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Multiple RT-PCR markers for the detection of circulating tumour cells of metastatic canine mammary tumours

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ABSTRACT

In humans, detection of circulating tumour cells (CTCs) using nucleic acid-based methods such as reverse transcription polymerase chain reaction (RT-PCR) has proven to be of prognostic relevance. However, similar procedures are still lacking in veterinary oncology. To assess the correlation of CTC markers with the metastatic potential of canine mammary tumours, 120 peripheral blood samples from bitches with mammary carcinomas with (group 1) and without (group 2) histological evidence of vascular invasion and/or presence of lymph node metastases and mammary adenomas (group 3) were analyzed. Blood samples were collected in EDTA tubes and RNA was extracted within 48 h. Subsequently, the samples were tested by RT-PCR for a panel of seven CTC mRNA markers.

CRYAB was the most sensitive single marker with a sensitivity of 35% and also the most specific marker with a specificity of 100% to detect group 1 blood samples. A multimarker assay combining four genes enhanced the sensitivity up to 77.5%, but decreased the specificity to 80%. CRYAB appeared to be highly specific but only moderately sensitive at detecting blood samples from dogs with metastatic tumours and detection significantly correlated with vascular invasion of primary mammary tumours. However, a multimarker assay of four genes significantly enhanced the sensitivity of the assay and is therefore preferable for CTC detection.

Introduction

Circulating tumour cells (CTCs) are defined as tumour cells in the peripheral blood (PB) which are originally shed either from the primary tumour or its metastases and thus possess antigenic and/or genetic characteristics of a specific tumour type (Criscitiello et al., 2010; Harris et al., 2007). CTCs are considered relatively rare among the intravascular cells and their concentration can be as low as one tumour cell/10^5–10^7 peripheral blood leukocytes (PBLs) (Alunni-Fabbroni and Sandri, 2010). CTCs are necessary for the development of distant, blood-borne metastases and it has therefore been hypothesized that they may be a useful biomarker for tumour spread (Allard et al., 2004; Attard and de Bono, 2011). Due to their very low concentrations, several highly sensitive methods (including cytological techniques) have been developed for their detection, although nucleic-acid based methods, such as reverse transcription polymerase chain reaction (RT-PCR), are commonly applied (Mostert et al., 2009).

In humans, CTC detection has been proven to have prognostic significance and has been considered helpful in monitoring therapeutic success in breast cancer patients. Several studies have been able to correlate the detection of CTCs with significantly decreased progression-free survival (PFS) and overall survival (OS) of women with metastatic breast cancer (Cristofanilli, 2006; Hayes et al., 2006; Weigelt et al., 2003). Specifically, Cristofanilli (2006) correlated the presence of five or more breast cancer CTCs per 7.5 mL PB with a poorer median PFS and OS. In addition, a decreasing breast cancer CTC number has also been correlated with therapeutic success (Hayes et al., 2006). The diagnostic value of CTC detection is, however, not restricted to mammary tumours and has also been shown for other types of malignancies, such as lung (Krebs et al., 2012; Naito et al., 2012; Nieva et al., 2012), colorectal (Cohen et al., 2008; Molnar et al., 2008), gastric (Cao et al., 2011), pancreatic (de Albuquerque et al., 2012; Khoja et al., 2012), prostate (de Bono et al., 2009; Saad and Pantel, 2012) and ovarian cancers (Poveda et al., 2011), as well as melanoma (Schuster et al., 2011).

In veterinary oncology, studies on the predictive value of CTCs are not yet available. Nevertheless, recent studies have been able to identify a set of potential CTCs or metastasis markers for canine mammary tumours (da Costa et al., 2011, 2010; Klopfleisch et al., 2010a; Klose et al., 2011). The CTC markers were identified out of a set of potential mRNA markers that included those known to

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be expressed in canine mammary tumours, mRNA markers previously used for breast cancer CTC detection, and newly discovered genes with canine mammary tumour-specific expression as identified by microarray technology. Finally, 11 of these potential markers revealed sensitivity to detect one canine mammary tumour CTC per 10^6 PBLs and were not expressed in the PB of healthy dogs (da Costa et al., 2011, 2010).

In the present study, the detection of seven of these markers in the PB of female dogs with mammary gland tumours was correlated with histological evidence of vascular invasion of the primary tumours or the presence of metastatic cells in the regional lymph node. Significantly increased detection of CRYAB and a multimarker pattern of four genes suggest that these markers may be valuable for the assessment of the metastatic potential of canine mammary tumours.

Materials and methods

Blood samples

PB samples (2–6 mL) of 120 female dogs with different types of mammary gland tumours as well as 10 PB samples from healthy female dogs were obtained in cooperation with several small animal clinics in Germany. The samples were preoperatively collected in EDTA tubes and stored at 4 °C. RNA extraction was immediately performed on the day of receipt. Blood samples were split into 2 mL aliquots and diluted 1:6 in erythrocyte lysis buffer (0.13 M NH_4CL, 0.1 M $KHC0_3$, and 1.2×10^{-4} M EDTA) to eliminate red blood cells. After incubation for 30 min at 4 °C, samples were centrifuged at 800 *g* for 15 min and the supernatant was discarded to obtain a pellet composed mainly of PBLs. The procedure was repeated until a clear, white pellet was obtained. Samples were preserved at −80 °C in lysis buffer (RA1 lysis buffer, Macherey–Nagel) containing 0.1% β-mercaptoethanol (Sigma–Aldrich) until RNA isolation within 1 week.

Blood samples were divided into four groups according to the histological criteria of the tumours. Group 1 ($n = 40$) included blood from dogs with malignant mammary gland carcinomas with histological evidence of vascular invasion of tumours cells or presence of metastatic cells in the regional lymph node; group 2 ($n = 40$) included blood from dogs with malignant mammary gland carcinomas without histological evidence of vascular invasion and metastasis free lymph nodes; group 3 ($n = 40$) included blood from bitches with benign mammary gland adenomas; and group 0 ($n = 10$) included blood from healthy female dogs without evidence of neoplastic disease.

Cell lines

The canine mammary carcinoma cell line CMM26 (kindly provided by F. Gärtner, IPATIMUP, University of Porto) was cultivated in RPMI1640 medium (Sigma) supplemented with 10% bovine fetal serum (Biowest). The cells were incubated in 75 cm^3 tissue culture flasks with air containing 5% CO_2 at 37 °C. After the formation of a confluent monolayer, cells were harvested by washing once with phosphate-buffered saline (PBS) pH 7.3 and incubation in PBS containing 0.02% EDTA and 0.25% trypsin (Biochrom) for 5 min at 37 °C and centrifuged at 800 *g*. Subsequently, pellets of these cells were prepared and preserved at −80 °C in lysis buffer (RA1 lysis buffer, Macherey–Nagel) containing 0.1% β-mercaptoethanol (Sigma–Aldrich) until RNA isolation within 1 week.

Total RNA extraction and cDNA synthesis

RNA was isolated from PBL pellets and cell lines using a commercial kit (Nucleo Spin RNAII, Macherey–Nagel) according to the manufacturer's instructions with subsequent DNAse treatment. RNA concentrations were determined by a spectrophotometer (Nanodrop ND-1000, Peqlab) reading at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed using a cDNA synthesis Kit (iScript cDNA Synthesis Kit; BioRad). Success of cDNA-synthesis was confirmed by RT-PCR amplification of the canine housekeeping gene ATP5B (Klopfleisch and Gruber, 2009a).

PCR

Canine genome specific primers were designed using Primer 3.0 or have been described before (Table 1) (Klopfleisch and Gruber, 2009b). To avoid amplification of genomic DNA, primers were designed to span an exon–exon border (Table 1). Each PCR reaction contained 5 μL buffer (10× TrueStart Hot Start Taq Buffer, Fermentas), 5 μL MgCl2 (25 mM), 5 μL nucleotide mix (10 mM), 0,25 μL from each primer (10 μmol/L), 2.5 μL template cDNA, 0.25 μL polymerase (TrueStart Hot Start Taq Buffer, Fermentas) and nuclease-free water to 50 μL. Samples were amplified with a precycling hold at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing 55.8–61.8 °C for 30 s, and extension 72 °C for 1 min. In addition to the blood samples, each PCR assay included a no template control as negative control and cDNA of the canine mammary carcinoma line CMM26 as positive control. Sequence sequencing of all PCR products confirmed specificity of the RT-PCR assays. Each sample was tested in three different rounds. A sample was considered positive when two positive PCR results out of the three rounds were obtained.

Statistical significance of differences in the numbers of positive PB samples between tumour groups was analyzed using the Kruskal–Wallis. $P < 0.05$ was considered statistically significant. Sensitivity was calculated as the ratio of true positive results per true positive plus false negative results. Specificity was calculated as the ratio of true negative results per true negative plus false positive results.

Results

Evaluation of differential expression, specificity and sensitivity of single markers

Expression of the seven tested markers (CLDN7, CRYAB, ELF3, SLC1A1, ATP8B1, EGFR and F3) differed between the three tumour groups (Fig. 1). All seven markers tested were not expressed in the PB of healthy donors (group 0). CRYAB and ELF3 were the markers with the highest percentage of positive blood samples in group 1 (35%) followed by ATP8B1 and EGFR, both with 27.5% positive blood samples. CLDN7, F3 and SLC1A1 showed lower percentages of positive blood samples, with 17.5%, 12.5%, and 2.5%, respectively (Fig. 1).

CRYAB and SLC1A1 were not detected in any of the blood samples from group 2 (Fig. 1). In group 2, ELF3 had the highest number of positive blood samples with 47.5%, followed by F3, ATP8B1, CLDN7 and EGFR with 22.5%, 20.0%, 15.5% and 15.0%, respectively. CRYAB, CLDN7 and ATP8B1 were not detected in any of the blood samples from group 3, while F3 and SLC1A1 were expressed in 2.5% and 5.0% of the cases, respectively. ELF3 was detected in 42.5% of the group 3 samples.

Statistical analysis confirmed significant differences in the number of CRYAB positive blood samples from dogs with carcinomas with vascular invasion (group 1) compared to dogs with carcinomas without vascular invasion (group 2) (Table 2). A statistically significant difference of the number of positive blood samples of group 1 compared to positive blood samples from dogs with adenomas (group 3) was identified for CRYAB, CLDN7, ATP8B1, EGFR, and F3. Finally, a significantly higher number of positive blood samples in group 2 compared to group 3 was identified for CLDN7, ATP8B1 and EGFR.

The sensitivity of the markers (i.e. the power to detect potentially positive blood markers) was rather low for all markers tested (Table 3; Fig. 2). CRYAB and ELF3 were the most sensitive markers with a sensitivity of 35%. CRYAB showed a specificity of 100% but in contrast, ELF3 had a rather low specificity of 55%. ATP8B1 and EGFR showed sensitivities of 32.5% and 27.5% and were also highly specific with 90% and 92.5%. F3 and SLC1A1 had low sensitivities with 12.5% and 2.5% and had specificities of 87.5% and 97.5%.

Evaluation of the sensitivity and specificity of multimarker assays

In a second step we established whether the testing of a combined expression pattern of the four markers with the highest sensitivity and specificity (namely CLDN7, CRYAB, ATP8B1 and EGFR) would enable a more sensitive and specific detection of potentially metastatic tumours. To this end, four multimarker (MM) patterns were established. Positivity of a blood sample was defined as one positive out of four tested genes in MM1, two positive out of four tested genes in MM2, three positive out of four tested genes in MM3 and four positive out of four tested genes in MM4.

Considering blood samples in group 1, 77.5% of the blood samples were positive in MM1, 27.5% in MM2 and in MM3 and MM4 5.0% and 2.5%, respectively. In group 2, 40.0% of the blood samples