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NEOPLASTIC DISEASE

Possible Role of Snail Expression as a Prognostic Factor in Canine Mammary Neoplasia

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Summary

Epithelial cells adhere tightly to each other by cell-to-cell adhesion and through the basement membrane barrier to prohibit movement. In carcinomas, neoplastic epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype during the epithelial–mesenchymal transition (EMT) for invasion and metastasis. The aim of this study was to identify Snail expression and examine the role of Snail protein in canine mammary tumour progression. Immunohistochemical expression of Snail, E-cadherin, oestrogen receptor, human epidermal growth factor receptor-2, cytokeratin 14 and p63 was analyzed in 54 samples of canine mammary epithelial tumours (11 adenomas and 43 carcinomas). Expression of mRNA encoding Snail was evaluated in seven samples (one normal mammary gland, two adenomas and four carcinomas) by reverse transcriptase-polymerase chain reaction. Snail mRNA was detected in all samples. Snail expression correlated significantly with histological type, grade and lymphatic invasion. However, there was no association between Snail expression and molecular subtype and between Snail expression and that of E-cadherin. Snail, a hallmark of EMT, might play an important role in invasion and metastasis of canine mammary carcinomas.

Keywords: dog; epithelial–mesenchymal transition; mammary carcinoma; Snail

Introduction

Tumours progress through multiple steps that involve initiation, promotion and progression (Kusewitt and Rush, 2007). Tumour progression is the final and most complex process; it occurs when malignant tumour cells move into adjacent stroma and distant sites and is a salient feature of malignant tumours that drives their spread and survival (Kusewitt and Rush, 2007).

Recent studies have described plasticity of epithelial cells that undergo the epithelial–mesenchymal transition (EMT) that is necessary for the invasion and metastasis of carcinomas (Vincent-Salomon and Thiery, 2003; Huber *et al.*, 2005; Pereira *et al.*, 2010). EMT was originally defined in gastrulation and neural crest delamination during embryonic morphogenetic remodelling (Reiman *et al.*, 2010). Normal epithelial cells are prohibited from moving due to their tight adherence to each other (Guarino, 2007). Inhibition of movement of epithelial cells is characterized by regularly spaced cell junctions and adhesions, the tight junction between individual cells and apico basal polarity (Thiery *et al.*, 2009). Mesenchymal cells have an irregular structure with no junction or focal adhesion, so they are motile and invasive (Guarino, 2007). During EMT, epithelial cell characteristics are down-regulated and mesenchymal cell characteristics are up-regulated (Tomaskovic-Crook *et al.*, 2009). Therefore, stationary carcinoma cells overcome the physical barrier and become mobile, enabling their entry into the stromal structure, which leads to invasion or metastasis (Tomaskovic-Crook *et al.*, 2009).

One of the key events of EMT is up-regulation of a zinc-finger transcription factor designated 'Snail'.

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Several pathways, such as those involving tyrosine kinase receptors, Wnt receptor, integrins and transforming growth factor (TGF)-β receptor, stimulate the activation of Snail, which is directly or indirectly correlated with extracellular matrix breakdown, loss of cell-to-cell junctions and cytoskeleton reorganization (Guarino, 2007). Furthermore, Snail induces repression of E-cadherin by binding to E-box elements in the E-cadherin promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000). Therefore, Snail leads to the transformation of epithelial cells into mesenchymal-like cells in EMT (Come *et al.*, 2006).

Numerous studies have implicated Snail expression in human cancers (Rosivatz *et al.*, 2006; Blechschmidt *et al.*, 2008; Schwock *et al.*, 2010), especially breast cancer (Vincent-Salomon and Thiery, 2003; Come *et al.*, 2006). Some studies have attempted to define the course of the EMT process in canine mammary tumours (Gartner *et al.*, 1999; Pereira *et al.*, 2010; Pang *et al.*, 2011). While the expression of Snail2 in long bone osteosarcoma was addressed by Sharili *et al.* (2011), no study has reported the function of Snail as a marker of EMT in canine mammary tumours. The aim of the present study was to examine Snail expression in canine mammary tumours and determine whether this expression was associated with clinicohistopathological features, molecular subtype or E-cadherin expression to clarify the role of the Snail protein in progression of these tumours.

Materials and Methods

Samples and Histopathology

Fifty-three samples of canine mammary epithelial tumours and three samples of normal canine mammary glands were obtained from the histopathological database of the Department of Veterinary Pathology, Konkuk University, Animal Teaching Hospital, Seoul, Korea. All tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 μm) were stained by haematoxylin and eosin (HE). Histological classification was performed according to the scheme proposed by Goldschmidt *et al.* (2011). Tumours were classified as grade I, II or III based on the evaluation of three morphological features: tubular formation, nuclear pleomorphism and mitotic index (Clemente *et al.*, 2010). Microscopical evidence of lymphatic invasion was identified.

Immunohistochemistry

Immunohistochemistry (IHC) was performed with formalin-fixed tissue sections with primary antibodies specific for Snail, E-cadherin, oestrogen receptor (OR), human epidermal growth factor receptor-2 (HER-2), cytokeratin 14 (CK14) and p63 (Table 1). Sections were de-waxed in xylene, hydrated through a graded ethanol series and washed three times in phosphate buffered saline (PBS; pH 7.4, 0.1 M). Endogenous peroxidase was blocked by incubation of sections in 3% H_2O_2 in PBS for 20 min at room temperature (RT). Sections were washed three times in PBS. Heat-induced epitope retrieval for primary antibodies except HER-2 was performed by immersion in boiling Tris-EDTA buffer (pH 9.0) using a microwave oven (650 W, high power). After the pretreatment, sections were washed three times in PBS. After the final wash, each section was overlaid with primary antibody. Isotype-matched immunoglobulins were used as negative controls. Normal mammary glands were used as positive controls for expression of E-cadherin, OR, CK14 and p63, and an example of a canine fibrosarcoma was used as a positive control for Snail. A two-step EnVision™ system (DAKO, Glostrup, Denmark) was applied to 'visualize' the immunolabelling (20 min at RT). Slides were subsequently washed four times in PBS and incubated with the supplied substrates to the desired colour intensity. The reaction was stopped with a distilled water wash. Sections were counterstained with

Table 1
Primary antibodies and immunolabelling protocols

Antibody	*Catalogue number*	*Supplier*	*Clone*	*Isotype*	*Dilution*	*Antigen retrieval*	*Incubation*
Snail	Ab85931,	AbCam, Cambridge, UK	Polyclonal antiserum	Rabbit IgG	1 in 500	Tris-EDTA (10 min)	3 h at RT
E-cadherin	M3612,	Dako, Glostrup, Denmark	NCH-38	Mouse IgG_1, kappa	1 in 100	Tris-EDTA (10 min)	4°C overnight
OR	MU368-UCE	BioGenex, San Ramon, California, USA	ER88	Mouse IgG_1	1 in 60	Tris-EDTA (10 min)	3 h at RT
HER-2	MU134-UCE	BioGenex	CB11	Mouse IgG_1	1 in 100	None	3 h at RT
CK14	Ab7800	AbCam	LL002	Mouse IgG_3	1 in 300	Tris-EDTA (10 min)	3 h at RT
p63	Sc-8431	Santa Cruz Biotechnology, Santa Cruz, California, USA	4A4	Mouse IgG_{2a}	1 in 100	Tris-EDTA (20 min)	4°C overnight