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Immunohistochemistry based approach to study cross reactivity of certain commercially available antibodies with mammary tumor cell proteins of dog

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ABSTRACT

A mammary tumor is a neoplasm in mammary gland. Immunohistochemical analysis of protein biomarkers like E-cadherin, Ki-67 and COX-2 etc. has a great importance in identifying the neoplastic cells. The current study aims to analyze the cross reactivity of the commercial antibodies against these proteins with Canine Mammary Tumor (CMT) tissue, which usually are used for detection of human mammary tumor. The present work was conducted with 20 clinical cases of surgically excised CMT using standard protocols. The expression of E-cadherin, Ki-67 and COX-2 proteins were observed in cell membranes, nucleus and cytoplasm respectively in the positive CMT tissue. These commercial antibodies cross reacted with the biomarker proteins of CMT tissue. The results suggest that, these proteins might have some conserved regions in their corresponding genes, which needs further study to understand the phenomenon. These commercial antibodies can also be used for diagnosis and prognosis of CMT in the dog.

Keywords: Biomarker, canine mammary tumor, diagnosis, neoplasm, prognosis

1. INTRODUCTION

A mammary tumor is a neoplasm associated with the mammary gland in the female dogs. They occur as single or multiple nodular forms in posterior mammary glands. Approximately 50% of CMT are malignant (Benavente et al., 2016). Different growth factors and hormones are associated with the development of neoplasm in mammary tissues. The rate of recurrence and metastasis leads to poor prognosis in malignant CMT. Hence early diagnosis of CMT using biomarkers could be an important tool for judging a patient's prognosis and designing therapy (Monteiro et al., 2016).

Recent advances in tumor biology have identified several biomarkers that augment the method of tumor classification (Kaszak et al., 2018). Several immunohistochemical markers in human, canine and feline mammary tissues are used to provide information regarding the histogenesis and prognosis of these tumors (Kaszak et al., 2018). Despite intensive clinico-pathological investigations, a little is known about the prognosis of canine mammary tumor. Therefore, accurate and additional prognostic aids are required to be identified. Immunohistochemical markers such as c-erbB-2, SFRP 2, Sialyl Lewis X and EGFR etc. have been associated with prognosis of CMT which are also found in women suffering from breast cancer (Dutra et al., 2004; Lee et al., 2004; Pinho et al., 2007; Gama et al., 2009).

E-cadherin is a calcium-dependent cell adhesion molecule and its low expression is associated with tumor identification and invasiveness. Epithelial Mesenchymal Transition (EMT) in tumor tissue correlates with loss of E-cadherin expression in cells (Li et al., 2017). Similarly, Ki-67 is a nuclear protein and associated with cell proliferation which can be detected during M phase of the cell cycle (Tokuda et al., 2017). High expression of p53 and Ki-67 leads to worse prognosis of the disease (Pan et al., 2017). Canine-specific ELISA kit has already been evaluated to study the efficacy of Ki-67 as a biomarker for tumor detection (Neumann et al., 2017). In human, Ki-67 and p53 biomarkers are mostly associated with human breast cancer (Pan et al., 2017). COX-2 expression and angiogenesis are also correlated with mammary carcinomas. Recent studies suggest that COX-2 expression is also associated with lymph node metastasis (Queiroga et al., 2011). It has been suggested that COX-2 inhibitors could be an alternative for treating CMT in female dogs

(Nunes et al., 2019). Keeping in view the above facts, the present work aimed to study the Immunohistochemical analysis of commercially available non-canine antibodies with CMT proteins of dogs.

2. MATERIALS AND METHODS

Ethical approval

Ethical approval is not required, as the study was conducted from the surgically removed tissue samples undertaken by the veterinary surgeons to cure the disease, taking adequate measures to minimize pain.

Sample collection

The present work was conducted on 20 clinical cases of surgically excised CMT in Labrador breed of dogs (4 to 10 years of age group) presented to the College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, India. The CMT tissues were surgically removed by the veterinary surgeons as a routine therapeutic practice to cure the dogs harboring mammary tumor. Preliminary diagnosis of mammary tumor was made based on clinical examination and histopathological findings. The list of chemicals and antibodies used in the study are given in table 1.

Tissue processing

The mammary tumor tissues were cut in to small pieces and kept in tissue cassettes. The cassettes were kept in 10% neutral buffered formalin solution and stored for 72 hrs. Tissue dehydration was done by putting the tissue sections in a series of increasing concentration of ethanol with 50%, 80%, 90% and 100% respectively for 1 hour each followed by treatment with acetone for 30 minutes and cleaning by xylene. Tissues were embedded in paraffin wax at 65°C. For further processing, 4-5 µ paraffin embedded tissue sections were cut using a microtome, kept on the slides and stained with routine Hematoxylin and Eosin stain, following the protocol by Luna,1968 (Luna, 1968).

Heat-Induced Epitope Retrieval

Heat-Induced Epitope Retrieval (HIER) of the antigenic proteins from the CMT tissues were done with different temperature and time setting as per the manufacturer's instructions (EZ-Retriever TM System, Biogenex, CA, USA)

Immunohistochemical analysis

The Paraffin was removed using xylene 2 times for 5 minutes each. The tissue samples were hydrated with ethyl alcohol (100%, 95%, 70%) for 5 minutes each followed by washing with distilled water. The antigens were retrieved for 20 minutes. The slides were cooled at room temperature and washed with Phosphate Buffer Saline with Tween-20 (PBST). The tissue slides were blocked with H_2O_2 for 15 minutes. The slides were again blocked with horse serum for 30 minutes. Respective primary antibodies diluted in 1:100 ratio with PBS, were treated to the tissue slide and kept at 4ºC overnight. Biotinylated secondary antibodies were treated, incubated at room temperature for 45 minutes and washed with PBST. Finally the slides were developed with DAB and stained with Hematoxylene for 3-5 minutes followed by washing with Scott's tap water.

3. RESULTS AND DISCUSSION

In all the CMT positive cases, less expression of E-cadherin was observed in cell membranes and in cytoplasm. The reaction in the membranes was strong in the ductular epithelial cells as shown in Figure 1-A and 1-B. Similarly Ki-67 expression was noticed and confirmed by nuclear staining as shown in Figure 2-C1 and 2-C2. The expression of COX-2 was also observed in the malignant tumors as shown in Figure 2-D1 and 2-D2.

The incidence rate of mammary tumor is high in female dogs of Teckel, Poodle and Labrador breeds of dogs in the range of 5 to 10 years of age groups (Benavente et al., 2016). The findings of our studies are similar to the previous study by Rutteman, 2001 (Rutteman et al., 2001). Our histopathological analysis confirmed malignancy of these tumors. In the current study, E-cadherin expression in CMT was tested irrespective of localization of the proteins in CMT positive cells and intensity of the staining. The results of our studies are similar with the reports of Li, et al. 2015 and Nowak, et al., 2017, since 80% of the neoplasms were malignant, which explains about less E-cadherin expression in neoplastic tissues (Li et al., 2017; Nowak et al., 2015). Similarly Ki-67 is an excellent biomarker for the diagnosis of malignancy in CMT, which usually is associated with cell proliferation and more often external and internal factors, influences its expression in different tissues (Jurikova et al., 2016). Thus, antibodies directed to Ki-67 can be used in immunohistochemical study of CMT (Neumann et al., 2017). Its localization within the nucleus during the cell cycle may be of diagnostic advantage. Our results are similar with the data, reporting Ki-67 as a marker for diagnosis of CMT (Tavasoly et al., 2013).

COX-2 is another biomarker associated with breast tumor. The role of COX-2 in tumor progression is still unknown. Incidence of breast cancer reduces with long-term use of NSAIDs, but no such information is available in dogs (de Pedro et al., 2015; Hugo et al., 2015). Further studies are needed to address this issue. Our results showed that CMT expresses significant amount of COX-2, suggesting its potential value in CMT diagnosis.

We overall suggest that all these antibodies which are meant for diagnosis of human breast tumor, can also be used for the diagnosis of CMT.

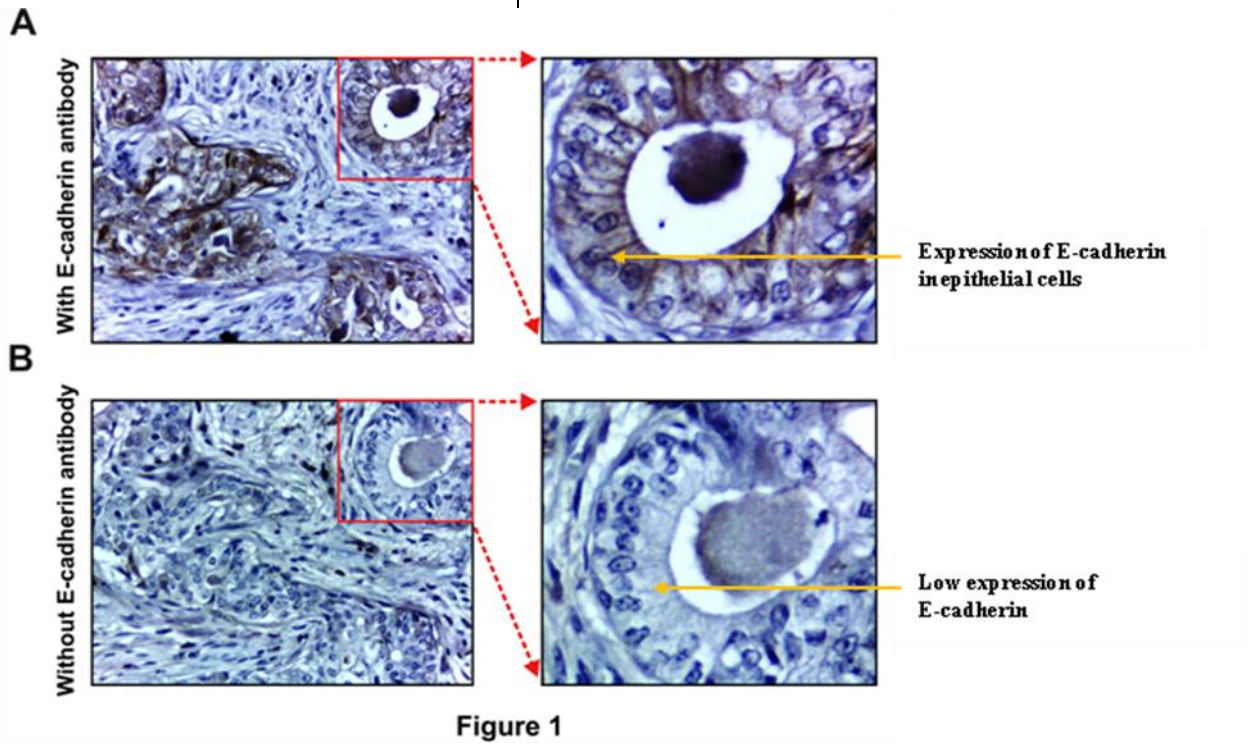

Figure 1: A: Reactivity of Non-canine E-cadherin specific antibody with CMT tissue; B: CMT tissue without treatment of Non-canine E-cadherin specific antibody.

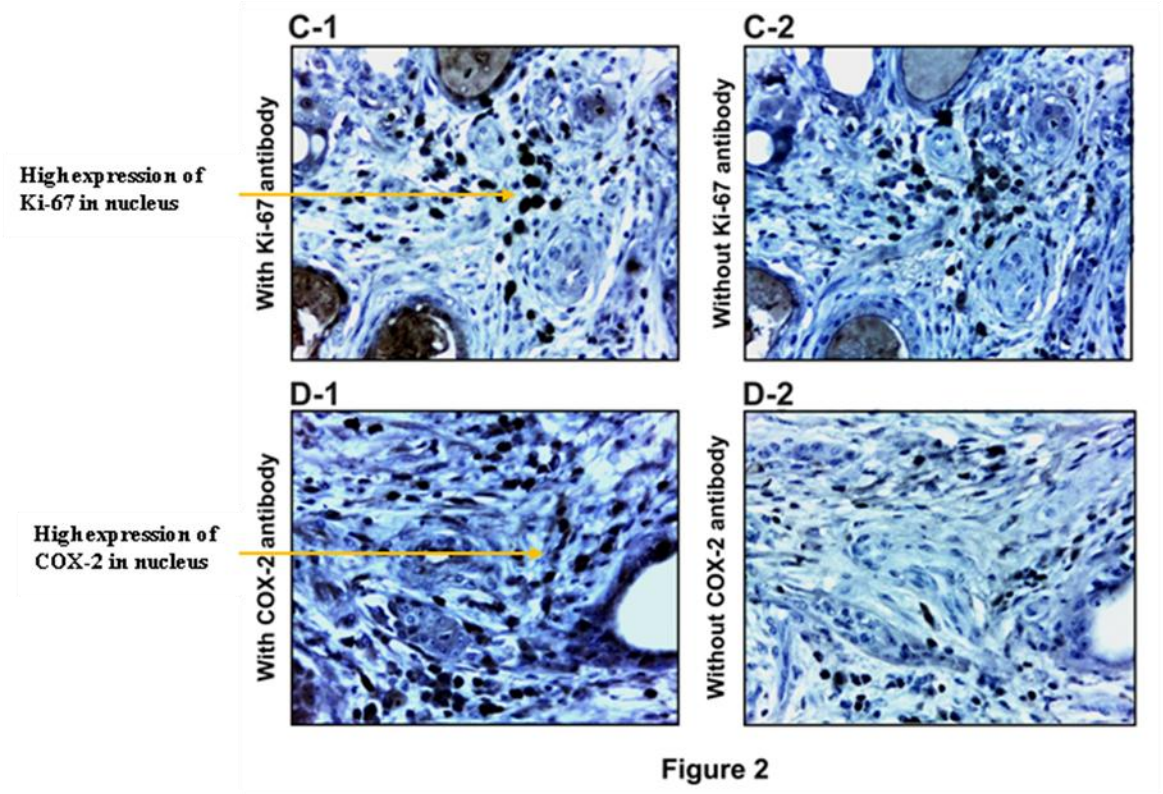

Figure 2: C-1, D-1: Reactivity of Non-canine Ki-67 and COX-2 specific antibody with CMT tissue respectively, C-2, D-2: CMT tissue without treatment of Non-canine Ki-67 and COX-2 specific antibody.

Table 1: List of chemicals and antibodies used in the study

Chemicals/Antibodies	Manufacturer
Paraffin wax	Leica Microsystem, Thermo Scientific, USA.
Hematoxylin and Eosin stain	Bio Lab Diagnostics Pvt. Ltd, Mumbai, India.
EZ-Retriever TM System	BioGenex Laboratories Inc., California, USA.
Antibody to E-cadherin	Cell Signaling Technology, Inc., United States.
Antibody to Ki-67	Vector Laboratories Inc., Burlingame, USA.
Antibody to COX-2	Novus Biologicals, LLC, Centennial, USA.
Anti-E-cadherin antibody	GeneTex, Inc. California, USA.
Anti-Ki-67 antibody	Boster Biological Technology, CA, USA.
Anti-COX-2 antibody	Boster Biological Technology, CA, USA.
Di-Amino Benzidine (DAB)	Sigma-Aldrich, Missouri, United States.
Scott's Tap Water	Sigma-Aldrich, Missouri, United States.

4. CONCLUSIONS

The current study showed that, non-canine commercial antibodies to E-cadherin, Ki-67 and COX-2 proteins raised in mouse or rabbit can also be used for molecular diagnosis of CMT in dogs. The reason for such cross reactivity may be attributed towards conserved domains of antigenic proteins irrespective of species, which needs further study to understand the phenomenon.

Conflict of interest: The authors declare that they have no conflict of interest.

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6. ACKNOWLEDGEMENTS

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