Arq. Bras. Med. Vet. Zootec., v.63, n.1, p.28-35, 2011

Immunohistochemical and molecular expression of laminin-332 gamma-2 chain in canine mammary tumors

[*Expressão imunoistoquímica e molecular da laminina-332 cadeia gama-2 em tumores mamários de cadelas*]

D.A.P.C. *Zuccari*[1], R. *Castro*[1], B.V. *Jardim*[1,2], U.M. *Mancini*[1], G.M. *Polachini*[1]

[1]Faculdade de Medicina de São José do Rio Preto
Av. Brigadeiro Faria Lima, 5416
15090-000 – São José do Rio Preto
[2]IBILCE - UNESP – São José do Rio Preto, SP

ABSTRACT

Forty-eight cases of canine mammary cancer were investigated to evaluate the immunohistochemical distribution of the γ2 chain of laminin-332. Tumor cells were compared to a pool of normal mammary tissues using quantitative RT-PCR. The western blot was performed in eight tumor samples as complementary test to evaluate protein integrity. Immunohistochemistry experiments showed negative, focal, and weak expression of laminin-332 γ2 in tumors with the worst prognosis. Quantitative PCR revealed downregulation of the gene in 27 (56.2%) of the animals. Out of the 16 dogs with γ2 chain overexpression, seven were still alive. The western blot results showed bands generation of 36, 50, and 98kDa, suggesting degradation of laminin-332 γ2 in malignant tumors. The results suggest that, in the future, low expression and/or degradation of laminin-332 γ2 chain in canine mammary tumors may be used as an indicator of malignant potential. However, further studies are necessary to corroborate these results.

Keywords: dog, adhesion molecule, cancer, western blot, RT-PCR

RESUMO

Para avaliar a expressão imunoistoquímica da cadeia γ2 da laminina-332, foram investigados 48 casos de câncer mamário canino. Comparou-se, por RT-PCR, a expressão gênica nas células tumorais com um pool de tecido mamário. Como teste complementar, em oito amostras tumorais, realizou-se western blot para avaliar a integridade da proteína laminina-332 γ2. A imunoistoquímica demonstrou expressão negativa, focal e fraca da laminina-332 γ2 nos tumores com pior prognóstico. A RT-PCR quantitativa revelou a baixa expressão do gene em 27 (56,2%) dos animais. Das 16 cadelas com superexpressão da cadeia γ2, sete ainda estavam vivas no final do estudo. O resultado do western blot mostrou bandas de 36, 50 e 98kDa, sugerindo uma degradação da laminina-332 γ2 em tumores malignos. Os resultados sugerem que, no futuro, a baixa expressão e/ou degradação da laminina-332 γ2 nos tumores mamários caninos podem ser utilizadas como indicadores de potencial maligno. No entanto, mais estudos são necessários para confirmar estes resultados.

Palavras-chave: cão, molécula de adesão, câncer, western blot, RT-PCR

INTRODUCTION

Laminin is a heterotrimeric glycoprotein. This major component of the basement membrane of epithelium is composed of three chains: α3, β3, and γ2. The γ2 chain is specific to isoform laminin-332, also called laminin-5. This protein interacts with at least two integrin receptors, expressed by epithelial cells α3β1 and α6β4, to regulate cell adhesion, migration, and morphogenesis (Colognato and Yurchenco, 2000; Kagesato et al., 2001; Tsuruta et al., 2008).

The main component of the basal lamina is type IV collagen followed by laminin. Laminin participates in the architecture of the basal lamina that surrounds the epithelial cells and plays an important mediation role in cell adhesion, growth, migration, proliferation, and differentiation (Hao et al., 2001; Miller et al., 2001; Tsuruta et al., 2008).

Recebido em 27 de setembro de 2010
Aceito em 15 de fevereiro de 2011
E-mail: debora.zuccari@famerp.br

Laminin-332 is a component of anchoring filaments, and failures to express the γ2 subunit may compromise the formation of anchoring filaments and hemidesmosomes. Failure in hemidesmosome formation makes epithelial-stromal junction less stable, thereby promoting malignant cells to invade and spread through adjacent structures (Hao et al., 1996).

The abnormal expression of laminin and its integrin receptors is a hallmark of certain tumor types. They are believed to promote invasion of colon, breast, and skin cancer cells (Tsuruta et al., 2008). Along with clinical and pathological information, it has been used to assess tumor invasiveness (Hao et al., 1996; Henning et al., 1999).

The integrin molecules are mainly involved in cell adherence to extracellular matrix, and they are components of the hemidesmosome that bind to a specific site (RGD) in the laminin-332, establishing the link between the basal area of epithelial cells and the basal lamina (Hao et al., 2001). In normal tissues, laminin-332 acts to tether cells and inhibits their movement by inducing assembly of stable adhesive devices called hemidesmosomes. In a variety of tumors, laminin-332 functions in a completely different fashion by promoting migration.

Epithelial tumors often secrete abundant amounts of extracellular laminin-332 and frequently express α6ß4integrin (Zahir et al., 2003).

Laminin-332 plays an important role in cell migration during tumor invasion and tissue remodeling. Some studies show that laminin-332 and products from its proteolytic degradation are found at the leading front of several tumors and likely induce and/or promote tumor cell migration (Lohi et al., 2001; Tsuruta et al., 2008).

Laminin-332 γ2 expression has been reported to be down regulated in colorectal carcinoma, breast cancer, prostate carcinoma, and oral squamous cell carcinoma. Because the γ2 chain of laminin-322 plays an important role in cancer, it was evaluated its expression in canine mammary neoplasms and its prognostic value.

MATERIAL AND METHODS

Mammary tumor samples were surgically removed from 56 bitches of pure and undefined breed, aged from 2 to 17 years (mean age=10 years). Two fragments were collected from each animal. The fragments used in the molecular assays were frozen immediately after surgery. The fragments used for immunohistochemical (IHC) assays were fixed in 10% buffered formalin solution. Tissue blocks were prepared by standard histological methods, embedded in paraffin wax, sectioned at 3μm, and stained with hematoxylin and eosin. Representative areas of the tumour were selected for IHC. The histopathological classification of tumor type was based on the World Health Organization (WHO) classification system for canine mammary tumors (World…, 2007). The histological study of canine mammary tumors was performed according to the criteria presented by Misdorp (1999) and supported by the World Health Organization / Institute of Pathology of the US Armed Forces. The system of histological grading was performed according to Elston and Ellis (1998), and the clinical stage as Owen (1980). Of the 56 animals, 48 carcinomas were selected for this study. The animals were followed-up for 18 months. The presence of metastasis, involvement of lymph nodes, and overall survival were also evaluated.

Tissues from normal mammary glands from 10 bitches euthanized in a Zoonosis Control Center were pooled to form a control group. This group was compared to the tumor cases using molecular assays. The phenotypic features of the control and experimental bitches were similar.

Laminin-332 γ2 IHC was based on polyclonal anti-Laminin-332 γ2 antibody (sc-7652, Santa Cruz Biotechnology Inc - Santa Cruz, CA, USA) diluted 1:50 in bovine serum albumin (BSA; Sigma-Aldrich - St Louis, MO, USA). A section from at least one representative block of each case was mounted onto silanized slides. After this, the slides were deparaffined, rehydrated through graded alcohol, and incubated with 3% hydrogen peroxidase for 30min to block endogenous peroxidase activity. Induced antigen retrieval in Pan Steam at 95°C with citric acid (pH 6.0, 35min) was made. After cooling down, the slides were blocked with 10% BSA for 30min and then incubated with the primary

antibody for 2h at room temperature and with avidin-biotin-peroxidase complex (ABC Erviegas, - São Paulo, Brazil) for 1h. A dose of 0.5% 3,3' diaminobenzidine tetrahydrochloride (Signet® Laboratories - Dedham, MA, USA) diluted in phosphate-buffered saline (PBS) was applied to the slides for 2-5min at 20-22°C. The slides were counterstained with Harris hematoxylin. All steps were performed as directed by the manufacturers. Negative controls were obtained by omitting the primary antibody, whereas normal renal tissue served as internal positive control in every assay.

Multiple fields were examined in each slide and positive immunostaining was indicated by distinct brown cytoplasmic staining. Immunohistochemical examination was blind (i.e. without knowledge of the histopathological diagnosis) and the results were based on the consensus of at least two observers. Expression level of the marker was recorded as proposed by Allred et al. (1998). Positive cell count was determined as a percentage of the total number of cells observed on each slide.

The total RNA was extracted from the mammary tissue and frozen by the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1993) with TRIZOL® (Invitrogen Life Technologies - São Paulo, Brazil) as recommended by the laboratory. The organic phase obtained from the RNA extraction was submitted to the isolation of DNA and proteins. The sample was incubated for 2-3min at 15-30ºC and the DNA was sedimented by centrifugation at 2,000g for 5min at 2-8ºC. The supernatant was centrifuged at 10,000 rpm for 3min at 4ºC. The supernatant was transferred to other tubes, the insoluble pellets were washed with 200µL lysis buffers, and the second supernatant was collected. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). The protein samples were stored at -70ºC.

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

Each transcript level was normalized by division with the expression values of the *HPRT1* used as internal control. Gene expression stability was analyzed by geNorm software. Transcript level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ΔCt was the difference between the threshold cycles of a target and the internal control and $\Delta\Delta Ct$ was the difference between the average ΔCt of the sample and the average ΔCt of the calibrator sample. The fold difference (relative abundance) was calculated using the $2^{-\Delta\Delta CT}$ formula and plotted as mean±SD of the triplicate reactions. At least 3-fold differences were considered significant.

A negative control was included in each reaction, and one sample was chosen for reaction calibration. Experiments were repeated when the coefficient of variation was higher than 5%. After each reaction, the products were analyzed on 2% agarose gel stained with ethidium bromide.

The gene was searched, selected in PUBMED (http://www.ncbi.nlm.nih.gov/entrez), and synthesized from canine messenger RNA already sequenced and confirmed. Its design was made using the program PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer 3_www. Cgi). The primers used for amplification were laminin-332 γ2 sense (5’ ATCATCTGGATGGGGGAAAC 3’) and antisense (5’ GCAGGAGACCCATTTCTTTG 3’) (163bp) and *HPRT1* sense (5' TTATAG TCAAGGGCATATCC 3') and antisense (5' AGCTTGCTGGTGAAAAGGAC 3').

Multivariate logistic regression analyses of prognostic factors were performed. The results were analyzed based on tumor diagnosis and patient outcome using histological expression of the antibodies by analysis of dependency (Cordeiro, 1987) and the chi-square test. The differences between group means were assessed by the t-test of Student. A value of $P<0.05$ was considered significant (two tail analysis). Statistical analysis was performed using

Microsoft Office XP Software. In addition, it was made a survival curve correlating laminin-332 γ2 expression and overall survival (Kaplan-Meier method).

For Western blot analysis, goat polyclonal anti-Laminin γ-2 antibody (sc-7652, Santa Cruz Biotechnology Inc) was used as antibody diluted 1:200. Proteins (70µg) under denaturing conditions were separated on SDS-PAGE (12% resolving gel with 5% stacking gel) at 110V for 60min (MiniProtean 3 Cell, BioRad - Hercules, CA, USA). The molecular weight ladders were the PageRuler™ Prestained Protein Ladder (SM0671; Fermentas Life Sciences - Burlington, Canada) and the See Blue Plus2 Pre-Stained Standard (Invitrogen Corp. - Carlsbad, CA, USA).

The proteins were transferred onto PVDF membranes (Immobilon-P, Millipore - Billerica, MA, USA) and immersed in transfer buffer (25mMTris, 0.2M glycine, 20% v/v methanol) at 325mA for 70min in the same electrophoresis system. The membranes were submitted to chromogenic staining using the Western Breeze Chromogenic Kit (Invitrogen) according to the protocol of the manufacturer. Finally, the membranes were photographed using the Gel Logic HP 2200 imaging system (Carestream Health Inc./Kodak Health Group - Rochester, NY, USA).

RESULTS AND DISCUSSION

Histopathological evaluation of the samples showed a predominance of carcinomas (48 of 56 animals, 85.7%) in the studied group. This disease is generally detected late because lack of symptoms at diagnosis, a situation that leads to inaccurate prognoses. However, most of the animals survived after surgery (26 of 48 animals or 54.2%).

The carcinomas were mainly simple (33 of 48 animals, 68.8%), followed by complex carcinoma in eight animals (16.6%), carcinoma in mixed tumor in six animals (12.5%), and carcinoma with squamous differentiation in one animal (2.1%). According to the WHO/AFIP classification, simple carcinomas are divided into tubulopapillary, solid, and anaplastic types. The prevailing cancer type in the present study was simple and histopathologically characterized as tubulopapillary.

Statistical analysis revealed important associations between IHC staining and diagnosis. Despite late diagnosis, choosing the best treatment option may improve prognosis. The tumors with the worst prognosis exhibited negative, focal, and weak expression (Fisher test, $P<0.05$) (Figure 1). In addition, diagnosis was correlated to overall survival ($P<0.05$). RT-PCR showed gene underexpression in 27 of the 48 bitches (56.2%).
As reported by Tsuruta et al. (2008), cancer is a malignant transformation of epithelial cells, and its infiltration is blocked by the extracellular matrix (ECM) of the basement membrane. However, ECM molecules are also co-opted by cancer cells as they migrate from an initial tumor and invade surrounding tissues.

According to Henning et al. (1999), the IHC demonstration of laminin-332 may serve as a marker of benignity in epithelial breast lesions. Fujita et al. (2005) observed that chains of laminin-332 were expressed in the ductal epithelium basement membranes of the breast and diminished with tumor progression. As expected, RT-PCR showed gene underexpression in most samples. Laminin-332 expression was observed by Korah et al. (2007) in benign and atypical dedifferentiation in mammary tissue samples. This protein was lost primarily with cancer transformation into an invasive stage. Also according to Korah et al. (2007), laminin-332 γ2 preserves ductal integrity during mammary carcinogenesis, and loss of expression corresponds to loss of ductal structure. Carpenter et al. (2008) suggest that laminin-332 expression in metaplastic carcinomas promotes aggressiveness and transition from an epithelial to a mesenchymal phenotype.

Out of the 16 animals with laminin-332 γ2 overexpression from the normal pool, seven were still alive 18 months postoperatively (Figure 2). There was not any correlation between laminin-332 γ2 expression and overall survival (Kaplan-Meier method, $P=0.676$) (Figure 3). This correlation could be significant ($P<0.05$) if the number of studied animals was higher.

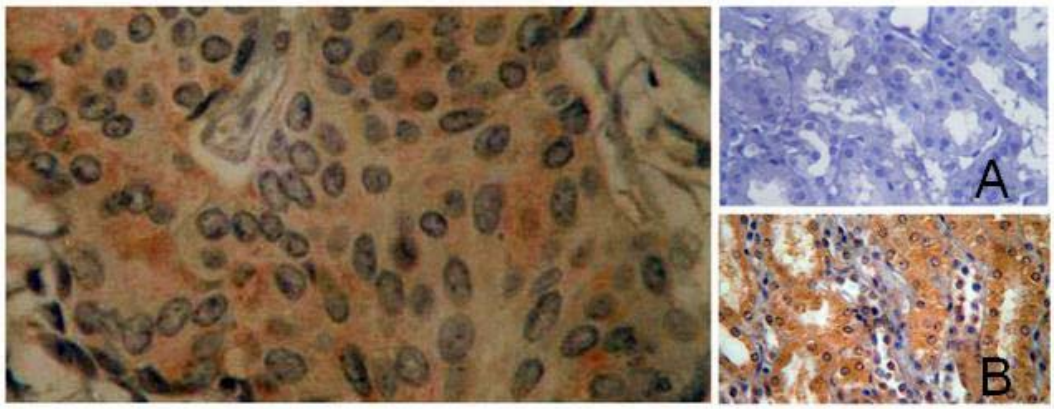

Figure 1. Photomicrography showing a moderate cytoplasmic expression of laminin-332 γ2 in canine tubulopapillary carcinoma. Avidin-biotin-peroxidase diaminobenzidinetetrahydrochloridechromogen, counterstained with Harris hematoxylin. 400X. A: negative control; B: positive control.

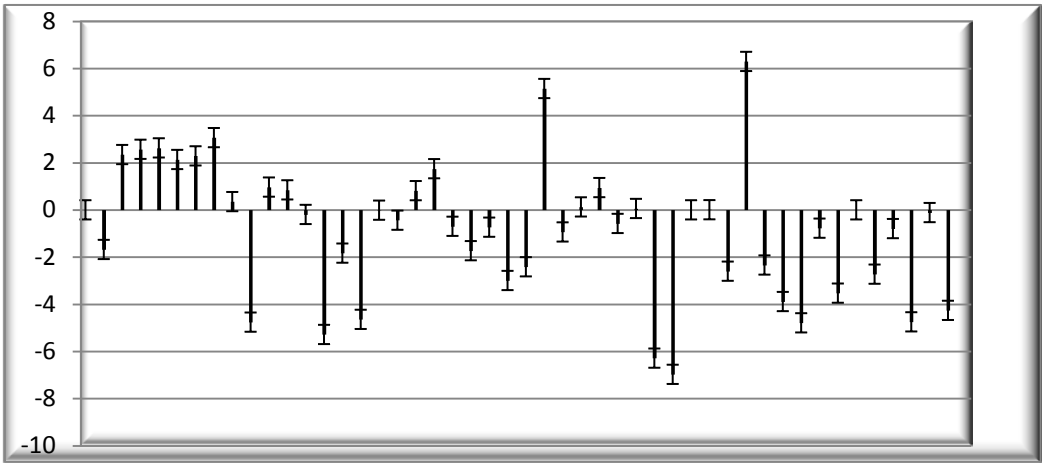

Figure 2. Quantitative gene expression of laminin-332 γ2 in samples of canine mammary cancer (black columns) and in a pool of normal mammary tissues (zero or 0).

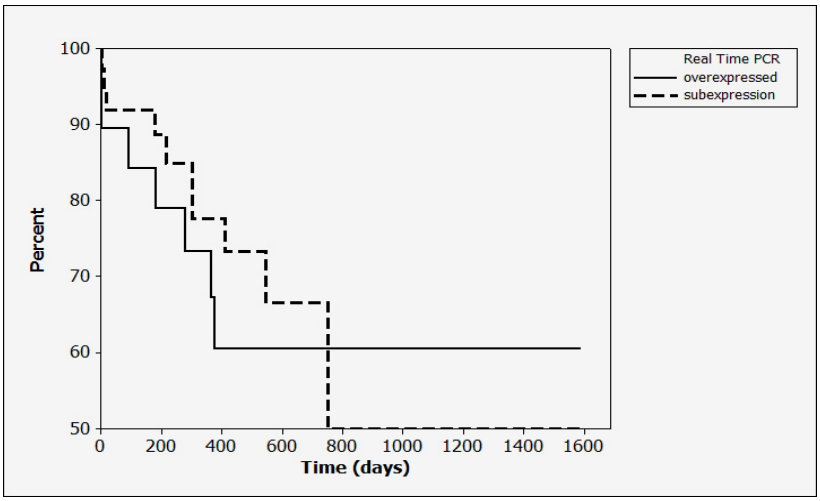

Figure 3. Survival curve [percent of live animals *versus* time (days)] according to Kaplan-Meier method (P-value=0.676).

A controversial role of laminin-332 in metastasis has emerged from investigations into various types of cancers. With regards to dogs that died with metastasis, eight (88.9%) showed low laminin-332 γ2 expression by IHC and four (44.4%) underexpression by RT-PCR.

Contrasting gene expression by RT-PCR with protein staining by IHC, it was found concordance in 66.3% (27 of 48 animals) of the results and 13 discordant results with gene overexpression and negative or weak protein staining.

The protein was absent in neoplastic tissues, but laminin-332 γ2 RNAm expression was detected, possibly because of post-transcriptional changes that affect protein synthesis. Therefore, the protein cannot be detected by IHC. At the protein level, laminin-332 seems to be both up and down-regulated in cancer. High expression of laminin-332 and its γ2 subunit in cancer is considered a poor prognostic factor and has been related to tumor invasiveness in several cancers, including breast cancer (Fukushima et al., 2001; Lohi et al., 2001; Aoki et al., 2002).

The expression of laminin-332 γ2 protein was observed in 100% of invasive or microinvasive carcinomas and in their related lymph node metastasis (Capt et al., 2003).

In contrast, cells or tissues that are adjacent to the tumor were also found to express the laminin γ2 chain or its fragments. The laminin γ2 fragment is also found in circulating blood in cancer patients and has been reported to be a tumor marker (Tsuruta et al., 2008).

The western blot was performed in eight tumor samples as a complementary test to evaluate protein integrity. All the carcinomas showed a band from 36 to 50kDa (around 40kDa). This preliminary result suggests degradation of laminin-332 γ2 in some mammary tumor types (one carcinoma in mixed tumor, three tubulopapillary, and three solid carcinomas). All analyzed tumor samples showed bands from 36 to 56kDa and one adenoma showed a 98kDa band suggesting a lower degradation when compared with the carcinomas. The adenoma exhibited only one band of 98kDa.

In invasive cancers, laminins usually become discontinuous or absent around tumor foci, due to either increased degradation or reduced synthesis (Fujita et al., 2005). There is the controversial role of laminin-332 in cancer, nonetheless, a general consensus is that laminin-332 is upregulated in many cancer types and it is often found at the migrating edge of tumor cells (Tsuruta et al., 2008).

Rajamäkiet al. (2006) asserted that the corresponding normal fragment of the laminin-332 γ2 chain has a molecular weight of 140kDa, and that broken fragments can vary from 36kDa to 53kDa. Ogawa et al. (2007) found proteolytic cleavage of the small γ2 chain arm of laminin-332 in human cell lines using the Western-blot method.

Koshikawa et al. (2005) suggest that membrane-type-1 matrix metalloproteinases processing of laminin-332 in human tumors may stimulate the epidermal growth factor receptor, increasing tumor cell scattering and migration, thereby enhancing their metastatic potential. According to Lenander et al. (2003), it has been claimed that laminin-332 promotes cell migration and/or invasion after the γ2 chain has been cleaved by metalloproteinases. The smaller bands (around 40kDa) may be a result of this proteolytic cleavage or degradation. According to other researchers Gagnoux-Palacios et al. (2001), laminin-332 undergoes extracellular proteolysis of the γ2 chain that removes the NH_2-terminal short arm of the polypeptide and reduces the size of this protein. The functional consequence of this event remains obscure, although evidences indicate that cleavage of the γ2 chain potently stimulated scattering and migration of keratinocytes and cancer cells.

Laminin-332 plays an important role in suppressing early tumor development *in vivo*. 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 laminin-332 as a tumor/transformation suppressor gene. Cases with increased laminin-332 expression likely involve the expression of other genes.

It can be hypothesize that instead of being an independent prognostic factor, laminin-332 γ2 must be evaluated along with the co-expressed proteins and the respective disease stage because

it likely has complex functions. This might finally explain the conflicting results of different scientific studies. Further investigations are warranted to understand the role of laminin-332 γ2 expression over a breast cancer course 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 laminin-332 γ2 expression itself.

With respect to test sensitivity, it was found that they are complimentary and have high concordance. The mechanisms underlying laminin-332 γ2 expression remain to be elucidated in most of the cases.

CONCLUSIONS

Findings suggest that laminin-332 γ2 degrades in canine mammary tumors. Univariate analysis revealed that laminin-332 γ2 underexpression was associated with low histological grade. However, multivariate analysis showed that laminin-332 γ2 was a significant prognostic factor for metastasis. All these findings can raise questions about the tumor-suppressive functions of laminin-332 γ2 in canine mammary cancer. Further studies are necessary to corroborate the oncogenic properties of laminin-332 γ2 in canine mammary tumors.

ACKNOWLEDGMENTS

We thank Prof. Dr. Lilian Castiglioni and Prof. Dr. Eloiza Helena Tajara da Silva for their help in the development of the project; Prof. Dr. Felipe Augusto Ruiz Sueiro, Prof. Dr. Noeme S. Rocha, and Prof. Dr. Cicero Meneghetti for their support in reading and analyzing the slides; and Prof. Dr. José Antônio Cordeiro for the statistical analysis of the data. We also thank Celso Pereira Reis Filho for technical support and Editora Scripta Ltda. for critically reading the English manuscript. This research was supported by FAPESP/Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant 05/51936-0).

REFERENCES

ALLRED, C.D.; HARVEY, J.M.; BERARDO, M. et al. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod. Pathol., v.11, p.155-168, 1998.

AOKI, S.; NAKANISHI, Y.; AKIMOTO, S. et al. Prognostic significance of laminin-5 gamma2 chain expression in colorectal carcinoma: immunohistochemical analysis of 103 cases. *Dis. Colon Rectum.*, v.45, p.1520-1527, 2002.

BRADFORD, M.M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, v.72, p.248-254, 1976.

CAPT, A.; SPIRITO, F.; GUYON, R. et al. Cloning of laminin gamma 2 cDNA and chromosome mapping of the genes for the dog adhesion ligand laminin 5. *Biochem. Biophys. Res. Commun*., v.321, p.1256-1265, 2003.

CARPENTER, P.M.; WANG-RODRIGUEZ, J.; CHAN, O.T. et al. Laminin 5 expression in metaplastic breast carcinomas. *Am. J. Surg. Pathol.*, v.32, p.345-353, 2008.

CHOMCZYNSKI, P.; SACCHI, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, v.162, p.156-159, 1987.

COLOGNATO, H.; YURCHENCO, P.D. Form and function: the laminin family of heterotrimers. Dev. Dyn., v.218, p.213-234, 2000.

CORDEIRO, J.A. *Analysis of dependence*. Campinas: Instituto de Matemática – UNICAMP, 1987. (Technical Report)

ELSTON, C.W.; ELLIS, I.O. (Eds). *The Breast.* London: Churchill Livingstone, 1998. p.365-384.

FUJITA, M.; KHAZENZON, N.M.; BOSE, S. et al. Overexpression of β1-chain-containing laminins in capillary basement membranes of human breast cancer and its metastases. *Breast Cancer Res.*, v.7, p.11-21, 2005.

FUKUSHIMA, N.; SAKAMOTO, M.; HIROHASHI, S. Expression of laminin-5-gamma-2 chain in intraductal papillary-mucinous and invasive ductal tumors of the pancreas. *Mod. Pathol.*, v.4, p.404-409, 2001.

GAGNOUX-PALACIOS, L.; ALLEGRA, M.; SPIRITO, F. et al. The short arm of the Lamininγ2 chain plays a pivotal role in the incorporation of laminin 5 into the extracellular matrix and in cell adhesion. *J. Cell Biol*., v.153, p.835-850, 2001.

HAO, J.; JACKSON, L.; CALALUCE, R. et al. Investigation into the mechanism of the loss of laminin 5 (α3β3γ2) expression in prostate cancer. *Am. J. Pathol.*, v.158, p.1129-1135, 2001.

HAO, J.; YANG, Y.; MCDANIEL, K.M. et al. Differential expression of laminin 5 (α3β3γ2) by human malignant and normal prostate. *Am. J. Pathol.*, v.49, p.1341-1349, 1996.

HENNING, K.; BERNDT, A.; KATENKAMP, D. et al. Loss of laminin-5 in the epithelium-stroma interface: an immunohistochemical maker of malignancy in epithelial lesions of the breast. *Histopathology*, v.34, p.305-309, 1999.

KAGESATO, Y.; MIZUSHIMA, H.; KOSHIKAWA, N. et al. Sole expression of laminin gamma 2 chain in invading tumor cells and its association with stromal fibrosis in lung adenocarcinomas. Jpn. J. Cancer Res., v.91, p.184-192, 2001.

KORAH, R.;DAS, K.;LINDY, M.E. et al. Coordinate loss of fibroblast growth factor 2 and laminin 5 expression during neoplastic progression of mammary duct epithelium. *Hum. Pathol.*, v.38, p.154-160, 2007.

KOSHIKAWA, N.; MINEGISHI, T.; SHARABI, A.et al. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin gamma 2 chain. *J. Biol. Chem.*, v.208, p.88-93, 2005.

LENANDER, C.; ROBLICK, U.J.; HABERMANN, J.K. et al. Laminin 5 gamma 2 chain expression: a marker of early invasiveness in colorectal adenomas. *Mol. Pathol*., v.56, p.342-346, 2003.

LIVAK, K.J.; SCHMITTGEN, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods*., v.25, p.402-408, 2001.

LOHI, J. Laminin-5 in the progression of carcinomas. *Int. J. Cancer*, v.94, p.763-767, 2001.

MILLER, K.A.;EKLUND, E.A.; PEDDINGHAUS, M.L. et al. Kruppel-like factor 4 regulates laminin α3A expression in mammary epithelial cells. *J. Biol. Chem.*, v.276, p.42863-42868, 2001.

MISDORP, W.; ELSE, R.W.; HELLMÉN, E. et al. *Histological classification of mammary tumors of the dog and the cat.* Washington, DC: World Health Organization., 1999. v.7, 59p.

OGAWA, T.; TSUBOTA, Y.; HASHIMOTO, J. et al. The short arm of laminin gamma2 chain of laminin-5 (laminin-332) binds syndecan-1 and regulates cellular adhesion and migration by suppressing phosphorylation of integrin beta4 chain. *Mol. Biol. Cell.*, v.18, p.1621-1633, 2007.

OWEN, L.N. *The TNM classification of tumors in domestic animals*. Geneva: World Health Organization, 1980. v.1, 53p.

RAJAMÄKI, M.M.; JÄRVINEN, A.K.; SORSA, T.A. et al. Elevated levels of fragmented laminin-5 gamma2-chain in bronchoalveolar lavage fluid from dogs with pulmonary eosinophilia. Vet. J., v.171, p.562-565, 2006.

TSURUTA, D.; KOBAYASHI, H.; IMANISHI, H. et al. Laminin-332-integrin interaction: a target for cancer therapy? Curr. Med. Chem., v.15, p. 1968-1975, 2008.

World Health Organization–Air Force Institute of Pathology classification system for canine mammary tumors, 2007. Available in: <http://www.who.int/en/>Accessed in: July 5, 2009.

ZAHIR, N.; LAKINS, J.N.; RUSSELL, A. et al. Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. *J. Cell Biol.*, v.163, p.1397-1407, 2003.