J. Comp. Path. 2014, Vol. 150, 169–174

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

www.elsevier.com/locate/jcpa

NEOPLASTIC DISEASE

# Reduced Expression of Claudin-2 is Associated with High Histological Grade and Metastasis of Feline Mammary Carcinomas

**A. R. Flores, A. Rêma, F. Carvalho, A. Faustino and P. Dias Pereira**

*Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal*

## Summary

Claudins (CLDNs) are a family of tight junction (TJ) proteins that play an important role in maintaining cell polarity, in controlling paracellular ion flux and in regulating cell proliferation and differentiation. There is a growing body of evidence that associates changes in CLDN expression with the development of human breast cancer. In the present study CLDN-2 expression was examined immunohistochemically in samples of normal feline mammary tissue ($n = 5$) and mammary carcinomas ($n = 52$), including metastatic lesions ($n = 29$). Seventy-seven percent of carcinomas showed reduced CLDN-2 expression compared with that observed in normal mammary gland. Reduced expression of CLDN-2 was significantly associated with a high histological grade of carcinoma ($P = 0.011$), with 88.6% of grade II/III carcinomas showing decreased expression. Furthermore, CLDN-2 down-regulation was significantly associated with metastatic disease ($P = 0.0027$), with 93.1% of cases with signs of metastasis showing decreased expression of this protein. CLDN-2 may constitute a molecular marker for identification of a subgroup of feline mammary carcinomas characterized by high histological grade and the development of metastasis.



*Keywords:* carcinoma; cat; claudin-2; mammary

## Introduction

Claudins (CLDNs) are a family of tight junction (TJ) proteins which, together with adherens junctions and desmosomes, maintain cell-to-cell adhesion and communication (Tokés *et al.*, 2005; Ouban and Ahmed, 2010). They play an important role in maintaining cell polarity, in controlling paracellular ion flux and in regulating cell proliferation and differentiation (Morin, 2005).

Several studies have reported changes in the expression pattern of CLDNs during the development of different types of human tumours, including mammary cancer (Martin and Jiang, 2009; Ouban and Ahmed, 2010). As constituents of TJs, CLDNs help to preserve the integrity of epithelium, thus changes in their expression pattern can be associated with cellular disorientation and detachment and invasion, processes commonly seen in cancer (Soini, 2004; Jakab *et al.*, 2008b). Although the exact role that CLDNs play in mammary carcinogenesis is still under investigation, these molecules clearly constitute an attractive potential target for cancer diagnosis, prognosis and therapy. The recently characterized 'Claudin-low' molecular subtype of human breast cancer, which is associated with an unfavourable prognosis, greatly increased the interest in these proteins (Prat *et al.*, 2010; Lu *et al.*, 2012).

In the past decade, some immunohistochemical studies have focused on the role of CLDN-2 in breast cancer, documenting loss of expression in carcinomas (Soini, 2005; Kim *et al.*, 2008; Szasz *et al.*, 2011; Tabariès *et al.*, 2011) and its association with unfavourable clinicopathological variables, namely high clinical stage and presence of metastasis (Kim

Correspondence to: P. Dias Pereira (e-mail: pdiaspereira@yahoo.com.br).

0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2013.09.004

*et al.*, 2008), suggesting that this molecule is implicated in the development and progression of breast carcinoma. More recently, CLDN-2 was identified as an important mediator of breast cancer metastasis formation (Tabariès *et al.*, 2011, 2012).

Feline mammary carcinomas are highly aggressive tumours, with an infiltrative growth pattern and frequent metastasis (Misdorp *et al.*, 1991). Several mechanisms are believed to be involved in their growth and progression, including loss of cell adhesion molecules. Previous studies demonstrated that cell adhesion molecules, namely E-cadherin and β-catenin, are important effectors in maintaining normal feline mammary tissue architecture, with changes in their expression pattern being associated with the development of neoplastic lesions and metastasis (Dias Pereira and Gärtner, 2003; Peñafiel-Verdu *et al.*, 2012; Zappulli *et al.*, 2012).

The aim of the present study was to evaluate immunohistochemically the expression of CLDN-2 in normal and neoplastic feline mammary tissue and in corresponding metastases, in order to study its involvement in the development and progression of feline mammary carcinomas.

**Table 1**
**Histological classification of the feline mammary tissues included in the study**

| *Histological classification* | *Number of samples* |
|---|---|
| Normal mammary gland | 5 |
| Malignant tumours | 52 |
| Tubulopapillary carcinoma | 32 |
| Grade I | 17 |
| Grade II | 10 |
| Grade III | 5 |
| Solid carcinoma | 12 |
| Grade II | 2 |
| Grade III | 10 |
| Squamous cell carcinoma | 5 |
| Grade II | 2 |
| Grade III | 3 |
| Anaplastic carcinoma | 2 |
| Grade II | 1 |
| Grade III | 1 |
| Lipid-rich carcinoma | 1 |
| Grade II | 1 |
| Metastatic lesions (neoplastic emboli/ lymph node metastasis) | 29 |

## Materials and Methods

Fifty-two spontaneously arising feline mammary carcinomas and associated metastatic lesions ($n = 29$), obtained from surgical excisions ($n = 49$) and from necropsy examinations ($n = 3$), were included in this study. Samples of normal mammary tissue were obtained at necropsy examination of five female cats that died from causes unrelated to mammary disease.

Tissue was dehydrated and embedded in paraffin wax and two consecutive histological sections (2 μm) were cut from each block. One was stained with haematoxylin and eosin (HE) for histological examination and the other was used for immunohistochemistry (IHC). All cases were classified (Table 1) by two independent observers (PDP and AF) according to the diagnostic criteria proposed by the World Health Organization (WHO) classification for mammary tumours of domestic animals (Misdorp *et al.*, 1999).

Histological grading of carcinomas was performed using a scheme similar to the Nottingham method proposed by Elston and Ellis (1996), based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and number of mitoses. For each feature a score of 1–3 was given: tubule formation (1, >75% of tumour area; 2, 10–75% of tumour area; 3, <10% of tumour area); nuclear pleomorphism (1, small, regular and uniform nuclei; 2, increased nuclear size and variation; 3, marked nuclear variation); mitoses per high-power field (1, 0–8 mitoses; 2, 9–17 mitoses; 3, >18 mitoses). The scores of three parameters were added to obtain the overall tumour grade: grade 1, 3–5 points (well-differentiated tumour); grade 2, 6–7 points (moderately differentiated tumour); and grade 3, 8–9 points (poorly differentiated tumour).

The expression of CLDN-2 was evaluated by IHC using a polymer-based system (Novacastra Novolink Polymer Detection System; Novocastra, Newcastle, UK) according to the manufacturer's instructions. Initially, sections were dewaxed in xylene, rehydrated in a series of decreasing concentrations of alcohol and subjected to antigen retrieval by immersion in citrate buffer (10 mM, pH 6.0) in a pressure cooker for 3 min. Endogenous peroxide activity was blocked with $H_2O_2$ 3% in methanol for 10 min. Slides were then incubated with anti-CLDN-2 mouse monoclonal antibody (12H12, Invitrogen, Carlsbad, California, USA), diluted 1 in 60 in Tris buffered saline (TBS) solution with 5% bovine serum albumin (BSA), overnight at 4°C. Positive immunolabelling was detected with a solution of 3, 3′-diaminobenzidine at room temperature. Sections were then counterstained with haematoxylin. In negative control sections the primary antibody was replaced by TBS. Positive controls consisted of sections of human breast tissue known to express CLDN-2.