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# Snai-1 and Epithelial-Mesenchymal Transition-Related Protein Immunoexpression in Canine Mammary Carcinomas

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## Abstract

**Epithelial-mesenchymal transition (EMT) is defined as switching of polarized epithelial cells to a migratory fibroblastoid phenotype. EMT is known to be involved in the progression and metastasis of various cancers in humans, but this specific process is still little explored in the veterinary literature. The aim of this research was to evaluate the expression of EMT-related proteins in canine mammary carcinomas (CMCs). The expression of six EMT-related proteins in 94 CMCs of female dogs was evaluated by immunohistochemistry using a tissue array method. Additionally, clinicopathological characteristics were compared with the expression of EMT-related proteins. Loss of epithelial protein and/or acquisition of the expression of mesenchymal proteins were observed in CMCs. Loss of epithelial protein and/or acquisition of the expression of mesenchymal proteins were observed, particularly in tumors with evidence of stromal invasion; however, significance was only observed between the S100A4 and vascular invasion. In addition, Snai-1 nuclear immunoexpression was significantly related to E-cadherin loss. In conclusion, loss of epithelial proteins and/or the acquisition of mesenchymal proteins are associated with EMT and may have an important role in the evaluation of CMC patients. The unique immunoexpression pattern of Snai-1 could help to distinguish between an adenoma and a non-metastatic carcinoma and seems to be related**

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**to conversion of myoepithelial cells to a complete mesenchymal-like phenotype. Loss of E-cadherin and cytokeratin and change of immunoexpression pattern of Snai-1, N-cadherin, S100A4 and MMP-2 indicate the occurrence of EMT in canine mammary carcinomas and should result in an en bloc resection or a close follow-up.**

## Keywords

**EMT, S100A4, Keratin, Mammary Tumors, Dog**

## 1. Introduction

Mammary gland tumors are the most common neoplasms of the female dog and represent a remarkably heterogeneous group in terms of morphology and biological behavior [1]. Consequently, the identification of reliable prognostic factors is very important in order to assess the individual risk and evaluate the clinical outcome. The prognosis of advanced mammary carcinoma patients is most likely related to the degree of metastatic spread. Although the process of cancer metastasis appears to be regulated by a variety of gene products, little is known about the molecular aspects of progression of canine mammary carcinoma (CMC) cells.

Recently, the conversion of epithelial cells to migratory fibroblastoid cells—known as epithelial-mesenchymal transition (EMT)—was suggested to be involved in metastasis. Such event is crucial during gastrulation and neural crest formation in embryogenesis [2], but has been suggested to be also involved in inflammation and neo-plastic progression. During EMT, cells lose epithelial polarity and acquire a spindle-shaped, highly motile mesenchymallike phenotype. Moreover, this transition involves loss or redistribution of tight- and adherence-junction proteins and a switch to mesenchymal gene expression which confers upon cells the ability to pass through the basement membrane [3]. This phenomenon is reactivated during the progression of numerous cancers and was demonstrated to be associated with poor histological differentiation, local invasiveness and distant metastasis [4] [5].

Different studies regarding expression of EMT-related proteins in canine mammary neoplasms were performed; however, none of them focused on the specific event of EMT and its implication in the diagnosis and prognosis of any canine tumor type. In the present study we therefore evaluated the expression of 6 established human breast cancer EMT markers (Snai-1, E-cadherin, N-cadherin, MMP-2, S100A4, and cytokeratin) in canine mammary carcinomas and compared their expression with different pathological parameters.

## 2. Materials and Methods

### 2.1. Tissue Samples

Consecutively collected, surgically resected 94 CMC tissue specimens were obtained from female dogs which underwent mastectomy at the São Paulo State University's Veterinary Hospital, Botucatu, Brazil, between March 2009 and September 2010. All patients had the tumor specimens surgically removed by radical mastectomy and none of them had received preoperative chemotherapy or radiotherapy. Tissue samples from these neoplasms were formalin-fixed and paraffin-embedded. For routine microscopic examination, 4 µm thick sections were obtained and stained with hematoxylin and eosin with subsequent evaluation under light microscopy.

### 2.2. Histologic Evaluation

Three independent pathologists were responsible for the evaluation of tissues and diagnosis of the neoplasms, according to the World Health Organization criteria for canine mammary tumors [6]. Malignant epithelial neoplasms were graded histologically in accordance with the Nottingham scoring system for mammary cancer [7], based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored on a scale of 1 - 3 to indicate whether it was present in slight, moderate or marked degree, giving a putative total of 3 - 9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points. Cases of mammary carcinomas were assessed for

mode of growth (expansive vs. infiltrative), presence of intratumoral necrosis, stromal and vascular invasion.

Additionally, lesions were classified according to standard diagnostic criteria provided by the World Health Organization [6] and classified using the Nottingham grading system for mammary neoplasms [7]. Presence of vascular invasion, invasion depth, mode of growth, and presence of intratumoral necrotic tissue were also evaluated. Tissue samples were arrayed for performing subsequent analysis.

## 2.3. Immunohistochemistry

Firstly, 3 µm thick histologic sections were obtained, deparaffinizated, and rehydrated. Immunohistochemistry was performed by using a polymeric labeling detection system (Novolink Polymer Detection System, Novocastra Laboratories, Newcastle, UK). Antigen retrieval was carried out by heat treatment in 10 mM citrate buffer pH 6.0 for all primary antibodies except for N-cadherin, for which the antigen retrieval was carried by heating the slides in a water bath using a Tris EDTA buffer pH 9.0. **Table 1** summarizes the used primary antibodies and staining procedures adopted for each antibody in this study and their immunoexpression patterns in non-neoplastic and CMC tissues. After cooling (20 minutes at room temperature), the sections were immersed for 30 minutes in a solution of 3% hydrogen peroxide diluted in methanol in order to block endogenous peroxidase activity. All slides were then incubated with a protein block reagent (Novocastra Laboratories, Newcastle, UK) for 10 minutes and subsequently overnight incubated at 4˚C with the specific primary antibodies. Then, the slides were immersed with the detection systems following the manufacturer's instructions. Subsequently, 3,3' diaminobenzidine tetrahydrochloride (DAB) was used as chromogen in order to allow the visualization of antigen-antibody reaction. Then, slides were counterstained using Harris's hematoxylin, dehydrated, and mounted for evaluation and light microscopy.

For immunollabeling evaluation, S100A4 was considered as positive when more than 10% of neoplastic cells revealed nuclear staining. N-cadherin was considered as positive when membranous immunostaining was observed in luminal neoplastic cells. On the other hand, E-cadherin was considered as negative when loss of membranous staining was observed in such cells. For cytokeratin tumor cells were considered as negative when loss of membranous/cytoplasmic staining was observed. For MMP-2, neoplastic cells were considered as positive when cytoplasmic/membranous staining was observed. The assessment of Snai-1 expression in canine mammary tissues was based on detectable immunoreactivity in nuclear region and on a semiquantitative analysis using the following scoring system: 0, no staining; 1+, nuclear staining in 1% to 25% of neoplastic cells; 2+, nuclear staining in 26% to 50% of neoplastic cells; and 3+: nuclear staining in more than 50% of neoplastic cells, as previously described (Hung *et al*., 2009).

## 2.4. Statistical Analysis

Differences in EMT-related proteins expression were compared using Fisher's exact test or Pearson's $X^2$ test for qualitative variables and using Student's t-test or analysis of variance for continuous variables. Survival curves were estimated using Kaplan-Meier product-limit method, and the significance of differences between survival curves was determined using the log rank text. Multivariate analysis was performed by Cox proportional hazards regression modeling. All statistical tests were two sided, and statistical significance was accepted at the $P < 0.05$ level. All analyses were performed using the Prism GraphPad software version 5.0 (San Diego, CA).

**Table 1.** Antibodies used, dilution, and expression patterns.

| *Antibody* | *Dilution* | *Source* | *Clone* | *Manufacturer* | *Expression pattern* |
|---|---|---|---|---|---|
| Snai-1 | 1:500 | Rabbit | Polyclonal | LifeSpan Biosciences | Nuclear |
| S100A4 | 1:1200 | Rabbit | Polyclonal | Abcam | Nuclear |
| MMP-2 | 1:100 | Mouse | 4D3 | Abcam | Cytoplasmic |
| E-Cadherin | 1:100 | Mouse | NCL-E-cad | Novocastra | Cytoplasmic/membrane |
| N-Cadherin | 1:50 | Mouse | 6G11 | Dako | Cytoplasmic/membrane |
| Cytokeratin | 1:500 | Mouse | AE1/AE3 | Dako | Cytoplasmic |

*MMP-2: matrix metalloproteinase 2.

## 3. Results

### 3.1. Snai-1 Immunoexpression Patterns in Canine Mammary Tissues

Snai-1 nuclear positive immunostaining was not observed in normal or hyperplastic mammary tissue adjacent to tumor areas and from female dogs that never developed mammary tumors. Dysplastic areas revealed only single positive cells. In mixed benign tumors, Snai-1 nuclear expression was observed in fibroblastoid cells, myoepithelial cells, and cells under chondroid/osteoid differentiation. In simple carcinomas, Snai-1 expression was observed in luminal cells (**Figure 1**), infiltrating cells of the invasive front and stromal cells, with absence of nuclear expression in myoepithelial cells. In complex carcinomas and carcinomas in mixed tumors, Snai-1 expression was observed in carcinomatous luminal cells, myoepithelial cells and cells under chondroid/osteoid differentiation (**Figure 2**). No or rare Snai-1 positive immunoreactivity (+) was observed in normal, hyperplastic, or dysplastic mammary tissue. A similar pattern was observed in mammary adenomas and within the benign luminal component of carcinomas in mixed tumors. On the other hand, high expression of Snai-1 was detected in mammary carcinomas. The results regarding the Snai-1 immunoexpression quantification are summarized in **Table 2**.

No relation with invasion depth, presence of intratumoral necrosis, vascular invasion, tumor mode of growth, HER-2 and hormone receptor status were statistically found.

### 3.2. Relation between Immunoexpression of EMT-Related Proteins and Pathologic Parameters

Epithelial protein loss frequencies were 25.53% (28/94) for cytokeratin and 38.29% (36/94) for E-cadherin, and aberrant mesenchymal protein expression frequencies were 59.57% (56/94) for N-cadherin, 36.17% (34/94) for matrix metalloproteinase 2, and 10.63% (10/94) for S100A4. Additionaly, 59.57% (56/94) highly expressed Snai-1. **Figure 2** shows a representative immunohistochemical result.

Expression loss of the epithelial protein E-cadherin was found to be significantly related to high nuclear expression of Snai-1 ($P = 0.045$). Novel mesenchymal protein expression of S100A4 was found to be related to vascular invasion ($P = 0.001$). Although in our study the number of Snai-1-highly-expressing cases increased with the occurrence of stromal invasion, a statistical significant correlation was not evident. Additionally, no relation between EMT related proteins immunoexpression and invasion depth, presence of intratumoral necrosis, vascular invasion, and tumor mode of growth was statistically found except for S100A4 and vascular invasion.

### 3.3. EMT-Related Protein Expression and Survival Analysis

Overall patient survival rates were determined using the log rank test with respect to expression of the six proteins. In terms of the epithelial proteins, cytokeratin loss ($P = 0.02$) (**Figure 3(a)**) was found to be significantly associated with a poor outcome. For mesenchymal proteins, the nuclear expression S100A4 ($P = 0.02$) (**Figure 3(d)**) was found to be significantly associated with an unfavorable prognosis. However, no significant difference in patient outcome was found with respect to E-cadherin loss (**Figure 3(b)**), N-cadherin expression (**Figure 3(c)**), Snai-1 expression (**Figure 3(e)**), or MMP-2 (**Figure 3(f)**) in the cases herein studied. Multivariate analysis was performed to determine relations between markers but no statistical significance was observed.

## 4. Discussion

Many recent studies have demonstrated the importance of EMT in various tumor types and humans [2]-[4] and, less extensively, in dogs [8] [9]. An important aspect of EMT is the loss of epithelial protein markers, *i.e.* cytokeratins and E-cadherin. E-cadherin is required for the formation of stable adherens junctions and, thus, for the maintenance of an epithelial phenotype [10]. Loss of epithelial proteins such as E-cadherin is a hallmark of metastatic carcinoma and, furthermore, proteomic analysis in breast cancer has revealed that circulating mammary tumor cells or those found in micrometastases reveal evidence of mesenchymal [10]. We observed both loss in the important epithelial proteins E-cadherin and cytokeratin expression. Loss in cytokeratin expression revealed to be related to overall survival ($P = 0.02$; risk four times higher than in dogs that did express cytokeratin) in dogs with CMCs and demonstrated to be an independent predictive factor in multivariate analysis. This feature is quite interesting since in cancer keratins are used as prognostic indicators in tumors and/or peripheral blood and several studies have provided evidence for active keratin involvement in cancer cell invasion and metastasis [11].

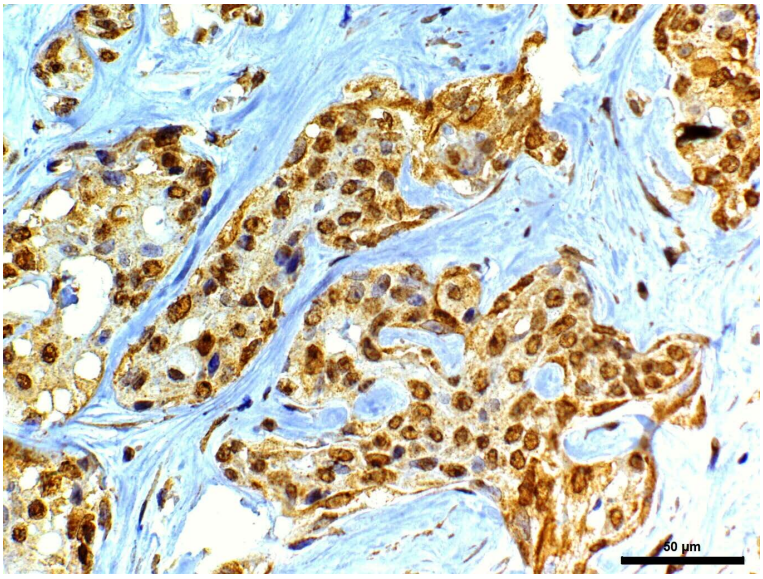


**Figure 1.** Nuclear Snai-1 expression in mammary luminal cells (DAB immunohistochemistry, Harris hematoxylin counterstain; bar = 50 µm).

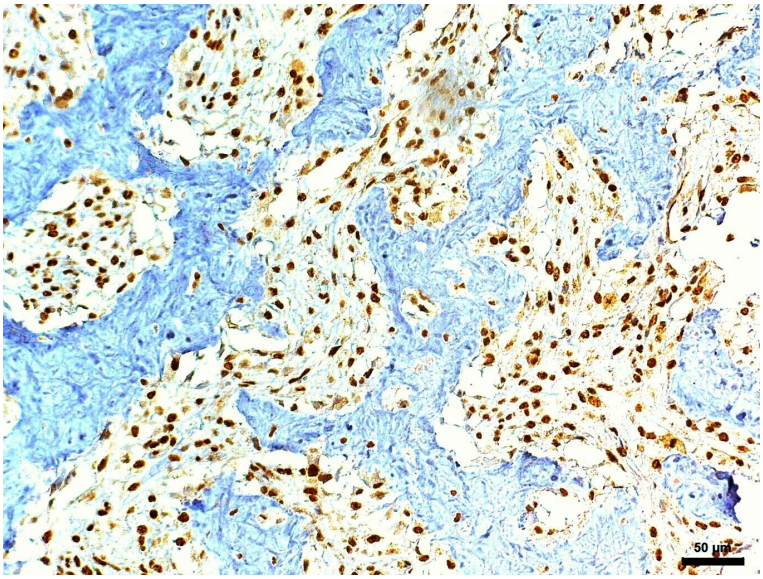


**Figure 2.** Nuclear Snai-1 expression in neoplastic cells under chondroid metaplasia/differentiation (DAB immunohistochemistry, Harris hematoxylin counterstain; bar = 50 µm).

**Table 2.** Snai-1 expression in luminal cells from mammary tissues and their relation to histological classification.

| | | *Snai-1 expression in luminal cells* | | |
|---|---|---|---|---|
| *Histological diagnosis* | *Number of samples* | −/+ | ++ | +++ |
| Lipid-rich carcinoma | 1 | 1 | 0 | 0 |
| Carcinoma arising in mixed tumor | 9 | 2 | 5 | 2 |
| Complex carcinoma | 34 | 7 | 19 | 8 |
| Tubulopapillary carcinoma | 34 | 18 | 15 | 8 |
| Solid carcinoma | 9 | 1 | 4 | 4 |

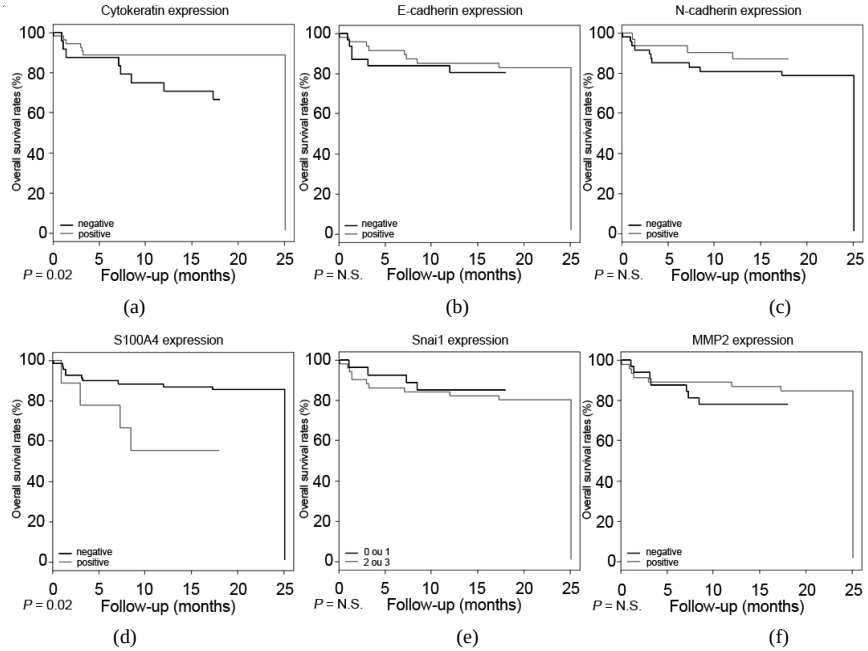


**Figure 3.** Survival curves using the Kaplan-Meier method by log rank test. (a) Cytokeratin expression; (b) E-cadherin expression; (c) N-cadherin expression; (d) S100A4 expression; (e) Snai-1 expression; (f) Matrix metalloproteinase 2 expression.

Another important aspect of EMT is the acquisition of mesenchymal protein markers, *i.e.* N-cadherin, Snai-1, S100A4, and MMP-2. In this study, the novel nuclear immunoexpression of S100A4 was significantly related with vascular invasion ($P = 0.0001$), features not previously demonstrated in dogs. Such S100A4 expression reveals it to be an interesting biomarker for evaluation of neoplastic progression in CMC, similarly to other authors' observations in humans. [12] [13] Additionally, it could be characterized as an independent predictive factor in multivariate analysis ($P = 0.01$; risk six times higher than in dogs that did not express nuclear S100A4), reinforcing its role as a useful biomarker for CMCs.

Novel Snai-1 expression was also detected and was related with E-cadherin loss ($P = 0.045$). These results indicate that EMT might also play an important role in the pathogenesis of CMCs. Previous studies in canine mammary tumors [9] did not reveal such relation in E-cadherin loss and Snai-1 expression, despite observing many EMT signs. This feature is supported by our finding of Snai-1 being highly expressed in the majority of CMCs, but not in benign tumors or non-neoplastic lesion. Since the loss of E-cadherin has already been documented as related to progression of non-infiltrating to highly infiltrating mammary carcinomas in dogs [14], consequently rising Snai-1 as a useful biomarker of neoplastic progression in CMCs.

Snai-1 was not consistently expressed in myoepithelial cells of non-neoplastic tissue and of simple carcinomas; however, we frequently observed expression of Snai-1 in myoepithelial cells from benign and malignant complex tumors, similarly to what was observed by other authors when analyzing metaplastic breast cancer in humans [15] [16]. These results suggest that Snai-1 may be important for the acquisition of a complete mesenchymal cell-like phenotype and can possibly lead to metaplastic differentiation in myoepithelial cells. It is also plausible to think that the proliferation of myoepithelial cells in myoepithelium disorders of mammary tissue can possibly be triggered by Snai-1, since the overexpression of this zinc-finger protein is related to the activation of important intracellular signaling pathways that regulate cell proliferation and differentiation such as the Wnt pathway [17].

Despite the fact that no statistical significance was observed in relation to the expression of other EMT markers and pathological characteristics, changes in the expression patterns of such proteins were detected in neoplastic cells and can represent an important change in the phenotype of CMC cells during the neoplastic progression.

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## References

[1] Nerurkar, V.R., Chitale, A.R., Jalnakurpar, B.V., Naik, S. and Lalitha, V.S. (1989) Comparative Pathology of Canine Mammary Turmours. *Journal of Comparative Pathology*, **101**, 389-397. http://dx.doi.org/10.1016/0021-9975(89)90022-4

[2] Larue, L. and Bellacosa, A. (2005) Epithelial-Mesenchymal Transition in Development and Cancer: Role of Phosphatidylinositol 3' Kinase/AKT Pathways. *Oncogene*, **24**, 7443-7454. http://dx.doi.org/10.1038/sj.onc.1209091

[3] Jechlinger, M., Grunert, S., Tamir, I.H., Janda, E., Lüdermann, S., Waerner, T., Seither, P., Weith, A., Beug, H. and Kraut, N. (2003) Expression Profiling of Epithelial Plasticity in Tumor Progression. *Oncogene*, **22**, 7155-7169. http://dx.doi.org/10.1038/sj.onc.1206887

[4] Thiery, J.P. (2002) Epithelial-Mesenchymal Transitions in Tumour Progression. Nature Reviews *Cancer*, **2**, 442-454. http://dx.doi.org/10.1038/nrc822

[5] Gotzmann, J., Mikula, M., Eger, A., Schulte-Hermann, R., Foisner, R., Beug, H. and Mikulitis, W. (2004) Molecular Aspects of Epithelial Cell Plasticity: Implications for Local Tumor Invasion and Metastasis. *Mutation Research*, **566**, 9-20. http://dx.doi.org/10.1016/S1383-5742(03)00033-4

[6] Misdorp, W., Else, R.W., Hellmen, E. and Lipscomb, T.P. (1999) Histological Classification of Mammary Tumors of the Dog and Cat. Armed Forces Institute of Pathology, Washington.

[7] Elston, C.W. and Ellis, I.O. (1998) Assessment of Histological Grade. In: *Systemic Pathology*, 3rd Edition, Churchill Livingstone, London, 365-384.

[8] Bongiovanni, L., D'Andrea, A., Romanucci, M., Malatesta, D., Candolini, M., Salda, L.D., Mechelli, L., Sforna, M. and Brachelente, C. (2013) Epithelial-Mesenchymal Transition: Immunohistochemical Investigation of Related Molecules in Canine Cutaneous Epithelial Tumours. *Veterinary Dermatology*, **24**, 195-203. http://dx.doi.org/10.1111/j.1365-3164.2012.01116.x

[9] Im, K.S., Kim, J.H., Kim, N.H., Yu, C.H., Hur, T.Y. and Sur, J.H. (2012) Possible Role of Snail Expression as a Prognostic Factor in Canine Mammary Neoplasia. *Journal of Comparative Pathology*, **147**, 121-128. http://dx.doi.org/10.1016/j.jcpa.2011.12.002

[10] Willipinski-Stapelfeldt, B., Riethdorf, S., Assmann, V., Woefle, U., Rau, T., Sauter, G., Heukeshoven, J. and Pantel, K. (2005) Changes in Cytoskeletal Protein Composition of an Epithlelial-Mesenchymal Transition in Human Micrometastatic and Primary Breast Carcinoma Cells. *Clinical Cancer Research*, **11**, 8006-8014. http://dx.doi.org/10.1158/1078-0432.CCR-05-0632

[11] Karantza, V. (2011) Keratins in Health and Cancer: More than Mere Epithelial Cell Markers. *Oncogene*, **30**, 127-138. http://dx.doi.org/10.1038/onc.2010.456

[12] Rudland, S.S., Martin, L., Roshanlall, C., Winstanley, J., Leinster, S., Platt-Higgins, A., Carroll, J., West, C., Barraclough, R. and Rudland, P. (2006) Association of S100A4 and Osteopontin with Specific Prognostic Factors and Survival of Patients with Minimally Invasive Breast Cancer. *Clinical Cancer Research*, **12**, 1192-1200. http://dx.doi.org/10.1158/1078-0432.CCR-05-1580

[13] Ismail, N.I., Kaur, G., Hashim, H. and Hassan, M.S. (2008) S100A4 Overexpression Proves to Be Independent Marker for Breast Cancer Progression. *Cancer Cell International*, **8**, 12. http://dx.doi.org/10.1186/1475-2867-8-12

[14] Brunetti, B., Sarli, G., Preziosi, R., Monari, I. and Benazzi, C. (2005) E-cadherin and $\beta$-Catenin Reduction Influence Invasion But Not Proliferation and Survival in Canine Malignant Mammary Tumors. *Veterinary Pathology*, **42**, 781-787. http://dx.doi.org/10.1354/vp.42-6-781

[15] Nassar, A., Sookhan, N., Santisteban, M., Bryant, S.C., Boughey, J.C., Giorgadze, T. and Degnim, A. (2010) Diagnostic Utility of Snail in Metaplastic Breast Carcinoma. *Diagnostic Pathology*, **5**, 76. http://dx.doi.org/10.1186/1746-1596-5-76

[16] Gwin, K., Buell-Gutbrod, R., Tretiakova, M. and Montag, A. (2010) Epithelial-to-Mesenchymal Transition in Metaplastic Breast Carcinomas with Chondroid Differentiation: Expression of the E-Cadherin Repressor Snail. *Applied Immunohistochemistry and Molecular Pathology*, **19**, 526-531. http://dx.doi.org/10.1097/PAI.0b013e3181e8d54b

[17] Stremmer, V., de Crane, B., Berx, G. and Behrens, J. (2008) Snail Promotes Wnt Target Gene Expression and Interacts with $\beta$-Catenin. *Oncogene*, **27**, 5075-5080. http://dx.doi.org/10.1038/onc.2008.140

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