 15 cases showed intracytoplasmic staining with one case showing granular cytoplasmic staining [15] which might be an indication of hyaluronate uptake required for invasion through extracellular matrix. Moreover, the problem of reduced expression was due to the existence of numerous CD44 isoforms, which might have caused the discrepancies [16] in solely stating that CD44 expression as an independent prognostic indicator [17] and others not [18] in human breast cancer. However, this association was not straightforward as demonstrated in the current study due to variable expression of CD44 in canine mammary tumours. So the potential role of CD44 expression, its variants and its receptor warrants further investigation.

CD24: Out of 24 cases subjected to triple immunohistochemistry, 18 (75%) cases were positive for CD24. The pattern of expression was apically accentuated membranous staining and also cytoplasmic staining was seen. CD24 was abundantly expressed in carcinomas with membranous staining intensity remarkably higher in malignant tumours [20]. Out of these, three cases had lung and lymph node metastases, two cases had lung metastasis and one case showed lymph node metastasis indicating an association between CD24 expression and metastases. CD24 could serve as a marker for human breast cancer and its expression played an important role in the metastasis of tumor cells through interaction with platelet or vascular endothelial cells [21] and shortened the patient overall survival and disease free survival with breast cancer [22]. However, six of the cases were negative for CD24 expression, amongst which four showed distant metastases which could be due to down regulation of CD 24 in invasive breast cell lines and might be
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Table 1: Histopathology, metastases and immunoreactivity of CD44, CD24, ESA, stem cells in mammary tumours of dogs

<table>
<thead>
<tr>
<th>Case No.</th>
<th>TNM Clinical staging</th>
<th>Elston &amp; Ellis Grading</th>
<th>Histopathology</th>
<th>Metastases</th>
<th>CD44 Subset cells</th>
<th>CD24 Subset cells</th>
<th>ESA Subset cells</th>
<th>CD44+/CD24+/Low / ESA+ STEM CELL</th>
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<td>NA</td>
<td>NA</td>
<td>-</td>
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</table>

The variation in CD24 immunolocalization from membranous to cytoplasmic staining differed in this study, possibly due to its highly heterogeneous expression within a single case [25]. However, further investigation is required to elucidate the role of CD24.

ESA: The current research on ESA expression is first of the kind so far reported in veterinary oncology literature especially in canine mammary tumours. Out of 24 cases 11 (45.83%) were positive for ESA expression which showed similarities with 41.7 to 48 per cent expression in human breast cancer [26]. The expression of ESA was membranous in the basolateral surface and cytoplasmic in all cases. However, majority of the cases in this study showed cytoplasmic expression possibly due to loss of membranous Ep-CAM expression, which is associated with a higher extent of tumor budding characteristics [27,28].

Explanation of Figures:

Fig. 1: Tubular Carcinoma CD44+(Brown). Fig. 2: Tubular Carcinoma CD24+(Fast Red). Fig. 3: Papillary cystic Adenocarcinoma-Basolateral Staining ESA+ (Vector Blue) Blue ESA+ (DAB). Fig. 4: Colocalized targets CD44 (Vector red) CD24 (Vector Brown). Fig. 5: Carcinoma-sarcoma Colocalized targets CD44+(DAB) CD24- (Fast Red) ESA+ (Vector Blue). Fig. 6: Carcinoma-sarcoma Colocalized targets CD44+ (Brown) CD24- (Fast Red) ESA+. Fig. 7: Carcinoma-sarcoma CD44+ (Brown) CD24- (Fast Red) ESA+ (Vector Blue). Fig 8: Carcinoma-sarcoma CD44+ (Brown) CD24-(Fast Red) ESA- (Vector Blue)
ESA expression was also seen in two of the cases showing thoracic and lymph node metastases; among which one case showed thoracic metastasis and one case with lymph node metastases indicating a correlation between ESA expression and clinico-pathological parameters like size and lymphnode metastasis [26]. There was no correlation between ESA expression and the tumor grade. Out of the 11 cases, ESA expression was seen in 6 cases which were in TNM stage III and four in stage IV, indicating a poor prognosis. However final proof for a direct cancer promoting role of ESA expression in canine mammary tumor needs to be investigated.

Cancer stem cell lineage marker / CD44+/CD24−/ESA−: Out of 24 cases subjected to triple immunohistochemistry two cases of carcinosarcomas were positive for stem cell lineage markers i.e., CD44+/CD24−/ESA− showing co localization expression of CD44 (membranous / cytoplasmic) and ESA with membranous staining. Stem cell lineage markers with the phenotype CD44+/CD24−/ESA− expression were also reported in canines with histological feature of Carcinosarcoma, Cystic papillary Adenocarcinoma and Tubular Adenocarcinoma respectively [8]. This expression in Carcinosarcoma was probably attributed to the induction of an Epithelial Mesenchymal Transition (EMT) as observed in transformed human mammary epithelial cells yielding cells with a CD44high/CD24low antigenic phenotype—precisely the antigenic phenotype that has been ascribed to neoplastic mammary stem cells [29].

However, ESA+ subset of CD44+/CD24− cells were known to be enriched for tumor repopulating capacity, as many as 200 of these cancer cells with the lineage of CD44+/CD24−/ESA− consistently formed tumours when inoculated into SCID/NOD mice and was concluded that CD44+/CD24−/ESA− lineage population had more than 50 fold ability to form tumors [7].

Two positive cases for stem cell markers had thoracic metastasis (Post surgical survival period 160 days) and one case had metastasis to liver and all regional lymph nodes (Post surgical survival period 84 days). Both the cases died and were in TNM clinical stage IV with a poor survivability and average survival period of 122 days.

However the cases in which cells which were positive for stem cells lineage markers i.e. CD44+/CD24−/ESA− as evident from this study, favored distant metastasis which is in agreement with other findings [30].

Cancer cells with CD44+/CD24−/ESA+ phenotype should be subjected to further charac-terization at single cell level, using in vivo, in vitro and ex vivo models to understand their pathway in canine mammary tumors that account for their tumourigenic potential and as possible tumor models for elucidating targets for therapeutic intervention.

REFERENCES