The Veterinary Journal 206 (2015) 191–196

Contents lists available at ScienceDirect

The Veterinary Journal

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

Significance of *caveolin-1* and *matrix metalloproteinase 14* gene expression in canine mammary tumours

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ARTICLE INFO

Article history:
Accepted 20 July 2015

Keywords:
Canine mammary tumours
Caveolin-1
Invadopodia
Malignancy

ABSTRACT

Canine mammary tumours (CMTs) are the most common neoplasms affecting female dogs. There is an urgent need for molecular biomarkers that can detect early stages of the disease in order to improve accuracy of CMT diagnosis. The aim of this study was to examine whether caveolin-1 (Cav-1) and matrix metalloproteinase 14 (MMP14) are associated with CMT histological malignancy and invasion. Sixty-five benign and malignant CMT samples and six normal canine mammary glands were analysed using quantitative reverse transcription–polymerase chain reaction.

Cav-1 and *MMP14* genes were highly expressed in CMT tissues compared to normal tissues. *Cav-1* especially was overexpressed in malignant and invasive CMT tissues. When a CMT cell line was cultured on fluorescent gelatin-coated coverslips, localisation of Cav-1 was observed at invadopodia-mediated degradation sites of the gelatin matrix. These findings suggest that Cav-1 may be involved in CMT invasion and that the markers may be useful for estimating CMT malignancy.

Introduction

Canine mammary tumours (CMTs) are the most common neoplasms affecting female dogs (Gilbertson et al., 1983; Egenvall et al., 2005). CMTs constitute about half of all tumours in female dogs, and approximately half of these are malignant (Fidler and Brodey, 1967). Breast cancer is the leading cause of cancer-related deaths in women, and as dogs share the same environment and are likely to have been exposed to the same carcinogens and cancer risk factors they are considered a suitable model for human breast cancer with naturally occurring tumours (Fidler and Brodey, 1967; Gilbertson et al., 1983; Egenvall et al., 2005).

Diagnosis of CMTs involves considerable conflict between histological classification and prognosis, and there is a critical need for the development of molecular biomarkers that can detect early stages of disease in order to more accurately diagnose the tumours (in humans and dogs) and facilitate prompt treatment. Many tumour markers have already been investigated as prognostic indicators for human breast cancer (Weigelt et al., 2005); oestrogen (ER), progesterone (PR) and c-erbB-2 (HER-2) receptors are proven and useful biomarkers for human breast cancer (Page, 1991; Bast et al., 2001; Weigelt et al., 2005) but specific biomarkers for CMT have not been clearly defined (MacEwen et al., 1982; Donnay et al., 1995; Chang et al., 2009; Hsu et al., 2009).

Caveolins (Cav) are integral membrane proteins that are the major component of caveolae membranes (Carver and Schnitzer, 2003). Caveolae are specialised invaginations of the plasma membrane that are implicated in various cellular functions including signal transduction, lipid regulation, endocytosis and tumorigenesis (Parton and Simons, 2007). The caveolin family is composed of three isoforms, namely, Cav-1, Cav-2 and Cav-3. Cav-1 regulates diverse cellular processes, including caveolae mediated endocytosis, vesicular transport, cell migration, signal transduction and invadopodia formation (Shatz and Liscovitch, 2008; Hehlgans and Cordes, 2011).

Clinical studies have revealed that *Cav-1* upregulation is associated with poor prognosis and the occurrence of metastases in several human neoplastic conditions including oesophageal (Kato et al., 2002), prostate (Thompson et al., 2010) and pancreatic cancers (Suzuoki et al., 2002). It has also been reported that Cav-1 regulates cell signalling, proliferation, migration, communication and invasion in human breast cancer cells (Bouras et al., 2004). Immunohistochemical studies have indicated that overexpression of canine Cav-1 is associated with CMT progression (Amorim et al., 2010; Pereira et al., 2010).

Invadopodia are enriched with proteins involved in degradation of the extracellular matrix (Yamaguchi and Oikawa, 2010). The matrix degradation activity is mainly mediated by the membrane type I matrix metalloproteinase (MT1 MMP/MMP14) at the surface

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http://dx.doi.org/10.1016/j.tvjl.2015.07.020
1090-0233/

of invadopodia (Poincloux et al., 2009) and MMP14 is thought to be an important protein for degradation of the extracellular matrix in CMT malignancy.

In this study, we examined whether gene expression analysis of *Cav-1* and *MMP14* was useful for the diagnosis of CMT malignancy.

Materials and methods

Cell lines and tissue samples

Human mammary tumour cell lines MDA-MB231 and MCF7 were purchased from the European Collection of Cell Cultures (ECACC). The CMT cell line CIP-M was supplied by Dr. Nobuo Sasaki (The University of Tokyo). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Nissui Pharmaceutical) supplemented with 8% $NaHCO_3$, 10% fetal bovine serum (Life Technologies) and Gluta-Max (Life Technologies) at 37 °C in a humidified atmosphere with 5% CO_2.

Tissue samples were obtained from 65 dogs with canine tumours that were admitted to the Veterinary Hospital of Rakuno Gakuen University, and the General Veterinary Hospital of Sapporo city, Hokkaido, Japan. All animal owners received and approved an informed client consent form. Clinical data are shown in Appendix: Supplementary Table S1 that includes breed, age, sex, tumour size(s) and location(s).

Six normal canine mammary glands from healthy dogs with no cancer at the time of mammary gland resections were used as normal control samples (Fukumoto et al., 2013). The control samples were collected from dogs according to the Laboratory Animal Control Guidelines at Kitasato (Ethics Committee protocol approval number 10-028, 15 March 2010).

Antibodies

Mouse anti-Cav-1 antibody (610407) was purchased from BD Biosciences. Rabbit anti-MMP14 antibody (ab88618), goat anti-mouse IgG (DyLight 594) secondary antibody (ab96873) and donkey anti-rabbit IgG (DyLight 650) secondary antibody (ab96894) were purchased from Abcam. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). Mouse IgG1-isotype control antibody (ab170190) for Cav-1 detection was purchased from Abcam. Normal rabbit IgG (PM035) for MMP14 detection was purchased from Medical and Biological Laboratories.

Histological classification

Tumour samples were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), and embedded in paraffin. Tissue sections were cut at 4 μm and stained with haematoxylin and eosin. Histological diagnosis of the sections was evaluated based on the World Health Organization (WHO) classification (Misdorp et al., 1999) by a veterinary pathologist. Following histopathological examination, CMTs were categorised into benign tumours or malignant, non-invasive or invasive. The non-invasive classification included benign and in situ carcinomas, and an invasive tumour was defined as one with infiltrative growth into the surrounding normal tissues or lymph and blood vessels.

Total RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy mini kit and RNase-free DNase 1 (Qiagen) from tissues and cells treated with RNAlater (Qiagen) to stabilise the RNA, according to the manufacturer's instructions. Total RNA was converted to cDNA using a ReverTra Ace (Toyobo) reverse transcriptase and oligo dT primers (Toyobo), according to the manufacturer's instructions.

Reverse transcription–polymerase chain reaction (RT-PCR)

PCR was performed with the following primers: *Cav-1* sense primer, 5′-GAGAAGCAGGTGTACGACGC-3′, and *Cav-1* antisense primer, 5′-TAATGCAG GGCACAACTGCC-3′; *MMP14* sense primer, 5′-CGATGCTGCTCTCTTCTGGATG-3′, and *MMP14* antisense primer, 5′-CCTGGCTCTACCTTCAGCTTC-3′, and *RPS19* sense primer 5′-CCTTCCTCAAAAAGTCTGGG-3′ and *RPS19* antisense primer 5′-GTTCTCA TCGTAGGGAGCAAG-3′.

The nucleotide sequences for these primers were designed using DNA sequences obtained from GenBank. PCR amplification was performed as follows: denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 1 min for 35 cycles. PCR products were separated by gel electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualised using UV light.

Quantitative RT-PCR

Cav-1, *MMP14* and *RPS19* were amplified from diluted cDNA and used for quantitative RT-PCR analysis. All of the amplified fragments for each gene were subcloned into pSTBlue-1 acceptor vector (Merck Millipore), T-vector and subsequently transformed into *Escherichia coli* DH5α. Plasmid vectors with each of the appropriate genes were prepared and quantified using a spectrophotometer. A standard curve for each gene was produced using 10- or 100-fold serial dilutions of the genes as a template (10^3–10^8 copies). The reaction was performed using a Quantitect SYBR Green PCR kit (Qiagen) and iQ5/MyiQ Single-Color (Bio-Rad), following the manufacturer's instructions. The copy number of each gene expressed in the mammary glands was calculated from their respective standard curves and normalised to that of *RPS19* (Figs. 1,2). Quantitative values are presented as means ± SE ($n = 3$).

Invadopodia assay

An invadopodia assay was performed as previously reported (Bowden et al., 2001). Thin layers of Oregon Green 488 dye gelatin were placed on a 35-mm glass base dish (AGC Techno Glass), dried for 30 min at room temperature and washed three times with PBS. Cells ($3–6 \times 10^5$ cells/mL) were plated directly onto a gelatin-coated 35-mm glass base dish and incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, cells were washed three times with PBS, fixed in 4% PFA for 5 min at room temperature and washed three times with PBS.

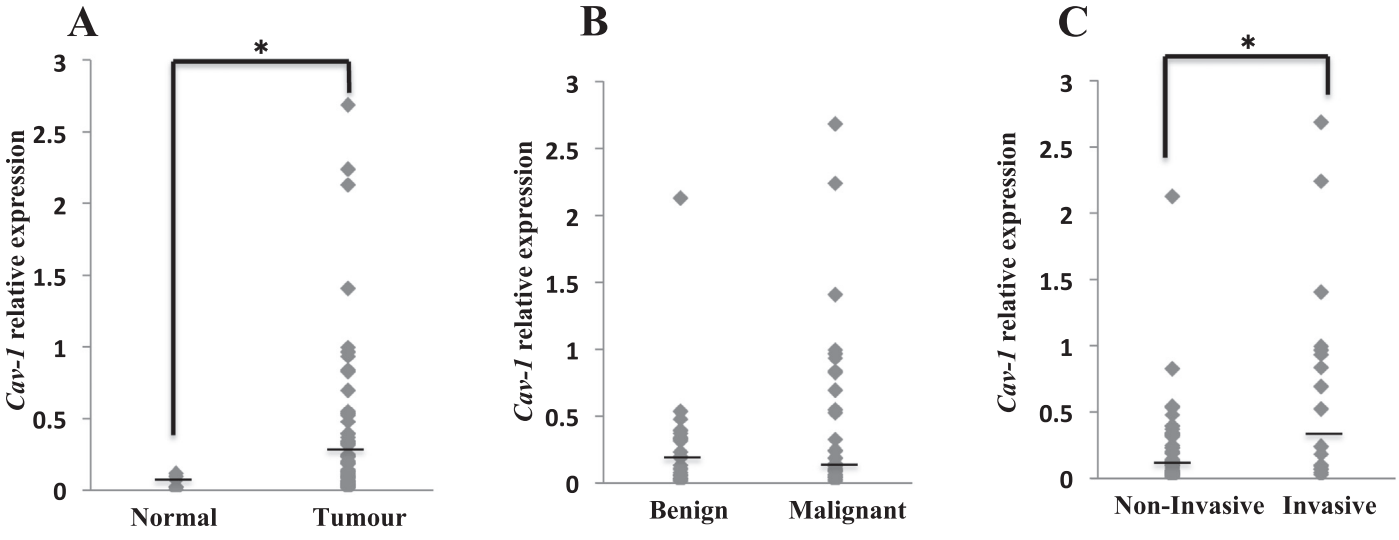

Fig. 1. Expression levels of *Cav-1* in canine mammary tumour tissue samples. (A) Expression levels of *Cav-1* in normal mammary glands ($n = 6$) and mammary tumours ($n = 64$). (B) Expression levels of *Cav-1* in benign ($n = 29$) and malignant ($n = 35$) mammary tumours. (C) Expression levels of *Cav-1* in non-invasive ($n = 46$) and invasive ($n = 18$) mammary tumours. Quantitative analysis of *Cav-1* mRNA was normalised to *RPS19*. The crossbar indicates the average expression level of *Cav-1* in each group. Statistical significance was confirmed using a Mann–Whitney *U* test (*$P < 0.01$).