proceedings

MDPI

Abstract

Cytomorphological, Molecular Diagnosis and Evaluation of Insertion of the LINE-1 Element in the C-MYC Gene in Canine Transmissible Venereal Tumor: Applicability in Veterinary Clinical Routine †

Faro Thamirys Aline Silva [1,2], Ferreira Wallax Augusto Silva [2], de Oliveira Edivaldo Herculano Correa [2,3]

[1] Postgraduate Program in Genetics and Molecular Biology (PPGBM), Institute of Biological Sciences, Universidade Federal do Pará, Belém, Pará, Brazil; thamirysfaro@outlook.com
[2] Laboratory of Tissue Culture and Cytogenetics; Evandro Chagas Institute, Ananindeua, Pará, Brazil; wallaxaugusto@gmail.com (F.W.A.S.); ehco@ufpa.com (D.O.E.H.)
[3] Institute of Exact and Natural Sciences, Faculty of Natural Sciences, Universidade Federal do Pará (UFPA), Belém, Pará, Brazil
* Correspondence:
† Presented at the 1st International Electronic Conference on Genes: Theoretical and Applied Genomics, 2–30 November 2020; Available online: https://iecge.sciforum.net/.

Received: date; Accepted: date; Published: date

Abstract: Canine transmissible venereal tumor **(**CTVT) is the oldest known cancer in the world. Cytomorphologically, the cases are classified into three types. Previous studies have shown that the insertion of the transposable element LINE-1 in the MYC gene has diagnostic importance. Therefore, we characterized the this insertion in samples of the three different cytomorphological types of CTVT, in order to verify if there were any differences concerning the presence, local of insertion and size of the fragment. Our results showed that LINE-1 was inserted in all the samples analyzed, in the same position and presenting the same size (400 bp). We conclude that even though it is important for the oncogenesis process, this insertion has no influence on the cytomorphological type and the observed clinical differences.

Keywords: CTVT; cytomorphological diagnosis; molecular diagnosis; dogs

1. Introduction

Canine transmissible venereal tumor (CTVT), also known as infectious sarcoma, venereal granuloma or Sticker tumor [1], is a cancer that occurs naturally and spreads directly through the transfer of cells between individuals [2]. It is the oldest known transmissible cancer in the world, since the tumor cells found in the animals affected today are correspondents of an animal from approximately 11 thousand years ago, probably when the dogs were first domesticated [3,4].

The disease manifests frequently in the external genitalia of animals, being transmitted by direct contact through coitus, and due to its anatomical location, is usually called genital CTVT [5]. Secondarily, because of the species communication and licking habits, the tumor can occur in the areas around the eyes, mouth and nasal cavity, being called extragenital CTVT [5,6]. This type, due to its atypical location, is difficult to diagnosis, with histiocytes, mastocytomas, amelanotic melanomas, lymphomas and poorly differentiated carcinomas as differential diagnoses [7].

Cytological and histopathological analyses in some cases are not sufficient to conclude the diagnosis of CTVT, as well as immunohistochemistry, since CTVT has the same origin as histiocytes [8]. Hence, the molecular method can be used for the definitive diagnosis, in which the insertion of

the transposable element LINE-1 in the MYC gene, which is characteristic of CTVT tumor cells, is identified [9].

Morphologically, CTVT is classified in three cytomorphological types: (1) Lymphocytic, characterized by more than 60% of round cells, with finely granular cytoplasm, central nucleus and few intracytoplasmic vacuoles; (2) Plasmacytic, characterized by containing more than 60% of cells with broad cytoplasm, eccentric nuclei and large amount of vacuoles; and (3) Mixed, presenting both lymphocytoid and plasmacytoid cells, neither of which exceed 59% [10,11]. Of them, plasmacytic type is the most common cytomorphological type, and is related to chemoresistance [10,12,13]. Hence, due to its prognostic and predictive value, the determination of the cytomorphological classification has gained importance in veterinary clinical practice.

Considering that the insertion of the transposable element LINE-1 in the MYC gene has been reported in every samples so far, the purpose of this work was to analyze if there anre any differences concerning size and insertion site between different cytomorphlogical types, which could be associated to their distinct clinical characteristics.

2. Materials and Methods

2.1. Ethical Aspects of the Research

This study is approved by the Ethics Committee on the Use of Animals (Protocol nº 2530101017—UFPA) and that the owners of the animals signed a Free and Informed Consent Term for donation of the samples.

2.2. Sample Collection

Biopsies were collected from 36 animals. Fragments of 1 cm from the affected genital region were collected and stored in RNAlater™ (Thermo Fisher). In addition, anamnesis and clinical examination of the animal were carried out and data concerning past and current information about the animal, time of neoplasia evolution, location, previous therapy, breeding and reproduction habits were obtained. Two samples of normal tissue from the penis and vulva of animals were also collected for necropsy.

2.3. Cytological Diagnosis and Cytomorphological Classification

For cytological diagnosis and classification, slides were prepared and fixed in methanol for five minutes and after fixation they were stained using Giemsa stain in a 1: 1 dilution, for 15 min. After a detailed scan of the slides, areas with the best pattern of cell distribution and staining were selected, and then at least ten fields were observed at 40× magnification, for CTVT cytomorphological classification according to the predominant cell type—plasmacytoid, lymphocytoid or mixed [10].

2.4. DNA Extraction, Amplification, Sequencing, and Alignment

For molecular analysis, genomic DNA (gDNA) from all tissue samples was isolated using the ReliaPrep™ gDNA Tissue MiniPrep System (Promega Corporation) following the manufacturer's instructions. The DNA samples were purified using EZ-10 Spin Column PCR Product Purification kit (Bio Basic/Ludwig Biotec) and quantified with Nano-Drop-2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and with with a Qubit™ 4 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and stored at −80°C. Genomic DNA was used as template for polymerase chain reaction (PCR) in order to amplify the LINE-1/MYC insertion. The amplification conditions and the primers used are detailed in researches [9,14]. Next, PCR products were analyzed by electrophoresis in 3% agarose and subsequently sequenced with the BigDye Terminator Cycle Sequencing Standard kit (Applied Biosystems) version 3.1, purified using a BigDye X Terminator Purification Kit (Applied Biosystems), and analyzed on an ABI 3130 automated sequencer (Applied

Biosystems). All sequence chromatograms were edited and aligned in BioEdit 7.2.6.1 software, using ClustalW algorithm.

3. Results and Discussion

Thirty six samples were collected from animals presenting different ages, sex and races. 12% of these animals were males and 67% females. 33% of the animals were elderly aged (7 years or older) and 67% were young animals (up to 6 years old). The cytological diagnosis as well as the cytomorphological classification showed that four samples were plasmocytic, five mixed type and 27 lymphocytic (Figure 1). Regarding breeds, 83% were mixed-breed animals, 14% Poodle and 3% Dachshund.

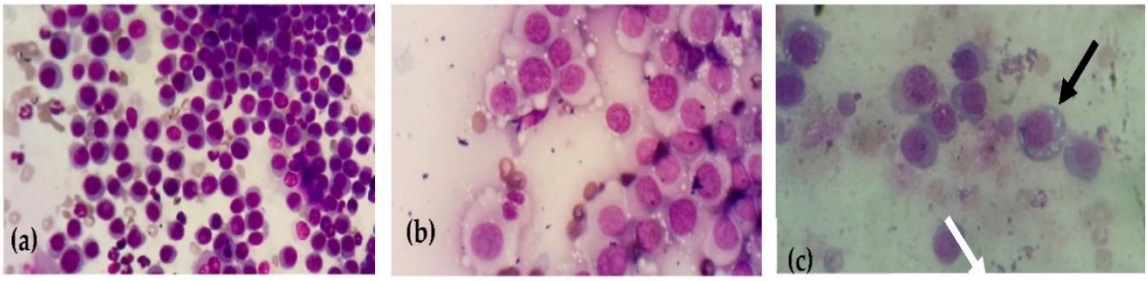

Figure 1. Cytology images of CTVT of different cytomorphological types: A: lymphocytic type—predominance of more than 60% of round cells, with little presence of cytoplasmic vacuoles and centralized nuclei. B: Plasmacytic type—predominance of more than 60% of ovoid cells with more abundant cytoplasms and eccentric nuclei. C: mixed type—there is the presence of both cell subtypes, however neither type exceeds 59% of the total cells analyzed in the slide. Black arrow—plasmacytoid cell; White arrow—lymphocytoid cell.

The molecular diagnosis, based on the identification of the insertion of the transposon LINE-1 in the MYC gene proved to be an efficient and definitive diagnosis, being found in all analyzed tumor samples, but not in control samples (Figure 2), as a fragment of approximately 400 bp, as later confirmed by sequencing samples.

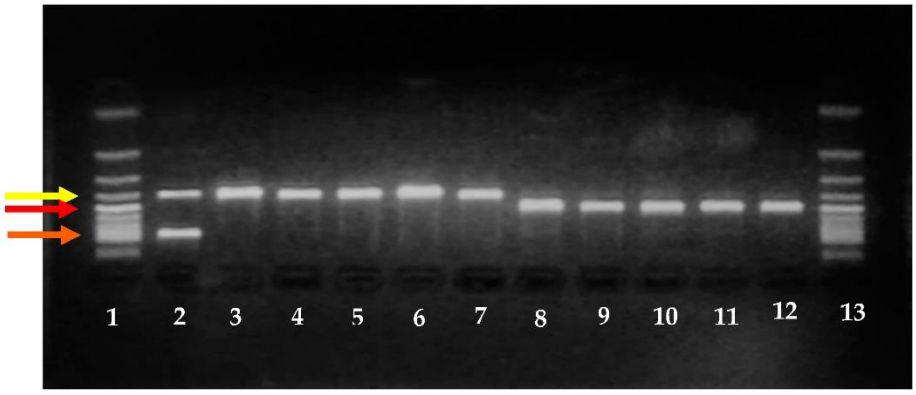

Figure 2. Molecular diagnosis of CTVT. Yellow arrow - 400bp; Red arrow - 500bp; Orange arrow -. 900bp; 1 and 13- Molecular weight of 1kb; 2- Dog testicle DNA as negative control of CTVT. There are two fragments of different sizes (900bp and ~ 400bp), respectively, transposon and MYC gene; 3- Sample of positive CTVT according to histopathological report; 4 to 7- CTVT samples showing the insertion of the transposon Line-1 (900bp) in the MYC gene (~ 400bp), in which there is only 1 fragment; 8 to 12- Positive internal sample controls (~ 480pb).

Precisely because there are different cell lines with different biological behaviors, cytomorphological diagnosis in cases of CTVT has gained importance in veterinary oncology in terms

of therapeutics and prognosis [15]. The percentage of cytomorphological types found in our study was different from those mentioned in the literature, as reported in Amaral et al. (2007) [10], in which the most frequent type was the plasmacytic type (52.53%), followed by the mixed type (29.11%) and finally, the lymphocytic type (18.36%) as well as in Gaspar's et al. (2010) [16], in which the frequency of the plasmacytic type was 50%, lymphocytic 19% and mixed 31% and in the research by Lima et al. (2016) the plasmacytic type represented 45% of the cases, followed by the lymphocytic type (30%) and lastly the mixed type (25%) [12].

Some researchers have already reported different subtypes according to the Brazilian regions in which the plasmacytic type was more frequent in the Southeast compared to the South [10]. A study carried out in the city of Mato-Grosso, the plasmacytic type was also the most prevalent (81.8%) and the lymphocytic type was the least found cytomorphological type (6.8%) [17], differing again from our data.

PCR results proved to be a high sensitive test for the detection of the rearrangement of the LINE-1 element in the MYC gene in CTVT, since all the samples of the transmissible tumors studied amplified a fragment of 400 pb, which characterizes such rearrangement, corroborating the observations by O'Neill (2011) [18]. There was no difference in the insertion site in the three cytomorphological types evaluated. Some studies have shown different insertion sizes in CTVT samples, but generally close to the 400 bp size [7,9,12,14].

4. Conclusions

We can conclude that even though it is important for the oncogenesis process, the rearrangement of the LINE-1 element in the MYC gene has no influence in the cytomorphological type and its clinical differences.

Acknowledgments: *This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001.*

References.

1. Goldschmidt, M.H.; Hendrick, M.J. Tumors of the skin and soft tissues. In *Tumors in Domestic Animals*, 4th ed.; Meuten, D.J., Ed.; Ames: Ames, IA, USA, 2002; chapter 2, pp. 45–118.
2. Ostrander, E.A.; Davis, B.W.; Ostrander, G.K. Transmissible Tumors: Breaking the Cancer Paradigm. *Trends Genet.* **2016**, *32*, 1–15.
3. Murgia, C.; Pritchard, J.K.; Kim, S.Y.; Fassati, A.; Weiss, R.A. Clonal Origin and Evolution of a Transmissible Cancer. *Cell* **2006**, *126*, 477–487.
4. Rebbeck, C.A.; Thomas, R.; Breen, M.; Leroi, A.M.; Burt, A. Origins and evolution of a transmissible cancer. *Evolution* **2009**, *63*, 2340–2349.
5. Setthawongsin, C.; Tangkawattana, S.; Rungsipipat, A.; Techangamsuwan, S. Computerized Cytomorphometric and Cytomorphological Analysis of Canine Transmissible Venereal Tumours. *J. Comp. Pathol.* **2018**, *163*, 18–22.
6. Strakova, A.; Murchison, E.P. The cancer which survived: Insights from the genome of an 11,000 year-old cancer. *Curr. Opin. Genet.* **2015**, *30*, 49–55.
7. Park, M.S.; Kim, Y.; Kang, M.S.; Oh, S.Y.; Cho, D.Y.; Shin, N.S.; Kim, D.Y. Disseminated transmissible venereal tumor in a dog. *J. Vet. Diagnost. Investig.* **2006**, *18*, 130–133.
8. Das, U.; Das, A.K. Review of canine transmissible venereal sarcoma. *Vet. Res. Commun.* **2000**, *24*, 545–556.
9. Fonseca, L.S.; Mota, L.S.; Colodel, M.M.; Ferreira, I.; Brandão, C.V.S.; Rocha, N.S. Spontaneous canine transmissible venereal tumor: Association between different phenotypes and the insertion LINE-1/c-myc. *Revista Colombiana de Ciencias Pecuarias* **2012**, *25*, 402–408.
10. Amaral, A.S.; Bassani-Silva, S.; Ferreira, I.; Fonseca, L.S.; Andrade, F.H.E.; Gaspar, L.F.J.; Rocha, N.S. Cytomorphological characterization of transmissible canine venereal tumor. *Rev. Port. Cienc. Vet.* **2007**, *102*, 253–260.
11. Flórez, M.M.; Fêo, H.B.; Da Silva, G.N.; Yamatogi, R.S.; Aguiar, A.J.; Araújo, J.P., Jr.; Rocha, N.S. Cell cycle kinetics, apoptosis rates and gene expressions of MDR-1, TP53, Bcl-2 and BAX in transmissible venereal tumor cells and their association with therapy response. *Vet. Comparat Oncol.* **2016**, *14*, 1–15.

12. Lima, C.R.O.; Faleiro, M.B.R.; Rabelo, R.E.; Vulcani, V.A.S.; Rubini, M.R.; Torres, F.A.G.; Moura, V.M.B.D. Insertion of the LINE-1 element in the C-MYC gene and immunoreactivity of C-MYC, p53, p21 and p27 proteins in different morphological patterns of the canine TVT. *Arq. Bras. Med. Vet. Zootec.* **2016**, *68*, 658–666.
13. Flórez, L.M.M. *Expressão dos Genes MDR-1, TP53, BCL-2 E BAX em Tumor venéreo Transmissível canino e sua Relação com a Agressividade e Resposta à Terapia*; Tese de doutorado: 2014; p. 81.
14. Spin, J.S.F.; Da Mota, L.S.; DA Castelli, L.S.L.S.; Silva, E.C.; Ferreira, S.B.; Rocha, I.; Sousa, N. Detecção molecular do rearranjo Line-1/c-MYC em tumores venéreos transmissíveis caninos espontâneos. *Clín. Vet.* **2010**, *15*, 64–68.
15. Silva, J.S.; Da Silva, D.M.M.; Silva, V.B.; Da Silva, D.A.; Silva, R.S.; De Lucena, R.B. Classificação citopatológica e epidemiologia do TVT nos cães atendidos no Hospital Veterinário da UFPB. 38º Congresso Brasileiro Da Anclivepa 2017. Anais do 38º CBA; p. 0808.
16. Gaspar, L.F.; Ferreira, I.; Colodel, M.M.; Brandão, C.V.; Rocha, N.S. Spontaneus canine transmissible venereal tumor: Cell morphology and influence on p-glycoprotein expression. Turk. *J. Vet. Anim. Sci.* **2010**, *34*, 447–454.
17. Valençola, R.A.; Antunes, T.R.; Sorgatto, S.; Oliveira, B.B.; Godoy, K.C.S.; De Souza, A.I. Aspectos citomorfológicos e frequência dos subtipos do tumor venéreo transmissível canino no município de Campo Grande, Mato Grosso do Sul, Brasil. *Acta Veterinaria Brasilica* **2015**, *9*, 82–86.
18. O'neill, I.D. Concise review: Transmissible animal tumors as models of the cancer stem-cell process. *Cancer Stem Cells* **2011**, *29*, 1909–1914.