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ORIGINAL PAPER

Protein Expression of *PI3K/AKT/mTOR* Pathway Targets Validated by Gene Expression and its Correlation with Prognosis in Canine Mammary Cancer

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Abstract

Mammary cancer is the main type of neoplasia in female dogs and is considered an adequate model for the biological and therapeutic study of cancer in women. The *PIK3CA/AKT/mTOR* pathway plays a central role in cellular homeostasis and is often dysregulated in cancer. The increased expression of PI3K protein in the literature is associated with a poor prognosis, and alterations in the *PIK3CA* gene can lead to changes in downstream pathways. Thus, the objective of this study was to validate the protein expression to confirm the gene expression of proteins belonging to the main pathway PI3K and PTEN, and their downstream pathways through ZEB1, ZEB2, HIF1A, VHL, CASP3 and PARP1 relating to prognosis in canine mammary cancer. For protein studies, the samples came from 58 female dogs with mammary neoplasia, immunohistochemistry was performed and its analysis by the histoscore method. For the genetic evaluation, the samples came from 13 patients, the DNA was extracted and the analysis for quantitative expression. Through immunohistochemistry, PI3K positivity was significantly associated with affected regional lymph node, distant metastasis, patients with HER2+, Triple Negative and Luminal B phenotypes, and the lowest survival rates. Through gene expression, we observed higher gene expression of *ZEB2* and *PARP1* both among patients who were alive and who died, which was not true for the expressions of *PIK3CA* and *HIF1A*. In conclusion, the data observed in this work are promising in the study of new molecular prognostic markers such as *PI3K*, *ZEB2* and *PARP1* for canine mammary cancer.

Keywords Angiogenesis · Apoptosis · H1047R · Mammary cancer · Metastasis

Introduction

Breast cancer is the second leading cause of death for women worldwide [1]. Mammary cancer is also extremely common in companion animals such as dogs and cats [2]. There is great interest in mammary neoplastic changes in dogs due to the similarity of the behavior of the neoplasm in women, such as the high rate of recurrence, metastasis and mortality [3, 4].

In both species, the prognosis of the disease is reserved due to tumor heterogeneity, characterized by the coexistence of different clones of neoplastic cells [5]. Advances have been made in recent years to improve the understanding of molecular changes in canine tumors [5–8]. As a result, the genetic panel and the molecular properties of these neoplasms have become clearer.

Genetic alterations can cause functional modifications of proteins that regulate intracellular signaling pathways, such as the *PI3K/AKT/mTOR* phosphatidylinositol 3-kinase pathway, which plays active roles in a wide range of important physiological processes. These include cell proliferation,

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survival, apoptosis, motility, adhesion, morphology, transformation and transport of proteins [6, 7].

Mutations in the *PIK3CA* gene have been found in several human cancers, such as glioblastoma, gastric cancer, lung cancer, colorectal cancer, and mammary cancer [7]. In veterinary medicine, mutations in this gene are often found; recent studies show that the H1047R mutation is the most frequently found in canine hemangiosarcoma [9] and canine mammary tumors [6, 7, 10].

The *PTEN* gene (phosphatase with tensin homology deleted in chromosome 10) is a tumor suppressor gene that functions as a negative regulator of cell proliferation [11, 12]. Alterations in this gene lead to failures in the signaling pathway and contribute to tumor growth through inhibition of apoptosis, neoangiogenesis and increased metastatic capacity [12–14]. In veterinary medicine, changes in *PTEN* expression have been investigated in canine melanoma [15], hemangiosarcoma [16], osteosarcoma [17], prostate cancer [18] and in canine mammary tumors [19] and feline mammary carcinomas [20] confirming the suppressive role between different species and neoplasms.

To overcome the stress signals, neoplastic cells often overexpress the *BCL-2* family proteins, which are anti-apoptotic. Consequently, the *Caspases-3* known as "executors" do not carry out the proteolytic cleavage of specific cell substrates that result in cell death [21]. Other proteins researched in cancer are specific proteins activated in response to DNA damage. Among these proteins, poly (ADP-Ribose) polymerase (*PARP*), *PARP1* is the most abundant protein of this family. In veterinary medicine, studies correlating *PARP1* with cancer survival are scarce [22–24].

PI3K signaling also contributes to cell migration and migratory cell polarization in various cell types [25]. The "Zinc-finger factors" family (*ZEB1* and *ZEB2*) is involved in oncogenesis through the *PI3K*/*AKT* pathway, in which its activation or inhibition promotes or suppresses the epithelial mesenchymal transition process (EMT) [26]. In the EMT process, they repress *E-cadherin* expression through direct binding in the promoter region, causing loss of *E-cadherin* expression and consequently promoting the development of metastatic properties, such as migration and invasion [27].

The *PI3K*/*AKT*/*mTOR* pathway also influences the angiogenesis process, contributing to the formation of new blood vessels. Hypoxia-inducible factor (*HIF-1α*) is a transcription factor that regulates oxygen concentration homeostasis. *HIF1A* expression is increased when the *PI3K*/*AKT*/*mTOR* pathway is activated [28]. Under normal oxygen conditions, *HIF1A* binds to Von Hippel-Lindau (p-*VHL*) protein and is degraded, however, under hypoxic conditions, *HIF1A* accumulates and translocates to the nucleus, regulating the transcription of their target genes [29].

The loss of *VHL* leads to the accumulation of *HIF1A* and consequently the activation of the angiogenesis cascade [30]. Therefore, low *VHL* expression impairs the degradation of *HIF1A*, promoting inappropriate activation of target genes downstream [31]. Zhang et al. [30] revealed a cancer proteogenomic atlas for *PI3K*/*AKT*/*mTOR* alterations that demonstrates that *VHL* mutations are associated with highly active *AKT*/*mTOR* signaling, conferring a more guarded prognosis.

Therefore, due to the high frequency of alterations in the *PI3K*/*AKT*/*mTOR* pathway reported in mammary cancer, its involvement in several important cellular processes, and because there are few studies exploring the expression patterns of the *PI3K*/*AKT*/*mTOR* pathway in canine mammary tumors [5–7, 10], this pathway is considered an important model for the study of proteins expressed by genes that are stimulated by *PIK3CA* and *PTEN*, related to metastasis (*ZEB1* and *ZEB2*), angiogenesis (*HIF1A* and *VHL*) and apoptosis (*CASP3* and *PARP1*). Thus, this work aimed to evaluate the gene and protein expression of molecular targets downstream of the *PI3K*/*AKT*/*mTOR* pathway, as well as to investigate their correlations with the prognosis of canine mammary cancer.

Materials and methods

Retrospective Study

Ethical Considerations

This study was approved by the Animal Experimentation Ethics Committee (CEUA) (Protocols nº 3231/2012) of the Faculdade de Medicina de São José do Rio Preto – FAMERP.

Samples

Samples of 58 adult female canines of different breeds and ages, affected by mammary neoplasia, from a previous study by our research group were used [8]. These fragments were submitted to standard histopathological processing and preserved in a paraffin block. The female dogs were monitored for 18 months to assess the occurrence of recurrences and metastases. For the immunohistochemical analysis, the paraffin TMA block was used.

Immunohistochemistry

Tissue fragments from selected female dogs were submitted to immunohistochemistry technique to evaluate the expression of target proteins PI3K, PTEN, ZEB1, ZEB2, HIF1A, VHL, CASP3 and PARP1. The "REVEAL Polyvalent

HRP-DAB Detection System" development kit, from Spring Bioscience (Pleasanton, CA, USA) was used for all procedures, being performed in a humid chamber. All immunoreactions were accompanied by a positive control for the antibody tested and a negative control (Supplementary Table 1).

At the end of the procedure, protein expression was quantified by the Histoscore (HS) technique using the ImageJ program with the "Immunohistochemistry (IHC) Image Analysis" tool. HS is a measure to convert classical immunohistochemistry into quantitative values and is based on staining intensity and percentages of stained cells and ranges from 0 to 300, being divided into four immunohistochemistry categories reported in percentage cells: stained negative cells (0), weak (1+), moderate (2+) and strong (3+). In each case, a histoscore with a potential range of 0-300 was calculated as follows: HistoScore = ((0 x % unstained cells) + (1 × % weakly stained cells) + (2 × % moderately stained cells) + (3 × % strongly stained cells)) [32].

Statistical Analysis

To perform the statistical analysis, the GraphPad Prism 8.0 program was used. Initially, to assess the assumption of normality, the Shapiro-Wilk test was performed.

The ROC curve was used in order to decide on the cutoff of the immunohistochemical staining index that integrates the best compromise between sensitivity and specificity, that is, when they are as high as possible. Soon after having this value, the survival curves were determined by the Kaplan-Meier method in order to calculate the cumulative probabilities of survival between the positive and negative labeling groups, followed by the logrank test to compare the survival curves. A value of $p < 0.05$ was considered statistically significant.

Finally, the Pearson Correlation test (r) was used to evaluate the correlations between the PI3K protein and the other proteins performed and classified according to the values of Dancey and Reidy [33].

Prospective Study

Ethical Considerations This study was approved by the Animal Experimentation Ethics Committee (CEUA) (nº 001-004391/2019) of the Faculdade de Medicina de São José do Rio Preto – FAMERP.

Sample Collection and Histopathological Evaluation for RT-PCR

Thirteen adult female canine patients of different breeds and ages were selected, affected by primary mammary neoplasia and followed up for 365 days, patients with cases of recurrence, were excluded. As a control group, six samples were collected, obtained from the mammaries of female dogs considered healthy on clinical examination. Exclusion criteria were: previous history of mammary cancer, pseudocyesis or any other lesion that demonstrates mammaries with an altered appearance on clinical examination. Race, clinical history and histopathological evaluation, including grading and tumor subtypes, which was performed based on the classification system by GOLDSCHMIDT et al. [34], can be seen in Supplementary Table 2.

Quantitative PCR (qPCR)

Total RNA was extracted from mammary fragments by the TRIZOL method (Invitrogen Life Technologies® - São Paulo, SP, Brazil), the concentration and purity of total RNA were analyzed using the NanoDrop 2000 C spectrophotometer (Thermo Fisher Scientific, Walthan, MA, USA). cDNA (single-stranded - complementary DNA) was synthesized by the High-Capacity cDNA Kit (Applied Biosystems®, Foster City, CA, USA) in a total volume of 20 µL of reaction mixtures at a final concentration of 100 ng/µL. Quantitative expression was performed by real-time PCR in triplicate using the Step One Plus system (Applied Biosystems®, Foster City, CA, USA), and a negative control was included in each reaction. PCR reactions contained 100ng of cDNA, 10µL of TaqMan Universal Master Mix, 8µL of DEPC water solution, 1µL of TaqMan Gene Expression for *PIK3CA*, *ZEB2*, *HIF1A* and *PARP1* which were subjected to the following amplification scheme: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The sequences used as endogenous controls have been previously described by Moschetta et al. (2019). For the analysis of the gene expression of the targets, the TaqMan assays (Applied Biosystems®, Foster City, CA, USA) were used: *PIK3CA*(Cf02705766_m1), *ZEB2* (Hs00207691_m1), *HIF1A* (Cf02741632_m1) and *PARP1* (Cf02630973_m1).

Relative quantification (RQ) was performed using the $2^{-\Delta\Delta Ct}$ [90] method. The relative expression of the samples was analyzed using the control group as a calibrator (RQ = 1). Results were expressed as median relative expression unit.

Results

Analysis of protein expression in mammary cancer samples in female dogs

Clinicopathological Characterization of the Population

In the retrospective study, which involved the analysis of protein expression, most female dogs diagnosed with mammary neoplasia were mixed breed (34.5%), followed by Poodles (16.1%); Coker Spaniel and Daschund (8.2%); Boxer and Miniature Pinscher (5.4%); Labrador and York Shire (2.7%); Pitbull, German Shepherd, Fox Paulistinha, Maltese, Beagle and Rotweiller (1.8%); Siberian Husky, Lhasa Apso, Shin Tzu, Akita, Basset Hound and Weimaraner (1%). In the prospective study, which comprised the analysis of gene expression, the majority were also of mixed race (76.9% %), followed by Poodles (15.3%) and Shih tzu (7.6%).

Fifty-eight samples of neoplastic mammary tissue from female dogs were analyzed and followed up for 540 days. At the end of this period, 44 patients remained in follow-up and 14 died, of which all had pulmonary and/or liver metastases and a mean survival of 274 days. These patients had been previously classified by histopathological examination according to Goldschimit et al. [34], performed the TNM staging and analysis of the tumor phenotype shown in Supplementary Tables 3, in addition to information on the presence of metastasis, death and survival in days.

In the histopathological analysis we observed: one solid mammary carcinoma grade I, four solid mammary carcinoma grade III, one mammary carcinosarcoma, one mammary carcinoma in situ, one anaplastic mammary carcinoma, one tubular mammary carcinoma grade II, one tubular mammary carcinoma grade III, two micropapillary mammary carcinomas, one tubulopapillary mammary carcinoma grade I, four tubulopapillary mammary carcinomas grade II, five tubulopapillary mammary carcinomas grade III, eight complex mammary carcinomas grade I, thirteen complex mammary carcinomas grade II, ten complex mammary carcinomas grade III, and two complex mammary carcinoma, one tubular mammary carcinoma and two tubulopapillary mammary carcinoma, all without grade classification.

Immunohistochemistry (IHC) test Analysis

Protein analyses were performed downstream of the PI3K pathway. IHC was performed on all 58 available tumor fragments, which revealed a median H-score for PI3K of 41, PTEN of 96, HIF1A of 25, VHL of 108, ZEB1 of 40, ZEB2 of 48, CASP3 of 92 and PARP1 of 40 (range 0 to 300). Figure 1 shows examples of staining results demonstrating positive and negative staining.

The cut-off points were determined from the Roc curve assays for each analyzed protein. Figure 2 shows the results; in all analyses $p < 0.05$. Among the mammary tumors, 17% (n = 10) were positive for PI3K. Among these, all patients progressed to the presence of metastasis and finally to death, in an average of 294 days. For its antagonist, PTEN, 43.5% (n = 20) of the tumors studied were negative, with 85% (n = 17) progressing to metastasis or recurrence and 70% (n = 14) to death.

In the analysis of the HIF1A protein, 25.9% (n = 15) of the tumors studied were positive, and only two patients (13%) remained alive and without metastasis. For VHL, 27.5% (n = 16) were negative, of which only 18.7% (n = 3) did not progress to metastasis and death. In the protein related to metastasis, for ZEB1, 29.3% (n = 17) of the patiens were positive. Of these, three remained alive and only one did not progress to metastasis or recurrence. For ZEB2, 27.5% (n = 16) were positive. Of these, four remained alive without metastasis or recurrence. Regarding proteins related to the apoptosis pathway, CASP3 was 46.5% (n = 27) positive, of which 51.8% (n = 14) evolved to death, and for PARP1, 24% (n = 14) were positive; of these, only three patients did not die.

Regarding the analyzed proteins, the H-score and outcome values are recorded in Supplementary Table 3. In Supplementary Tables 4, the best sensitivity and specificity values can be observed in relation to the cutoff value.

Association of PIK3CA with clinicopathological parameters

Table 1 summarizes the associations between PI3K expression and clinicopathological variables. PI3K positivity was significantly associated with regional lymph node involvement and distant metastasis (Fisher test $p < 0.0001$). The positive expression of PI3K was not associated with tumor size and grade however it is important to emphasize that the tubulopapillary, complex, and solid tumor types were the most frequently observed in PI3K positive patients.

Association of PI3K in Different Immunohistochemically Defined Molecular Subtypes of Mammary cancer

The phenotypic characterization of these patients was previously performed by Varallo et al. [8]. In this study, it was observed that patients with HER2+, Triple Negative and Luminal B phenotypes associated with PI3K positivity resulted in a lower overall survival rate. In addition, all patients with Luminal A phenotypes were negative for PI3K, as seen in Fig. 3.

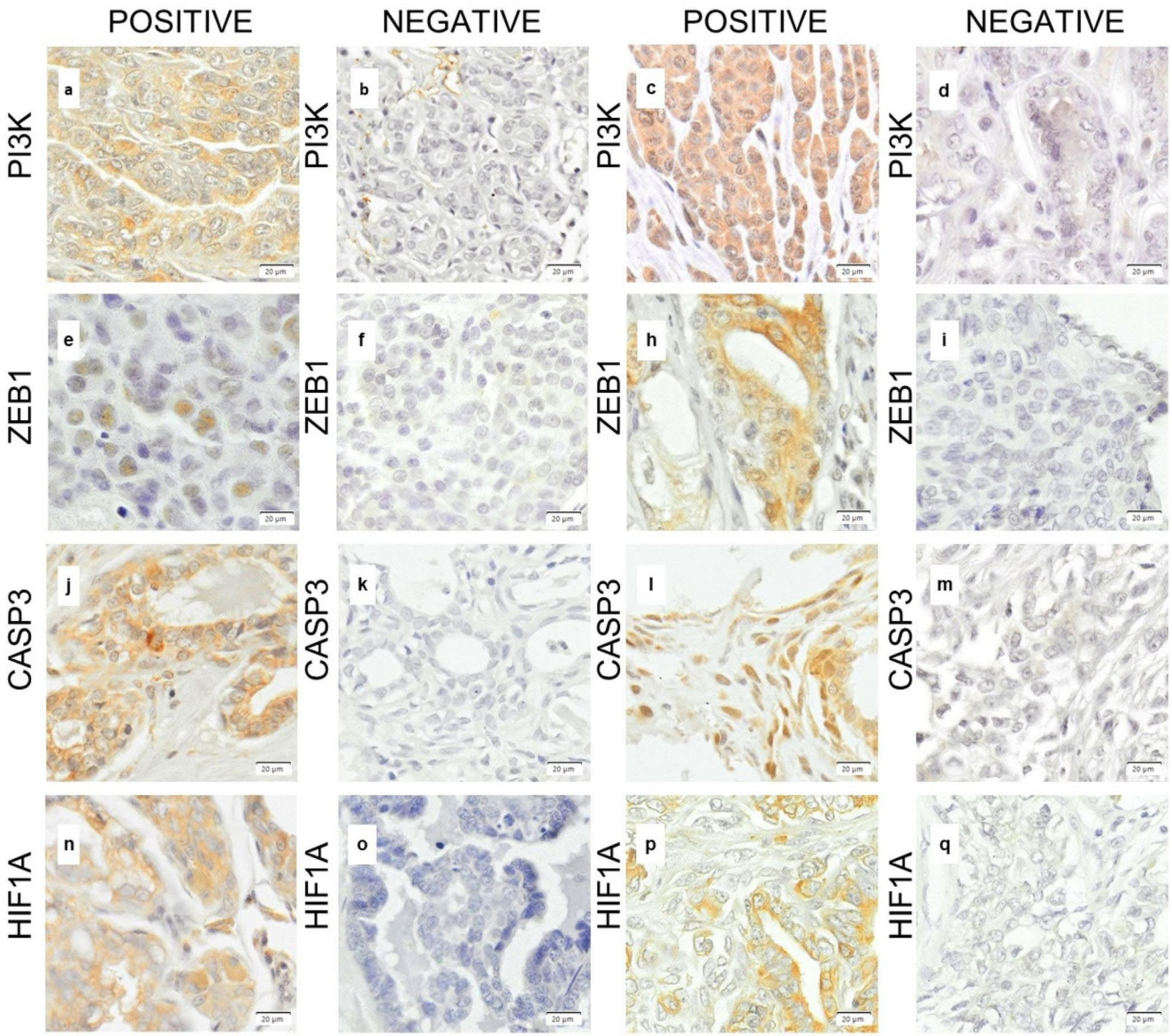

Fig. 1 Photomicrographs of mammary tissue samples from patients. (a,c,h,j,n,p) Positive cytoplasmic immunostaining with anti-PI3K p110α, anti-PTEN, anti-ZEB2, anti-CASP3, anti-HIF1A and anti-VHL in mammary carcinomas (scale bar, 20 nm). (e,l) Nuclear immunostaining with anti-ZEB1 and anti-PARP1 in mammary carcinomas (scale bar, 20 nm). (b,d,f,i,k,m,o,q) Negative immunostaining with anti-PI3K p110α, anti-PTEN, anti-ZEB1, anti-ZEB2, anti-CASP3, anti-PARP1, anti-HIF1A and anti-VHL in mammary carcinomas (scale bar, 20 nm)

Association Between PI3K and Survival

The mean overall survival of patients was 475.9 days, with a minimum of 60 days and a maximum of 540 days (median 540 days and 95% CI). Univariate survival analysis showed that patients with PI3K-positive mammary tumors had shorter survival [log rank (LR) = 50.26; $p < 0.0001$, Hazard ratio (HR) = 0.05314, 95% CI = 0.01043 to 0.2707; Fig. 4].

Among the PI3K positive female dogs, all ten (10/10) died as a result of the disease. The negative patients were kept in follow-up, during which four female dogs (4/48) had metastasis or local recurrence without death, and three (3/48) died. The other patients (40/48) did not have complications associated with the disease in the 540 days of follow-up. The Supplementary Table 5 shows the mean survival curve of patients for each protein studied.

Association Between PI3K and Other Proteins

Statistically applying Pearson's Correlation to evaluate the relationship of PI3K histoscore values with the histoscore values of the other proteins, we observed that PI3K has a

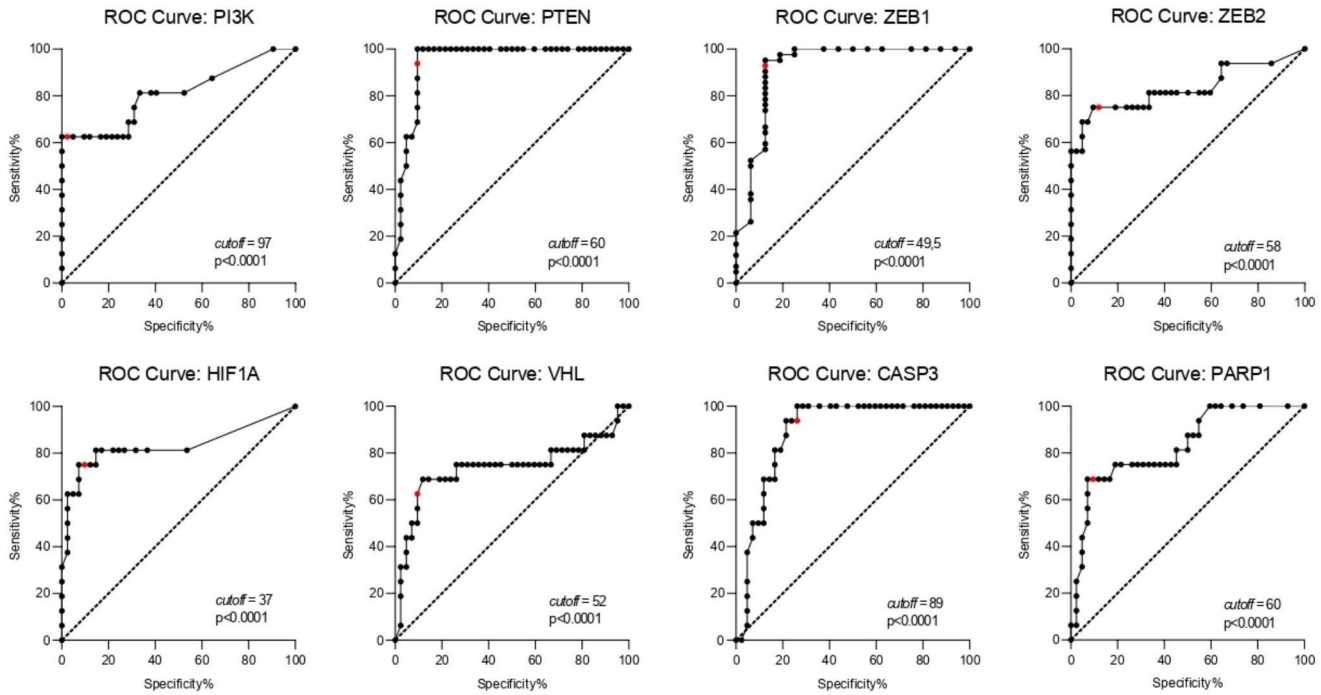

Fig. 2 Receptor Operating Characteristic Curve (ROC) for assessing the discriminatory potential of proteins considering living and dying patients. Labeling index ROC curves of proteins PI3K, PTEN, HIF-1α, VHL, ZEB1, ZEB2, CASP3 and PARP1, for all $p < 0.0001$

Table 1 Association between clinicopathological parameters of analyzed patients and positive or negative PI3K staining

Parameters	Expression PI3K		Significance	
	Positive (%)	Negative (%)	x^2	p value
Histopathological grade				
I e II	5 (8.6%)	29 (50.0%)		0.7261
III and specials	2 (8.6%)	19 (32.0%)		
Tumor Size				
T1	2 (3.5%)	23 (39.6%)		0.1626
T2 e T3	8 (13.8%)	25 (43.1%)		
Affected Regional Lymph Node				
Absent	2 (3.5%)	44 (77.2%)		0.0003
Present	6 (10.5%)	5 (8.7%)		
Distant metastasis				
Absent	0 (0%)	42 (72.4%)		< 0.0001
Present	10 (17.3%)	6 (10.3%)		

moderate negative correlation with PTEN, a weak negative correlation with VHL and CASP3, and a moderate positive correlation with HIF1A, ZEB1, ZEB2 and PARP1. The values of the correlation coefficient (r) and significance (p) can be observed in Supplementary Table 6.

Validation of canine mammary carcinoma markers by real-time RT-PCR

Gene Expression of PIK3CA, ZEB2, HIF1A and PARP1

Real-time quantitative RT-PCR (qRT-PCR) was used to validate mammary tumor gene expression in female dogs by an independent method. Figure 4 illustrates the expression levels of *PIK3CA*, *ZEB2*, *HIF1A* and *PARP1* (Fig. 5a). In this study, we did not observe overexpression of any of the genes when compared to the relative control (1.0; $p = 0.6355$; $p = 0.4973$; $p = 0.5879$ and $p = 0.5879$, respectively).

However, we observed higher gene expression of *ZEB2* (Fig. 5c) and *PARP1* (Fig. 5e) in patients who died (2.9 and 2.2) compared to those without metastasis (-0 0.5336 and 0.49; $p = 0.0429$ and $p = 0.041$ respectively). In addition, there was no significant difference between patients with mammary cancer who were alive or who died considering the expression of *PIK3CA* (0.63 and 0.78, respectively; $p = 0.8873$ – Fig. 5b) and *HIF1A* (0 0.31 and 1.3, respectively; $p = 0.2075$ - Fig. 5d).

Discussion

The *PI3K*/*AKT*/*mTOR* signaling pathway participates in the regulation of processes such as growth, proliferation, survival, metastasis and chemotherapy resistance, thus

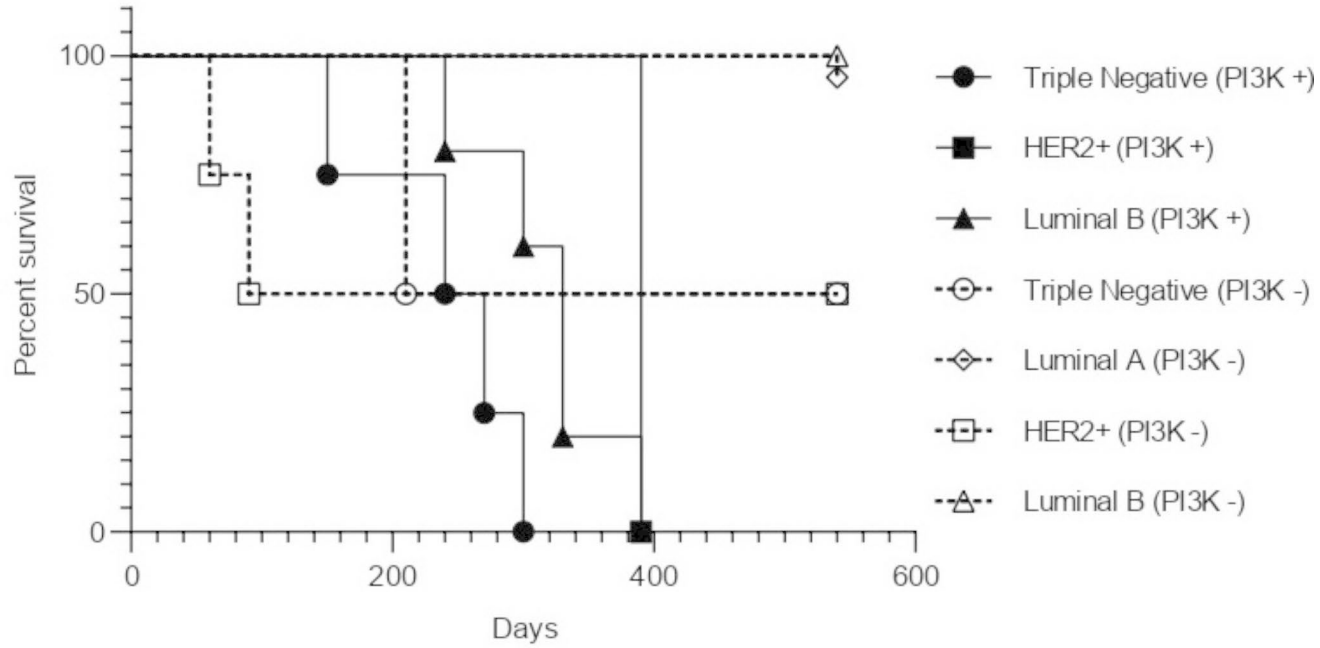

Fig. 3 Association between fenotype results and positive or negative staining of PI3K. Kaplan-Meier curve comparing the estimated survival, in days, and phenotypic profile of sick patients with negative and positive staining for PI3K ($p < 0.0001$)

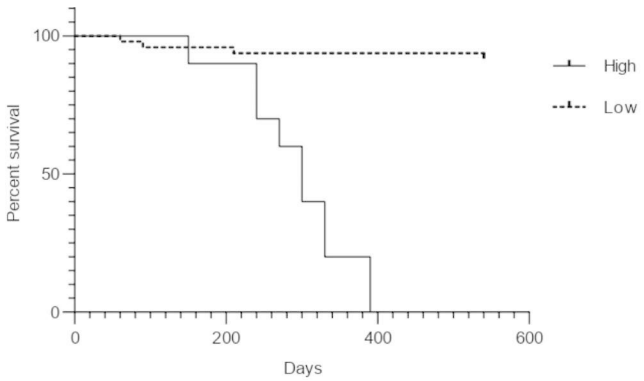

Fig. 4 Mean overall survival curve of PI3K positive and negative patients. Kaplan-Meier curves comparing the estimated survival, in days, of sick patients with negative and positive labeling ($p < 0.0001$)

participating in the promotion and progression of mammary cancer [35]. In this study, genetic variants related to angiogenesis, survival and metastasis associated with the *PI3K* pathway were analyzed, aiming to clarify their association with gene and protein expression levels, tumor subtype, survival and metastasis in female dogs with mammary carcinoma.

In our study, we observed that all patients with increased protein expression of PI3K had a decrease in overall survival, progressing to metastasis and death. We also noted a positive correlation of high levels of *PI3K*, with lymphatic invasion and metastasis. However, we did not observe significant correlations between positivity of PI3K, tumor size and histopathological classification, results partially similar to those observed by [7], who did not observe correlation with affected lymph node.

The *PI3K* signal promotes the growth of estrogen receptor positive mammary cancer in an estrogen-independent manner [7]. In our analyses we observed a significant association between PI3K expression, not only between the Luminal molecular classes B and HER2+, but also for Triple Negative; results similar to those observed by Aleskandarany et al. [36] where HER2+, Luminal B and Triple Negative TN showed significantly increased proportions in cases of overexpression of PI3K.

In veterinary science, there are few studies that have studied the PI3K pathway, as well as the correlation of different clinicopathological parameters and their downstream pathways [5–7, 10]. On the other hand, in humans, Aleskandarany et al. [36], Garcia-Escudero et al. [37] and Tapia et al. [38], studying breast cancer, head and neck cancer and gastric cancer, respectively, observed that patients with tumors carrying mutations in the PIK3CA pathway had shorter survival and shorter disease-free time. Thus, studies in the veterinary PIK3CA pathway are encouraged, this gene is an excellent candidate as a therapeutic and prognostic target.

In the protein expression analysis of this study, all PI3K positive patients also had under-expressed PTEN. PTEN loss is reported in human and canine cancers, derived from epigenetic silencing, mutation and transcriptional repression [7, 39]. Studies carried out with feline mammary carcinoma observed that cats with loss of PTEN had a worse prognosis [11, 20]. Asproni et al. [12] observed that the expression

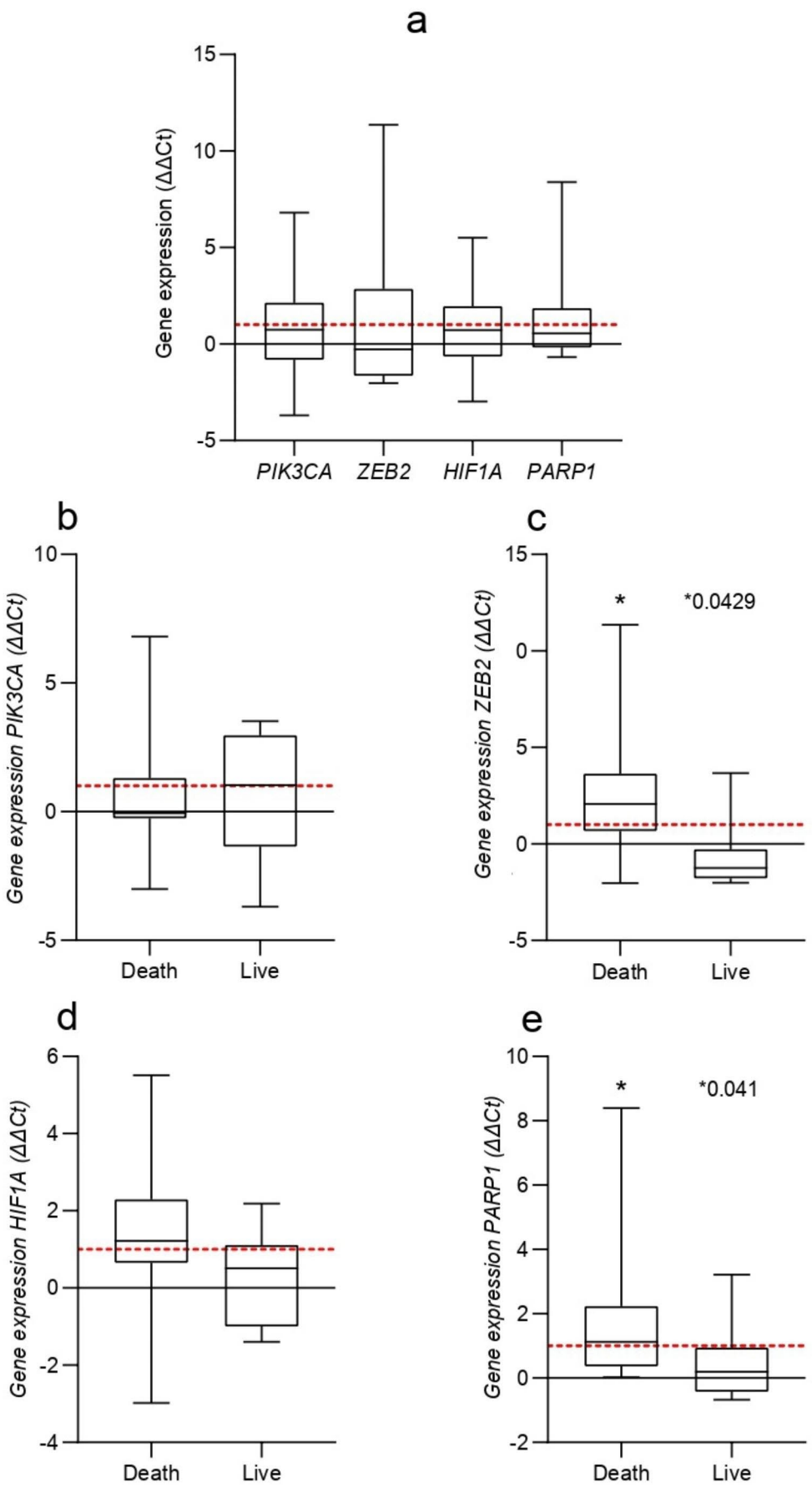

Fig. 5 Relative gene expression of*PIK3CA*, *ZEB2*, *HIF1A*and-*PARP1*. Relative gene expression of *PIK3CA*, *ZEB2*, *HIF1A* and *PARP1* in patients with mammary carcinoma compared to the control group (a) and relative gene expression of these genes in patients with mammary carcinoma alive and dead (b, c, d and e)

of PTEN was inversely correlated with the malignancy of the neoplasm, being more expressed in adenomas than in canine carcinomas. Thus, the combination of PI3K and PTEN could be considered as a potential prognostic marker in mammary tumors.

In the literature, there is a relationship of overexpression of the PI3K/AKT/mTOR pathway with the angiogenesis-promoting pathway [28]. In this study, we observed a significant correlation between protein expression of PI3K and HIF1A, which consequently causes a negative correlation of VHL. A significant correlation between PI3K and HIF1A was also observed by Sitaram et al. [40] in human kidney cancer. In the same work, they observed that the loss of PTEN also influences the loss of VHL.

Studies exploring the relationship between mammary cancer and HIF1A in veterinary medicine are scarce. However, some studies have related the increase in HIF1A protein expression in mammary tissue of female dogs with poor prognosis [41–44].

In our study, we observed a significant positive correlation between PI3K and ZEB1 and 2, which corroborates studies found in the literature. In a study with metastatic bladder carcinoma, the authors observed that in the process of recolonization of neoplastic cells in bone tissue, the PI3K pathway was aberrantly activated, causing the activation of ZEB1 transcription [26]. In another study, also in humans, it was observed that the invasion of intrahepatic cholangiocarcinoma cells was mediated by the upregulation of ZEB1 through PI3K/AKT signaling [45].

With ZEB2, Wu et al. [46] observed that metastasis in lung cancer mediated by the downregulation of E-cadherin through the PI3K/AKT signaling pathway, decreased survival in these patients. In another study, they observed the existence of a PI3K/AKT-GSK-3β-ZEB2 signaling pathway, which promotes IGF-I-induced EMT in gastric cancer cells, being considered an excellent clinical biomarker [47].

In this study, we observed a significant correlation between PI3K and the apoptosis-related proteins CASP3 and PARP1. The *PI3K/AKT/mTOR* lifetime pathway is widely studied in human works mainly in relation to therapy. An example is WANG et al. [48] who observed that the combination of PI3K and PARP1 inhibitory drugs resulted in reduced proliferation and significantly increased apoptotic cell death. In another study linking PARP1, CASP3 and PI3K with therapy and survival, the authors observed that the drug induced apoptosis in human osteosarcoma cancer cells via the PI3K/AKT pathway [49].

In the analyses of this work, there was a significant difference in the gene expression of *ZEB2* and *PARP1* between the groups of living and dead patients, confirming the data found in the retrospective study. The *ZEB2* gene is a DNA-binding transcriptional regulator, which dimerizes with E-box in different promoters, such as E-cadherin, and negatively regulates this and other epithelial genes. Information available in the literature on the expression of *ZEB2* in veterinary mammary cancer is scarce. However, Xavier et al. [50] observed a higher expression of *ZEB2* in the canine lineage CF41.Mg, the most malignant cell line in the experiment. These data corroborate our findings, in which patients with protein and gene overexpression of *ZEB2* had a worse prognosis. *PARP* is a nuclear protein responsible for the DNA repair process that can collaborate with tumor resistance [35]. The *PARP* gene and protein overexpression observed in this work are similar to those observed in the literature, where *PARP* is considered a marker for the repair process, being increased in cancer cells [51]. This result is promising, as this gene has been studied as a targeted therapy for mammary cancer in women and female dogs [52, 53].

Thus, according to our data, the *ZEB2* and *PARP1* genes may constitute new biomarkers of metastasis and survival in canine mammary cancer.

On the other hand, the gene expressions of *PIK3CA* and *HIF1A* did not reveal a significant difference between the groups of living and deceased patients, not confirming the alterations found in the retrospective study. *HIF1A* is a gene that controls angiogenesis and is constitutively expressed in human cells and, despite being scarce, there are studies that report increased expression in the mammary tissue of female dogs [44, 54] which was not observed in the gene expression of this study, even when comparing living and deceased patients.

PIK3CA, is considered a proto-oncogene related to several types of cancer, mainly in breast cancer [55], being actively studied in recent years in canine mammary cancer [5, 7, 10]. In this study, the data observed in gene expression were not similar to those observed in protein expression or to the data found in the literature. Thus, for the results of this study, *PIK3CA* gene expression does not define prognosis.

One of the reasons for the non-correlation of protein and genetic alterations in this work may be the fact that protein alterations do not always come from genetic alterations, because due to errors that accumulate over the various processes that occur from DNA to protein synthesis, gene expression may not interfere with protein synthesis [56]. In addition, our sample number for the analysis of gene expression was small, which may have compromised the results of this analysis. Thus, further studies with a larger sample size are interesting to clarify these results.

Therefore, by studying the protein expression of the patients, our results agree with the literature, in which alterations in the *PIK3CA* gene can lead to alterations in the downstream signaling pathways studied. However, for

PIK3CA and *HIF1A* genes, this was not confirmed in the analysis of gene expression.

In conclusion, the data observed in this work are promising in the study of new molecular markers of prognosis suitable for the development of new therapies. Further research aimed at clarifying and validating the relationships between the *PI3K*/*AKT*/*mTOR* pathway and its downstream pathways are necessary to understand the progression of canine mammary carcinoma and confirm new molecular markers of suitable prognosis and for the development of new therapies.

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Declarations

Conflict of interest There are no conflicts of interest.

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