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QUALITATIVE MORPHOLOGICAL ASSESSEMENT OF TUMOR ASSOCIATED LYMPHATIC VASCULATURE IN MAMMARY GLAND NEOPLASIA OF FEMALE DOG IN RELATION WITH SENTINEL LYMPH NODES METASTATIC INFILTRATION

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Abstract

Metastasis, the spread of tumor cells from the primary site to lymph nodes and the distant organs is the most aggressive and specific feature of malignant cancer. The mechanisms by which malignant cells leave the primary tumor, invade lymphatics and metastasize are complex and interconnected being directly related to biological behavior of tumor. However, the lymphatic vasculature is often neglected. The aim of this study is to establish if there is a correlation between the peritumoral and intratumoral lymphatic vascular density and the presence of metastatic infiltration in sentinel lymph nodes of mammary gland tumor. Injecting the coloring solution in mammary gland tumor of nine female dogs it was noted the pattern of lymphatic vessels at the injection site, their density, size distribution area, their trajectory to the first lymph node. Also the status of the tumor draining lymph node was histological assessed. The architecture and density of intratumoral and peritumoral lymphatic vessels was determined by their function in absorbing interstitial fluid together with the tumoral cells, due to their permeability. The coloring solution show the sinuous pattern of peritumoral lymphatic vessels, with a rich chaotic vascular network compared with reticular or plexiform pattern of lymphatic vasculature of healthy mammary gland. The size of peritumoral lymphatic colored area was dependant on the histological type of the tumor and on its size. Also, a malignant tumor size >1cm was associated with the presence of the metastatic infiltration in the first tumor draining lymph node. The density of intratumoral lymphatic vessels was low compared with the peritumoral lymphatics. In conclusion, qualitative morphological assessment of lymphatic vasculature of malignant mammary gland tumors of female dog revealed an increased density of lymphatic vessels in the peritumoral region and a lesser degree intratumoraly. The size of peritumoral lymphatic area was directly related with the presence of metastases in sentinel lymph nodes. Although great progression has been made in revealing the lymphangiogenic markers, additional studies are required to understand the paradoxical significance of intratumoral and peritumoral lymphatics density and lymph nodes metastases for prognosis and development of metastases in vital organs.

Key words: *lymphatic, metastasis, mammary gland tumor, dog.*

INTRODUCTION

Mammary gland is a common site of malignancies in female dogs (Sorenmo et al., 2011; Santos et al., 2014). Although the importance of the lymphatic system in tumor dissemination is fully recognized, lymphatic vessels were not considered as active factors involved in tumor progression. In canine mammary tumors characteristics of lymphangiogenesis is not fully known, and its role in tumor progression and metastasis is not completely understood.

Tumor lymphangiogenesis in most human cancers were associated with increased metastatic potential and lymphatic vessels density becomes another prognostic factor in overall survival of patients (Steven et al., 2014, Alitalo and Detmar 2012). More recently, lymph nodes lymphangiogenesis itself proved to be another circumstance that contributes to the dissemination of tumor cells.

Considering the above, there seems to be multiple causes for the apparition of metastasis (Tamela and Alitalo, 2010).

However, two concepts are conveyed in this direction: one directly related to the tumor type (Sorenmo et al., 2011) and the second connected to the anatomical and functional particularities of mammary drainage (Pereira et al., 2003; Stan 2009, 2012).

Therefore, our study analyzes the morphology and density of peritumoral lymphatic vessels, and their correlation with clinicopatological and staging parameters, namely the presence of metastases in mammary glands sentinel lymph nodes.

MATERIALS AND METHODS

The study was conducted on a group of nine subjects: two female dogs without pathology of mammary glands and seven female dogs who have visible tumors in cranial thoracic, cranial and caudal abdominal mammary gland and in inguinal mammary glands. One female presented mammary tumors on the entire left mammary chain, with impairment of contra lateral cranial abdominal (A1), cranial caudal (A2), and inguinal (I) mammary gland. Subjects with mammary gland tumors received a peritumoral 0.5% Blue Dye injection, in four points: cranial, caudal, medial and lateral of interest area. The same injection was performed subareolar in healthy subjects. The total amount of dye was 0.2ml on the injected point. To facilitate dye diffusion, a gentle massage of injected area was made. Previous to injection, the subjects were sedated, using 0.2mg/kg/bw of ketamine. Subjects were continuously monitored. Twenty four hours later euthanasia was made by IV administration of Euthasol (Virbach AH Inc.), 0.22ml/kg/bw. Regional stratigraphic dissection was performed. Histopatological examination of sentinel lymph nodes was made.

RESULTS

In case of malignancy, lymphatic vessels appear with an aberrant morphology, and a sinuous route, with relatively visible lumen and numerous branches (Fig. 1). Evaluation of lymphatic vessel density was achieved for

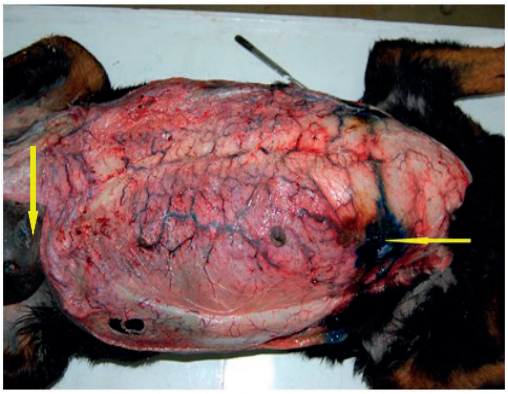

Fig. 1 Numerous branch of lymphatic vessels with contra lateral anastomosis in a subject with cranial thoracic mammary gland and inguinal mammary gland-arrows

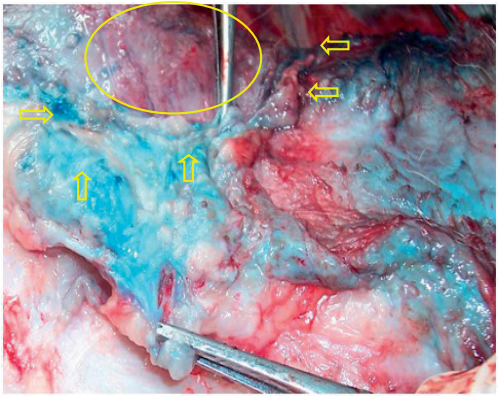

Fig. 2 Chaotic distribution of lymphatic vessels-arrows-around a cranial abdominal mammary gland tumor-oval shape

neoplastic mammary glands differentiated in two areas: a) we noted the presence of numerous well stained peritumoral lymphatic vessels, having a winding pattern, seemingly to supplement tumors lymphatic vessels in adjacent territory (Fig. 2).

These vessels were located especially at tumor-parenchyma interface, with chaotic distribution, without a typical morphology and uneven walls.

The corresponding area was also unclear delimitated (Fig.3); b) within the tumor, lymph vessels have a very low density relative to the peritumoral area or normal parenchyma, with small diameter, that appeared to be lacking in content. Around the tumor we noted a higher density of lymphatic vessels compared to less numerous lymphatic vessels inside the tumors.

Lymphatic's of healthy mammary glands

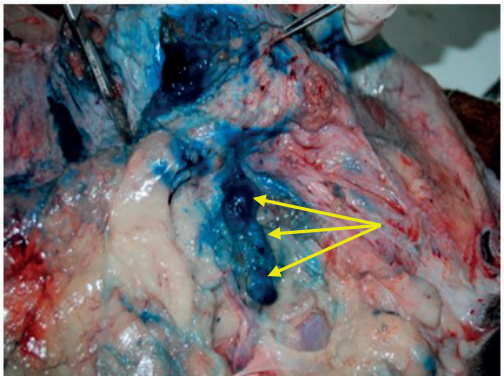

Fig. 3 The inguinal superficial lymph nodes draining a inguinal tumoral mammary gland-small arrows. Well defined lymphatic vessels are seen toward to the sentinel lymph nodes-joined arrow

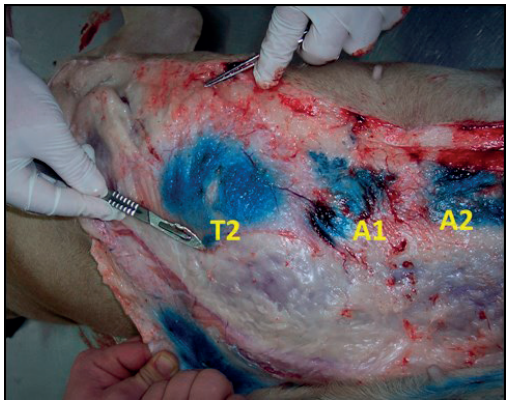

Fig. 4 Well defined lymphatic vascular area in healthy mammary gland. Note the ordered distribution of lymphatics of superficial dermis toward to the areola

were well stained highlighting the two drainage areas: deep and superficial (Fig.4).
Deep injection in mammary gland parenchyma revealed the lymphatic vessels that arise around glandular lobes, with an upward centripetal trajectory, accompanying milk ducts, toward the areola.
Periareolar injection of dye, colored the numerous subareolar lymphatic vessels, which made multiple anastomoses with parenchyma and dermis lymphatic vessels. These lymphatics had a relatively superficial centrifugal path, towards the mammary gland periphery. In absence of pathology, the parenchyma mammary lymphatic vessels were orderly distributed, showing a reticular model in deep parenchyma and a plexiform model toward the surface (Fig.4). In healthy subjects the superficial lymphatic vessels that were stained after dye injection realized well circumscribed areas around the mammary areola. The lymphatic vessels in their path to the corresponding lymph nodes were followed. Lymphatic vessels that drained the neoplastic cranial abdominal mammary gland (A1) and inguinal mammary gland (I) leave the peritumoral area, confluencing and creating well defined afferent lymphatic

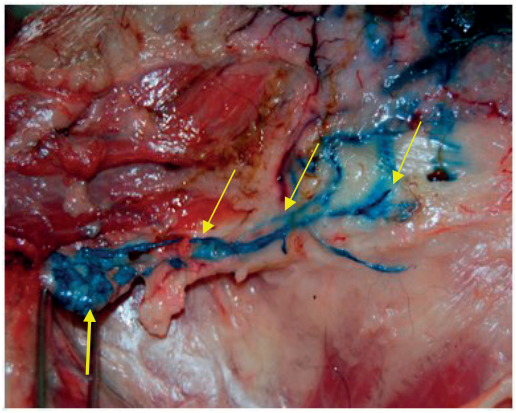

Fig. 5 Axillary lymph center draining cranial thoracic tumoral mammary gland. Vizible lymphatic paths to the proper axillary lymph node

vessels draining into inguinal lymph nodes. This aspect was seen in five subjects. In two subjects, when neoplastic T1 mammary gland was injected, we note that lymphatic vessels has not achieved so much confluence, rather had a separate route to the axillary lymph node (Fig. 5). Regarding lymphatic drainage of healthy mammary glands, axillary lymph center was well stained. In one case it consists in two lymph nodes, proper and accessories respectively, and in the other subject it was colored one axillary lymph node. In caudal direction it was obvious colored inguinal lymph nodes as sentinel lymph nodes for healthy A1 and inguinal mammary glands. In the present research the same lymph nodes were sentinel lymph nodes to neoplastic mammary glands. In a case of neoplastic A1 mammary gland, lymphatic vessels had only a cranial route toward axillary lymph center, without caudal direction to inguinal lymph nodes. Moreover, lymphatic vessels of apparently healthy neighborhood A2 mammary gland were stained.
Table 1 present the study protocol and the results concerning each subject of the study.

Table 1. The study protocol and the results related to the lymphatic drainage of injected mammary gland and the presence of metastatic infiltration into draining lymph nodes

Dog	Characteristics and tumor location	Site of dye injection	Draining lymph nodes	Lymph node metastatic infiltration
1	No tumor present	Left subareolarT1,A1 and right I	Left axillary ln and right superficial inguinal ln	Absent
2	No tumor present	Subareolar Left and right A1	Axillary ln	Absent
3	Right T1,T2	Peritumoral T1	Right axilary and cranial sternal ln	Present
4	Right A1,A2,I	Peritumoral A1	Right axillary ln	Present
5	Left A2,I	Peritumoral A2	Left superficial inguinal ln	Present
6	Left I	Peritumoral I	Left superficial inguinal ln	Absent
7	Left I	Peritumoral I	Left superficial inguinal ln	Present
8	Right and left I	Peritumoral I	Left and right superficial inguinal ln and left popliteal ln	Present
9	Left mammary chain and contralateral A1,A2,I	Left peritumoral T1, I and right peritumoral A1	Left and right superficial inguinal ln, popliteal ln and a lymphatic plexus on the medial aspect of the left tigh	Present in all mentioned draining sites

T1-cranial thoracic; T2-caudal thoracic; A1-cranial abdominal; A2-caudal abdominal; I-inguinal mammary gland; ln-lymph node

Histopathological examinations of lymph nodes which drained the neoplastic mammary glands confirm the presence of metastatic infiltration in suspicious lymph nodes (Fig. 6).

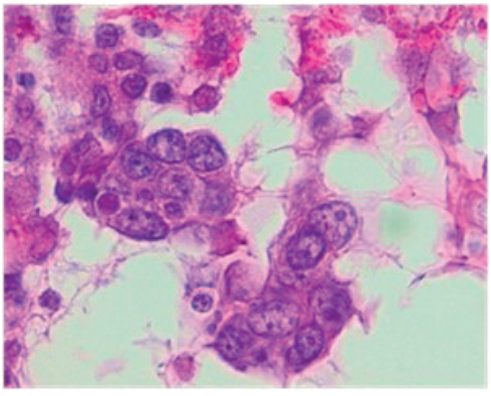

Fig.6 Metastatic infiltration of lymph nodes. The large poliglonal tumor cells possess finely granular cytoplasm. H&E40x

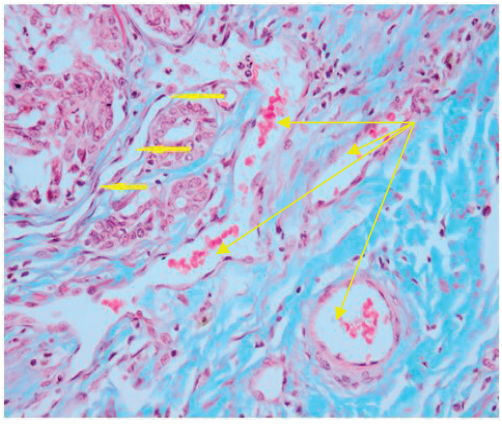

Fig. 7 Lymphatic channel into a mammary carcinoma (arrows) and numerous intratumoral blood vessels-(joined arrows). Goldner Trichrome 40x

Inside the tumor the lymphatic vessels appears like tiny channels with no content (Fig. 7). Contrary, the blood vessels were numerous proving high angiogenesis.

DISCUSSIONS

It is well known that mammary tumor metastasis into regional lymph nodes, their status being considered a major criterion to prognosis and lead to a different approach in therapy (Patsikas et al., 2006; Stan 2012). This feature is due to certain tumors type. Most of mammary tumors are carcinomas, a small percentage sarcomas, fibrosarcomas and osteosarcomas. Epithelial malignant mammary tumors as carcinomas metastasize through lymphatic vessels, while mesenchymal tumors metastasize through blood vessels (Restucci et al., 2003; Sorenmo 2011). We can say that this phenotype feature is due to lymphangiogenesis which is typical in malignancies, involving formation of new vessels from preexisting lymphatic vessel (Tamela and Alitalo 2010, Fidler 2011). In our study, peritumoral lymphatic vessel density assessment showed that there are numerous lymphatic vessels at the tumor periphery, in the adjacent tumor area, at the tumor-mammary parenchyma connection, compared with low lymphatic vessels density inside of the tumor. All these tumors metastasize through the lymphatic system, the first quantifiable station being the sentinel lymph node. But until the appearance of metastasis in sentinel lymph nodes, the tumor cells must leave the primary tumor and through the lymphatic vessels, via lymph nodes, populate another distance sites (Achen 2006, Ungheforen et al., 2011). In current veterinary practice, if a mammary gland tumor is diagnosed, the surgical treatment eradicates entire mammary chain together with the sentinel lymph nodes, if they are identified. This is not an appropriate attitude for several reasons: lymph nodes excision carries a significant morbidity with complications such as lymphoedema, pain, numbness, and limited member movement. Nevertheless, if the sentinel lymph nodes are positive for tumor cells, there is a 40% risk that higher next lymph nodes may also be involved in metastatic disease (Stan 2009). Fine needle aspiration biopsy of sentinel lymph node is a minim invasive alternative to surgical excision. But in carnivores it is difficult to specify which nodes are really the sentinel ones. Numerous studies have been conducted by Patsikas et al, related to the lymphatic drainage in healthy and neoplastic mammary glands in female dogs. Their results showed that healthy mammary glands are drained by the ipsilateral lymph centers, cranial by axillary lymph center and caudal by inguinal lymph nodes. They revealed no connection between contralateral lymph nodes or mammary glands. On the contrary, Pereira et al., 2003, have shown that in the presence of mammary tumor the lymph drainage is completely changed, both in terms of recruitment of new lymph nodes and establishment of new connections. Similar results were obtained by our team in a study regarding the cranial thoracic mammary gland drainage, in which we identify the cranial sternal lymph node as a sentinel lymph node simultaneously with axillary lymph nodes (Stan 2009). Also, it was demonstrated the presence of lymphatic connections between contralateral inguinal lymph nodes (Stan 2012). In addition our results are consistent with those of other researchers, about the existence of a lymphatic plexus, located at the medial aspect of the thigh, involved in lymphatic drainage of neoplastic inguinal mammary gland. (Pereira et al., 2003; Patsikas et al., 2006). Based on these findings, it can be stated that the lymphatic drainage of mammary glands in female dog, shows a great variability. These findings are among the few, who studied the lymphatic vasculature in carnivores' neoplastic mammary glands. Therefore, there is insufficient anatomic data concerning lymphatic vascularization in neoplastic mammary glands in the bitches and is a lack of comparative studies focusing on lymphatic vessels in mammary neoplasia. To metastasize in various locations, tumor cells have to cross lymphatic system barriers. If we considered that initial lymphatic's are blind ended, without basement membrane, being fenestrated, it can be said this features are real facilities to tumor dissemination, compared to

blood vessels (Olivier, 2004). Furthermore, our results showing the aberrant distribution, high peritumoral density and sinuous route of the lymphatic vessels, these features could be the morphological explanation of easy entrance of the tumoral cells into the peritumoral lymphatic vessels. In our research, within the tumor the lymphatics appear to be dysfunctional with no content. The explanation is logical if we consider that inside of tumor the interstitial pressure is high due to uncontrolled multiplication of tumor cells. Under these conditions, entry of tumor cells could be partially affected. Another aspect which worth taking into account to explain the low density of intratumoral lymphatic vessels is the possibility that intratumoral lymphangiogenesis is inhibited rather than induced. Padera et al., reported the absence of intratumoral lymph vessels in an experimental induced tumor model in rodents. Therefore, metastatic cells can easily invade preexisting lymphatic vessels or the new formed peritumoral vessels due to induction of lymphangiogenesis by tumor itself. There are studies showing that tumor cells can use as transporting agents chemokines or lymphocyte or antigen presenting cells to gain access into lymphatic vessels, thus increasing the dissemination possibility. Many types of tumors express themselves vascular endothelial growth factors VEGF- C and VEGF-D and the presence of these factors induce active lymphangiogenesis, sentinel lymph nodes metastasis and distant metastasis (Saharinen et al., 2010). All these are leading to a poor prognostic. Level of VEGF-C and VEGF-D and their corresponding receptor are increased in determination made in the presence of breast tumor in woman (Kodera et al., 2011). There is not a correlation between tumor angiogenesis and lymphangiogenesis as long as each process is mediated by the specific markers (VEGF-A and VEGF-B for angiogenesis and VEGF-C and VEGF-D for lymphangiogenesis). Studying dogs and cats carcinomas, it was showed that VEGF is strongly expressed in the cytoplasm of tumor cells, occasionally in carcinoma stroma cells and infrequent in endothelial cells of tumor vessels without a correlation between VEGF and lymphatic involvement. These results are not in agreement with those obtained by Restucci et al, 2010, who showed existence of a strong correlation between VEGF expression and increased density of blood tumor vessels. The same question arises in case of lymphatic dissemination. Both, human and animal experimental models on clinicopathological data, indicate that adjacent tumor lymphangiogenesis is associated with sentinel lymph nodes metastasis (Padera et al., 2002, Sleeckx et al., 2014). These findings are similar to our results of the present research in which we found a high density of peritumoral lymphatic vessels on a large area, all these findings being associated with presence of metastatic infiltration in sentinel lymph nodes. Also, morphological changes of newly formed vessels from the tumor vicinity, with sinuous feature and multiple anastomoses, increased the lymphatic endothelial properties in adhesion of tumor cells, facilitating the spread of cancer.

More recently, it was found that VEGFs secreted by primary tumor, induce lymphangiogenesis in sentinel lymph nodes that drained the tumor territory even before the spread of tumor cells (Steven et al., 2014). Based on these findings and considering our results from present research, we hypothesized that the lymphatic network is already established when the tumor becomes invasive.

Many anti-lymphangiogennic therapies have been proposed, especially in women breast cancer. Kodera et al, have realized a study on VEGF receptors blocking activity, clinically proven to be an inhibitor of tumor angiogenesis, concluded that the same therapy may be beneficial in breast cancer, by suppressing lymphangiogenesis and axillary metastasis. The therapy consists of administration of Sunitinib, an VEGF-3 activity inhibitor. Not only VEGF and their ligands VEGF-C and VEGF-D are involved in lymphatic dissemination (Fidler 2011). Based on the findings that dilatation and spreading of tumor cells are inhibited by administration of non steroidal anti-inflamatory drugs (NSAIDs). Karnezi et al., 2012, have shown a direct link between regulation and involvement of prostaglandins in metastatic dissemination. All these studies

lead to the creation of a new therapy, anti-angiogenic therapy, whose direct target are VEGF, especially the VEGF-C and VEGF-D which has been shown to inhibit lymphangiogenesis and lymph nodes metastases. Therefore, it can be said that lymphatic vessels which are actively involved, both morphologically and functionally in metastatic dissemination, may be considered therapeutic targets in inhibition of tumor dissemination.

CONCLUSIONS

Our results demonstrate that increased peritumoral lymphatic density and a large area of peritumoral lymphatic vessels are associated with the presence of metastasis in sentinel lymph nodes. These features could be considered as prognostic factors in development of mammary tumor in female dog. In veterinary medicine, assessing the efficiency of anti-angiogenic and anti-lymphangiogenic therapy, alone or in combination with chemotherapy, as potential inhibitors of tumor growth and metastasis would be of great interest.

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