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PATHOLOGY

Expression of LIN28A/B and Let-7 miRNAs in canine mammary carcinomas

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ABSTRACT: *LIN28 is a RNA-binding protein including two highly conserved homologous, LIN28A and LIN28B. Proto-oncogenes such as LIN28A and LIN28B are generally targeted by the let-7 miRNAs in different types of human cancers. Here, we determined the expression of LIN28A in canine mammary tumor samples and the LIN28/let-7 pathway in canine mammary cell lines. In those cell lines, we identified a functional LIN28/let-7 pathway which exhibited high expression of let-7 members and low expression of its targets, including LIN28A and LIN28B. However, the mammary carcinoma tissue samples showed a frequent expression of LIN28A being expressed mainly in the epithelial cells. No association was observed between LIN28A expression and histopathological classification and grade, TNM and survival time. Our results suggested a possible role of the LIN28A protein in the development of canine mammary carcinomas due to the high frequency observed in the tumor samples (28 of 32). The in vitro experiments suggested that the LIN28/let-7 pathway is active in the tumor cells evaluated. However, more studies are necessary to elucidate the exact role of LIN28/let-7 pathway in canine mammary carcinomas.*
Key words: *canine mammary carcinomas, LIN28, let-7, veterinary oncology.*

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RESUMO: *LIN28 é uma proteína de ligação ao RNA, com duas formas homólogas altamente conservadas, LIN28A e LIN28B. Os proto-oncogenes LIN28A e LIN28B são regulados pela família de miRNAs let-7 em diferentes tipos de cânceres em humanos. No presente trabalho, o objetivo foi determinar a expressão de LIN28A em amostras de tumor mamário de cadelas e a via LIN28/let-7 em linhagens celulares mamárias caninas. Nestas linhagens, através das técnicas de qPCR e RNAseq, foi identificado que a via LIN28/let-7 apresenta-se funcional, com alta expressão dos membros da família let-7 e baixa expressão de seus alvos, entre eles LIN28A e LIN28B. No entanto, as amostras de tecidos de carcinomas mamários caninos demonstraram expressão frequente de LIN28A, sendo observada principalmente em células epiteliais. Não foram observadas associações entre expressão de LIN28A com classificação e gradação histopatológicas, TNM e tempo de sobrevida. Nossos resultados sugerem uma possível relação da proteína LIN28A no desenvolvimento de carcinomas mamários caninos devido à alta frequência observada nas amostras tumorais (28 de 32). Os experimentos in vitro sugerem que a via LIN28/let-7 é ativa nas linhagens celulares caninas avaliadas. Entretanto, estudos funcionais ainda são necessários para elucidar a função exata da via LIN28/let-7 nos carcinomas mamários caninos.*
Palavras-chave: *carcinoma mamário canino, LIN28, let-7, oncologia veterinária.*

INTRODUCTION

The *LIN28A* and *LIN28B* genes have been studied in several types of human cancer such as breast (PISKOUNOVA et al., 2011; XIONG et al., 2017), colon (KING et al., 2011), gastric (HU et al., 2014), ovarian (LU et al., 2012) and prostate cancer (ALBINO et al., 2016). These genes are frequently found with high expression in these tumors exhibiting a key role in transformation, progression, and malignancy (HAMANO et al., 2012; ZHOU et al., 2013). Furthermore, high expression of LIN28A/B in human malignant tumors is related to Epithelial-Mesenchymal Transition (EMT) and the Cancer Stem Cell (CSCs) phenotype (LIU et al., 2013; VISWANATHAN & DALEY, 2010) being associated to high rate of metastasis, relapse, and reduced survival time (WANG et al., 2015).

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The family of let-7 miRNAs plays an important function during carcinogenesis acting as a tumor suppressor and regulating the expression of a large number of oncogenes including *LIN28A/B* (CAI et al., 2013; KOLENDA et al., 2014; LV et al., 2012; THORNTON & GREGORY, 2012). Furthermore, the presence of let-7 is related to cellular differentiation, proliferation and self-renewal developing a key role in the regulation of genes like *c-MYC*, *RAS* and *HMGA2* (SAKURAI et al., 2012; WANG et al., 2012). Thus, let-7 miRNAs are post-transcriptionally downregulated in various types of cancer such as pancreatic cancer, ovarian cancer, prostate cancer, breast cancer and melanoma (WANG etal.,2015) mostly targeted by LIN28A/B in a double-negative feedback, through distinct mechanisms.

Only few studies have addressed the *LIN28/*let-7 pathway in canine cancers. STERENCZAK et al. (2014) observed a small frequency of *LIN28* gene expression in oral squamous cell carcinoma (OSCC). In the same tissues, let-7 expression was constant and higher in the OSCC samples in comparison with non-neoplastic control samples indicating a possible *LIN28* regulation mediated by let-7, in contrast to studies in human cancer (STERENCZAK et al., 2014). Therefore, more detail studies need to be performed to elucidate the role of *LIN28/*let-7 pathway in canine cancer in general.

Here, we evaluated the presence and frequency of LIN28A protein in canine mammary carcinomas, as well as the mRNA expression of *LIN28A/B* proto-oncogenes and the let-7 miRNA family members in cell lines isolated from canine mammary carcinomas to observe the presence of the LIN28/let-7 pathway in the canine mammary cancer cells.

MATERIALS AND METHODS

Cell lines

Two cell lines were used in this study: M5 and M25, previously isolated from canine mammary tumors and characterized regarding to morphology, cytoskeleton filaments markers and malignant potential. Both lines present spindle cell shape and cytokeratin, vimentin and α-smooth actin expression, characteristic immunomarkers of myoepithelial-like cells. Furthermore, these cell lines were positive for invasion and tumor sphere assays. Molecular validation of cell lines was also performed previously (CORDEIRO et al., 2018; XAVIER et al., 2019). M5 and M25 cells were maintained in 75 cm^2 flasks at 37 °C and 5% CO_2 with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Passaging was performed when cells were 85% confluent. Culture evolution was evaluated daily by optical microscopy (Axio Vert A1, Zeiss, Germany). All reagents used for cell culture were purchased from Thermo Fisher Scientific, USA.

RNA extraction, cDNA synthesis and Real Time PCR

Gene expression of *LIN28A*, *LIN28B*, let-7a, let-7b, let-7c, let-7e, and let-7g miRNAs was evaluated by qPCR. The total RNA of M5 and M25 cell lines was extracted using TRIzol Reagent (Thermo Fisher Scientific, USA), following the manufacturer's recommendation. RNA samples were quantified and the 260/280 and 260/230 ratio were determined by NanoDrop 2000™ (Thermo Fisher Scientific, USA). Reverse transcription to obtain cDNA was performed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA), according to manufacturer's protocol. The reverse transcription of the miRNAs was performed using the miScript II RT kit (QIAGEN, Germany). Specific primers were designed using Primer3 Plus program (UNTERGASSER et al., 2007). *LIN28A/B* qPCR reactions were carried out using Fast SYBR Green Master Mix (Thermo Fisher Scientific, USA). Conditions for quantitative qPCR were as follows: 95 °C for 20 seconds; 40 cycles at 95 °C for 3 seconds for denaturation, 60 °C for 30 seconds for anneal/extend; melt curve analysis was performed at 95 °C for 15 seconds and 60 °C for 60 seconds. Let-7 members family qPCR reactions were carried out using miScript SYBR green PCR kit (QIAGEN, Germany). Conditions for qPCR were as follows: 95 °C for 15 minutes; 40 cycles at 94 °C for 15 seconds for denaturation, 55 °C for 30 seconds for annealing, 70 °C for 30 seconds for extension. The housekeeping genes used were *18S* ribosomal RNA for *LIN28A/B* reactions and the *RNT43 snoRNA* and *Hm/Ms/Rnt-1* snRNA for let-7 reactions. The analysis of relative gene expression data was performed according to the ΔΔCt method (LIVAK & SCHMITTGEN, 2001). The experiment was performed using three biological triplicates and analyzed in technical duplicates.

Prediction of let-7 targets

The target prediction of the let-7 family was performed using *in silico* analyses by the database miRTarBase (CHOU et al., 2018). This tool provides experimentally validated target genes of specific miRNAs; however, this database is designed for

human miRNAs. Thus, to validate if the genes can also be target by let-7 in canine species, the 3'UTR homology of these genes in both species was evaluated using TargetScan tool (AGARWAL et al., 2015).

Gene expression of let-7 targets by RNA-seq data analysis

Evaluation of the expression of predicted let-7 targets was performed from the results obtained from the global analyses of gene expression from M5 and M25 cell lines. Extraction of RNA, preparation of mRNA libraries, sequencing and sequencing quality, aligments and analysis to determine FPKM are described in CORDEIRO et al., 2018. Once we obtained a high sequencing quality, each sample was aligned against the reference genome (CanFam3.1) using TopHat 2.0.9 and Bowtie 2.1.0_ENREF_11. PCR duplicates, not primary alignments and low-quality reads were removed with Sam tools and the abundance of the transcripts that aligned with the reference genome was determined using Cufflinks 2.2.1, providing the fragments per kilobase million (FPKM) values. The FPKM values for each let-7 target were analyzed.

Tissues samples and Immunohistochemistry evaluation of LIN28A protein

Thirty samples of formalin-fixed and paraffin-embedded tissues (FFPE) from canine mammary carcinomas attended in the Veterinary Hospital of the Metropolitan University of Santos (UNIMES), under the supervision of Prof. Dr. Luiz Roberto Biondi, were used. In addition, were included tissue samples obtained in private veterinary clinics, from which M5 and M25 cell lines were derived. All these hospitals and clinics agreed to participate in the present study according to the protocol approved by the Ethics Committee on Animal Use, University of Sao Paulo, protocol number CEUA/3094061014. Samples were classified according to histological type and tumor grading following the criteria proposed by Goldschmidt 2011 (GOLDSCHMIDT et al., 2011). The information about patient survival time and TNM tumor staging were classified according the criteria proposed by OWEN (1980) and by the World Health Organization (WHO), according to LANA et al. (2007).

For the immunohistochemistry of LIN28A, FFPE tissues were cut into 3 µm thick fragments and mounted in silanized glass slides. Antigen retrieval was performed with 0.01M Sodium Citrate, pH 6.0 on steam heating at approximately 96 °C for 20 minutes. Then, peroxidase blockade was performed using 3% hydrogen peroxide for 20 minutes covered from light and at room temperature. Next, a second blockade using 5% skimmed milk at room temperature for one hour was performed. A primary rabbit polyclonal antibody against LIN28A (ab155542, Abcam, EUA) was used at 1:1000 concentration and the slides were incubated at 4 °C in a wet chamber overnight. Next, the slides were subjected to the polymer secondary antibody complex using the EasyLink One kit (EP-12-20502, EasyPath) for 15 minutes covered from light. Staining was performed using DAB chromogen – Liquid 20X Concentrated (EP-12-20542, EasyPath) for 5 minutes. Standardization of immunohistochemistry protocol was performed in canine testis slides (LEE et al., 2017). Negative control samples were processed in the same conditions as the positive samples, with replacement of the primary antibody for normal rabbit IgG (sc-2027, Santa Cruz, EUA) or PBS. Staining was evaluated according to intensity, varying from 1 to 3, being 1, low intensity; 2, moderate intensity; and 3, high intensity. According to percentage of stained cells, it varied from 0 to 3, being 0, no tumor cell stained; 1, 1 – 10% of tumor cells stained; 2, 10 – 50% of tumor cells stained, and 3, more than 50% of tumor cells stained. The final score was obtained by multiplying the intensity score with the percentage score, resulting in values from 0 to 9 (HSU et al., 2015).

Statistical analysis

Statistical analysis and graphs were made with GraphPad Prism® 6.0 Software (San Diego, CA, USA). The comparison between the let-7 members expression in the two cell lines was performed by Kruskal-Wallis test. Association between LIN28A score and histopathological type was performed by ANOVA. The correlations between LIN28A immunostaining score with histological grading and TNM staging were performed by Spearman correlation. The relation between LIN28A score and patients' survival time was assessed using the Kaplan-Meier curve. Significant statistical differences were considered when $P < 0.05$.

RESULTS

Gene expression of let-7 miRNAs family in cell lines

In order to verify the expression levels of let-7 miRNAs in M5 and M25 myoepithelial-like cells, we performed qPCR analysis. Let-7a, let-7b, let-7c, let-7e, and let-7g miRNAs were highly expressed in both cell lines, with no significant statistical difference between M5 and M25 cell lines (Table 1).

Table 1 - Gene expression levels of Let-7a, Let-7b, Let-7c, Let-7e and Let-7g miRNAs evaluated by real-time PCR. No significant statistical difference was observed between the M5 and M25 cell lines (P < 0.05).

	M5	M25
CT *Rnt-1*	10.95 ± 0.35	10.65 ± 0.23
CT *let-7a*	18.88 ± 0.44	18.23 ± 0.22
2ΔΔCT *let-7a*	1.00 ± 0.06	1.31 ± 0.41
CT *let-7b*	19.97 ± 1.01	18.72 ± 0.23
2ΔΔCT *let-7b*	1.15 ± 0.64	1.93 ± 0.29
CT *let-7c*	18.49 ± 0.56	18.10 ± 0.15
2ΔΔCT *let-7c*	1.01 ± 0.18	1.07 ± 0.27
CT *let-7e*	18.60 ± 0.56	18.14 ± 0.05
2ΔΔCT *let-7e*	1.00 ± 0.16	1.12 ± 0.21
CT *let-7g*	22.47 ± 0.28	22.63 ± 0.04
2ΔΔCT *let-7g*	1.38 ± 0.08	1.00 ± 0.13

However, we observed differences regarding the let-7 expression in each cell line, where the let-7g is the less expressed both in the M5 and M25 cell lines (P < 0.05; Figure 1).

Gene expression of let-7 targets evaluated by RNA-seq

Besides *LIN28A/B*, let-7 miRNAs have several other targets described (BÜSSING et al, 2008). First, we performed an *in silico* analysis using MirTarBase version 7 algorithm that predicted 126 experimentally validated target genes of let-7 miRNA family. From these 126 targets, 65 genes showed homology at the seed region between the human and canine species, as observed using TargetScan version 7.2. Finally, the RNA-Seq data confirmed low expression of some let-7 target genes in M5 and M25 cell lines. From 65 targets, 10 genes exhibited low expression with FPKM values equal to or close to zero, including *IL10*, *PRDM1*, *KLK10*, *MYCN*, *FASLG*, *IGF1*, *IGF2BP1* and *TRIM71* (Table 2). These results suggested that the *LIN28*/let-7 pathway is potentially functional in M5 and M25 cell lines.

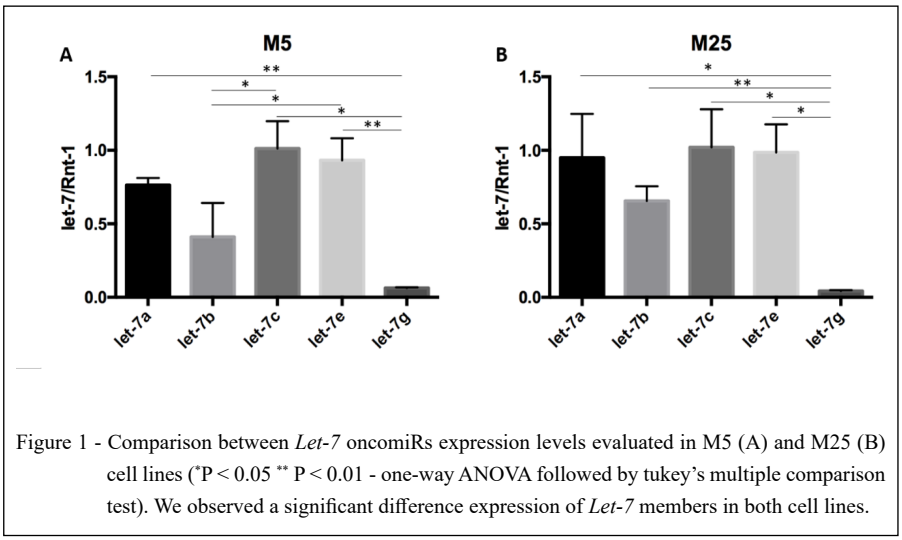

Figure 1 - Comparison between *Let-7* oncomiRs expression levels evaluated in M5 (A) and M25 (B) cell lines (*P < 0.05 ** P < 0.01 - one-way ANOVA followed by tukey's multiple comparison test). We observed a significant difference expression of *Let-7* members in both cell lines.

Table 2 - From the 65 *Let-7*-target genes, 10 genes exhibited very low expression with FPKM (fragments per kilobase million) values equal to or close to zero.

miRTarBase ID	miRNA	Target Gene	Ensemble code (Canine Species)	FPKM (M5)	FPKM (M25)
MIRT054365	hsa-let-7c-5p	*IL10*	ENSCAFG00000011443	0.31	0
MIRT005514	hsa-let-7f-5p	*PRDM1*	ENSCAFG00000003694	0	0
MIRT000455	hsa-let-7f-5p	*KLK10*	ENSCAFG00000029529	0	0
MIRT032100	hsa-let-7e-5p	*MYCN*	ENSCAFG00000003715	0	0
MIRT731263	hsa-let-7e-5p	*FASLG*	ENSCAFG00000014678	0	0
MIRT054579	hsa-let-7e-5p	*IGF1*	ENSCAFG00000007304	0.57	0.34
MIRT006055	hsa-let-7a-5p	*IGF2BP1*	ENSCAFG00000016907	0.61	0
MIRT004801	hsa-let-7a-5p	*LIN28A*	ENSCAFG00000012488	0	0
MIRT003834	hsa-let-7b-5p	*LIN28B*	ENSCAFG00000003598	0	0
MIRT002077	hsa-let-7a-5p	*TRIM71*	ENSCAFG00000025466	0.31	0.85

LIN28A protein expression in canine mammary carcinoma tissues

In order to evaluate the presence of LIN28A protein expression in canine mammary carcinoma, 32 samples of different histopathological types were analyzed by immunohistochemistry. The LIN28A protein expression was observed in 28 of 32 mammary carcinomas samples. Positive samples exhibited final staining score ranging between 1 and 9 according to their intensity and percentage of stained cells (HSU et al., 2015). Samples 5952, 12709, 16786 and 17773 were negative for LIN28A presenting final score 0 (Table 3). The LIN28A expression was observed in the nucleus and/or cytoplasm of parenchymal cells, predominantly in epithelial cells. The stromal tissue showed less or no expression of LIN28A. In addition, 23 samples exhibited variable expression of LIN28A in myoepithelial cells. LIN28A expression was also frequently observed in endothelial cells. No significant statistical correlation was observed between staining score and histopathological type, histological grading, TNM staging and survival time. (Table 3 and figure 2).

DISCUSSION

In the present study, we evaluated the expression of five members of the let-7 miRNAs family in two myoepithelial-like cell lines isolated from canine mammary carcinomas. The results showed that all let-7 members were highly expressed in both cell lines. In addition, we observed low expression of let-7 targets genes including the homologues *LIN28A* and *LIN28B*, suggesting that the *LIN28*/let-7 pathway may be functional in these canine mammary cancer cell lines. However, in contrast to the results reported in cell lines, we showed a frequent and heterogeneous expression of LIN28A protein in different histopathological canine mammary carcinomas tissues by immunohistochemistry.

In mammals, LIN28A/B develop a key role in many biological processes including stem cell differentiation, cell proliferation and glucose metabolism (CHANG & DALEY, 2013; ZHANG et al., 2016). In human tumors, LIN28 expression is generally upregulated, correlating with advanced disease and poor prognosis for many cancer subtypes (WANG et al., 2015). Conversely, this is the first description of LIN28 protein expression in canine mammary cancers. We determined the presence of the LIN28A protein in tissue samples from canine mammary carcinomas. The protein was expressed in 87.5% of the samples, mainly in the epithelial cancerous cells. However, the myoepithelial cells and other stromal cells showed modest or absent expression of LIN28A protein. Some studies in humans described the expression of LIN28A and LIN28B mainly in cancer cells presenting a mesenchymal phenotype, since they are directly associated with epithelial-mesenchymal transition

Table 3 - Evaluation of LIN28A expression in 32 samples of canine mammary carcinomas. LIN28A protein expression was observed in 28 of 32 canine mammary carcinoma samples, exhibiting final score staining ranging from 1 to 9. Samples exhibiting final score 0 were considered negative.

		----------LIN28A Staining----------					
Histopathological type (GOLDSCHMIDT et a., 2011)	Samples	Intensity	Percentage	Final Score	Stroma	Myoepithelial cells	Endothelial cells
Tubular	12318	1	3	3	Yes	Yes	Yes
Tubular	17695	1	2	2	No	No	No
Tubular	17704	1	2	2	Yes	No	No
Tubular	17939	1	1	1	No	No	Yes
Tubular	18690	2	3	6	Yes	Yes	Yes
Tubular	18997	2	2	4	Yes	Yes	Yes
Tubulopapillary	5952	0	0	0	No	No	No
Tubulopapillary	16766	2	3	6	Yes	Yes	Yes
Tubulopapillary	17577	2	1	2	No	No	Yes
Tubulopapillary	17629	2	3	6	Yes	Yes	Yes
Tubulopapillary	17773	0	0	0	No	No	Yes
Tubulopapillary	18719	2	3	6	Yes	Yes	Yes
Complex	16461	2	3	6	Yes	Yes	Yes
Complex	16770	3	3	9	Yes	Yes	Yes
Complex	16786	0	0	0	No	No	Yes
Complex	17621	2	3	6	Yes	Yes	Yes
Complex	17641	2	2	4	Yes	Yes	Yes
Complex	18127	2	3	6	Yes	Yes	Yes
Complex	18558	2	2	4	Yes	Yes	Yes
Mixed	16160	3	3	9	Yes	Yes	Yes
Mixed	17564	1	3	4	Yes	Yes	Yes
Mixed	17630	1	2	2	Yes	No	Yes
Mixed	18141	2	3	6	Yes	Yes	Yes
Mixed	18341	1	1	1	No	Yes	Yes
Mixed	19136	3	2	6	Yes	Yes	Yes
Mixed	025/14	3	3	9	Yes	Yes	Yes
Comedocarcinoma	11025	2	3	6	Yes	Yes	Yes
Comedocarcinoma	005/13	3	2	6	Yes	Yes	Yes
Solid	12709	0	0	0	No	No	Yes
Solid	17524	2	3	6	Yes	Yes	Yes
Solid	18062	2	3	6	Yes	Yes	Yes
Inflammatory	17570	2	2	4	Yes	Yes	Yes

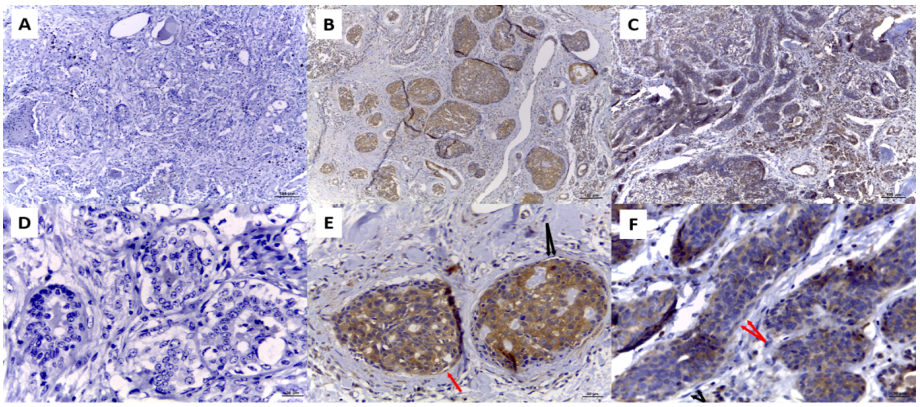

Figure 2 - Photomicrographs of canine mammary carcinomas. (A and D) carcinoma mixed-type used as a negative control; (B and E) tissue sample 005/13 (carcinoma mixed-type) with moderate percentage and high intensity of stained cells, resulting in final score 6; (C and F) tissue sample 025/14 (comedocarcinoma) with high percentage and moderate intensity of stained cells, resulting in final score 6. In both samples, around the malignant epithelial formations it is observed myoepithelial-like cells that do not stain for LIN28A. Magnification: 10X in A, B and C, 40X in E, F and G. Red arrow: myoepithelial-like cells expressing LIN28A protein. Black arrow: myoepithelial-like cells with no expression of LIN28A protein.

(EMT) (LIU et al., 2013). Studies also demonstrate that LIN28A is also present in mammal epithelial cells poorly differentiated of lung, kidney, and intestine during the embryonic development but with the progression of gene differentiation is downregulated (TSIALIKAS & ROMER-SEIBERT, 2015; YANG & MOSS, 2003).

The cellular distribution of LIN28A in cancer cells has also been widely studied. It has been reported that LIN28A is predominantly present in the cytoplasm. The localization of LIN28A is probably related to its function, which inhibits the processing of let-7 precursors by Dicer in the cytoplasm (PISKOUNOVA et al., 2011). Nevertheless, it is also reported that LIN28A proteins can be reported in a lesser extent in the nucleus and nucleolus of the cells (BALZER & MOSS, 2007; CHANG & DALEY, 2013; VOGT et al., 2012). In the canine cancer samples evaluated here, the localization of the LIN28A protein was also observed basically in the cell cytoplasm which corroborated with previous studies in human cancer (CHO et al., 2012; THORNTON & GREGORY, 2012).

In the present study, we also evaluated the presence of *LIN28*/let-7 pathway in two canine mammary cancer cell lines established in our laboratory (CORDEIRO et al., 2018). Although most of the tissue samples from canine mammary carcinomas, including the tissues that originated the M5 and M25 cell lines (005/13 and 025/14), showed significant expression of LIN28A protein, the gene expression of both *LIN28A* and *LIN28B* were not detected in the cell lines. This could be explained by two hypotheses: 1) the tumor dissociation process and cell culture of our conditions selected myoepithelial cells. In fact, both cell lines present a myoepithelial-like phenotype, and some cells with this phenotype did not originally express the protein LIN28A in the tissues, as it was demonstrated in the LIN28A immunohistochemistry assay; 2) when cells were dissociated from the primary tumor and cultured, the cell gene expression profile changed leading to the loss of some malignancy characteristics. This can justify the high let-7 miRNAs gene expression and the downregulation of *LIN28A*, *LIN28B* and other gene targets. Taken together, these possibilities may justify why the cell lines used in this experiment did not express *LIN28A* and *LIN28B* genes.

So far, only one study about *LIN28*/let-7 pathway in canine cancer could be found. STERENCZAK et al. (2014) observed that in seven tissue samples of OSCC, only one showed *LIN28* expression evaluated by real-time PCR. Conversely, we observed LIN28A protein expression in 87.5% of our canine mammary carcinoma samples. This result suggested that LIN28A could play a more

relevant role in mammary carcinomas in dogs. Lastly, regard to the cell culture, STERENCZAK et al. (2014) demonstrated that the two canine OSCC cell lines expressed let-7a but did not express *LIN28A*, similar to what we reported in the present research. Thus, despite the previous results, the present study contributed to the identification of a possible role of *LIN28* genes in canine mammary carcinomas.

CONCLUSION

In conclusion, our findings demonstrated the presence of LIN28A expression in the canine mammary cancer tissues and the probable functionality of *LIN28*/let-7 pathway in canine mammary carcinoma cells, with let-7 members possibly regulating the expression of its targets. Despite the results observed here, more studies are necessary to determine the role of LIN28A and LIN28B in cancer development and progression in canine species.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHOR'S CONTRIBUTIONS

Conceptualization, H.F., R.L.G. and J.C.S.; Methodology, H.F., R.L.G., P.L.P.X., Y.G.C., R.F.S. and L.R.B; Formal Analysis: H.F., R.L.G., P.L.P.X; Resources: H.F., R.F.S., J.S.C., and L.R.B. Writing - Original Draft: H.F., R.L.G., and P.L.P.X; Writing – Review & Editing: all authors; Supervision: H.F.; Project Administration: H.F.; Funding Acquisition: H.F.

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