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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).

the software Feature Extraction v10.7 (Agilent Technologies, Santa Clara, CA, USA).

Data analysis was performed using the program *Nexus version 5.0* (Biodiscovery - www.biodiscovery.com), and the cases were selected based on the fulfillment of the quality criteria of the software (values up to 0.3). The algorithm *FASST Segmentation*, which is based on the *Hidden Markov Model* (significance threshold of 5.00E-5), and the maximum space between adjacent probes prior to the break of a 1 Mb segment were used. Five consecutive altered probes were needed for a segment to be considered as altered in regards to the number of copies. The threshold for the gain of a copy, gain of 2 or more copies (high level of gain), loss of a copy and loss of two copies (homozygous deletion) were 0.3, 1.14, −0.3 and −1.1, respectively. X and Y alterations were not analyzed.

Detailed analysis was carried out in regions that showed variations in >20% of samples (representative regions). Characterized regions were considered to be those that had named genes with identified functions. Descriptive statistical analysis of the genes was also performed.

## 3. Results

### 3.1. Genomic alterations in canine mast cell tumors

Five hundred and fifty-five CNVs were detected in 10 MCT, with an average of 55.5 per case. The average CNVs of Group ST < 6 was 85 ± 38.5, while that of Group ST > 12 was 11.33 ± 3.3.

The tumors analyzed had genomic alterations that varied from 4 to 246 CNVs per MCT sample, which were divided into gains (1 additional copy), losses (1 missing copy), high level of gains (2 or more additional copies) and homozygous losses (2 missing copies). It was observed to be 251 gains (45.22%), 293 losses (52.79%), 3 high level of gains (0.54%) and 8 homozygous losses (1.44%).

Chromosomes 6 and 11 showed the greatest number of affected sequences, with 47 CNVs (30 losses and 17 gains) and 50 CNVs (48 losses, 1 homozygous loss and 1 gain), respectively. The chromosomes with the lowest number of alterations were chromosomes 12, 28 and 34, each with a single alteration present (gain, gain and loss, respectively).

Chromosome 11 showed the greatest number of sequences affected by loss (48), and chromosome 14 the greatest number of gains (35).

The alterations observed in the total set of chromosomes from the tumor samples analyzed, and their respective losses (red) and gains (blue) are illustrated in Fig. 1. The areas of gain and loss in the chromosomes of each MCT patient are illustrated in Fig. 2.

### 3.2. Minimum common regions (MCRs) analysis

Only variations present in at least 20% of MCT samples were considered representative, despite the survival time of patients.

Within the total number of cases, 153 genes showed variations in >20% of the samples (112 genes with a gain and 41 genes with a loss in copy number). Out of this total number of genes, 91 had characterized functions. The chromosomes that showed representative copy number variation were chromosomes 1, 2, 5, 6, 8, 9, 16, 18, 19, 26, 27 and 36. Chromosome 8 had the greatest number of representative copy number variation (3 variations – 2 losses and 1 gain); however, these were related to non-characterized regions.

In Group ST < 6, there were 75 characterized genes that showed gains and 16 characterized genes that showed copy number losses. In Group ST > 12, there were 24 genes with gains (these genes were also present in Group ST < 6) but no genes with losses in copy number (Table 2).

From the genes currently characterized, those that were known to be related to tumor development were selected and correlated to the groups based on the survival time of patients.

The main genes found in the regions of copy number losses were located in chromosome 26. In total, 16 genes were found in regions of loss, all of which have already been characterized. The main genes in this region that are related to neoplasia development, and their correlation to the patients from Group ST > 12 and ST < 6 are detailed in Table 3.

### 3.3. Genomic pattern of aberration correlated with clinical outcomes

The *PTEN* gene (*Phosphatase and tensin homolog*), present in chromosome 26, showed a reduction in copy number in 50% of the cases in Group ST < 6, one in which had a homozygous loss (both alleles). In Group ST > 12, there were no copy number variations of this gene (Table 3).

*MINPP1* gene showed a loss in copy number in 50% of patients from Group ST < 6 group, but no alteration was observed in Group ST > 12 (Table 3). The *FAS* gene, also located in chromosome 26, showed a reduction in copy number (2 losses and 1 homozygous loss) in 50% of patients from Group ST < 6 group, but no alteration was observed in Group ST > 12 (Table 3).

The most significant genes found in the regions of copy gain that were present in >20% of the samples were located in chromosomes 1, 2, 6, 16, 18 and 27.

Important CNVs were observed in chromosome 27, in which 33% of the tumors from Group ST < 6 showed a copy number gain in the area

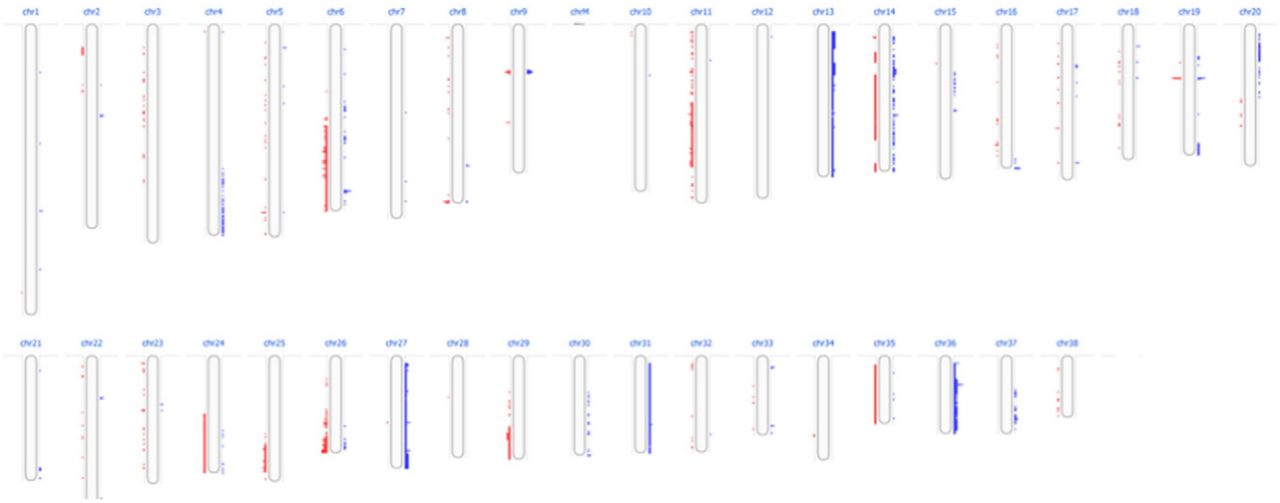

**Fig. 1.** Graphical representation of the changes found in the set of chromosomes evaluated in canine cutaneous mast cell tumors. The gains are in blue and the losses are in red.

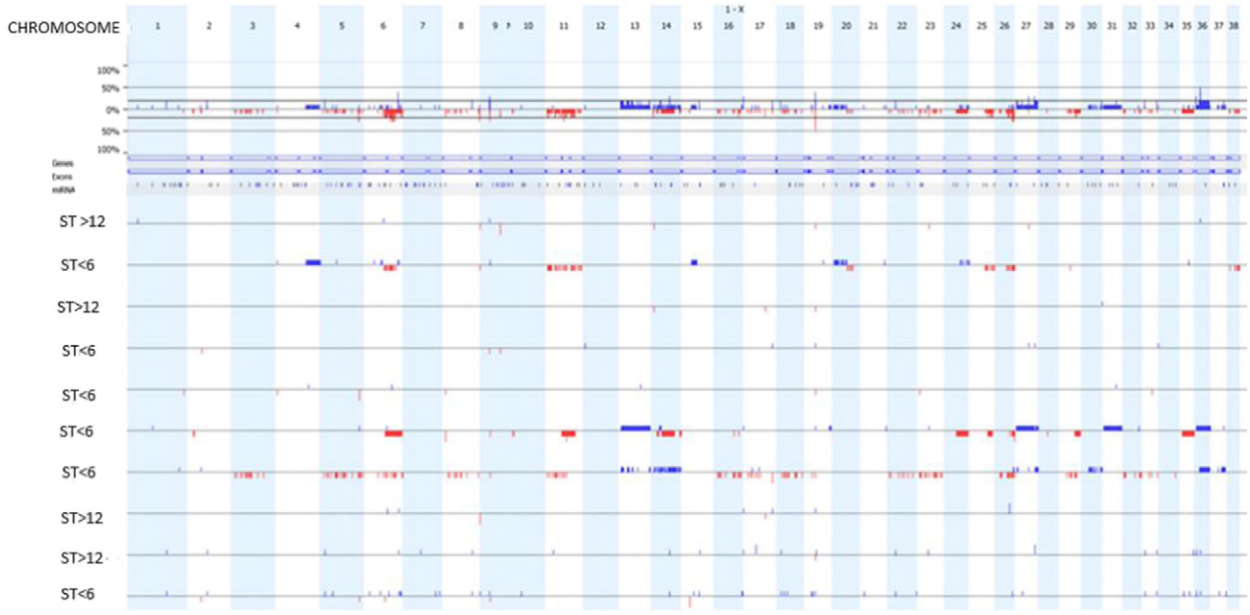


**Fig. 2.** Graphical representation of change in chromosome (gain – blue and loss – red) according with each case and survival time in 10 dogs with mast cell tumors evaluated by aCGH array. *Nexusversion 5.0.*

corresponding to *MAPK3*, *CCND2*, *WNT5B*, *FOXM1*, *RAD51AP1*, *FGF 6* and *FGF23* genes. No variations in these genes were observed in Group ST > 12 (Table 3).

The only altered gene in chromosome 1 was the *TLE-1*, which codifies the transducin-like enhancer protein 1. It showed a copy gain in 25% and 16.6% of patients from Group ST > 12 and ST < 6, respectively (Table 3).

Chromosome 2 showed variation in copy number in the region corresponding to the *GIPC2* gene, which is part of the *GIPC* family. Gain was observed in 50% and 33% of the cases from Group ST > 12 and ST < 6, respectively.

## 4. Discussion

Knowledge of genomic copy number variations and the genes involved in the altered regions could help in the understanding of the clinical behavior of MCT and the establishment of a prognosis, as described for other tumors (Thomas et al., 2009; Tang et al., 2010).

As expected, dogs from Group ST < 6 showed more complex genomic alterations than those from Group ST > 12.

Although several factors may influence the survival time of patients with MCT, because this is considered a neoplasia with highly varied biological behavior and there is a difficulty in performing studies with homogeneous groups, we chose to divide the groups according to survival time; we excluded patients with an intermediate survival time (6–12 months) and all animals in the group ST < 6 that died from MCT.

Differences in the CNVs between groups of distinctive prognoses have also been observed in canine colorectal tumors, where animals with adenomas had a mean CNV significantly inferior to those with colorectal adenocarcinoma, suggesting that a relationship exists between the number of CNVs and the progression of the disease (Tang et al., 2010). This is in agreement with the findings from the present study.

The regions with genomic copy number variations in MCT identified important genes related to tumor development. The main genes found in the regions of loss were *PTEN*, *FAS* and *MINPP-1*, present in 50% of Group ST < 6.

*PTEN* and *FAS* are known to be involved in the development of several tumors (Carracedo et al., 2011; Hanahan and Weinberg, 2011). The *PTEN* gene is an important tumor suppressor, and it is involved in several cellular processes, including cell growth, cell survival, inhibition of cell cycle progression and angiogenesis, and stimulation of apoptosis (Tsugawa et al., 2002). Thus, a reduction in *PTEN* gene activity could lead to an increase in cell proliferation and a reduction in cellular death, both of which are essential conditions for tumor development (Tsugawa et al., 2002; Keniry and Parsons, 2008; Carracedo et al., 2011).

The involvement of *PTEN* on the development of several neoplasias in dogs and cats has been reported (Koenig et al., 2002; Levine et al., 2002; Ressel et al., 2009). In canine mammary tumors, loss of PTEN protein expression has been correlated with lymphovascular invasion,

**Table 2**
Chromosome changes and the related genes in canine cutaneous mast cell tumors evaluated by aCGH array.

| Chromosome | Change | Frequency of change (%) | *p*-Value | Genes |
|---|---|---|---|---|
| 1 | Gain | 20 | 0,021 | *TLE-1* |
| 2 | Gain | 20 | 0,023 | *ARAP3*, *DIAPH1*, *FCHSD1*, *PCDHGA10*, *PCDHGA6*, *PCDHGA7*, *PCDHGA8*, *PCDHGB3*, *PCDHGB4*, *PCDHGB5*, *PCDHGB6*, *PCDHGB7*, *PCDHGC3*, *RELL2* |
| 6 | Gain | 30 | 0,034 | *DNAJB4*, *GIPC2* |
| 16 | Gain | 30 | 0,0 | *CBD103*, *CBD104*, *SPAG11*, *SPAG11E* |
| 18 | Gain | 20 | 0,038 | *BLVRA*, *HECW1*, *STK17A* |
| 26 | Loss | 30 | 0,0 | *ACTA2*, *ANKRD22*, *ATAD1*, *CH25H*, *FAS*, *LIPA*, *LIPF*, *LIPJ*, *LIPK*, *LIPM*, *MINPP1*, *PAPSS2*, *PTEN*, *RNLS*, *STAMBPL1* |
| 27 | Gain | 20 | 0,0 | *ADIPOR2*, *AKAP3*, *ANO2*, *ATP6V1E1*, *B4GALNT3*, *BCL2L13*, *CACNA1C*, *CACNA2D4*, *CCDC77*, *CCND2*, *DCP1B*, *DDX11*, *EFCAB4B*, *ERC1*, *FBXL14*, *FGF23*, *FGF6*, *FKBP4*, *FOXM1*, *FYTTD1*, *GALNT8*, *IL17RA*, *IQSEC3*, *ITFG2*, *KCNA1*, *KCNA5*, *KCNA6*, *KDM5A*, *LRTM2*, *MAP3K*, *MICAL3*, *NDUFA9*, *NINJ2*, *NRIP2*, *NTF3*, *PARP11*, *PEX26*, *PRMT8*, *RAD51AP1*, *RAD52*, *SLC25A18*, *SLC6A12*, *SLC6A13*, *TEAD4*, *TSPAN11*, *TSPAN9*, *TUBA8*, *TULP3*, *USP18*, *WASH1*, *WNK1*, *WNT5B* |

**Table 3**
Copy number variation in characterized genes and the relationship with survival time.

| | Group ST > 12 | Group ST < 6 |
|---|---|---|
| Total genes change | 24 | 91 |
| Total of characterized genes located in gain regions | 24 | 75 |
| Total of characterized genes located in loss regions | 0 | 16 |
| Characterized genes located in gain regions | *TLE-1* (25%)<br>*GIPC2* (50%) | *TLE-1* (16,6%)<br>*GIPC2* (33%)<br>*MAPK3* (33%)<br>*FOXM1* (33%)<br>*FGF23* (33%)<br>*FGF 6* (33%)<br>*CCND2* (33%)<br>*WNTB5* (33%) |
| Characterized genes located in loss region | – | *PTEN* (50%)<br>*FAS* (50%)<br>*MINPP1* (50%) |

metastasis in lymph nodes, distant metastasis, tumoral recurrence and shorter survival time. It has also been suggested that deletion of the *PTEN* gene has an effective role on the development of canine osteosarcoma (Levine et al., 2002; Thomas et al., 2009b).

Copy number loss in *FAS* was observed in 50% of the cases from Group ST < 6 but was not detected in Group ST > 12. Genomic copy number loss in areas of the *FAS* gene could result in a reduction in neoplastic cell apoptosis, leading to the progression of the disease and accumulation of mutations essential for tumor development (Abrahans et al., 2003). This may have contributed to the more aggressive behavior of the neoplasia in Group ST < 6.

In regards to the genes located in regions of copy number gain, the *MAPK3* gene (*Mitogen-activated protein kinases*), previously known as *ERK1/2*, was altered in 33% of the cases from Group ST < 6. The activation of the *MAPK/ERK* pathway is a frequent event in carcinogenesis and is related to cell migration, apoptosis regulation and angiogenesis, all of which are essential during metastasis (SEBER and KREBS, 1995; Reddy et al., 2003; Rubinfield and Seger, 2005).

Another important gene found in the region of gain in 33% of cases from Group ST < 6 was *WNT5B* from the *WNT* family. The WNT signaling pathway has a key role in carcinogenesis and embryogenesis, and WNT signaling molecules are potent targets in the prevention, diagnosis and treatment of cancer (Saitoh and Katoh, 2002).

*FGF*6 and *FGF* 23 showed gains in 33% of the mast cell tumors in Group ST < 6. It has been reported that fibroblast growth factors (FGFs) control a great number of biological functions, including the regulation of cellular proliferation, migration and differentiation (Turner and Grose, 2010). Thus, the genes from this family are important therapeutic targets in antineoplastic treatment, through the production of drugs capable of inhibiting the activation of FGF receptors (Knights and Cook, 2010).

Two genes, *FOXM1* and *RAD51AP1*, showed copy number gains in 33% of MCT from Group ST < 6 but no alterations in Group ST > 12. *FOXM1* is a transcription gene that is not expressed in quiescent or differentiated cells but is found in high levels in neoplastic cells undergoing rapid multiplication in several human neoplasia (Zhang et al., 2016). Giantin et al. (2014), in a study of gene expression in canine mast cell tumors and its correlation with the degree of differentiation, observed that low grade MCT showed higher expression of FOXM1 than high grade MCT. Giantin et al. (2014) reported that animals with low grade mast cell tumors had significantly lower RAD51 expressions than those with poorly differentiated ones. Our results are in agreement with these reports, as we found gains of both of these genes in the ST < 6 group.

The critical point of our work is the number of samples used. Therefore, we considered these results as a pilot study, specifically concerning the need to validate these results at the protein level.

## 5. Conclusion

In conclusion, the application of CGHarray in canine cutaneous MCT contributed to identifying CNVs that can be related to prognosis. This analysis is a starting point for future investigations with more detailed studies in larger populations of animals for the identification of possible candidate genes that can be used as prognostic factors and therapeutic targets.

## Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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