The Veterinary Journal 191 (2012) 203–207

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Sequence variation and mRNA expression of the *TWIST1* gene in cats with mammary hyperplasia and neoplasia

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ARTICLE INFO

Article history:
Accepted 15 January 2011

Keywords:
Feline
TWIST1 gene
Mammary gland
Hyperplasia
Neoplasia
Sequence variation
mRNA expression

ABSTRACT

In humans, a germline mutation (c.309C>G) in the *TWIST1* oncogene may predispose to breast cancer and its expression has been associated with tumour progression and metastasis. In this study, the feline *TWIST1* gene was screened for sequence variations in 37 neoplastic and eight hyperplastic mammary gland lesions from cats. In addition, mRNA levels were examined in 15 mammary tumours and three cases of mammary hyperplasia by quantitative real-time reverse-transcriptase PCR. Feline mammary carcinomas had significantly lower levels of expression of *TWIST1* mRNA than benign mammary tumours. No variations were identified in the *TWIST1* coding region in feline mammary tumours and the mutation described in humans was not detected. However, two germline variants in the *TWIST1* gene intron were identified in four and three carcinomas, respectively: GQ167299:g.535delG and GQ167299:g.460C>T. There was no association between these sequence alterations and *TWIST1* mRNA levels.

Introduction

Mammary tumours account for 17% of tumours in female cats and usually exhibit more aggressive biological behaviour than mammary tumours in humans or dogs, with 85–93% of tumours being malignant (Schmidt and Langham, 1967; Hayes et al., 1981; Bostock, 1986). Due to similarities in pattern of metastasis, molecular characteristics and histopathological appearance, feline mammary carcinoma has been proposed as a comparative model to study hormone-independent human breast carcinomas (Zappulli et al., 2005).

TWIST1 is an oncogene encoding the Twist-1 protein, a basic helix-loop-helix DNA-binding transcription factor (Yang et al., 2004). This protein regulates embryonic morphogenesis, enhances cell survival in response to cytotoxic stress and promotes invasive behaviour during tumour progression (Yang et al., 2004; Foubert et al., 2010). *TWIST1* may also contribute to acquired paclitaxel and vincristine resistance (Wang et al., 2004; Cheng et al., 2007).

Several mutations in the coding sequence of the human *TWIST1* gene, leading to haploinsufficiency, have been identified in Saethre-Chotzen syndrome (SCS) (Yousfi et al., 2002), human paediatric osteosarcomas (Entz-Werle et al., 2005) and some cases of Baller-Gerold syndrome (Gripp et al., 1999). Sahlin et al. (2007) reported that women with SCS had an increased risk of breast cancer, although this association was not confirmed in another study (James et al., 2009). A germline *TWIST1* mutation c.309C>G has been identified as a disease-specific mutation in patients with SCS (El Ghouzzi et al., 1997; Paznekas et al., 1998). This single nucleotide change generates a stop codon at amino acid residue 103, resulting in premature termination of the Twist-1 protein and loss of function (Paznekas et al., 1998).

Gort et al. (2008) found no clear differences in *TWIST1* mRNA levels between normal and malignant breast tissue in humans. Other studies have suggested that increased *TWIST1* mRNA expression may play a role in mammary carcinogenesis (Watanabe et al., 2004; Yang et al., 2004), including an association with decreased survival time (Martin et al., 2005) and early systemic tumour re-

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doi:10.1016/j.tvjl.2011.01.011

Table 1
Clinicopathological features and sequence variations in 45 feline mammary masses.

Breed	Number of lesions	Histological classification (WHO criteria)	Lymph node metastasis	Sequence variation[a]
Domestic shorthaired	Single	Fibroadenomatous change	NA	Absent (960 bp)
Siamese	Single	Fibroadenomatous change	NA	Absent (266 bp)
Domestic shorthaired	Multiple	Fibroadenomatous change	NA	Absent (316 bp)
Domestic shorthaired	Multiple	Fibroadenomatous change	NA	Absent (316 bp)
Domestic shorthaired	Multiple	Fibroadenomatous change	NA	Absent (266 bp)
Domestic shorthaired	Multiple	Fibroadenomatous change	NA	Absent (266 bp)
Domestic shorthaired	Multiple	Fibroadenomatous change	NA	Absent (266 bp)
Domestic shorthaired	*	Fibroadenomatous change	NA	Absent (266 bp + 431 bp)
Domestic shorthaired	Multiple	Low-cellularity Fibroadenoma	–	Absent (316 bp)
Domestic shorthaired	Multiple	Low-cellularity Fibroadenoma	NE	Absent (266 bp + 431 bp)
Domestic shorthaired	Single	Low-cellularity Fibroadenoma	NE	Absent (266 bp)
Domestic shorthaired	Multiple	Carcinoma	+	g.535delG (960 bp)
Domestic shorthaired	Multiple	Carcinoma	+	g.460C>T; g.535delG (960 bp)
Domestic shorthaired	Multiple	Carcinoma	+	g.460C>T; g.535delG (960 bp)
Domestic shorthaired	*	Carcinoma	+	Absent (266 bp + 431 bp)
Domestic shorthaired	Multiple	Carcinoma	NE	Absent (266 bp + 431 bp)
Siamese	Single	Cribriform carcinoma	+	Absent (316 bp)
Domestic shorthaired	Multiple	Cribriform carcinoma	NE	Absent (316 bp)
Turkish Angora	Single	Cribriform/solid carcinoma	NE	Absent (316 bp)
Domestic shorthaired	Single	Cribriform/solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Multiple	Solid carcinoma	–	Absent (316 bp)
Domestic shorthaired	Single	Solid carcinoma	–	Absent (266 bp)
Domestic shorthaired	Multiple	Solid carcinoma	+	Absent (316 bp)
Persian	Multiple	Solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Single	Solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Multiple	Solid carcinoma	NE	Absent (316 bp)
Domestic shorthaired	Single	Solid carcinoma	NE	Absent (316 bp)
Siamese	Multiple	Solid carcinoma	NE	Absent (266 bp)
Domestic shorthaired	Single	Solid carcinoma	+	Absent (266 bp)
Domestic shorthaired	Multiple	Solid carcinoma	–	Absent (266 bp)
Domestic shorthaired	Multiple	Tubular/solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Single	Tubular/solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Multiple	Tubular carcinoma	NE	Absent (266 bp)
Domestic shorthaired	Multiple	Tubular carcinoma	–	Absent (266 bp + 431 bp)
Domestic shorthaired	Single	Papillary carcinoma	NE	Absent (316 bp)
Domestic shorthaired	Single	Complex carcinoma	NA	Absent (316 bp)
Siamese	Multiple	Tubulopapillary/solid carcinoma	+	Absent (316 bp)
Domestic shorthaired	Multiple	Tubulopapillary/solid carcinoma	–	Absent (316 bp)
Siamese	Single	Tubulopapillary/solid carcinoma	+	Absent (960 bp)
Domestic shorthaired	Multiple	Tubulopapillary carcinoma	–	g.460C>T; g.535delG (960 bp)
Domestic shorthaired	Single	Tubulopapillary carcinoma	NE	Absent (316 bp)
Domestic shorthaired	Multiple	Tubulopapillary carcinoma	NE	Absent (316 bp)
Domestic shorthaired	Multiple	Tubulopapillary carcinoma	NE	Absent (316 bp)
Siamese	Single	Tubulopapillary carcinoma	–	Absent (316 bp)
Domestic shorthaired	Multiple	Tubulopapillary carcinoma	NE	Absent (316 bp)

*, Unknown; +, present; –, absent; NE, not evaluated; NA, not applicable; bp, base pair.
[a] The length (bp) refers the number of nucleotides sequenced and analysed in each sample. Nucleotide numbers refer to the DNA sequence available in GenBank (GQ167299). The nomenclature for sequence alterations is according to the recommendations from the Human Genome Variations Society (Ogino et al., 2007).

lapse (Watson et al., 2007; Tjensvoll et al., 2010). Low expression of Twist-1 protein and *TWIST1* mRNA in triple negative (oestrogen receptor, progesterone receptor and HER-2 negative) invasive ductal carcinomas of the breast have been correlated with poor overall survival (Montserrat et al., 2011).

Previously, we sequenced part of the *TWIST1* gene in the cat (GenBank GQ167299) and demonstrated a high similarity with the corresponding human sequence (Baptista et al., 2010). In the present study, we screened 45 feline mammary gland lesions, including hyperplasia and benign and malignant neoplasia, for sequence variations in the *TWIST1* gene, in particular looking for the c.309C>G mutation described in human breast cancer (Sahlin et al., 2007). Additionally, we examined the expression pattern of *TWIST1* mRNA in 15 mammary gland tumours and three cases of mammary gland hyperplasia by quantitative real-time reverse-transcriptase PCR (qRT-PCR).

Materials and methods

Source of samples

Direct PCR sequencing of the *TWIST1* gene was performed on DNA extracted from eight hyperplastic mammary lesions, three benign mammary neoplasms and 34 malignant mammary tumours from 45 queens (median age 9.5 years, range 3–15 years). Of these 45 mammary masses, 11 were fresh tissues collected during surgery and the remaining 34 samples were formalin-fixed and paraffin-embedded (FFPE) tissues from diagnostic biopsies. A control group consisted of DNA extracted from four normal mammary glands and three blood samples obtained from seven queens >5 years of age with no evidence of clinical disease. The blood samples were collected by venipuncture into heparin-treated tubes after the owners' consent. Four unaffected skin samples, biopsied during surgery, were collected from animals also bearing a mammary gland carcinoma. Mammary lesions were characterised histologically according to Misdorp et al. (1999).

To measure *TWIST1* specific transcripts by qRT-PCR, 25 normal, hyperplastic and neoplastic feline mammary gland tissues were collected and stored at −80 °C in RNALater (Ambion). Hyperplastic ($n = 3$) and neoplastic ($n = 15$) mammary gland lesions were collected at routine surgery from 15 queens (3 speyed, 12 intact) with a mean age of 10.5 years (range 4–20 years). One of these queens was initially diagnosed with fibroadenomatous change (sample 1) and subsequently developed two carcinomas (samples 15 and 16). Normal mammary gland samples ($n = 7$) were collected postmortem from seven different queens (3 speyed, 4 intact) >5 years of age with no evidence of clinical disease that were humanely euthanased as part of the national stray cat control programme. All samples were collected in accordance with EU Directive 2010/63/EU (Ethic Commission of Porto University approval number EC/12-04/POCI/CVT/62940/2004).

Extraction of DNA

DNA was extracted from fresh mammary tissue samples using the Quick-Gene DNA Tissue Kit S (Fujifilm Life Science) and from FFPE tissues as described by Santos et al. (2009). DNA was also extracted from 200 µL total blood using the Quickgene-