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Evaluation of a genetic assay for canine transmissible venereal tumour diagnosis in Brazil

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Abstract

The canine transmissible venereal tumour (CTVT) is a transmissible cancer that is spread between dogs by the allogeneic transfer of living cancer cells. The infectious agents in CTVT are the living cancer cells themselves, which are transmitted between dogs during coitus. CTVT first arose several thousand years ago and the disease has a global distribution and is frequently observed in dogs from Brazil. We evaluated the utility of a LINE-MYC quantitative polymerase chain reaction for diagnosis of CTVT cases in Brazil. Our analysis indicated that the LINE-MYC rearrangement was detectable in all CTVT samples but not in their corresponding hosts. This genetic assay proves to be a useful tool for providing a definitive molecular diagnosis of CTVT, which presents with varying degrees of aggressiveness and invasiveness in different host dogs and can therefore be a diagnostic challenge in some specific cases.

Keywords
canine transmissible venereal tumour, LINE-MYC, molecular diagnosis, oncology, transmissible cancer

Introduction

The canine transmissible venereal tumour (CTVT) is a naturally occurring transmissible cancer that affects the external genitalia of both male and female dogs. The disease is spread by the allogeneic transfer of living cancer cells mainly during coitus but also through licking, biting and scratching.[1–3] CTVT is the oldest known mammalian somatic cell lineage and genetic studies indicate that it originated more than 10 000 years ago.[4–6] Rather than dying together with its original host, the cells of this specific cancer are still alive today through acquiring adaptations for cell transmission between hosts and for survival as an allogeneic graft.[3] CTVT is frequently observed in dogs from Brazil, however, this disease has a global distribution and has been documented to be present in at least 90 countries on all six inhabited continents and its distribution is linked to the presence of free-roaming dogs.[7] Important evidence for clonal transmission of CTVT was provided by the identification of a genomic rearrangement involving the insertion of a repetitive 1.5 kb DNA segment derived from a long interspersed nuclear element (LINE) upstream of the first exon of the c-myc gene.[8] This genomic rearrangement has been identified in a large set of globally distributed CTVT tumours, but it has not been found in normal dogs, and it is considered to be diagnostic evidence for CTVT.[4,5,9–16] It is possible that this rearrangement was present in the germline of the CTVT founder (i.e. the animal from whose somatic cells CTVT first emerged), that it occurred somatically during the development of the founding CTVT tumour, or that it occurred somatically in a CTVT clone that has subsequently achieved global distribution. [17] The purpose of this study was to evaluate the utility of a LINE-MYC quantitative PCR (qPCR) for diagnosis of CTVT cases in Brazil.

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Table 1. Summary of samples used in this study

Sample	Breed	Sex	Age (years)	Tumour location
1	Pit bull	F	2	Vagina
2	Mixed	F	10	Vagina
3	Mixed	M	4	Penis
4	Mixed	F	5	Vagina
5	Mixed	F	3	Vagina
6	Mixed	F	5	Vagina
7	Mixed	M	4	Penis
8	Mixed	F	5	Vagina
9	Shih Tzu	F	4	Vagina
10	Border Collie	F	4	Vulva/vagina
11	Shih Tzu	F	7	Vagina
12	Mixed	M	3	Penis
13	Mixed	F	6	Vagina
14	Mixed	F	4	Vulva/vagina
15	Mixed	M	3	Penis
16	Poodle	F	4	Vagina
17	Mixed	M	5	Penis
18	Mixed	F	5	Vagina
19	Mixed	M	4	Nasal cavity
20	Border Collie	M	3	Penis/prepuce/eyelid

Tumour tissue was collected from all 20 animals; matched blood was collected from 17 animals.

Materials and methods

Tumour and blood specimens

Samples were obtained from 20 unrelated dogs with spontaneous CTVT diagnosed at the Veterinary Hospital 'Dr Halim Atique' – Centro Universitário de Rio Preto (UNIRP), São José do Rio Preto, São Paulo, Brazil. The dogs were of different breeds, age and sex, as specified in Table 1. Tumour samples with an average size of 1 cm^3 were collected by surgical excision from all 20 dogs and immediately stabilized by freezing in liquid nitrogen. These samples were collected before starting chemotherapy treatment. 2–5 mL of peripheral blood were also collected from 17 of these dogs and mixed immediately with RNA later solution in the proportion of 1:3 (Blood:RNAlater solution). This study was approved by the Animal Research Ethics Committee of the São Paulo State University at Jaboticabal, São Paulo, Brazil, under protocol number 24674/2012.

Genomic DNA extraction

DNeasy blood & tissue kit® (product number 69506; Qiagen, Hilden, Germany) was used to extract genomic DNA from both blood and tumour tissue according to the manufacturer's protocol. The DNA was quantified using a Nanodrop ND-1000 spectrophotometer and Qubit® assay (product number Q32851; Life Technologies, Carlsbad, California, USA).

Table 2. Primer sequences for qPCR of LINE-MYC and B-ACTIN

	Forward	Reverse
B-ACTIN	CTCCATCATGAAG TGTGACGTTG	CGATGATCTTGA TCTTCATTGTGC
LINE-MYC	AGGGTTTCCCATCCT TTAACATT	AGATAAGAAGCTT TTGCACAGCAA

Table 3. qPCR reaction conditions

qPCR Reagent	Volume per reaction (µL)
SYBR green mix	10
Primers (5µM/primer)	2.4
gDNA (20ng/µL)	0.5
Water	7.1
Total volume	20

Table 4. qPCR amplification conditions[a]

Stage	Temperature (°C)	Time
Initial denaturation	95	10 min
40 cycles	95	15 s
	60	60 s

[a]Fluorescence was detected each cycle.

Quantitative PCR

The quantitative PCR (qPCR) was performed with two primer sets (see Table 2): one primer set was specific for B-ACTIN, and was used as a control for normalization; the second pair of primers spanned the LINE-MYC rearrangement junction, and their product is thus specific to CTVT.[18] The qPCR was performed with SYBR® Green Mix (product number 4312704; Life Technologies) with conditions described in Tables 3 and 4. qPCR was performed using an 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, California, USA). Standard curves for LINE-MYC and B-ACTIN were generated using a CTVT sample, 29T. Relative LINE-MYC and B-ACTIN amplification values

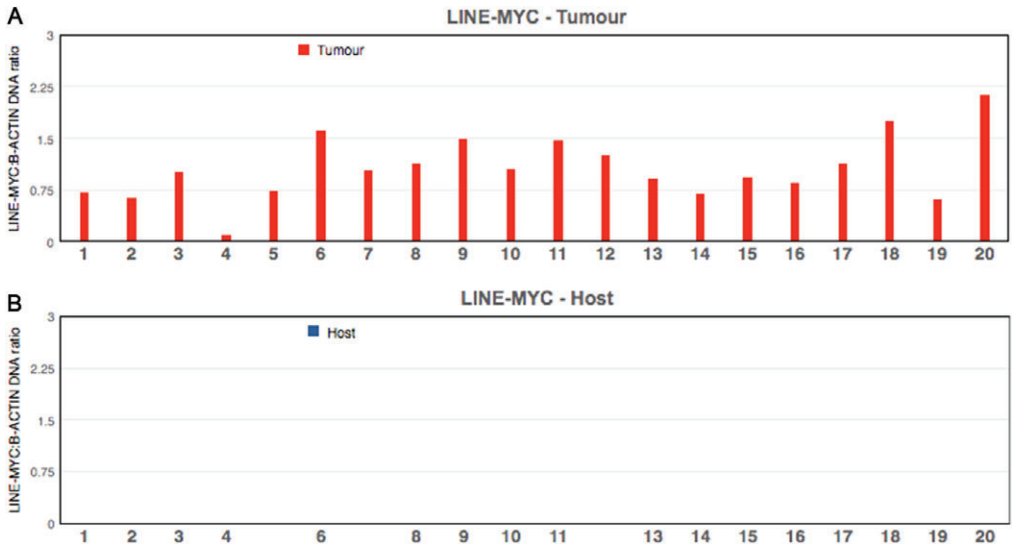

Figure 1. The bars represent LINE-MYC:B-ACTIN DNA ratio measured by qPCR in tumours (red) and hosts (blue). The LINE-MYC rearrangement was (A) detectable in all 20 CTVT samples and (B) absent in all hosts. Missing data from some hosts due to unavailability of samples are represented by absence of label (5, 7 and 12).

were estimated for each sample using the standard curve.

Results

LINE-MYC rearrangement

In order to confirm the CTVT status of all 20 samples, qPCR was performed for the LINE-MYC rearrangement. This procedure was conducted with CTVT and host samples. Results showed that the LINE-MYC rearrangement was detectable in all 20 (20/20) CTVT samples but not in their corresponding hosts (0/17) (Fig. 1A,B). The mean LINE-MYC:B-ACTIN ratio was 1.2527 in tumours and 0.0002 in hosts.

Discussion/conclusion

CTVT represents the oldest known malignant cell in continuous propagation whereby a single malignant clone of cells has colonized dogs worldwide. Clinical history, signalment and cytological/histological features are often sufficient for diagnosis of CTVT. However, molecular biology can also be helpful in cases with an atypical presentation. The identification of a LINE element insertion near the MYC locus in the CTVT genome by PCR amplification is of diagnostic importance. The presence of detectable levels of LINE-MYC rearrangement observed in all CTVT samples and absence in their corresponding hosts confirm that this assay provided a diagnosis of CTVT. Our findings support published data indicating that the LINE-MYC rearrangement is conserved in CTVT and can be used clinically as a definitive diagnosis of CTVT.[4,5,19] The variation in observed LINE-MYC:B-ACTIN DNA ratios between tumours identified in this study is probably due to variable levels of normal host cells present in tumours; however, it is also possible that there is variation in LINE-MYC and/or B-ACTIN copy number between tumours. It will be important to determine the limit of sensitivity of this assay to detect CTVT tumours with a high proportion of host cells. The mean ratio of LINE-MYC to B-ACTIN in CTVT tumours was 1.2527, and probably reflects non-diploid copy number of LINE-MYC and/or B-ACTIN in CTVT tumours, together with the presence of host cells. Molecular diagnosis of CTVT may be particularly useful in cases of CTVT with atypical presentation. For instance, CTVT with extragenital occurrence and generalized metastases, often become a diagnostic and clinical challenge, as it may be difficult to distinguish between CTVT and other canine round cell neoplasms. This genetic assay may, therefore,

prove to be a very useful tool for providing a definitive diagnosis in these cases. In conclusion, as a specific molecular alteration in CTVT, the rearrangement of LINE-MYC was also identified in CTVT cells in dogs from Brazil and this genetic assay can be useful as definitive diagnosis of CTVT.

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