

WT1 Expression as a Potential Biomarker of Malignancy in Canine Breast Tumor

La Expresión de WT1 como un Biomarcador Potencial Maligno en un Tumor de Mama Canino

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SUMMARY: Veterinary oncology is very important nowadays to get a better understanding of human carcinogenesis. Estrogen receptor, progesterone receptor and Human Epidermal Growth Factor receptor 2 are frequently evaluated by immunohistochemistry (IHC) in human breast tumor. WT1 is an oncogene, its overexpression has been detected in leukemia and diverse solid tumors like breast cancer, lung cancer and mesothelioma in humans. WT1 expression was evaluated in 15 canine breast tumors (CBT) diagnosed by histopathological analysis to find its relationship with neoplasia and malignancy. IHC and RT-PCR were performed in CBT tissues. Fisher's test was used to analyze WT1 relationship with malignancy. Of the 15 tumors, 9 (60 %) were diagnosed as benign and 6 (40 %) were malignant. With IHC, WT1 expression was positive only in biopsies diagnosed as malignant. Expression of WT1 by RT-PCR was detected in 14 of the 15 tumors (93.33 %) as well as in control healthy mammary gland. Clinical significance: This study describes for the first time a close correlation between CBT and a positive result for WT1 expression with IHC; hence, it can be used as a biomarker for this neoplasia and as an indicator of malignancy. RT-PCR analysis also showed to be good option to detect WT1 expression. These results will be useful to further investigations to elucidate WT1-related signaling pathways in CBT. Also to know molecules that regulate the translation of this protein as a marker for tumor progression.

KEY WORDS: Canine Breast Tumor; WT1; Histological type; Malignancy; Immunohistochemistry.

INTRODUCTION

Veterinary oncology is of vital importance nowadays to extend our knowledge in molecular, preventive, epidemiological and therapeutic aspects of carcinogenesis due to the shared similarities among species of mammals (Fürdös *et al.*, 2015).

The canine breast tumor (CBT) is very frequent, representing 42 % of all tumors in female dogs (Zatloukal *et al.*, 2005), approximately 50 % of these are diagnosed as malignant and metastasis is the main cause of death (Klopfleisch *et al.*, 2011). The risk factors in CBT are age,

breed, genetic predisposition, hormones and growth factors, cyclooxygenase 2 expression and diet (Sleeckx *et al.*, 2011; Sorenmo *et al.*, 2011) which are very similar to those in human breast cancer.

The expression of markers such as estrogen receptor (ER), progesterone receptor (PR), and receptor 2 of epidermal growth factor (HER2) are frequently evaluated by immunohistochemistry (IHC) in human breast tumors (Reis-Filho & Tutt, 2008).

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In CBT, the expression of different genes is responsible for tumor growth such as epidermal growth factor, growth inhibitors, cell proliferation, antiapoptotic, DNA repair and angiogenic genes have been previously identified and related to this neoplasia (Klopfeisch *et al.*).

WT1 (Wilm's tumor gene), is a transcription factor that modulates the expression of genes involved in sexual differentiation, cell proliferation and apoptosis (Hewitt *et al.*, 1995), in embryogenesis it regulates the genitourinary system (Fernández *et al.*, 2009), whereas in adulthood is found also in CNS and hematopoietic tissues (Yang *et al.*, 2007). Currently this gene is recognized in humans as an oncogene because it is overexpressed in leukemia and various solid tumors such as breast cancer, lung cancer and mesothelioma (Xu *et al.*, 2013). The presence of WT1 has been detected by immunohistochemistry in 494 gastrointestinal, pancreatic, urinary tract, male and female genital organs, lung, brain, skin, soft tissue and bone tumors (Nakatsuka *et al.*, 2006).

Recent reports on WT1 expression analysis on human breast cancer by immunohistochemistry and RT-PCR show controversial results, Silberstein *et al.* (1997) found that expression was null in both normal mammary tissue and neoplastic cells, whilst, Loeb *et al.* (2001) detected WT1 in neoplastic cells, specifically carcinomas and noted absence in normal mammary tissue, moreover, it has been shown that WT1 expression in breast cancer is related to a poor prognosis (Miyoshi *et al.*, 2002) and silencing of WT1 with siRNA causes a decrease in cell proliferation, making it a promising therapeutic target for this neoplasia (Zapata-Benavides *et al.*, 2002). The objective of the present study was to analyze the expression of WT1 in samples of CBT by PCR and IHC to find the relation between this transcription factor with the tumor malignancy.

MATERIAL AND METHOD

Tissue samples: Collection of CBT biopsies was carried out in veterinary clinics of small species. The protocol was endorsed by the local committee of bioethics and animal welfare, following the national animal welfare act (NOM-062-ZOO-1999). Also, normal mammary tissue and kidney were collected as controls. Patient's age and ovariohysterectomy data were collected at the time of specimen collection. An informed and signed consent letter was obtained from the owners of the dogs that participated in the study.

Histopathological and IHC analysis. Each biopsy was divided in 2 sections, one was submerged in 10 % formaldehyde for histopathological studies and the other was stored at -20 °C in PBS 1X (pH: 7.4) for RNA extraction.

Fixation were in 10 % formaldehyde during 24 h, later, dehydration was performed with an ethanol gradient (70, 90, 96 and 100 %, 2 h each), 2 h in xylene and embedded in paraffin blocks at 60 °C for 2 h. Slides of 5 mm thickness were made and rehydrated for hematoxylin and eosin staining.

Also, the CBT biopsies were analyzed by IHC using a mouse monoclonal antibody anti-WT1 (1:100, clone: 6F-H2, Dako Cytomation Inc®). Mouse and rabbit specific HRP/DAB (ABC) detection IHC kit (ab64264, Abcam®) was used. Positivity was identified with 3, 3'diaminobenzidine (DAB), and nuclei were counterstaining with Mayer 's hematoxylin. Sections of kidney were used as positive controls. Samples were analyzed by light microscopy.

Molecular analysis. For the molecular analysis, we use frozen sections of the biopsies, 50 mg of tissue were used to extract the RNA with Trizol® Reagent (Life Technologies, Invitrogen)™ following the manufacturer's instructions. Integrity was assured by spectrophotometry at 260 nm of absorbance and by electrophoresis at 90 Volts for 15 minutes in an 0.8 % agarose gel.

Conversion to complementary DNA (cDNA) was performed using the enzyme Reverse Transcriptase (RT, Life Technologies, Invitrogen™) under the manufacturer's instructions. Subsequently, 1 ml of cDNA was amplified to detect WT1 and b-actin gene was used as internal control. For the amplification of WT1 the following pair of primers were used: Fw5'GAG AACA CCA TAC CAG TGT GA-'3, and Rw5'GTT TTA CCT GTA TGA GTC CT-'3 using previously established conditions (Brouillette *et al.*, 2000) (800 bp product). For canine b-actin gene the primer set was Fw5'GTG GGG CGC CCC AGG CAC CA-3' and Rw5'GTC CTT AAT GTC ACG CAC GAT TTC-3' (200 pb). Then PCR products of WT1 and b-actin were analyzed by 1 % agarose gel electrophoresis.

Statistical analysis. The correlation between WT1 expression and malignancy of the tumor were performed using contingency tables using Chi-square test and/or Fisher's exact test, all analyzes were performed using the statistical software SPSS version 15.0. For all tests, the significance level was $P < 0.05$, 1-b = 80 % and two-tailed curves.

RESULTS

In this study, 17 biopsies were collected. The animals studied showed an average age of 10 years and 26 % had ovariectomy.

In the histopathological study of the 17 biopsies, 15 were diagnosed as CBT, 9 (60 %) were classified as benign and 6 (40 %) malignant, 5 (55.55 %) benign tumors were from whole females and 4 (44.44 %) from castrated ones and all samples diagnosed as malignant were from whole females ($P = 0.103$, Fisher's Exact). Four types of CBT were identified: benign mixed tumor and adenoma (benign tumors) and Carcinoma (simple, solid and squamous) and osteosarcoma (malignant tumors), which are shown in Figure 1.

On the other hand, HIC analysis showed that WT1 expression was positive in all biopsies diagnosed as malignant and in kidney tissue (Fig. 2A-D), whilst, most of benign tumors and healthy mammary gland showed negative results (Fig. 2E-H).

Molecular analysis showed that b - actin expression (200 bp) was detected in all biopsies, whereas WT1 expression (800 bp) was observed in 14 of the 15 tumors (93.33 %) and in healthy mammary gland, moreover, no amplification was observed in kidney tissue (control), these analyses are detailed in Figure 3.

Finally, Fisher's exact test confirm that WT1 analysis by IHC correlates efficiently with the malignant nature of the tumors ($P = 0.002$), whereas the molecular technique of RT-PCR was not useful for this purpose ($P = 1,000$).

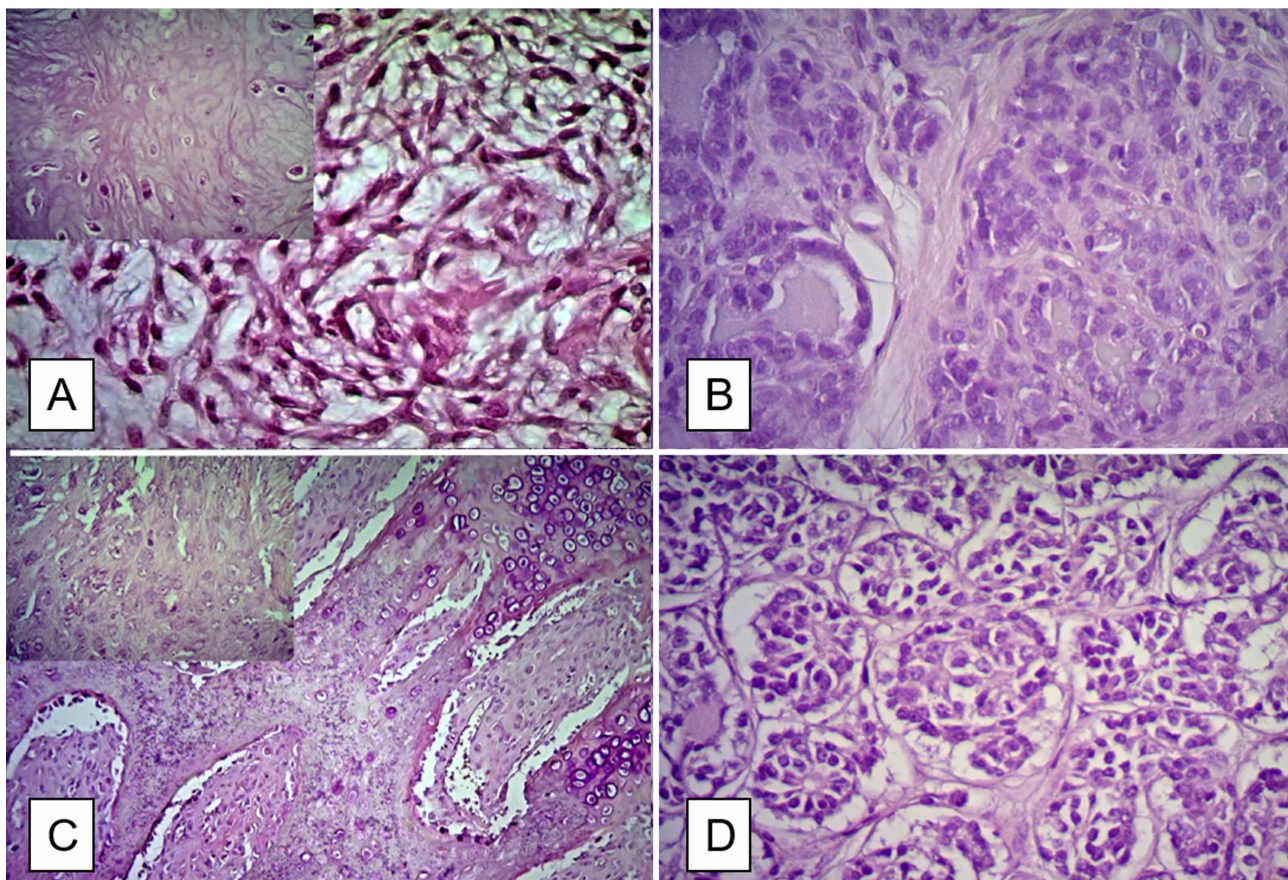


Fig.1. Micrographs of canine breast tumor biopsies analyzed. A) Mixed breast tumor, box: chondroid tissue, H & E. B) Simple adenoma, H & E. C) Osteosarcoma, box: pleomorphic myoepithelial tissue, H & E. D) Simple carcinoma, H & E. Original objective 10x, box: 40x

DISCUSSION

Approximately 6 million dogs are diagnosed with cancer each year in the U.S. and 27 % will die from it (Printz,

2011). Recent studies report that about 50 % of the analyzed biopsies are classified as malignant (Gupta *et al.*, 2012), our

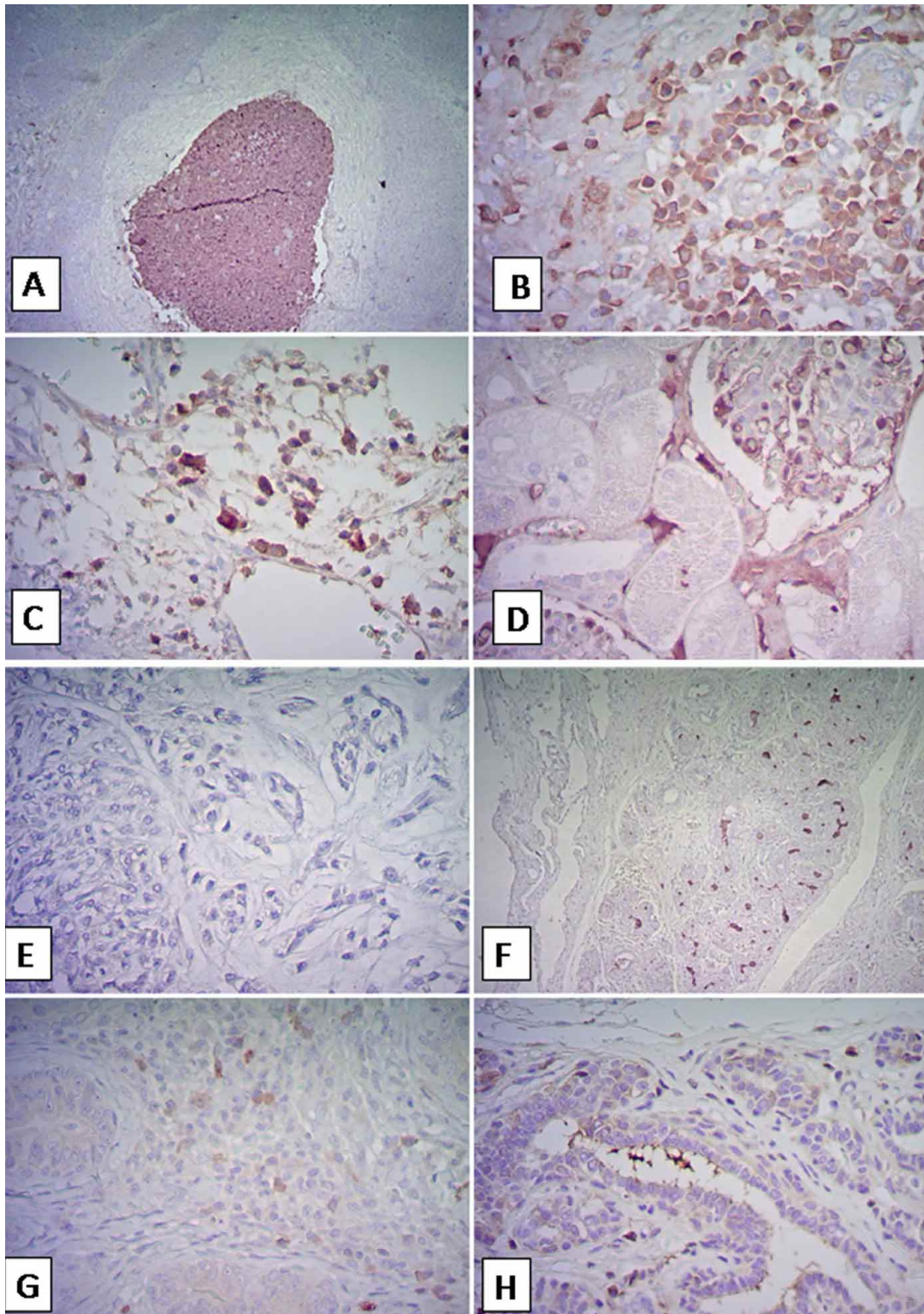


Fig. 2. Micrographs of the IHC analysis of WT1 expression in canine breast tumor biopsies with positive immunoreactivity. A) Solid carcinoma. B) Solid carcinoma. C) Osteosarcoma. D) Kidney Tissue. Micrographs of the analysis of WT1 expression in canine breast tumor biopsies with negative or non-specific immunoreactivity. E) Benign mixed tumor. F) Benign mixed tumor (reactivity in proteinaceous matter). G) Simple adenoma (reactivity to lymphocytes and macrophages). H) Healthy mammary gland (reactivity in the lumen). Original objective 10x.

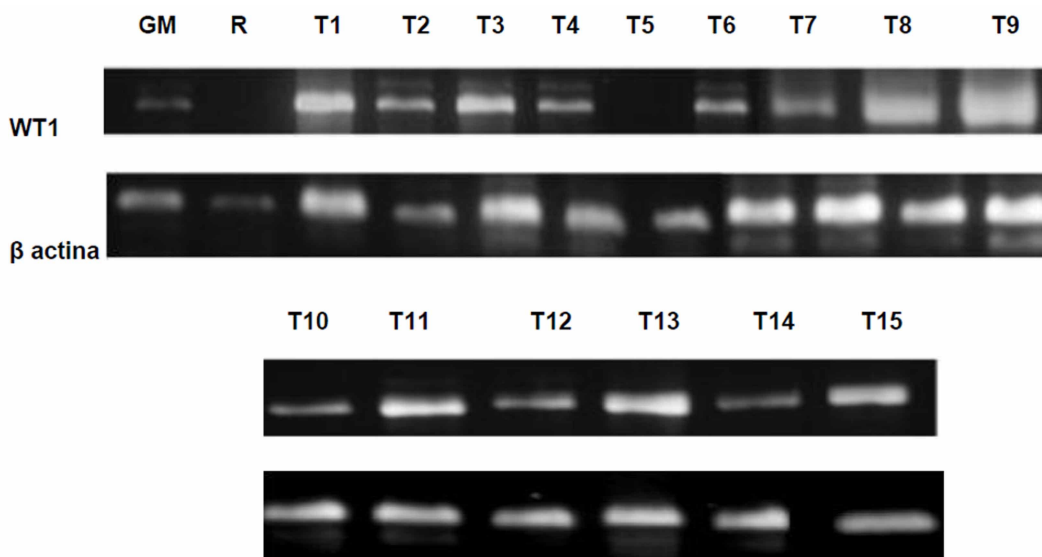


Fig. 3. PCR Analysis of WT1 expression in canine breast tumor biopsies. Benign tumors: T1-T9, malignant tumors: T10-T15.

findings in this study show 40 % of malignant CBT, this difference may be due to the high number of castrated females included in our study, it has been shown that sterilization has a protective effect against malignant tumors, being 3 to 7 times less frequent than in whole females (Perez Alenza *et al.*, 2000), moreover, castrated females with CBT are diagnosed more frequently as benign tumor (Sorenmo *et al.*).

On the other hand, the most frequent CBTs are carcinoma in the case of malignant and mixed tumor on the benign ones (Sleeckx *et al.*), which coincides with the results obtained in this study.

In this study, we evaluated the relationship between WT1 gene expression and the malignant nature of CBT, because it is now considered an oncogene, appearing in several types of solid tumors (Xu *et al.*). WT1 expression was detected in both benign and malignant tumors, as well as in healthy mammary gland. Recent studies report WT1 expression in neoplastic tissue, but not in healthy mammary gland (Miyoshi *et al.*), however, other authors have observed expression in both tumors and healthy tissue (Silberstein *et al.*), including mammary gland (Yang *et al.*).

Nowadays, IHC has been shown to be efficient to evidence the presence of biomarkers associated with carcinomas. WT1 protein expression has been found in various breast tumors (Nakatsuka *et al.*). In this study, we proved a positive cytoplasmic immunoreactivity in malignant CBT biopsies, formerly, a positive result for WT1 in

immunohistochemistry must be at the nuclear level due to its transcription function, however, this has changed because the phosphorylation in the WT1 DNA binding domain alters the DNA affinity and cellular distribution of WT1, resulting in a retention in the cytoplasm (Nakatsuka *et al.*).

We observe a close correlation between malignant CBT and a positive result for WT1 expression with IHC, hence, it can be used as a biomarker for this neoplasia and as an indicator of malignancy. On the other hand, the RT-PCR technique showed expression in all samples including healthy breast tissue, representing an unhelpful option for diagnostic and prognostic purposes. We infer that there is some interruption in the translation process in the tissues of benign mammary tumor and normal breast tissue. Therefore, we consider necessary to further investigate WT1-related signaling pathways in tumor tissues to know molecules that regulate the translation of this protein and to evaluate its efficiency as a marker for tumor progression.

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RESUMEN: La oncología veterinaria es muy importante hoy en día para comprender mejor la carcinogénesis humana. El receptor de estrógeno, el receptor de progesterona y el receptor 2 del factor de crecimiento epidérmico humano se evalúan con fre-

cuencia mediante inmunohistoquímica (IHC) en tumores de mama humanos. WT1 es un oncogén, su sobreexpresión se ha detectado en leucemia y en diversos tumores sólidos como el cáncer de mama, cáncer de pulmón y mesotelioma en humanos. La expresión de WT1 se evaluó en 15 tumores de mama caninos (TCC) diagnosticados mediante análisis histopatológico para encontrar su relación con la neoplasia y la malignidad. IHC y RT-PCR se realizaron en tejidos CBT. La prueba de Fisher se utilizó para analizar la relación de WT1 con la malignidad. De los 15 tumores, 9 (60 %) fueron diagnosticados como benignos y 6 (40 %) fueron malignos. Con IHC, la expresión de WT1 fue positiva solo en biopsias diagnosticadas como malignas. La expresión de WT1 por RT-PCR se detectó en 14 de los 15 tumores (93,33 %), así como en el control de la glándula mamaria sana. Importancia clínica: este estudio describe por primera vez una estrecha correlación entre la TCC y un resultado positivo para la expresión de WT1 con IHC; por lo tanto, se puede utilizar como un biomarcador para esta neoplasia y como un indicador de malignidad. El análisis por RT-PCR también demostró ser una buena opción para detectar la expresión de WT1. Estos resultados serán útiles para futuras investigaciones para dilucidar las vías de señalización relacionadas con WT1 en la TCC. También para conocer moléculas que regulan la traducción de esta proteína como marcador de progresión tumoral.

PALABRAS CLAVE: Tumor de mama canino; WT1; Tipo histológico; Malignidad; Inmunohistoquímica.

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