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Genomic copy number variation associated with clinical outcome in canine cutaneous mast cell tumors

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ABSTRACT

Mast cell tumors are the most common malignant cutaneous tumors in dogs. Although there are several prognostic factors involved, the clinical and biological behavior of this type of tumor varies greatly, making the best choice of treatment challenging. Molecular techniques can be used to evaluate a large number of genes involved in the neoplastic process and aid in the selection of candidate genes related to prognostic and predicting factors. Identification of the genes associated with tumor development and progression can be performed through the analysis of numerical and structural changes in DNA isolated from tumor cells by array comparative genomic hybridization (aCGH). The aim of this study was to compare copy number variations (CNVs) in cutaneous mast cell tumors of dogs that survived less than six (ST < 6) and >12 months (ST > 12) from the date of diagnosis. Ten animals were used: four from Group ST > 12 and six from Group ST < 6. Genomic DNA was extracted, and aCGH was performed using Agilent *Canine Genome CGH Microarray* 4 × 180 (ID-252 552 – Agilent, USA). Data analysis was carried out using *Nexus program version 5.0* (Biodiscovery, USA). The group ST > 12 presented 11 ± 3.3 CNVs, while the ST < 6 group presented 85 ± 38.5 CNVs. Regions of loss in *PTEN* and *FAS* as well as regions of gains in *MAPK3*, *WNT5B*, *FGF*, *FOXM1* and *RAD51* were detected in mast cell tumors with shorter survival times, and thus, worst prognoses, allowing for the identification of potential candidate genes for more detailed studies.

1. Introduction

Mast cell tumors (MCT) are of particular interest in veterinary oncology for being the most frequent cutaneous neoplasia in dogs and for having variable characteristics and biological behavior within the same histopathological grade, which makes it difficult to provide a prognosis based on these features (Kiupel et al., 2011).

The characterization of the canine genome, enabled through the use of various methods that often involve a limited number of genes, can be used for the identification of several genes related to the carcinogenic process (Breen, 2009; Ojopi and Neto-Dias, 2004). Array comparative genomic hybridization (aCGH) is a technique used to identify genomic alterations and is an important tool in the evaluation of biological processes through the wide analysis of genes from a specific tissue (Krol et al., 2008).

The use of large-scale techniques allows for the selection of candidate genes related to the carcinogenic process, prognosis or even predictive factors. The aCGH technique enables the analysis of copy number variations (CNVs) of a tissue and the identification of genes associated with the neoplastic process (Arico et al., 2014).

Although this is a technique vastly used in human medicine, few studies have been performed in veterinary medicine. This technique has been described for other canine tumors, such as lymphoma, osteosarcoma, colorectal tumors and transmissible venereal tumors; however, there is scarce information available on its use in mast cell tumors. Due to the lack of information in this area and the importance of mast cell tumors in dogs, the aim of this study was to characterize genomic imbalances in mast cells tumors of dogs with different prognoses based on survival time to identify genes of prognostic and/or therapeutic potential.

2. Material and methods

2.1. MCT samples

All procedures were approved by the Ethics Committee for Animal Experimentation (CETEA, protocol number 003412/13) of the São Paulo State University – UNESP – Jaboticabal – SP – Brazil.

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Ten primary mast cell tumors from ten different dogs were obtained immediately after surgical excision at the "Governador Laudo Natel" Veterinary Hospital, UNESP – Univ Estadual Paulista, Campus Jaboticabal in SP, Brazil. None of the animals received any treatment (chemotherapy or tyrosine kinase inhibitor therapy) prior to surgery. In animals for which surgery was not an option (2 animals) due to the extension of the disease or the location of the tumor, the samples were obtained by excisional biopsy before the beginning of treatment. Four animals had metastases at the time of diagnosis (lymph node and/or distant metastasis).

Two or more 1 cm^3 fragments were obtained from each tumor. Part of each sample collected was stored in RNAse and DNAse-free cryotubes, kept in liquid nitrogen at −196 °C and subsequently stored at −80 °C. The remaining samples were fixed in 10% formalin for histopathology diagnostic and tumor grading, according to Kiupel et al. (2011).

Using a cryostat (LEICA), the frozen MCT samples were serially sectioned (5 μm), mounted onto slides, and stained with Hematoxylin and Eosin to confirm the presence of at least 80% of neoplastic cells in the sample. If two nodules were present (3 animals), the one with the higher histopathological grade (worse prognosis) was selected for DNA extraction.

Staging was performed by image analysis (thoracic radiography and abdominal ultrasonography) and by cytological examination of sentinel lymph nodes or any lymph node or organ believed to be affected by the disease after clinical examination or image analysis.

The animals were subjected to chemotherapy after tumor removal and evaluated weekly by hematology tests and every 2 months by image analysis and fine needle aspiration biopsy when necessary. Animals that did not receive adjuvant chemotherapy were checked every 2 months by hematology and image analysis.

Dogs diagnosed with MCT were divided into two groups according to survival time (ST): survival > 12 months (Group ST > 12) and survival up to 6 months (Group ST < 6). In animals that died as a result of MCT during the analysis period, and in which post-mortem examination could not be performed but progressive disease was confirmed by cytology and the worsening of clinical signs, death was considered as a consequence of local disease, metastasis or related neoplastic syndromes. Two animals were euthanized.

Out of the 10 dogs with MCT, four belonging to Group ST > 12 (all animals were alive by the end of the experiment) and six to Group ST < 6 (mean survival time ± standard error of 3.4 ± 2.04 months) died as a consequence of MCT. The clinical data of the patients, the neoplastic staging and the treatments used are detailed in Table 1.

2.2. Control samples

Control DNA (normal) was obtained from a leukocyte suspension of peripheral blood of 20 clinically healthy dogs. These animals were subjected to clinical examination and full CBC and chemistry. The extracted DNA were grouped into a genomic DNA pool, resulting in a final concentration of 500 ng of control genomic DNA after dilution.

DNA extraction and quantification.

Tumor samples were placed in tubes containing magnetic beads and homogenized in Precellys R (Bio America Inc., Florida, EUA). DNA extraction of tumor samples was performed using Qiagen Dneasy blood and Tissue kit (N° - 69504, Qiagen, Germany), while DNA extraction of blood leucocyte samples (controls) was carried out using a commercial kit GE – Illustra Blood Genomic Prep Mini Spin (N-289042-64, GE, UK) according to the manufacturer's instructions.

Subsequent to extraction, DNA was quantified using *Nano Drop* (ND-1000 Spectrophotometer v.3.0.1, Labtrad). The quality and integrity of the extracted DNA was analyzed using 0.8% agar gel.

2.3. aCGH protocol

After extraction and quantification, the tumor and control DNA were diluted in MilliQ water to a final concentration of 500 ng/μl and final volume of 20.2 μl. Subsequently, the samples underwent a digesting stage by the addition of water, Buffer C, acetylated BSA, *ALu* I and *Rsa* I and incubated for 2 h at 37 °C and then 20 min at 65 °C. Cyanines Cy3- and Cy5-dCTPs (Amersham Biosciences, Buckinghamshire, UK) were incorporated by random primer labelling (Bioprime DNA Labelling Kit, Invitrogen, Carlsbad, CA). The tumor samples were labeled with Cy3, and the controls with Cy5. Purification, hybridization and washing steps were carried out according to the enzyme labelling protocol recommended by the manufacturer (Agilent Technologies, Santa Clara, CA, USA).

Labelling intensity and reaction yield were analyzed in all samples using Nano Drop 1000 (Thermo Fisher Scientific Inc.). Test and reference samples of similar labelling efficiency were then paired. Human Cot DNA, 10× Blocking Agent and 2× Hi-RPM Hybridization Solution were added to each reaction. The hybridization mixture was denatured at 95 °C for 3 min and incubated at 37 °C for 30 min in a dry bath. CGH block (27 μl) was added to the labeled reactions, and 100 μl of the end solution was transferred to the glass slides and incubated at 65 °C for 24 h. The data on the variation in the number of DNA copies were obtained through the use of the *Canine Genome CGH Microarray* 4x180K slide (252552 - Agilent). This platform has approximately 180,000 probes that map well-characterized genes, especially those involved in neoplasia, as well as codifying and non-codifying sequences (http://www.chem.agilent.com). After hybridization, the slides were washed in Wash Buffer 1 for 5 min, Wash Buffer 2 for 1 min, acetonitrile for 10 s, and Stabilization and Drying Solution for 30 s.

Images were obtained with a Microarray Scanner System (Agilent G2565CA) and Scan Control Software 8.1. Data were extracted using

Table 1
Clinical presentation, clinical stage, histologic grading, treatment and outcomes of 10 dogs with mast cell tumors.

Group	Breed	Sex	Age (years)	Clinical presentation	Ulceration	Metastasis	Clinical stage[a]	Histologic grading[b]	Treatment	Survival time
ST > 12	Boxer	F	6	Single	No	No	I	Low	Surgery	Alive[c]
ST > 12	Pitbull	F	10	Single	No	No	I	Low	Surgery	Alive[c]
ST > 12	Labrador	M	7	Single	No	No	III	Low	Surgery + chemotherapy	Alive[c]
ST > 12	Pitbull	F	4	Single	No	No	I	Low	Surgery	Alive[c]
ST < 6	Labrador	M	8	Single	No	No	III	High	Surgery + chemotherapy	6 months
ST < 6	Labrador	M	13	Single	Yes	Spleen	V	Low	Surgery	3 months
ST < 6	Brazilian Fox Terrier	F	7	Multiple	No	Submandibular lymph node	IV	High	Surgery + chemotherapy + TKI	6 months
ST < 6	Pitbull	M	8	Single	Yes	No	III	High	Surgery + chemotherapy	2 months
ST < 6	Pitbull	F	5	Multiple	Yes	Iliac lymph node	V	High	Chemotherapy	2 months
ST < 6	Shar-pei	M	11	Multiple	Yes	Iliac lymph node and spleen	V	High	TKI	1,5 months

TKI - Tyrosine kinase inhibitor.
[a] Clinical stage: Consensus Panel on Diagnosis, Staging, Grading and Therapy of Mast Cell Disease. - ESVONC/VCS, Copenhagen, 2008.
[b] Histologic grading - Kiupel et al. (2011).
[c] Alive (follow up at least 12 months).