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RESEARCH ARTICLE

# *PGAM1* and *TP53* mRNA levels in canine mammary carcinomas – Short communication

TÁBATA MAUÉS[1]*, TÁYA FIGUEIREDO DE OLIVEIRA[1], KÊNIA BALBI EL-JAICK[2], AGNES MARIE SÁ FIGUEIREDO[3], MARIA DE LOURDES GONÇALVES FERREIRA[1] and ANA MARIA REIS FERREIRA[1]

[1] Department of Pathology and Veterinary Clinic, Faculty of Veterinary, Universidade Federal Fluminense, Av. Alm. Ary Parreiras, 507, Icaraí, 24220-000, Niterói, RJ, Brazil

[2] Department of Genetics and Molecular Biology, Universidade Federal do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

[3] Department of Medical Microbiology, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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**ABSTRACT**

*TP53* and *PGAM1* genes play a key role in glycolysis which is an essential metabolic pathway of cancer cells for obtaining energy. The purpose of this work was to evaluate *PGAM1* and *TP53* mRNA expressions in canine mammary carcinomas (CMC) and to correlate them with animal data and tumour histological features. None of the nine samples analysed revealed *PGAM1* DNA sequence variations. *PGAM1* and *TP53* RNA expressions from 21 CMC were analysed using a one-step reverse transcription-PCR kit and its platform system. Most CMC samples had low levels of *PGAM1* mRNA (71.5%) and normal expression of *TP53* mRNA (95.2%). Our results suggest a different feature of the Warburg effect on canine mammary cancer cells compared to human cells.

**KEYWORDS**

cancer, dog, glycolysis, phosphoglycerate mutase, p53

Cancer cells are characterised by unregulated cell proliferation and the blood vessels that form within tumours are usually structurally and functionally abnormal, resulting in severe hypoxia. The activity of hypoxia-induced factors mediates angiogenesis, epithelial–mesenchymal transition, stem cell maintenance, invasion, metastasis, and tumour resistance (Semenza, 2012). Additionally, there is a significant correlation between malignancy and anaerobic glycolysis rate (Mikawa et al., 2014a, 2014b). The glycolytic pathway is essential for energy production in cells and its increase, known as the Warburg effect, is often observed in cancers (Warburg, 1956), being one of the ten hallmarks of cancer (Hanahan and Weinberg, 2011). However, little is known about the mechanisms of the Warburg effect in canine cancers. Some genes such as *TP53* and *PGAM1* play a key role in glycolysis. Besides its already known cell cycle inhibition and pro-apoptotic function, it has been discovered that the p53 protein also participates in the control of metabolic pathways (Shen et al., 2012). The p53 protein activates p53-induced glycolysis and apoptosis regulator, decreasing glycolytic metabolism. However, when p53 activity is reduced, cell proliferation can still be maintained through the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) (Hitosugi et al., 2012). The enzyme phosphoglycerate mutase (PGAM) has two subunits that form three isoenzymes, BB-PGAM (PGAM1), MM-PGAM (PGAM2) and MB-PGAM (PGAM3). PGAM1 is found mainly in brain, liver, kidneys, blood vessels and white adipose tissue (Ren et al., 2010; Mikawa et al., 2014a). The role of PGAM1 in glycolysis makes it an attractive therapeutic target for cancer treatment, since its inhibition may interfere with the basic energy uptake of cancer cells (Qu et al., 2017; Jin and Zhou, 2019).

*Corresponding author. Tel.: +55 21 2629 9505.
E-mail: tabatamaues@id.uff.br

The purpose of this work was to evaluate *PGAM1* and *TP53* mRNA expressions in canine mammary carcinomas (CMC) and to correlate them with animal and tumour features (age, breed, histological type, grade and histopathological characteristics such as cellular atypia, mitotic index, ulceration, haemorrhage, inflammation, neovascularisation, pleomorphism, local invasion, and regional lymph node metastasis.

This study was approved by the Ethics Committee on the Use of Animals of Universidade Federal Fluminense (verdict number 693/2015).

A total of 21 CMC tissue samples from 14 female dogs undergoing therapeutic mastectomy were collected. Following mastectomy, mammary tumour samples were reserved for histopathological, DNA and RNA analyses. Small tumour tissue fragments of about 0.5 $cm^3$ in size, with no macroscopic evidence of necrosis or devitalised areas, were selected and stored at –20 °C for DNA extraction and other fragments were stored in liquid nitrogen at –196 °C for further RNA extraction. The remaining mammary chain was fixed in 10% buffered formalin solution for further histological preparation and evaluation. Meantime, a healthy mammary tissue for RTq-PCR calibrator was obtained from a 3-year-old female Yorkshire Terrier dog that had undergone elective spay surgery.

Genomic DNA was extracted from 9 out of 21 CMC tissue samples (DNeasy Blood & Tissue Kit, Qiagen®, CA). Forward (5′-ATTCTGATTCCAGAGTGGACCTG-3′) and reverse (3′-GCTCAATGGGACTAGGCAGATAC-5′) primers were designed for *PGAM1* gene exon 2 amplification (the region that contains the only variant reported in dogs) using the NCBI genomic sequence NC_006610.3 as reference. PCR reactions were held according to a standard reaction with an annealing temperature of 58 °C. PCR products of 442 bp were analysed by Sanger DNA sequencing method (Applied Biosystems™).

RNA from 21 CMC was extracted and purified using the RNeasy mini kit (Qiagen™, Germantown). The expression of *PGAM1* was analysed using a one-step reverse transcription-PCR kit (Thermo Fisher Scientific, Waltham™) and its platform system (Step One real-time PCR system, Thermo Fisher Scientific). The forward (5′-GATCAGCTAC CCTCCTGTGAG-3′) and reverse (3′-CTCCAGATGCTT GACAATGCC-5′) primers were designed for *PGAM1* RNA analysis. Primers for *TP53* and *ATP5B* (reference gene) analyses were described previously (Klopfleisch et al., 2010; Costa et al., 2011). The relative quantity (RQ) of transcript levels was calculated using the comparative ΔΔCT method. In order to measure *PGAM1* and *TP53* expression changes, the mean RQ values for one healthy canine mammary tissue sample was set as 1.0 (calibrator) by statistical normalisation. Cut-off values were defined as fold change (FC) $\geq$ 2.0 for *PGAM1* or *TP53* overexpression, $\leq$ 0.5 for reduced and > 0.5 to < 2.0 for normal expression (Dalgin and DeLisi, 2005; Shi et al., 2008).

Table 1 shows that 15 tumours (71.5%, $n$ = 15/21) had low *PGAM1* expression, four (19.0%, $n$ = 4/21) were considered to be within the normal range, while two carcinomas (9.5%, $n$ = 2/21) had high levels. Both tumours with increased *PGAM1* mean RQ are from animals number 2 and 11, which presented carcinomas in a mixed tumour grade I with normal levels of *TP53* mRNA.

Regarding the expression of *TP53*, only one CMC (4.8%, $n$ = 1/21) from animal number 5 showed increased levels of mRNA. All other CMC (95.2%, $n$ = 20/21) presented a normal standard of mRNA transcript.

The modified TNM clinical staging based on the WHO Classification (Owen, 1980) was used. The animals' data collected included age, breed and clinical stage, while the tumors were analysed regarding histological type, grade and histopathological characteristics (cellular atypia, mitotic index, ulceration, haemorrhage, inflammation, neovascularisation, pleomorphism, local invasion, regional lymph node metastasis), and none of them presented statistically significant differences ($P$ > 0.05) in relation to *PGAM1* or *TP53* mean RQ values of transcript levels by Pearson's two-sided Chi-Square test. Nevertheless, the present study has some limitations that may have hampered the statistical analysis and the drawing of more accurate conclusions. The biases include (1) the small number of animals and tumours, (2) the heterogeneity of mammary tumours, and (3) the lack of evaluation of cell proliferation factors as well as p53 protein profiles and *TP53* mutations.

None of the nine CMC tested showed DNA variants in *PGAM1* exon 2. The mean RQ was high in one, normal in three and reduced in five of these nine samples also tested by sequencing for *PGAM1* variants. The absence of *PGAM1* exon 2 variants suggests that mRNA expression changes are related to *PGAM1* interactions with other gene products that regulate its expression, such as p53. Studies demonstrate the ability of p53 to regulate the expression of several genes, including *PGAM1* (Ruiz-Lozano et al., 1999; Qu et al., 2017). The *PGAM1* gene contains a p53 response element, which mediates the activation of *PGAM1* transcription, as demonstrated in cardiomyocytes (Kondoh et al., 2005). A wild-type p53 was reported to inhibit *PGAM1* expression, whereas a mutant p53 induced it (Kondoh et al., 2005). Therefore, *PGAM1* could be differently regulated in cancer (Mikawa et al., 2014a, 2014b).

In humans, increased *PGAM1* expression was associated with human breast cancer (Durany et al., 2000). Besides, high expression of *PGAM1* is associated with metastasis, advanced clinical stage, and poor prognosis in some human cancers (Zhang et al., 2017; Liu et al., 2018; Feng et al., 2020). One study identified a significant increase of *PGAM1* transcripts in the serum of dogs with mammary cancer (Zamani-Ahmadmahmudi et al., 2014). Hussain et al. (2018) found overexpression of PGAM1 in CMC tissues, especially in the myoepithelia and moderate immunostaining in the tubular epithelia. Nevertheless, the results reported by those authors were not significantly different from those of dogs with benign mammary tumours. Our study showed high *PGAM1* expression in only two CMC samples, but it is noteworthy that we did not evaluate any case of complex carcinoma, which has myoepithelial origin.

*Table 1.* Animal number, breed, age, tumour staging by the TNM system (stage, tumour size in cm, regional lymph node metastasis and distant metastasis), type and histological grade of mammary carcinomas, and *PGAM1* and *TP53* mean RQ of each tumour

| An[1] | Breed | Age (years) | TNM System | | | | Histological | | Mean RQ[2] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Stage | T[3] (cm) | N[4] | M[5] | Type | Grade | *PGAM1* | *TP53* |
| 1 | D[6] | 12 | II | 3–5 | – | – | CMiT[7] | 2 | 1.115 | 1.079 |
| | | | | <3 | – | – | CMiT[7] | 2 | 0.390 ↓ | 0.613 |
| | | | | <3 | – | – | CMiT[7] | 3 | 0.444 ↓ | 0.532 |
| 2 | D[6] | 12 | I | <3 | – | – | CMiT[7] | 1 | 2.130 ↑ | 1.577 |
| 3 | MD[8] | 8 | III | >5–10 | – | – | PC[9] | 2 | 1.598 | 1.632 |
| 4 | YT[10] | 9 | III | >5–10 | – | – | CMiT[7] | 2 | 0.453 ↓ | 0.531 |
| 5 | LR[11] | 7 | IV | 3–5 | + | – | SC[12] | 3 | 0.013 ↓ | 2.001 ↑ |
| 6 | MD[8] | 11 | III | >10–15 | – | – | CS[13] | 2 | 1.189 | 1.112 |
| 7 | P[14] | 9 | III | >5–10 | – | – | CMiT[7] | 2 | 0.019 ↓ | 0.584 |
| 8 | D[6] | 11 | III | >5–10 | – | – | PC[9] | 1 | 0.291 ↓ | 0.751 |
| 9 | MD[8] | 10 | III | >15 | – | – | CS[13] | 2 | 0.258 ↓ | 0.712 |
| 10 | MD[8] | 10 | I | <3 | – | – | PC[9] | 1 | 1.657 | 1.192 |
| 11 | MD[8] | 11 | II | 3–5 | – | – | CMiT[7] | 1 | 3.145 ↑ | 1.114 |
| 12 | YT[10] | 9 | I | <3 | – | – | CMiT[7] | 1 | 0.012 ↓ | 0.732 |
| | | | | <3 | – | | CMiT[7] | 2 | 0.044 ↓ | 1.794 |
| | | | | <3 | – | | CMiT[7] | 1 | 0.015 ↓ | 1.367 |
| 13 | MD[8] | 10 | III | >5–10 | – | – | CMiT[7] | 1 | 0.022 ↓ | 0.598 |
| | | | | 3–5 | – | | CMiT[7] | 1 | 0.024 ↓ | 0.871 |
| 14 | MD[8] | 8 | IV | >5–10 | + | – | TC[15] | 1 | 0.263 ↓ | 1.040 |
| | | | | >5–10 | + | | TC[15] | 2 | 0.461 ↓ | 1.243 |
| | | | | <3 | + | | CMiT[7] | 2 | 0.252 ↓ | 1.617 |

[1]An = Animal number; [2]RQ = Relative Quantity; [3]T = Tumour diameter size; [4]N = Regional lymph node metastasis; [5]M = Distant metastasis; [6]D = Dachshund; [7]CMiT = Carcinoma in a Mixed Tumour; [8]MD = Mixed Breed Dog; [9]PC = Papillary Carcinoma; [10]YT = Yorkshire Terrier; [11]LR = Labrador Retriever; [12]SC = Solid Carcinoma; [13]CS = Carcinosarcoma; [14]P = Poodle; [15]TC = Tubular Carcinoma.

It is still not well understood how *TP53* regulates certain aspects of metabolism in distinct types of cells and tissues, as well as in response to different stress signals, including glucose starvation, nutritional deprivation, DNA damage, or oncogene activation. As far as it is known, mutant and wild-type p53 proteins often regulate the same cellular biological processes with opposite effects. Thus, in metabolic regulation, wild-type p53 inhibits glycolysis while mutant p53 protein promotes glycolysis through distinct mechanisms (Zhang et al., 2013).

Some hypotheses could be considered in order to justify the reduced expression of *PGAM1* in the CMC samples identified in this study. Perhaps this *TP53* tumour suppressor mechanism that we suggest in our study, might be related to the performance of a competent wild-type *TP53*, since it is reported to block *PGAM1* expression (Kondoh et al., 2005). The reduction of *PGAM1* mRNA levels may be a consequence of glycolytic pathway inhibition in order to reduce the energy available to tumour cells. However, neither p53 protein profiles nor *TP53* mutations were evaluated in our experiment, so it is not possible to assert the link between *PGAM1* and *TP53*.

Little is known about the mechanisms of the Warburg effect in canine tumours. Interestingly, some reports suggest an unexpected aspect of the Warburg effect in canines which could evidence a diversity in cell metabolism (Gutte et al., 2015). Clemmensen et al. (2020) visualised the Warburg effect by clinical molecular imaging in soft tissue sarcoma, melanoma, osteosarcoma, and thyroid carcinomas in dogs. They found that [1-$^{13}$C]lactate generation showed a large degree of heterogeneity across tumour types and also spatially within tumours. Sarcomas tended to have a higher [1-$^{13}$C]lactate ratio compared to carcinomas. Our study also suggested reduced glycolysis in carcinomas due to the low expression of *PGAM1* in most samples (71.5%, $n$ = 15/21), but it is worthy of mention that we have not analysed sarcomas.

To conclude, these findings support that glycolysis for energy production in CMC might unfold differently from human cancer. Further studies with a higher number of samples are warranted to better understand the metabolic pathways of cancer cells in dogs.

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