



Immunohistochemical and molecular analysis of caveolin-1 expression in canine mammary tumors

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ABSTRACT. Caveolin-1 (Cav-1) is a structural protein present in invaginations of the cell membrane. In human breast cancer, the *cav-1* gene is believed to be a tumor suppressor gene associated with inhibition of tumor metastasis. However, little is known about its expression, regulation and function in canine mammary tumors. Expression levels of *cav-1* were investigated using real-time PCR and immunohistochemical detection with an anti-human Cav-1 antibody. Gene expression stability of different samples was analyzed using the geNorm software. Mammary tumors from 51 female dogs were

compared to normal mammary tissue from 10 female dogs. Malignant mammary cells showed a loss of *Cav-1* expression by quantitative RT-PCR and weak Cav-1 staining by immunohistochemistry compared to normal mammary gland tissue. There was a significant relationship between outcome and immunostaining as well as with tumor size, indicating that caveolin subexpression has a positive predictive value and is related to higher survival and smaller tumor size. Our findings indicate that Cav-1 is a potential prognostic marker for canine mammary tumors.

Key words: Mammary cancer; Prognosis; Immunohistochemistry; Quantitative PCR

INTRODUCTION

Caveolin-1 (Cav-1) is a structural protein of 50 to 100 nm present in invaginations of the cell membrane (caveolae) (Bat'ová et al., 2003; Williams and Lisanti, 2005; Sotgia et al., 2006; Williams et al., 2006; Shajahan et al., 2007). Caveolae are enriched by sphingolipids and cholesterol (Zhu et al., 2008) and are directly involved in vesicular trafficking and signal transduction (Bouras et al., 2004; Chen et al., 2004; Elsheikh et al., 2008). Caveolae and caveolins have several functions including vesicular transport, cholesterol homeostasis, signal transduction, and tumor suppression (Bat'ová et al., 2003). Caveolin-1 is a phosphorylated tyrosine with a molecular mass of 22 kDa (Williams and Lisanti, 2005) and it primarily acts as an inhibitor of mitotic proliferation in different cell types, including mammary gland epithelial cells (Bouras et al., 2004; Chen et al., 2004; Sloan et al., 2004; Williams and Lisanti, 2005; Glait et al., 2006). Decreased *Cav-1* expression has been reported in oncogenic transformation of fibroblasts (Mercier et al., 2008).

In humans, the *caveolin-1* gene (*cav-1*) is believed to be a tumor suppressor gene (Williams et al., 2006; Sotgia et al., 2006; Savage et al., 2007) and decreased *Cav-1* expression has been reported in breast cancer. The gene is located on chromosome 7, locus D7S522, with fragile site q31.1 (Williams et al., 2006). A deletion in the 7q31.1/D7S522 region of *cav-1* has been associated with human breast cancer (Lee et al., 2002). In contrast, a significant increase in *caveolin-1* expression has been found in one third of evaluated invasive breast cancers in women when compared with benign lesions and healthy mammary gland tissue, by another group of researchers (Liedtke et al., 2007). In cultured cells, *Cav-1* expression levels are reduced, unchanged or up-regulated depending on the tumor cell type (Williams and Lisanti, 2005). For instance, Cav-1 down-regulation is typical for ovarian, lung, and mammary carcinomas as well as for mesenchymal sarcomas. On the other hand, Cav-1 has been reported to be up-regulated in bladder, esophagus, thyroid (papillary subtype), and prostate carcinomas (Williams and Lisanti, 2005).

However, Williams and Lisanti (2005) and Amorim et al. (2010) found that caveolin-1 overexpression was correlated with metastasis in esophageal squamous cell carcinoma, clear cell renal cell carcinoma, mammary adenocarcinoma, and metastatic cell lines derived from lung adenocarcinoma. These results have implications for human cancers where Cav-1 is consistently up-regulated and suggest that the up-regulation of Cav-1 may represent an

acquired feature that contributes to a metastatic phenotype. This concept of a “biphasic” expression pattern for Cav-1 was elegantly demonstrated in colon carcinoma cells after selection for metastatic variants (Williams et al., 2004; Williams and Lisanti, 2005).

Caveolin-1 expression patterns have not been reported in canine mammary tumors. The aim of this study was to evaluate *Cav-1* expression in these tumors and suggest its value as a prognostic marker.

MATERIAL AND METHODS

Specimens

Mammary tumors from 51 female dogs undergoing surgical extirpation were evaluated and followed-up for 18 months. The dogs ranged from age 2 to 17 years (mean 10 years) and most were of mixed breeds. According to Sorenmo et al. (2009) and Ferreira et al. (2009) models, the staging tests included routine bloodwork (complete blood count, serum chemistry profile), urinalysis, fine needle aspirates of palpable draining lymph nodes, and thoracic radiological examinations. The mammary glands were examined carefully and all tumors were recorded and measured. The surgical resection was performed according to standard practice, with the aim of removing all tumor/tumors with complete margins. The type of surgery, i.e., lumpectomy, simple mastectomy or regional mastectomy depended on the tumor size, location and the number of tumors present in the individual dog. If necessary, the surgeries were staged in two phases in order to prevent excessive tissue tension and wound dehiscence in dogs with multiple large tumors.

Normal mammary gland tissues from 10 female dogs euthanized at the Zoonosis Control Center were used as a pool control in the molecular study and as controls for immunohistochemistry. In addition a human endothelial tissue fragment was used as immunohistochemical control.

Histopathology

Two samples were collected from each animal. The sample used in the molecular assays was frozen immediately after surgery, and the sample for immunohistochemistry was fixed in 10% buffered formaldehyde solution. Tissue blocks were prepared by standard histological methods, embedded in paraffin wax, sectioned at 5 μ m and stained with hematoxylin and eosin. Representative areas of the tumor were selected for immunohistochemistry.

All tumors were submitted for histopathological examination. Two independent pathologists performed the evaluations blinded to each other and all clinical information about the case. In cases of discordance a third pathologist was consulted and the chosen result was retained. The tumors were classified according to type of tissue present (epithelial, myoepithelial and/or connective tissue) and whether they were malignant or benign. The malignant sample epithelial tumors were grouped together as carcinomas for the purpose of this study and included tubulopapillary carcinoma, solid carcinomas and complex carcinomas. The diagnosis also included histopathological assessment of the entire section including dysplastic changes and was not limited to the neoplastic changes alone. The animals were followed-up

for 18 months and the survival time was defined as the period (days) between surgical tumor removal and date of death caused by the disease (end point). The cause of death was confirmed by clinical data.

The histopathological classification was based on the World Health Organization classification system for canine mammary tumors (WHO, 2007); in addition, a TNM classification was used to evaluate tumor size (T): T1 (0-3 cm), T2 (3-5 cm) and T3 (>5 cm), to regional lymph nodes (N) metastasis: N1 (presence) and N0 (absence) and to the occurrence of distant metastasis (M): M1 (presence) and M0 (absence) (Ferreira et al., 2009).

Clinical follow-up studies

The dogs were reexamined regularly every 4 months and until 18 months after the surgery. They were **diagnosed with metastasis or recurrence confirmed by chest X-ray, abdominal ultrasound, needle aspiration biopsy of the new lump, or necropsy, where the dog had died.**

Caveolin-1 immunostaining

A section of at least one tissue block representing a characteristic area of the tumor of each of the 51 cases was mounted on silanized glass slides (Sigma-Aldrich, St. Louis, MO, USA). The slides were deparaffinized, rehydrated in graded alcohols, and incubated with 3% hydrogen peroxidase (Farmax, Divinópolis, Brazil) for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed on Pan Steam (Arno, São Paulo, Brazil) at 95°C with citric acid buffer, pH 6.0, for 35 min. After cooling to room temperature at 25°C, the slides were covered with bovine serum albumin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min before incubation with the primary antibody for 2 h at room temperature, and the avidin-biotin-peroxidase complex (ABC) for 1 h. A mouse monoclonal anti-human Cav-1 (clone 2297) (Transduction Labs, Lexington, KY, USA) diluted 1:150 in bovine serum albumin (BSA) was used as the primary antibody. Chromogen, 3,3'-diaminobenzidine tetrahydrochloride 0.5% (Signet®, Dedham, MA, USA) diluted in phosphate-buffered saline was applied to the slides for 2-5 min at 20-22°C. Slides were subsequently counterstained with Harris's hematoxylin.

Negative controls were obtained by omitting the primary antibody, whereas normal mammary gland tissue was used as an internal positive control in each assay. Expression of the marker was evaluated according to the grading by Allred et al. (1998).

Evaluation of immunohistochemical data

Multiple fields of each slide were examined and positive immunostaining was indicated by the presence of distinct brown cytoplasmatic staining (Figure 1). Immunohistochemical examination was blinded (i.e., without knowledge of the histopathological diagnosis) and the results were based on the consensus of at least two observers. A number of 1000 cells were evaluated in 10 fields (400X magnification) to assess positive cell (PC) percentage. The results were grouped into semiquantitative scores as negative, focal (<10% PC), weak (10-25% PC), moderate (25-50% PC), or strong (>50% PC).

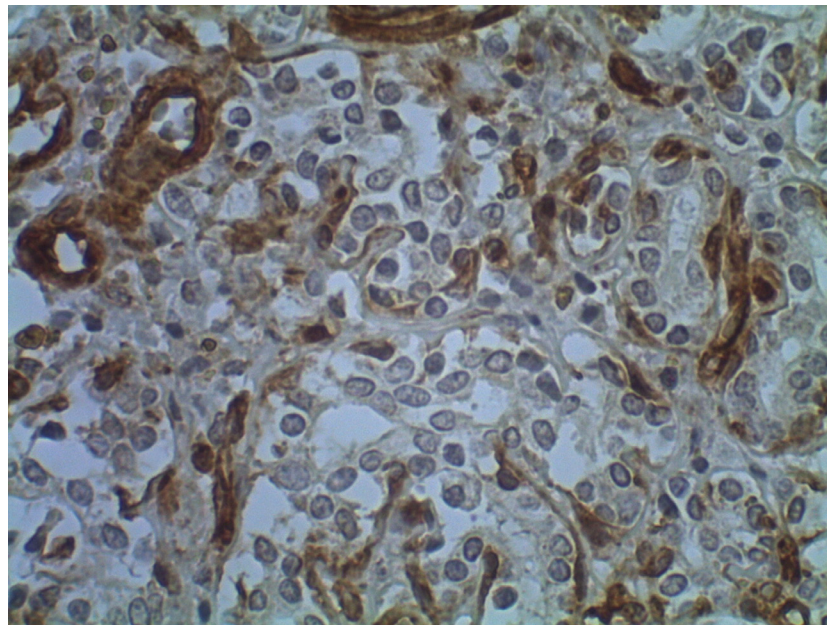


Figure 1. Immunohistochemistry of a solid carcinoma (Dog 28) with strong expression of *caveolin-1*. Notice the dark cytoplasmic staining. Mouse monoclonal anti-human *caveolin*, avidin-biotin-peroxidase, diaminobenzidine tetrahydrochloride chromogen, counterstained with Harris's hematoxylin. Magnification = 40X.

Quantitative RT-PCR

A quantitative real-time (RT) PCR was performed in triplicate using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Briefly, the reactions were performed in 20 μ L with 10 μ L Power SYBR Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil), 250 nM of each primer and 10 ng cDNA. The PCR conditions were 50°C for 2 min, 95°C for 10 min followed by 35 cycles of 95°C for 15 s, 60°C for 1 min. Following the PCR, dissociation curve analysis was performed to confirm the desired single-gene product: 1 cycle of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s.

Expression of five housekeeping genes (*ACTB*, *GAPD*, *GUSB*, *PPIA*, and *HPRT1*) was first tested in 15 breast cancer samples (Vandesompele et al., 2002) and expression of *HPRT1* and was considered to be the most stable among samples. *HPRT1* was then chosen as a reference gene. Investigated transcripts showed high real-time PCR efficiency rates (approximately 1.0) and relative expression was calculated as $2^{-\Delta\Delta CT}$. Results were then transformed into their natural logarithm values. Gene expression stability was analyzed by the geNorm software.

All the reactions were performed with a negative control and one sample was selected as the calibrator control for the reactions. Analyses were repeated when the coefficient of variation was higher than 5%. After each reaction, the products were analyzed on a 2% agarose gel stained with ethidium bromide.

Primers used for amplification were: *CAVEOLIN-1* sense (5'-ACAGCAAGCGG TAAAACCAG-3') and antisense (5'-ACACCAAGGAAATCGACCTG-3'); and *HPRT1* (en-

ogenous control), sense (5'-TTA TAG TCA AGG GCA TAT CC-3') and antisense (5'-AGC TTG CTG GTG AAA AGG AC-3'). They were classified into underexpressed (the samples obtained less than -1 Log3 measurement) and overexpressed (the samples with quantification over 1 Log3).

Statistical analysis

Multivariate logistic regression analyses of prognostic factors were performed. Results were analyzed on the basis of tumor diagnosis and animal outcome compared to immunohistochemical expression of the antibodies by analysis of dependency (Cordeiro, 1987). The differences of means between groups were assessed by the Student *t*-test. $P < 0.05$ was considered to be significant (two-tail analyses). The Kaplan Meier analysis was performed for survival based on the results of RT-PCR (overexpression/subexpression) and immunohistochemistry (moderate or severe/focal or weak) (Figure 2). All statistical analysis was performed using the Microsoft office XP Software (Minitab, version 12.22).

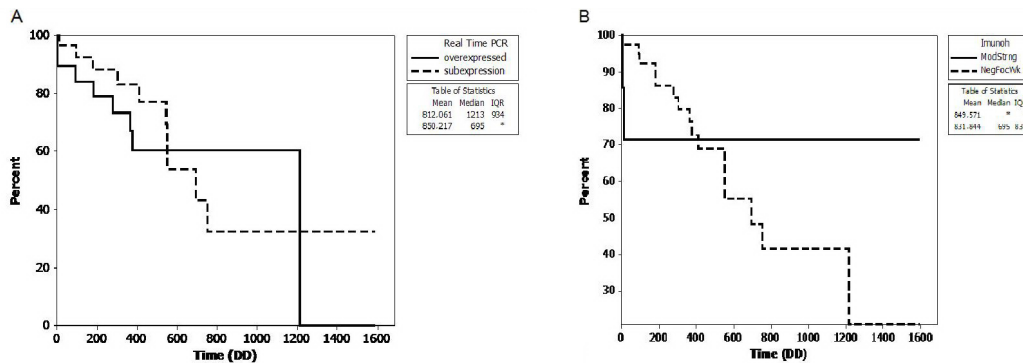


Figure 2. Kaplan Meier method showing no significance in the expression of the *cav-1* gene by real-time PCR (A) and by immunohistochemistry (B) related to overall survival.

RESULTS

Evaluation of normal mammary tissue showed no expression of Cav-1 by luminal epithelial cells, while the basal/myoepithelial cells always exhibited strong labeling in both lobular and ductal units. *Cav-1* expression was also consistently found in adipocytes, endothelial cells and stromal fibroblasts around the mammary glandular tissue; therefore, these structures were used as positive internal controls (Pereira et al., 2010).

The complete data on the 51 dogs based on individual histopathological diagnoses, immunohistochemistry, RT-PCR, clinical status 18 months after surgery, time period between surgery and reoccurrence of disease and TNM classification are available in Table 1 (individual data). The histopathological evaluation of the benign mammary tumors showed two mixed benign, one adenoma and one ductal hyperplasia. The histopathological evaluation of the malignant mammary tumors showed a predominance of carcinomas (N = 44). Simple-type carcinomas dominated in the group (N = 31) consisting of both tubulopapillary (N = 19) and

solid (N = 12) subtypes. Complex carcinomas were found in 7 dogs, while one had a carcinoma with squamous cell differentiation. Carcinosarcomas were diagnosed in 5 dogs based on the presence of malignancy cellular criteria and metaplasia. Sarcoma was diagnosed in three cases.

Table 1. Immunohistochemistry and molecular expression of *caveolin-1*, histopathological diagnosis, outcome of the disease and disease-free interval of mammary tumors from 51 dogs.

Sample	Histopatologic diagnosis	Immunohistochemistry*	Real-time PCR	Outcome	Disease-free interval	TNM
01	Tubulopapillary carcinoma	9% Focal	Subexpressed	Death-metastasis	Between 6-18 months	T2N1M0
02	Carcinosarcoma	5.5% Focal	Subexpressed	Death-metastasis	Less than 6 months	T3N0M0
03	Tubulopapillary carcinoma	2% Focal	Overexpressed	Alive	More than 18 months	T3N0M0
04	Solid carcinoma	0.3% Focal	Overexpressed	Death-metastasis	Between 6-18 months	T2N0M0
05	Complex carcinoma	3.6% Focal	Overexpressed	Alive-recurrence	More than 18 months	T1N0M0
06	Solid carcinoma	0.8% Focal	Overexpressed	Death-recurrence	More than 18 months	T2N0M0
07	Fibrosarcoma	0.2% Focal	Overexpressed	Alive	More than 18 months	T3N0M1
08	Tubulopapillary carcinoma	4.7% Focal	Subexpressed	Death	More than 18 months	T1N2N1
09	Carcinosarcoma	0.3% Focal	Subexpressed	Death-metastasis	Less than 6 months	T1N0M0
10	Solid carcinoma	20.2% Weak	Subexpressed	Death-metastasis	Less than 6 months	T2N2M1
11	Solid carcinoma	8.2% Focal	Subexpressed	Death	More than 18 months	T3N0M0
12	Carcinosarcoma	Negative	Subexpressed	Alive	More than 18 months	T3N0M0
13	Complex carcinoma	16.4% Weak	Subexpressed	Alive	More than 18 months	T1N0M0
14	Solid carcinoma	Negative	Subexpressed	Death-metastasis	More than 18 months	T2N0M1
15	Solid carcinoma	13% Weak	Subexpressed	Death-metastasis	Between 6-18 months	T3N0M1
16	Tubulopapillary carcinoma	0.3% Focal	Subexpressed	Alive	More than 18 months	T3N0M0
17	Spindle cell sarcoma	32.4% Moderate	Subexpressed	Alive	More than 18 months	T3N0M0
18	Fibrosarcoma	5.6% Focal	Subexpressed	Alive	More than 18 months	T1N0M0
19	Solid carcinoma	7% Focal	Subexpressed	Alive	More than 18 months	T1N0M0
20	Tubulopapillary carcinoma	52.7% Strong	Subexpressed	Alive	More than 18 months	T1N0M0
21	Solid carcinoma	22% Weak	Overexpressed	Death-recurrence	Less than 6 months	T2N1M0
22	Tubulopapillary carcinoma	3% Focal	Subexpressed	Alive	More than 18 months	T3N0M0
23	Tubulopapillary carcinoma	19.1% Weak	Subexpressed	Death-recurrence	More than 18 months	T3N1M0
24	Complex carcinoma	32.7% Moderate	Subexpressed	Alive	More than 18 months	T2N0M0
25	Complex carcinoma	12.7% Weak	Subexpressed	Death-metastasis	More than 18 months	T3N0M1
26	Tubulopapillary carcinoma	22.7% Weak	Subexpressed	Alive-recurrence	More than 18 months	T3N1M1
27	Tubulopapillary carcinoma	7.4% Focal	Subexpressed	Death-recurrence	Less than 6 months	T3N0M0
28	Solid carcinoma	93.8% Strong	Subexpressed	Alive	More than 18 months	T1N0M0
29	Complex carcinoma	16.7% Weak	Subexpressed	Death-recurrence	Between 6-18 months	T2NxMx
30	Carcinoma with squamous diff.	1% Focal	Subexpressed	Death-recurrence	More than 18 months	T2N0M0
31	Tubulopapillary carcinoma	25.3% Moderate	Subexpressed	Alive	More than 18 months	T1N0M0
32	Tubulopapillary carcinoma	60.5% Strong	Subexpressed	Alive	More than 18 months	T2N0M0
33	Complex carcinoma	35.3% Moderate	Subexpressed	Alive	More than 18 months	T1N0M0
34	Tubulopapillary carcinoma	39.6% Moderate	Subexpressed	Death-recurrence	More than 18 months	T3N0M0
35	Carcinosarcoma	9.7% Focal	Subexpressed	Alive	More than 18 months	T3N0M0
36	Complex carcinoma	21.3% Weak	Overexpressed	Alive	More than 18 months	T1N0M0
37	Solid carcinoma	13.4% Weak	Subexpressed	Alive	More than 18 months	T2N0M0
38	Tubulopapillary carcinoma	45.7% Moderate	Subexpressed	Alive	More than 18 months	T1N0M0
39	Tubulopapillary carcinoma	35.1% Moderate	Subexpressed	Alive	More than 18 months	T1N0M0
40	Tubulopapillary carcinoma	0.2% Focal	Subexpressed	Alive	More than 18 months	T2N0M0
41	Tubulopapillary carcinoma	24.8% Weak	Subexpressed	Alive	More than 18 months	T1N0M0
42	Tubulopapillary carcinoma	7.2% Focal	Subexpressed	Alive	More than 18 months	T1N0M0
43	Solid carcinoma	78.1% Strong	Overexpressed	Alive	More than 18 months	T1N0M0
44	Solid carcinoma	32.4% Moderate	Subexpressed	Alive	More than 18 months	T2N0M0
45	Tubulopapillary carcinoma	10.7% Weak	Subexpressed	Alive	More than 18 months	T1N0M0
46	Carcinosarcoma	15.6% Weak	Subexpressed	Death-metastasis	Less than 6 months	T3N0M0
47	Tubulopapillary carcinoma	49.6% Moderate	Subexpressed	Alive	More than 18 months	T1N1M0
48	Benign	Strong	Subexpressed	Alive	More than 18 months	T2N0M0
49	Benign	Weak	Subexpressed	Alive	More than 18 months	T2N0M0
50	Benign	Weak	Subexpressed	Alive	More than 18 months	T2N0M0
51	Benign	Focal	Subexpressed	Alive	More than 18 months	T1M0N0

*Percentage of caveolin-1 expression in 1000 cells counted according to Allred et al. (1998) as: (-) negative or no cells stained; (+) values until 10% of cells stained; (++) values between 10 to 25%; (+++) values between 25 to 50%, and (+++++) values above 50%.

The immunohistochemical examinations of *Cav-1* demonstrated a correlation between the *Cav-1* expression and histopathological diagnosis (Table 2). Carcinomas and sarcomas were either predominantly focal or weak. A comparison between the overall survival time and the histopathological diagnosis did not demonstrate a statistical positive concordance (Table 3).

Table 2. Correlation between histopathological diagnosis and caveolin-1 expression in 51 canine mammary gland tumors.

Histopathological diagnosis	Immunohistochemical staining*	
	Negative/focal/weak	Moderate/strong
Simple	21	10
Complex	10	2
Sarcoma	2	1
Squamous	1	0
Benign	3	1
All	37	14

*Grading is based on semiquantitative score.

Table 3. Correlation between histopathological diagnosis and overall survival time for 51 dogs having mammary gland neoplasia.

Histopathological diagnosis	Overall survival time in months		
	<6	6-18	>18
Simple	3	3	25
Complex	3	1	8
Sarcoma	0	0	3
Squamous	0	0	1
Benign	0	0	4
All	6	4	41

As described in Table 4, the relationship between outcome and tumor size was statistically significant ($P = 0.011$). Also, the relationship between outcome and immunostaining demonstrated a positive concordance ($P = 0.005$) (Table 5).

Table 4. Statistical correlation between outcome and tumor size.

Outcome	Tumor size			P
	T1	T2	T3	
Alive	17	8	8	0.011*
Death	2	8	8	
All	19	16	16	

*Chi-square P value.

Table 5. Statistical correlation between outcome and immunostaining.

Immunostaining	Outcome		P
	Alive	Death	
Moderate/Strong	13	1	0.005*/0.010#
Negative/focal/weak	20	17	
All	33	18	

*Chi-square P value. #Fisher exact test P value.

Eighteen females died after recurrence or metastasis and the remaining females were followed-up for at least 18 months after surgery (Table 1).

Analysis of the gene expression by RT-PCR demonstrated subexpression in 43 dogs, while 8 dogs overexpressed *Cav-1* when compared to a pool of normal mammary gland tissue (Figure 3). These females were diagnosed as solid carcinomas (4), complex carcinoma (2), fibrosarcoma (1), and a tubulopapillary carcinoma without any correlation. A comparison between RT-PCR and survival time showed that for the overexpressed samples, two had died and 6 were alive after 18 months. For the 43 subexpressed samples, 8 had died and 35 were alive after 18 months. These figures show a high percentage (84.3%) of animals with a subexpression of the *cav-1* gene (Table 6).

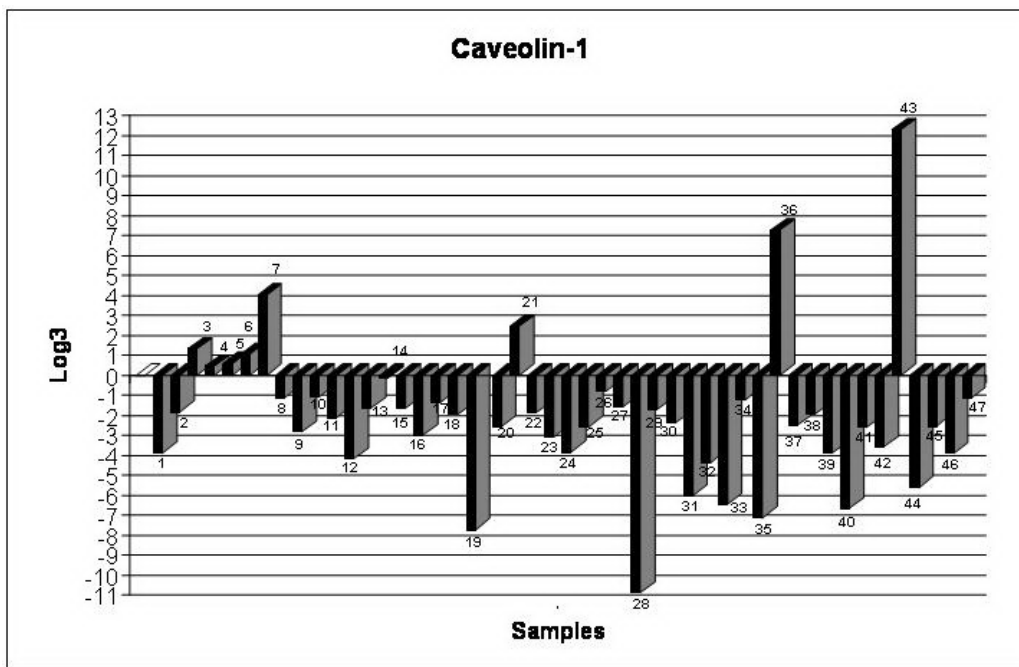


Figure 3. Quantitative gene expression of *caveolin-1* in 47 canine mammary gland neoplasms (black). A pool of normal canine mammary gland (control), plotted as the first bar (white), is equal to zero.

Table 6. Correlation between expression of *caveolin-1* determined by real-time PCR and overall survival time for 51 dogs with mammary gland neoplasia.

Caveolin-1 expression	Overall survival time in months		
	<6	6-18	>18
Subexpressed	5	3	36
Overexpressed	1	1	6
All	6	4	41

Metastasis occurred in 9 dogs within 18 months following diagnosis. Immunohistochemical examination showed low *Cav-1* expression (graded as negative, focal or weak) in all 9 dogs. Similarly, RT-PCR demonstrated that 8 of these had subexpression of *Cav-1*.

Analysis of the correlation between the *cav-1* gene expression as determined by RT-PCR and expression of Cav-1 examined by immunohistochemistry demonstrated a genuine trend with no statistical correlation.

DISCUSSION AND CONCLUSION

According to Liedtke et al. (2007), evaluation of normal breast tissue samples showed no expression of Cav-1 in breast epithelial cells. In myoepithelial cells of ducts and lobuli as well as in blood vessels, expression of Cav-1 was consistently found in normal breast tissue as well as both benign breast tumors and DCIS (ductal carcinoma *in situ*). Moreover, high *Cav-1* expression levels were again observed in mammary myoepithelial cells. Using a monoclonal antibody, no expression of Cav-1 could be found in the epithelial cell component of normal breast tissue or in luminal epithelial cells of benign breast lesions such as radial scars. However, luminal epithelial cells demonstrated *Cav-1* expression in 13.4% of DCIS and 9.4% of invasive breast cancer specimens.

As expected, there was a prevalence of carcinomas in the group studied. The diagnoses were made at a late stage and many animals showed signs of malignancy at the time of diagnosis, which led to a bad prognosis. Moreover, only three sarcomas were diagnosed. However, when surgery was performed, the majority of bitches survived (61.7%). There was a significant correlation between the outcome and the tumor size, which confirms this statement. The majority of cancers studied were simple, which characterized them histopathologically as tubulopapillary.

Although there was a trend, the statistical analysis did not show any significant results based on the assessment of diagnosis and survival. It was observed that if the diagnosis was delayed, the choice of the best practice seemed to help improve the prognosis. There was no statistical correlation between the immunohistochemical marking and the diagnosis. However, this technique showed an important correlation with the outcome, which may render this marker significant.

The caveolin was reduced in this group in accordance with the worst diagnoses. As expected, the molecular study showed subexpression in most samples.

It has been reported that Cav-1 is expressed in normal human mammary epithelial cells and it is down regulated during the progression of cancer. There is a low *Cav-1* expression in non-metastatic primary tumors and a lower expression in highly metastatic tumors. Cells that expressed higher levels of Cav-1 showed a reduced capacity to invade other tissues and produce metastasis (Sloan et al., 2004), which indicates its role as a tumor suppressor (Sloan et al., 2004; Sotgia et al., 2006; Savage et al., 2007). The *Cav-1* expression was significantly reduced in breast cancer cells compared with that observed in normal breast tissue and the overexpression of Cav-1 resulted in substantial growth inhibition (Wang et al., 2008). When comparing the expression of the marker and the presence of metastasis, subexpression was observed in these animals, which suggested a significant marker for metastasis, confirmed in both the molecular and immunohistochemical study.

In some cases, overexpression was observed accompanied by high survival rate. How-

ever, the role of Cav-1 in mammary carcinogenesis is still far from being completely understood. Scientific evidence of a tumor suppressive role of Cav-1 in breast cancer supported by some researchers (Williams and Lisanti, 2005) is contrasted by recent results that strengthen the role of Cav-1 overexpression to promote certain steps of tumorigenesis (Liedtke et al., 2007). When confronted with this controversy one has to assume that the role of Cav-1 as both tumor suppressor and promoter might be context-dependent. While being down regulated in early stage malignancies and thereby mediating growth promoting effects, up-regulation of Cav-1 in the late stage disease might promote resistance against chemotherapeutic agents in colon cancer as well as metastatic properties in prostate cancer (Liedtke et al., 2007). Our results showed a very low expression of the majority of the group in both methods, which could include *Cav-1*, a suppressor gene in canine mammary tumors as well.

A controversial role for Cav-1 in metastasis has emerged from investigations into various types of cancers (Williams et al., 2004). In addition, this group showed a low *Cav-1* immunohistochemical expression in all animals that had metastasis and 8 of 9 (88.8%) gene subexpression in molecular study. This is justified by the possibility of gene mutation occurrence, which alters the protein synthesis. Furthermore, from the nine animals that presented overexpression in relation to the normal sample pool, only three had a bad prognosis and six, 66.7%, were still alive. Williams et al. (2004) had shown that the presence of Cav-1 in mammary tumor cells suppresses tumor growth and metastasis. Complete ablation of Cav-1 (-/-) clearly accelerates tumor onset and growth, as well as the appearance of metastatic disease. In agreement with our results, these authors had previously shown that Cav-1 functions to suppress the processes of tumorigenesis and metastasis in the mammary gland. It was also observed by Sloan et al. (2004) that Cav-1 reduces both primary tumor growth and spontaneous metastasis to lung and bone.

Cav-1 plays an important *in vivo* role in suppressing early tumor development. Its absence facilitates the appearance and growth of mammary intraepithelial neoplasia lesions. These findings directly support a wealth of clinical, genetic, and cellular evidence implicating Cav-1 as a tumor/transformation suppressor gene (Williams et al., 2003). In cases where there was super-expression this must be related to the expression of other genes.

Liedtke et al. (2007) hypothesized that instead of being an independent prognostic factor alone, Cav-1 might exhibit a more complex function that needs to be evaluated in context with the co-expressed proteins as well as in view of the respective disease stage. This might finally explain the conflicting results described in the scientific literature. Further studies are warranted to understand the role of *Cav-1* expression in the disease course of breast cancer as well as its potential as a therapeutic target. Thus, its distinct role in cellular processes may depend on the combination of proteins expressed in the cells rather than on *Cav-1* expression itself.

There are only two studies regarding canine mammary tumors and Cav-1 that recognize this marker as a malignant factor, which is in contrast to our study. Amorim et al. (2010) findings are concordant with data previously reported on human breast cancer, documenting Cav-1 overexpression in tumors with unfavorable behavior. For these authors there is a clear association between increased expression of Cav-1 and neoplastic transformation of the epithelium and the data suggest that *Cav-1* expression is associated with a more malignant phenotype of canine mammary neoplasia and with vascular invasion and regional lymph node metastasis. In a second article, Pereira et al. (2010) also associated *Cav-1* expression with malignant neoplastic transformation in the canine mammary gland. *Cav-1* expression may be

associated with highly malignant subtypes of mammary tumors (basal-like carcinoma), invasion and metastasis, and thus it may play a critical role in the epithelial-mesenchymal transition process. Furthermore, *Cav-1* expression is associated with an unfavorable prognosis and is associated with shorter overall survival in canine mammary carcinomas.

The underlying mechanism of in the majority of cases remains to be elucidated. In summary, our findings show that *Cav-1* is consistently expressed in canine mammary tumors, contradicting the results presented by Pereira et al. (2010) and Amorin et al. (2010). On univariate analysis, subexpression of *Cav-1* was associated with low histologic grade. However, on multivariate analysis, *Cav-1* was a significant prognostic factor for metastasis and its down regulation. All these findings may call into question the tumor-suppressive functions of *Cav-1* in canine mammary cancer. Further studies are necessary to corroborate the oncogenic properties of *Cav-1* in canine mammary tumors (Savage et al., 2007).

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REFERENCES

- Allred CD, Harvey JM, Berardo M and Clark GM (1998). Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod. Pathol.* 11: 155-168.
- Amorim I, Lopes CC, Faustino AM and Pereira PD (2010). Immunohistochemical expression of caveolin-1 in normal and neoplastic canine mammary tissue. *J. Comp. Pathol.* 143: 39-44.
- Bat'ová Z, Bittnerová M, Krenek P and Kyselovic J (2003). Caveolar functions and caveolin structural proteins. *Cesk. Fysiol.* 52: 166-171.
- Bouras T, Lisanti MP and Pestell RG (2004). Caveolin-1 in breast cancer. *Cancer Biol. Ther.* 3: 931-941.
- Chen ST, Lin SY, Yeh KT, Kuo SJ, et al. (2004). Mutational, epigenetic and expression analyses of caveolin-1 gene in breast cancers. *Int. J. Mol. Med.* 14: 577-582.
- Cordeiro JA (1987). Analysis of Dependency. Relatório Técnico N° 48/87. Instituto de Matemática e Estatística, Unicamp, Campinas.
- Elsheikh SE, Green AR, Rakha EA, Samaka RM, et al. (2008). Caveolin 1 and Caveolin 2 are associated with breast cancer basal-like and triple-negative immunophenotype. *Br. J. Cancer* 99: 327-334.
- Ferreira E, Bertagnolli AC, Cavalcanti MF, Schmitt FC, et al. (2009). The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours. *Vet. Comp. Oncol.* 7: 230-235.
- Glait C, Tencer L, Ravid D, Sarfstein R, et al. (2006). Caveolin-1 up-regulates IGF-I receptor gene transcription in breast cancer cells via Sp1- and p53-dependent pathways. *Exp. Cell Res.* 312: 3899-3908.
- Lee H, Park DS, Razani B, Russell RG, et al. (2002). Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. *Am. J. Pathol.* 161: 1357-1369.
- Liedtke C, Kersting C, Burger H, Kiesel L, et al. (2007). Caveolin-1 expression in benign and malignant lesions of the breast. *World J. Surg. Oncol.* 5: 110.
- Mercier I, Casimiro MC, Wang C, Rosenberg AL, et al. (2008). Human breast cancer-associated fibroblasts (CAFs) show caveolin-1 downregulation and RB tumor suppressor functional inactivation: Implications for the response to hormonal therapy. *Cancer Biol. Ther.* 7: 1212-1225.
- Pereira PD, Lopes CC, Matos AJ, Cortez PP, et al. (2010). Caveolin-1 in diagnosis and prognosis of canine mammary

- tumours: comparison of evaluation systems. *J. Comp. Pathol.* 143: 87-93.
- Savage K, Lambros MB, Robertson D, Jones RL, et al. (2007). Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. *Clin. Cancer Res.* 13: 90-101.
- Shajahan AN, Wang A, Decker M, Minshall RD, et al. (2007). Caveolin-1 tyrosine phosphorylation enhances paclitaxel-mediated cytotoxicity. *J. Biol. Chem.* 282: 5934-5943.
- Sloan EK, Stanley KL and Anderson RL (2004). Caveolin-1 inhibits breast cancer growth and metastasis. *Oncogene* 23: 7893-7897.
- Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, et al. (2009). Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. *Vet. Comp. Oncol.* 7: 162-172.
- Sotgia F, Rui H, Bonuccelli G, Mercier I, et al. (2006). Caveolin-1, mammary stem cells, and estrogen-dependent breast cancers. *Cancer Res.* 66: 10647-10651.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: RESEARCH0034.
- Wang Y, Yu J and Zhan Q (2008). BRCA1 regulates caveolin-1 expression and inhibits cell invasiveness. *Biochem. Biophys. Res. Commun.* 370: 201-206.
- WHO (2007). World Health Organization-Air Force Institute of Pathology Classification System for Canine Mammary Tumors. Available at [<http://www.who.int/en/>]. Accessed July 5, 2009.
- Williams TM and Lisanti MP (2005). Caveolin-1 in oncogenic transformation, cancer, and metastasis. *Am. J. Physiol. Cell Physiol.* 288: C494-C506.
- Williams TM, Cheung MW, Park DS, Razani B, et al. (2003). Loss of caveolin-1 gene expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice. *Mol. Biol. Cell* 14: 1027-1042.
- Williams TM, Medina F, Badano I, Hazan RB, et al. (2004). Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis *in vivo*. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. *J. Biol. Chem.* 279: 51630-51646.
- Williams TM, Sotgia F, Lee H, Hassan G, et al. (2006). Stromal and epithelial caveolin-1 both confer a protective effect against mammary hyperplasia and tumorigenesis: Caveolin-1 antagonizes cyclin D1 function in mammary epithelial cells. *Am. J. Pathol.* 169: 1784-1801.
- Zhu H, Weisleder N, Wu P, Cai C, et al. (2008). Caveolae/caveolin-1 are important modulators of store-operated calcium entry in Hs578/T breast cancer cells. *J. Pharmacol. Sci.* 106: 287-294.