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Expression Pattern of *Cdkn2b* and Its Regulators in Canine Mammary Tumors

THAMIRYS ALINE SILVA FARO[1,2*], DANILO DO ROSÁRIO PINHEIRO[1*], DANIELLE QUEIROZ CALCAGNO[3], WASHINGTON LUIZ ASSUNÇÃO PEREIRA[4], LUCIEN ROBERTA VALENTE MIRANDA DE AGUIRRA[4], ROMMEL RODRIGUEZ BURBANO[5,6], MARIA LÚCIA HARADA[1] and BÁRBARA DO NASCIMENTO BORGES[1]

[1]*Molecular Biology Laboratory, Biological Science Institute, Federal University of Pará, Belém, Brazil;*
[2]*Tissue Culture and Cytogenetics Laboratory, Evandro Chagas Institute, Belém, Brazil;*
[3]*Oncology Research Center, Federal University of Pará, Belem, Brazil;*
[4]*Animal Pathology Laboratory, Health and Animal Production Institute, Federal Rural University of Amazonia, Belém, Brazil;*
[5]*Molecular Biology Laboratory, Ophir Loyola Hospital, Belém, Brazil*
[6]*Molecular Biology and Human Cytogenetics Laboratory, Federal University of Pará, Belém, Brazil*

Abstract. *Background/Aim: In female dogs, mammary cancer is the most frequent cancer type, accounting for 50% of all tumors affecting these animals. Amongst the commonly altered genes in cancer is the cell-cycle regulator cyclin-dependent kinase inhibitor 2B (Cdkn2b), whose expression is negatively regulated by protein products of BMI1 proto-oncogene (Bmi1), MYC proto-oncogene (Myc) and T-box gene transcription factor 2 (Tbx2) genes. Considering this, the aim of this study was to evaluate the expression pattern of the Cdkn2b gene and these regulators in canine mammary tumors of dogs from Northern Brazil (Belém, Pará). Material and Methods: Gene expression in samples from 33 animals was assessed by real-time polymerase chain reaction. To check the influence of methylation on gene expression, bisulfite sequencing polymerase chain reaction was also performed. Results: All studied genes, except Cdkn2b, were found at increased expression levels in tumor tissue when compared with control samples. No correlation between expression and methylation data was observed. Conclusion: Our results suggest these markers may have a diagnostic value in the veterinary clinic.*

*These Authors contributed equally to this study.

Correspondence to: Bárbara do Nascimento Borges, Laboratório de Biologia Molecular "Francisco Mauro Salzano", Instituto de Ciências Biológicas, Universidade Federal do Pará, Cidade Universitária Prof. José da Silveira Netto, Rua Augusto Correa, 01, Zip Code: 66075-970-Belém, Pará, Brazil. Tel/Fax: +55 9132017585, e-mail: bnborges@ufpa.br

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As in women, mammary cancer in dogs represents a serious problem in veterinary medicine, being responsible for about 50% of all cancers that affect canines, especially females (1).

Cyclin-dependent kinase inhibitor 2B (*Cdkn2b*) is a tumor-suppressor gene of the *Ink4/Arf* locus, located on canine chromosome 11, which interacts with the CDK4–cyclin D complex, suppressing the phosphorylation of retinoblastoma protein (Rb), thus preventing the progression of the cell cycle (2). For unknown reasons, mutations in *CDKN2B* are uncommon in several types of human tumors (3, 4).

The loss of *CDKN2B* expression occurs mainly by alterations of its gene methylation pattern, as observed in several tumor types in humans, such as leukemia and rectal cancer (5-7). However, regulatory proteins that negatively control the expression of the locus, such as BMI1 proto-oncogene (*BMI1*) (8), MYC proto-oncogene (*MYC*) (9) and T-box gene transcription factor 2 (*TXB2*) (10, 11) have also been identified.

Canine mammary cancer provides a comparative model for study of breast cancer in women (12). The aim of this study was to characterize the expression and methylation patterns of *Cdkn2b* gene and its negative regulators in order to understand their influence on mammary tumorigenesis in female dogs.

Materials and Methods

Samples and study approval by the Research Ethics Committee. Sixty-seven samples (34 neoplastic and 33 adjacent non-tumoral tissues) from 33 animals undergoing mastectomy at the Veterinary Hospital of Federal Rural University of Amazonia, plus two samples from non-affected individuals used as controls were analyzed. All samples were collected and stored in microtubes containing RNA Later® solution (Ambion Inc., Austin, TX, USA). This study had the approval of the

Table I. *Primers used in the methylation analysis and their respective annealing temperatures.*

Gene name	Primer	Annealing temperature (˚C)	Primer sequence 5'-3'	Fragment size (base pairs)	CpG sites analyzed
Cyclin-dependent kinase inhibitor 2B	*Cdkn2b* Met F	59	GTGAGGTTGTGGGGTTTAG	354	15
	Cdkn2b Met R		AACCTCCCAATACAAATAATTCA		
BMI1 proto-oncogene	*Bmi1* Met F	52	GTAAAATTTTTTTATGGATTTT	334	28
	Bmi1 Met R		AATATAAATTACTATAAAAACCCC		
MYC proto-oncogene	*Myc* Met F	55	GGAGAAGTTGGTTTTTTATTAG	444	37
	Myc Met R		TTTCCCTTCCTAAAACTAAAA		
T-Box gene transcription factor 2	*Tbx2* Met F	59	TGGTGTGGTGTTTATTGTGT	328	26
	Tbx2 Met R		TTACATACCAAATCCAAATATACAAC		

Ethics Committee of the Federal Rural University of Amazonia (Protocol 001/2013 CEUA-23084.000265/2013-53 UFRA) and the animals' owners signed an informed consent to donation of samples.

DNA and RNA extraction and quantification. The DNA and RNA samples were obtained using All Prep kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Preparation of cDNA was carried out using High Capacity cDNA Reverse Transcription kit (Life Technologies, Austin, TX, USA). DNA and RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA), and the integrity of samples was assessed visually on 1% and 3% ethidium bromide-stained agarose gels, respectively.

Bisulfite sequencing polymerase chain reaction (BSP). For the methylation analysis, the DNA was subjected to bisulfite modification using EZ DNA Methylation Lightning kit (Zymo Research, Irvine, CA, USA). Primers were designed using Methyl Primer Express software v. 1.0 (Applied Biosystems, Foster City, CA, USA) (Table I). The purified polymerase chain reaction product was sequenced by didesoxyterminal method according to Sanger *et al*. (13), using Big Dye Terminator Kit Cycle Sequencing Standard (Life Technologies) version 3.1, followed by capillary electrophoresis in an ABI 3130 automatic sequencer (Life Technologies). The analysis of methylation pattern was assessed in BIQ Analyzer software (14), and samples with at least 20% of methylated sites were considered hypermethylated (15).

Real-time PCR (RT-qPCR). The expression level of each gene was detected with Taqman probes (Life Technologies), and the emission of the fluorescence was captured using an ABI PRISM 7500 Sequence Detection System (Life Technologies). The expression values of cDNA were normalized using the expression levels of hypoxanthine phosphoribosyltransferase-guanine (*Hprt*) and ribosomal protein L32 (*Rpl32*), as constitutive controls.

Statistical analysis. The analysis of the association of methylation patterns with gene expression levels, tumor progression and other clinicopathological characteristics were performed in GraphPad Prism version 5.01 (www.graphpad.com) using Chi-square (with Yates correction) and odds ratio tests, and the results were considered significant when $p<0.05$.

Results and Discussion

Of the 33 studied animals, 18 were mongrel (54.6%) and 15 of pure breed (45.4%). The mean age at diagnosis was 10.7 years old. According to Dalek *et al*. (16), canine mammary cancer is usually detected in middle-aged patients (7-12 years old). This could be due to the several estrous cycles which such patients have undergone, resulting in physiological hormonal changes, which makes the individuals more prone to carcinogenesis (17).

Of the four analyzed genes, *Myc* and *Bmi1* showed no difference in the methylation pattern in tumors compared with their nontumor counterparts (data not shown). Only one sample presented hypomethylation of *Txb2* compared with its non-tumor counterpart, while for *Cdkn2b*, two samples showed differences (one hypomethylated and one hypermethylated) in the methylation pattern when compared to their non-tumor counterparts. No statistically significant differences in methylation according to the patient characteristics (Table II) or gene expression levels were observed.

As far as we are aware, no studies about the methylation pattern of these target genes for canine mammary cancer are available in the literature, therefore our results are discussed based on research on human cancer.

There are few studies in the literature concerning *CDKN2B* methylation in human breast cancer. However, some authors suggest that hypermethylation of human *CDKN2B*, even at a low frequency, may be an early event of breast carcinogenesis (18, 19).

The regulation of expression of *MYC, BMI1* and *TBX2* genes is still poorly understood. Concerning MYC, many signaling pathways, transcription factors, epigenetic events (*i.e*. promoter methylation) and other regulatory elements are responsible for its expression (20-22). Although there is no correlation between *TBX2* methylation pattern and gene expression in humans, studies suggest a correlation between

Table II. *Clinical and pathological characteristics of the studied animals.*

Characteristic	N (%)
Tumor type	
Carcinoma	28 (84.8)
Sarcoma	5 (15.2)
Breed	
Pure	15 (45.4)
Mongrel	18 (54.6)
Age at diagnosis	
>7 Years	31 (94)
≤7 Years	1 (3)
Not known	1 (3)
Contraceptive use	
Yes	7 (21.2)
No	13 (39.4)
Not known	13 (39.4)
Pseudopregnancy	
Yes	5 (15.2)
No	13 (39.4)
Not known	15 (45.4)
Spayed	
Yes	3 (9.1)
No	17 (51.5)
Not known	13 (39.4)
Parity	
0	11 (33.3)
1	2 (6.1)
2 or more	7 (21.2)
Not known	13 (39.4)
Pulmonary metastasis	
Yes	3 (9.1)
No	17 (51.5)
Not known	13 (39.4)

hypermethylation and a worse prognosis for patients with hepatocellular carcinoma (23), endometrial tumors (24) and bladder cancer (25). No data were available for *BMI1* gene regulation in the literature. Therefore, we can postulate the lack of correlation between methylation and gene expression of the analyzed genes in canine mammary cancer.

All genes studied here presented elevated expression levels when comparing tumor and nontumor counterparts with mammary tissues from control animals except *Cdkn2b* (Figure 1). No statistically significant data for any gene in relation to clinical characteristics such as age, pregnancy, use of exogenous progestogens, and overall survival were found. However, some statistically significant correlations were observed, except for *Cdkn2b* gene, where a 1.54-fold and a 1.16-fold increase in its expression in tumoral and non-tumoral samples, respectively, were observed when compared with the control samples.

When we considered the expression level of *Myc*, we observed a 9.4-fold (p=0.0145) and 10.1-fold (p=0.0256) increase in the expression between tumoral and non-tumoral tissues in relation to the control samples. According to Aulmann *et al*. (26) and Chen and Olopade (27), in human breast cancer, *MYC* was overexpressed in samples of invasive carcinoma and ductal carcinoma *in situ*. Our data are in agreement with this as we observed overexpression of *Myc* in comparison with control samples. Moreover, to our knowledge, this is the first report of MYC expression in canine mammary cancer where a significant difference in expression from the control samples was observed, suggesting that *Myc* overexpression might be a marker for canine mammary carcinogenesis independent of the tumor type.

The same pattern described for *Myc* was observed for *Tbx2* (8.70-fold increase, p=0.0002; and 9.78-fold increase, p=0.0004, for tumoral and non tumoral samples, respectively) and *Bmi1* (2.47-fold increase, p=0.0006; and 2.97-fold increase, p=0.0032, for tumoral and non-tumoral samples, respectively), suggesting these alterations are early events in canine mammary carcinogenesis. A statistically higher expression level was observed in tumoral samples with no pulmonary metastasis at diagnosis (p=0.0307), suggesting that *Bmi1* is not involved in the metastatic process.

In humans, the *TBX2* gene is widely expressed and is involved in the morphogenesis of a variety of tissues and organs, including the heart, lungs, kidneys, mammary glands, testis and cranial structures (28, 29). This gene is involved in the normal development of the breast and studies have shown that its altered expression is correlated with the pathogenesis of breast cancer (28, 30, 31), is associated with a poor prognosis (32), as is responsible for the promotion of deregulation at key points of cell proliferation (33, 34).

BMI1 is a protein associated with the epithelial–mesenchymal transition (EMT), a complex physiological process, and in various pathological states, such as tumor tissue fibrosis. During EMT, epithelial cells lose their functions and shape, developing mesenchymal characteristics, thus increasing their motility and invasion (35, 36). In humans, altered expression of BMI1 was suggested as a prognostic biomarker in several tumor types, including breast cancer, associated with the initiation and progression of cancer, and with a poor prognosis (32, 37-42). On the other hand, our results suggest that a high BMI1 expression is not associated with pulmonary metastasis at diagnosis. This might be for two reasons: i) in canine mammary cancer, BMI1 expression is associated with a better prognosis, similar to the scenario described for human breast cancer (43) and non-squamous cell lung carcinoma (44), or ii) BMI1 expression was associated with micrometastases which were not initially identified in the patients.

Together, our results suggest that deregulation of *Cdkn2b, Tbx2, Myc* and *Bmi1* expression is not associated with changes in the methylation pattern. Our results also suggest that *Tbx2, Myc* and *Bmi1* expression might be used in the

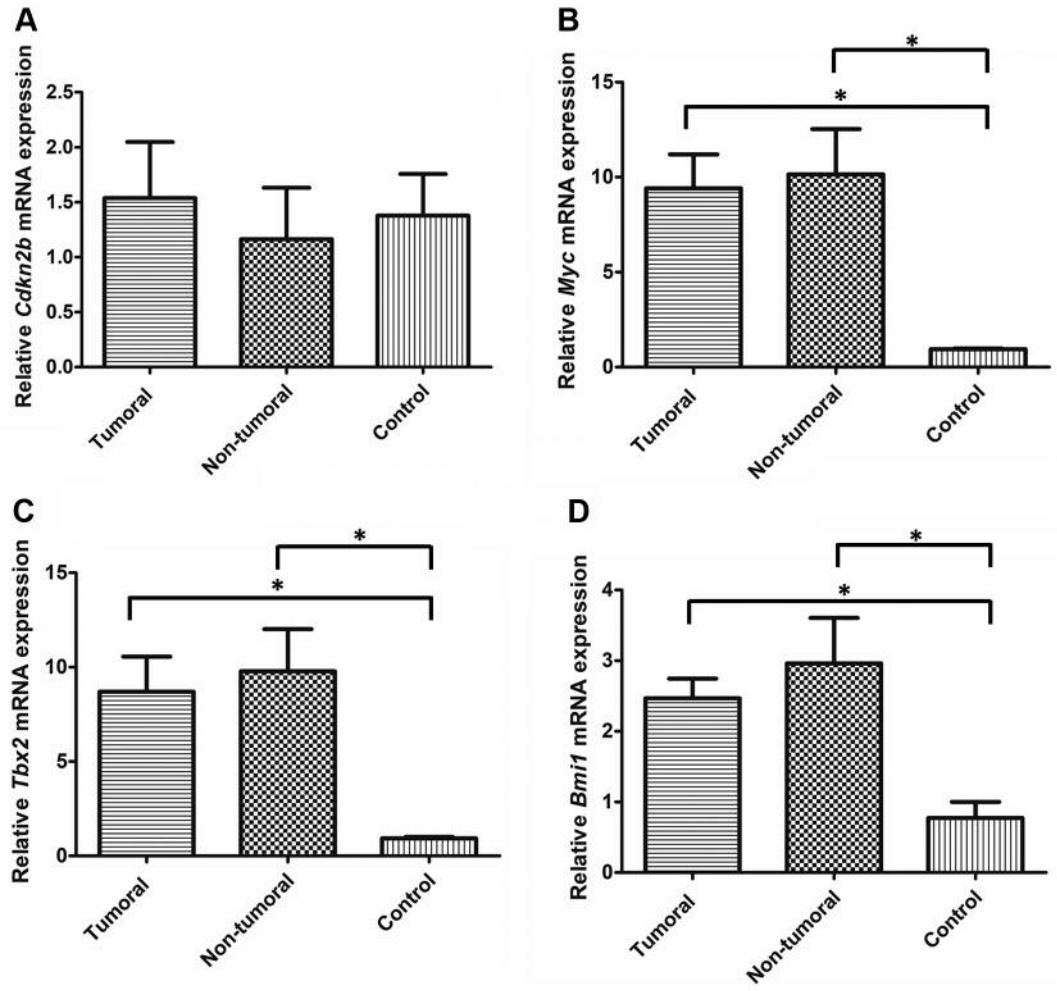

Figure 1. *Tumoral and non-tumoral mRNA expression levels in canine mammary cancer. Values were normalized based on hypoxanthine phosphoribosyltransferase-guanine (Hprt) and ribosomal protein L32 (Rpl32) expression levels. Animals without history of cancer were used as controls. Data expressed as mean±SD. Expression levels of A: Cyclin-dependent kinase inhibitor 2B (Cdkn2b), B: MYC proto-oncogene (Myc), C: T-box gene transcription factor 2 (Tbx2) and D: BMI1 proto-oncogene (Bmi1) mRNA. Data are the mean±SD. *Significantly different at p<0.05.*

diagnosis of animals with mammary cancer. *Bmi1* overexpression should be better evaluated regarding its role in the metastatic process in canine mammary cancer. As far as we are aware, this is the first study involving these genes in canine mammary cancer.

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