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cDNA microarray profiles of canine mammary tumour cell lines reveal deregulated pathways pertaining to their phenotype

N. A. S. Rao*, M. E. van Wolferen*, R. van den Ham*, D. van Leenen†, M. J. A. Groot Koerkamp†, F. C. P. Holstege† and J. A. Mol*

*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.
†Department of Physiological Chemistry, University Medical Centre, Utrecht, The Netherlands

Summary

Mammary cancer is the most common type of cancer in female dogs with a lifetime risk of over 24% when dogs are not spayed. The elucidation of the complete canine genome opens new areas for development of cancer therapies. These should be tested first by *in vitro* models such as cell lines. However, to date, no canine mammary cell lines have been characterized by expression profiling. In this study, canine mammary tumour cell lines with histologically distinct primary tumours of origin were characterized using a newly developed canine cDNA microarray. Comparisons of gene expression profiles showed enrichment for distinct biological pathways and were related to biological properties of the cell lines such as growth rate and *in vitro* tumourigenicity. Additionally, gene expression profiles of cell lines also showed correspondence to their tumour of origin. Major differences were found in Wnt, cell cycle, cytokine/Rho-GTPase, alternative complement and integrin signalling pathways. Because these pathways show an overlap at the molecular level with those found in human breast cancer, the expression profiling of spontaneous canine mammary cancer may also function as a biological sieve to identify conserved gene expression or pathway profiles of evolutionary significance that are involved in tumourigenesis. These results are the basis for further characterization of canine mammary carcinomas and development of new therapies directed towards specific pathways. In addition these cell lines can be used to further investigate identified deregulated pathways and characterize until now unannotated genes.

Keywords canine, cell lines, gene expression, mammary tumour, pathway.

Introduction

Mammary cancer is the most common type of cancer in female dogs where its incidence and mortality rates are at least twice as high as in humans (MacEwen 1990). About 50% of canine mammary tumours are classified as malignant, and most of the treatment strategies (such as combinations of chemotherapies, radiation and immunotherapy) are not very effective at preventing recurrence (MacEwen 1990). Early ovariectomy is reported to reduce the lifetime mammary cancer risk from 24% to 0.05% and even when performed on older dogs, partially protects them against mammary tumour formation (Schneider *et al.* 1969; Misdorp 1991) in agreement with the hormone dependence of the disease. In dogs, progestins play a major role in the incidence of mammary tumours, at least in part through stimulation of mammary growth hormone expression (Selman *et al.* 1994; Mol *et al.* 1996; Bhatti *et al.* 2007).

Breast cancer is also the most common cancer in women, comprising 23% of all female cancers (Parkin *et al.* 2005). The aetiology of breast cancer is very complex and not very well understood. In addition to diverse genetic, dietary, environmental and carcinogenic entities leading to tumourigenesis, breast cancer has long been recognized as a hormone-dependent malignancy (Russo & Russo 1998). The role of ovarian hormones, predominantly oestrogens and to a lesser extent progestins in mammary gland development, tumourigenesis and tumour progression has been very well documented (for a review, see Russo & Russo 1998).

Address for correspondence

J. A. Mol, Department of Clinical Sciences of Companion Animals, Yalelaan 104, 3584 CM Utrecht, The Netherlands.
E-mail: j.a.mol@uu.nl

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Although the advent of DNA microarray technology has led to numerous gene expression profiling studies of (human) breast tumours and cell lines that have been reported recently (Perou *et al.* 2000; Sorlie *et al.* 2001, 2003), in the dog, no gene expression profiling studies have yet been documented. There are very few well characterized (at the molecular level) canine mammary tumour cell lines (CMTs) available for mammary cancer research in dogs, but no studies have identified main signalling pathways (in CMTs) or make cross-species comparisons of pathway profiles to identify molecular similarities of the disease across species.

As a first step in this study, we developed a dog-specific cDNA microarray containing 20 160 independent genes and studied the gene expression signatures of three CMTs originating from a primary atypical benign mixed tumour (CMT-U229), a primary mammary osteosarcoma (CMT-U335) and a primary mammary anaplastic carcinoma (P114). Several differentially expressed gene clusters were identified and were related to various signalling pathways; these were compared to gene expression profiles in human breast cancer. These results are the basis for further characterization of canine mammary carcinomas.

Materials and methods

Cell lines and cell culture

The cell line CMT-U335 was isolated from a canine mammary osteosarcoma, CMT-U229 from a canine mammary atypical benign mixed tumour and P114 from a highly malignant canine anaplastic carcinoma. Isolation and characterization of these three cell lines were reported previously (Hellmen 1992; Van Leeuwen *et al.* 1996). Cell lines were grown in DMEM-F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Harlan Sera-Lab) at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were conducted at early passages of cell lines (3–22 passages).

Growth rate experiments were performed in triplicates. For this, 5×10^4 cells were seeded into each well of six-well plates (Becton Dickenson). Cells were counted manually after being treated with trypsin every 24 h for 4 days. The experiment was repeated five to six times and the average doubling time of each cell line was estimated. These results were validated by estimating the doubling time using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay as described previously with minor modification to the original protocol (Mosmann 1983). In brief, cell lines were seeded in 96-well plates (Becton Dickenson) at a density of 1500–3000 cells/well. After 24, 48 and 72 h, the cells were incubated with MTT (0.45 mg/ml final concentration) for 1.5 h. After 1.5 h the cell culture medium with MTT was removed, cells were lysed with acidic isopropanol (100 μl/well) and the absorbance of the dissolved formazan was measured at 595 nm with 650 nm as reference wavelength using a microplate reader (BioRad, Model 3550). Cell number for every time point was deduced from absorbance using the standard curve (serial dilution of cells) for each cell line.

Growth characteristics of the cell lines were documented by growing cell lines in reconstituted Matrigel matrix (Becton Dickenson). For this, cell lines were seeded at 5×10^4 cells per well in Matrigel-coated 12-well plates (Becton Dickenson) and the morphology of cells was assessed under an IMT-2 phase contrast microscope fitted with an E-330 digital camera (Olympus) after 1 week. Experiments were repeated five to six times.

Invasion assay

Invasion assays were performed as described earlier (Trusolino *et al.* 2000) using Transwell chambers (Corning) with minor alterations to the original protocol. The 8-μm pore membranes of the upper chambers were coated with 75 μg (50 μl) of Matrigel (Becton Dickenson) and placed into a well containing DMEM-F12 supplemented with 10% FBS. About 1×10^5 cells in media containing 1% FBS were seeded into the upper Transwell chamber. After 20 h of incubation, cells were mechanically removed from the upper compartment of the Transwell chamber by using cotton swabs. The number of cells that passed through the Matrigel and filter membrane of the Transwell into the lower chamber was estimated using the DNA-based sensitive Cyquant cell proliferation kit (Invitrogen). Standard curves were plotted by serially diluting the cell lines and recording the corresponding fluorescence signal of each sample. The total number of invaded cells was deduced for each cell line from their respective standard curves. Invaded cells were also visually inspected under the phase contrast microscope. All assays were repeated three to four times.

Colony formation in soft agar

The anchorage-independent growth ability of cell lines was determined by colony formation in soft agar as described earlier (Galbiati *et al.* 1998) with minor modifications. A single-cell suspension of each cell line was prepared from monolayer culture by briefly treating them with trypsin. Cells (3×10^4 cells) suspended in 1.5 ml of DMEM-F12 containing 10% FBS and 0.5% low melting point agarose (Promega) were seeded into each well of six-well plates precoated with a 1.5-ml layer of solidified DMEM-F12/10% FBS/1% agarose. The cell culture medium was refreshed every 3–4 days. Two weeks later, colonies were photographed under 10–12 random focus areas using the IMT-2 phase contrast microscope fitted with an E-330 digital camera (Olympus) at 10× magnification. The experiments were performed in triplicate and repeated four times. Individual colonies were scored for each focus area and the average number of colonies for each area was calculated.

Canine-specific microarray production

A canine-specific collection of 20 160 non-redundant clones of 3′-UTR cDNA fragments inserted in pBluescript II KS and cloned into *E. coli*-DH10B were purchased from Lion Bioscience GmbH. Individual clones were grown overnight in LB medium supplemented with 50 μg/ml ampicillin and heated at 95 °C for 10 min. The bacterial culture (4 μl) was subsequently used for PCR amplification of the clone inserts using plasmid-specific sense (5′-AG CGTGGTCGCGGCCGAGGT-3′) and antisense (5′-TCGAGC GGCCGCCCGGGCAGGT-3′) primers and 35 cycles of denaturation (94 °C, 30 s), annealing (66 °C, 30 s) and extension (72 °C, 1 min) using standard PCR protocols. PCRs with 100-μl reaction volumes were performed in triplicate for each clone in order to obtain enough PCR products for microarray production. Each PCR product was checked for contamination using gel electrophoresis and flagged if no or more than one PCR product was detected. PCR products were purified using 96-well MultiScreen plates with FB filters according to the manufacturer's protocol (Millipore). After measuring the concentration, PCR products were air-dried, reconstituted to 150 ng/μl (final concentration) in print buffer (150 mM phosphate buffer pH 8.5) and 10 μl of each product was transferred to a 384-well plate. PCR products that did not reach the desired concentration were flagged. PCR products were spotted on UltraGAPS slides (Corning) using the MicroGRID II arrayer (Genomic Solutions), air-dried and immobilized by UV cross-linking using a Stratalinker2400 (Stratagene) for 10 min at 2400 mJ. Microarrays were stored at room temperature (RT) in desiccators until needed. The microarray platform has been deposited in the public database [GEO: GPL5117].

RNA isolation and cRNA synthesis

Labelled cRNA was prepared according to a previously published protocol (van de Peppel *et al.* 2003; Roepman *et al.* 2005). RNA isolation and purification was performed with the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA was isolated from cells that were grown to near confluence in 75 cm^2 Cellstar tissue culture flasks (Greiner Bio-One GmbH). Total RNA from three to four independent passages of each cell line was used for cRNA synthesis. About 2 μg of total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and T7 promoter-coupled poly-T primers (5′-GGCCAGTGAATTGTAATACGACTCACCTATAGGGAGG CGGT-3′). After second-strand synthesis, cDNA products were purified using the QIAquick PCR Purification Kit (Qiagen), followed by transcription using the T7 Megascript kit (Ambion) and a UTP to 5-(3-aminoallyl)-UTP ratio of 1/3.5. The RNeasy mini kit (Qiagen) was used to purify the resulting cRNA. The quality of the total RNA and the amplified cRNA was analysed using a bioanalyser (Agilent Technologies). cRNA (3 μg) that met the quality criteria was coupled to Cy3 and Cy5 fluorophores (GE Healthcare). Labelled cRNA was purified using ChromaSpin-30 columns (Clontech-Takara Bio), and dye incorporation efficiency was measured using a ND-1000 spectrophotometer (Isogen). Equal quantities of labelled cRNA samples with specific activity of 2–5% dye-labelled nucleosides were used for microarray hybridization.

Microarray hybridization and scanning

Microarray slides were washed for 1 min in 2× SSC containing 0.05% SDS at RT, incubated in 0.25% sodium borohydride and 0.25% SDS in 2× SSC for 30 min at 42 °C and then rinsed five times in double-distilled water at RT. Slides were incubated for 45 min at 42 °C in pre-warmed pre-hybridization buffer (2×) consisting of 10× SSC, 25% formamide, 0.1% SDS and 1% bovine serum albumin (BSA). Slides were washed five times in water and then dipped five times in isopropanol at RT and air dried. To a 40-μl mixture containing 2.5 μg each of Cy3- and Cy5-labelled RNA, 40 μl of hybridization buffer consisting of 50% formamide, 10× SSC, 0.2% SDS and 200 μg/ml sheared herring sperm DNA was added. The mixture was heated at 95 °C for 5 min, centrifuged for 2 min at 12 000***g*** and hybridized to the slides at 42 °C for 16–18 h using hybridization chambers (Corning) in a water bath. After hybridization, slides were washed four times: first a low-stringency wash for 4 min at RT in 1× SSC and 0.2% SDS, next a high-stringency wash in 0.1× SSC and 0.2% SDS for 4 min and finally two washes in 0.1× SSC at RT. Slides were blown dry using compressed nitrogen and scanned using an Agilent G2565AA DNA microarray scanner (100% laser power, 30% photomultiplier tube).

Data analysis

Image analysis was carried out using IMAGENE 5.0 software (Biodiscovery Inc.). Defective spots were flagged and data were normalized based on Lowess print-tip normalization (Yang *et al.* 2002) by using the gene spots. Normalized data were subjected to logarithmic (base 2) transformation before further statistical analysis. Differentially expressed genes between cell lines were extracted by performing a two-class unpaired *t*-test using SIGNIFICANCE ANALYSIS FOR MICROARRAYS (SAM) software with the mean false discovery rate set at 5% (Tusher *et al.* 2001). Gene expression data were clustered and visualized using GENESPRING version 7 (Agilent Technologies). SAM-generated and -annotated differentially expressed genes were subjected to pathway analysis in the framework of gene ontology (GO) and signalling networks using the PANTHER pathway analysis tool (Mi *et al.* 2005). Major pathways over-represented by the statistically significant genes (random overlapping *P*-value <0.001) were

identified as important signalling pathways. The microarray data files have been deposited in the public database [GEO: GSE7659].

Real-time quantitative PCR

Synthesis of cDNA was carried out with 1.5 μg total RNA in 60-μl reaction volumes using the iScript™ cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad). Primers (Table 1) were designed using PRIMER SELECT software from DNAStar according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product. The *HPRT* and *RPS19* genes were used as the non-regulated reference genes for normalization of target gene expression (Brinkhof *et al.* 2006). Real-time quantitative PCR (RT-PCR) was performed as described previously using the Bio-Rad MyIQ detection system (BioRad) with the SYBR green fluorophore. Data analysis was carried out using the pairwise fixed reallocation and randomization test incorporated in the software program REST-XL (Pfaffl *et al.* 2002) at a 5% level of significance. The estimated fold change for each gene was plotted against the same estimated from the microarray analysis.

Results

Growth characteristics of canine mammary cell lines

Canine mammary cell lines showed a clear difference in growth rate when grown on plastic cell culture bottles (Fig. 1). CMT-U229 showed the highest doubling times (18 ± 2 h) followed by P114 (22 ± 2 h) and CMT-U335 (30 ± 2 h). When grown in laminin-rich Matrigel matrix, cell line P114 formed branching structures (Fig. 1). Although CMT-U229 and CMT-U335 formed colonies in Matrigel, they showed no signs of branching morphogenesis. Compared to CMT-U229 and CMT-U335, P114 was observed to be highly tumourigenic with greater invasion ability in the Transwell invasion assay and exhibited higher anchorage-independent growth ability when grown in soft agar (Fig. 2).

Gene expression profiling of canine mammary cell lines

The three canine mammary cell lines that we characterized using the canine-specific cDNA microarray originated from histologically distinct primary tumours. Statistical analysis of raw data using SAM with a false discovery rate

Table 1 Nucleotide sequences of canine-specific primer pairs used for quantitative real-time PCR analysis.

Gene name	Primer orientation	Primer sequences (5′–3′)	T (°C)	Product size (bp)
SFRP1	U	AGCGAGTTTGCATTGAGGAT	60	106
	L	TCTTGATGGGTCCCAACTTC		
CRYAB	U	CCATGCACCTCAATCACATC	58	104
	L	ATGCGTCTGGAGAAGGACAG		
BF	U	AGGGACACGAAACCTGTATG	62	106
	L	ACGCTGACCTTGATTGAGTG		
KTR8	U	CCTTAGGCGGGTCTCTCGTA	63	149
	L	GGGAAGCTGGTGTCTGAGTC		
DDR1	U	CTGGGGTCAGGAGGTGATT	58	135
	L	ACCCGCAGACAGACACTCAT		
CDKN1A	U	ATGAAATGGGGGAAGGGTAG	58	116
	L	AATCTGTCAGGGGCGTATTG		
RGC32	U	CATCTCTGTCGGCTCTGGTAG	58	99
	L	AAGTTCTGGGTCCTTTCATCAT		
TMEFF2	U	TAGTCCAGCCACTGTGCAAC	58	141
	L	CCTCTGCATCACCAGGAAAT		
GP80	U	CCGAGAGGAATGAGATGTGAA	57	111
	L	CACTTGGTGACATGCAGAGC		
DKK3	U	CATCCAGTCCAGTGCTCTCA	58	140
	L	GGGCCAGGATTGTAAGTGAA		
PAK1	U	CCCAAGGTTGACATCTGGTC	58	110
	L	TTGGTGGCAATGAGGTACAA		
RPS19	U	CCTTCCTCAAAAAGTCTGGG	61	95
	L	GTTCTCATCGTAGGGAGCAAG		
HPRT	U	AGCTTGCTGGTGAAAAGGAC	56	100
	L	TTATAGTCAAGGGCATATCC		

U, forward primer; L, reverse primer; T, optimum annealing temperature.

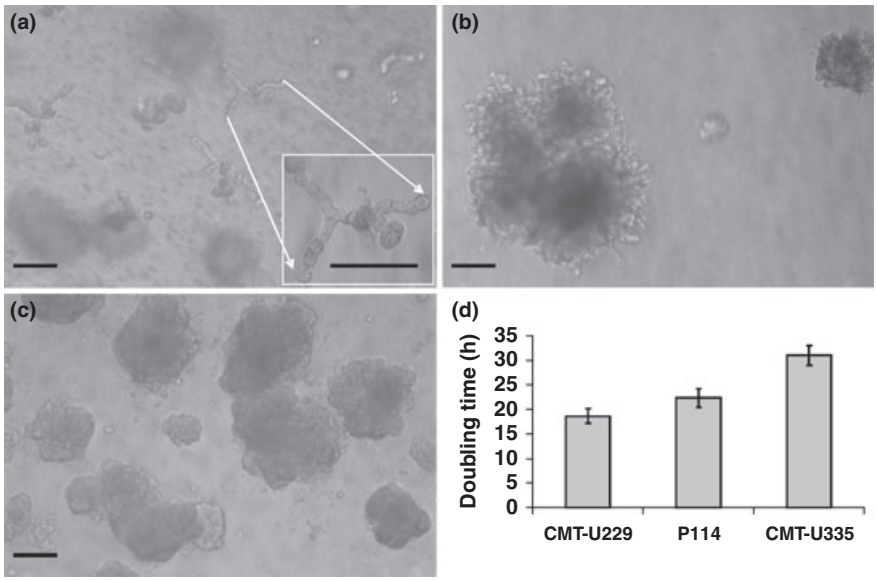

Figure 1 Growth characteristics of CMTs: Phase contrast micrograph of CMTs grown on Matrigel matrix for a week (— bar = 300 μm). (a) P114, insert = 20× magnification; (b) CMT-U335; (c) CMT-U229; (d) doubling time of CMTs when grown on plastic growth surface.

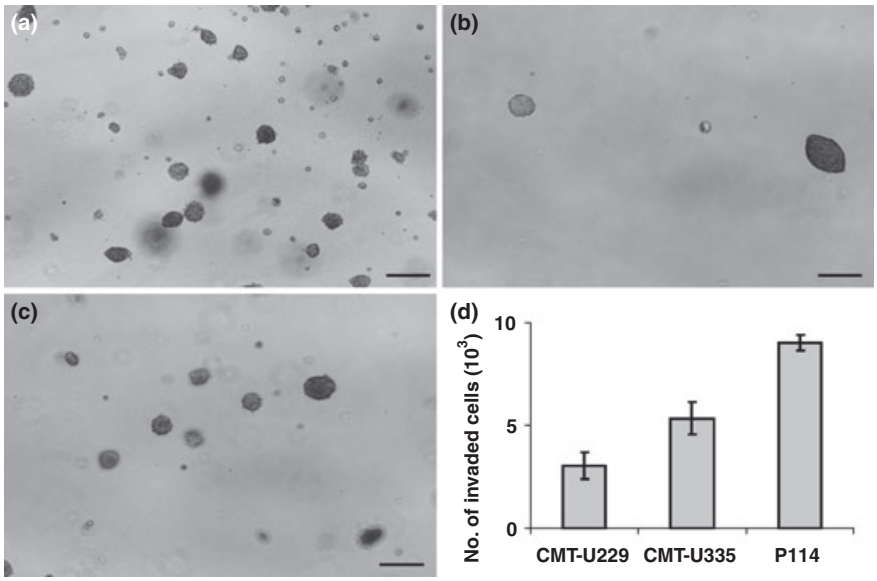

Figure 2 Tumourigenicity of CMTs: Anchorage-independent growth ability of CMTs grown in soft agar over 2 weeks. Representative phase contrast micrographs are presented (— bar = 300 μm). (a) P114; (b) CMT-U229; (c) CMT-U335. (d) Invasiveness of CMTs estimated as number of cells invaded through Matrigel-coated Transwell chambers in 20 h over a serum gradient.

set below 5% generated a clear set of genes that were differentially expressed between cell lines (Table S1). CMT-U229 overexpressed 70 genes against P114 and 179 genes against CMT-U335. The cell line P114 overexpressed 118 genes compared to CMT-U335 and 70 genes compared to CMT-U229. The cell line CMT-U335 overexpressed 165 compared to CMT-U229 and 93 genes compared to P114. The statistically significant gene lists obtained from SAM analysis were further analysed and visualized using GENESPRING software. Dendrograms generated by hierarchical clustering of gene expression data revealed a clear clustering of replicate experiments and their dye swaps (Fig. 3).

Unique gene lists, which are the lists of genes that are differentially expressed in a particular cell line compared to the other two cell lines, were generated. In CMT-U229, 32 unique upregulated and seven downregulated genes were found. P114 showed 36 upregulated and 15 downregulated genes. CMT-U335 had 53 upregulated and 45 downregulated genes (Table S2).

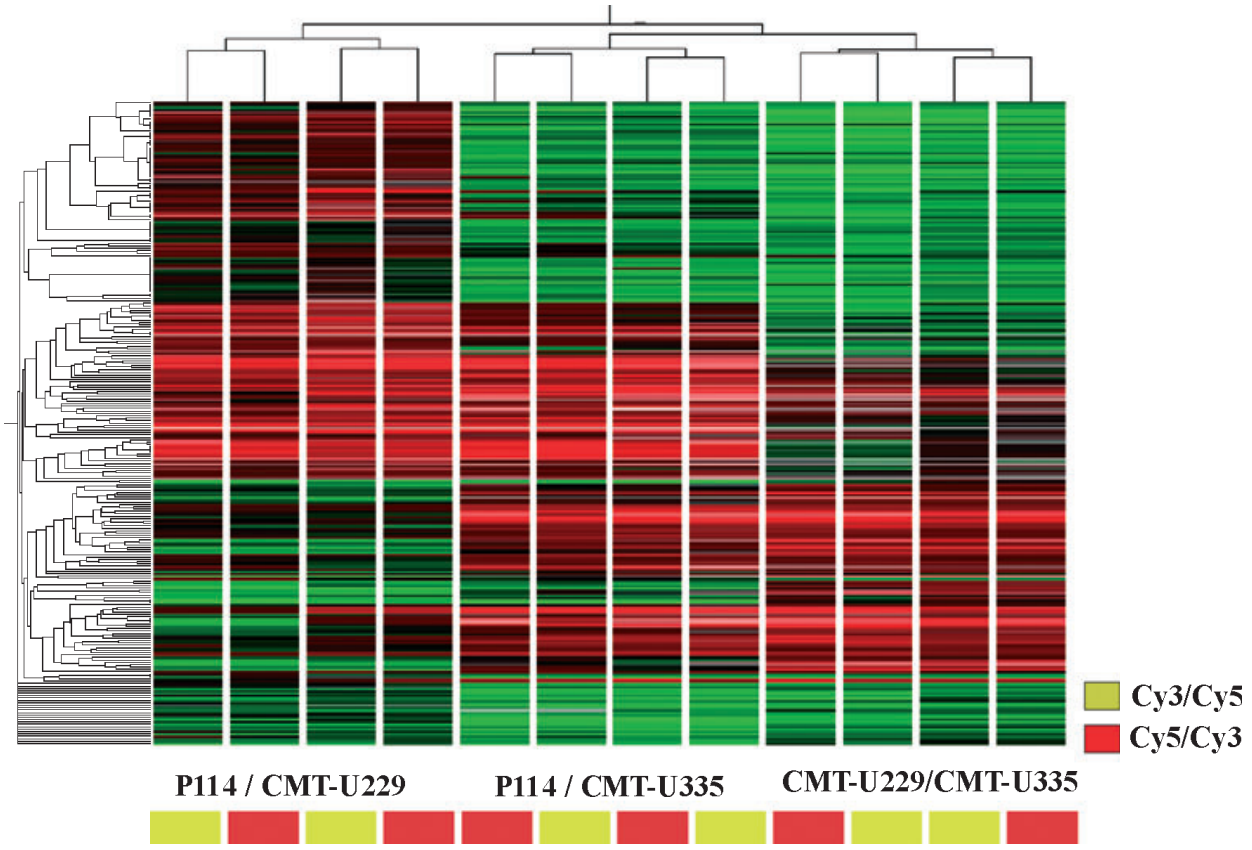

Figure 3 This dendrogram generated (in GENESPRING) by hierarchical clustering of all differentially expressed genes in CMTs found by SAM analysis revealed a clear clustering of replicate experiments and their dye swaps.

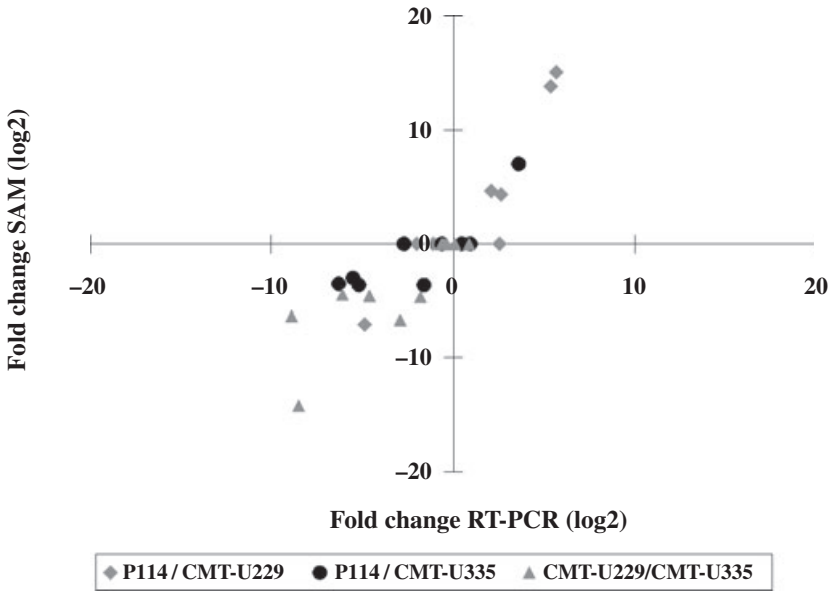

Figure 4 Correlation between gene expression values of a few candidate genes obtained by microarray analysis (SAM) and quantitative real-time PCR (log scale).

Gene expression data were validated by RT-PCR on differentially expressed candidate genes using cell line total RNA used for microarray analysis as well as RNA derived from different biological replicates of each cell line. A good correlation ($R^2 = 0.753$) was found between the fold change values obtained by microarray analysis and

Table 2 Statistically significant differentially expressed genes in CMTs with known functional annotation were sub-grouped under relevant biological pathways. Only important signalling pathways represented by a large number of differentially expressed genes are shown: (↓) = gene downregulated against one cell line; (↓↓) = gene downregulated against two cell lines; (↑) = gene upregulated against one cell line; (↑↑) = gene upregulated against two cell lines; (+) = stimulation of the pathway or its effectors; (−) = inhibition of the pathway or its effectors.

				Cell lines		
Gene ID	Ref-seq-HS	Gene name	Regulation	CMT-U229	P114	CMT-U335
Wnt-signalling						
DG2-66o13	NM_000944	*PPP3CA*	+	↑	≈	↓
DG9-288i19	NM_002074	*GNB1*	+	↓	≈	↑
DG40-163k12	NM_001892	*CSNK1A1*	+	↑	↑	↓
DG32-147a19	NM_053056	*CCND1*	+	↑	↑	↓
DG11-72g24	NM_004284	*CHD1L*	+	≈	↑	↓
DG9-113g15	NM_001257	*CDH13*	+	↓	↑	↑
DG2-88c13	NM_001904	*CTNNB1*	+	↓	↑	↓
DG2-7i22	NM_004932	*CDH6*	+	↓	↓	↑
DG32-213c20	NM_007236	*CHP*	−	↑	≈	↓
DG2-126g23	NM_001329	*CTBP2*	−	≈	↑	↓
DG32-129f24	NM_005077	*TLE1*	−	↓	↑	↑↑
DG2-102p15	NM_013253	*DKK3*	−	↑	↓	↓↓
DG9-275n20	NM_003012	*SFRP1*	−	↑	↓↓	↓
DG8-49118	NM_001954	*DDR1*	+	↓	↑	↓
DG2-102m21	NM_000582	*OPN/SPP1*	+	↓	↑	↓
Cell cycle and alternative complement cascade						
DG32-147a19	NM_053056	*CCND1*	+	↑	↑	↓
DG33-101c10	NM_004060	*CCNG1*	+	↑	↑	↓
DG11-192m15	NM_006835	*CCNI*	+	↑	↓	↑
DG2-41a10	NM_000321	*RB1*	−	≈	↑	↓
DG2-34n17	NM_000389	*CDKN1A*	−	↓	↑	↓↓
DG12-1a17	NM_001710	*BF*	+	↑	↓	↓
DG10-1p13	NM_001710	*BF*	+	↑	↓	↓
DG2-3618	NM_014059	*RGC32*	−	↑	↓	↓↓
DG11-4j12	NM_014059	*RGC32*	−	↑	↓	↓↓
DG32-152k14	NM_004048	*B2M*	−	↑	↓	↓
DG2-93n14	NM_001831	*CLU*	−	↑	↓	↓
DG2-10i18	NM_014011	*SOCS5*	−	↑	↓	↓
Integrin-signalling						
DG33-90p14	NM_000090	*COL3A1*	+	↓	↑	≈
DG8-53o24	NM_001753	*CAV1*	−	↓	↑	≈
DG2-66a5	NM_005717	*ARPC5*	+	≈	↓	↑
DG11-126m15	NM_002213	*ITGB5*	+	↓	≈	↑
DG2-133o11	NM_001315	*MAPK14*	+	↓	≈	↑
DG33-2h3	NM_003373	*VCL*	+	↓	≈	↑
DG9-140122	NM_004370	*COL12A1*	+	↓	↓	↑
DG40-161p20	NM_000089	*COL1A2*	+	↓↓	↓	↑
DG2-64i21	NM_001012267	*OGN*	+	↓↓	≈	↑
DG14-240i5	NM_001845	*COL4A1*	+	↓↓	↓	↑
DG2-28j3	NM_001010942	*RAP1B*	+	↓	↑	↑↑
DG42-169k17	NM_002026	*FN1*	+	≈	↑	↓
DG14-194i10	NM_001848	*COL6A1*	+	↑	≈	↓
DG33-32n23	NM_000954	*LAMB2*	+	↑	≈	↓
DG14-5c14	NM_000210	*ITGA6*	+	↑	≈	↓
DG8-173b12	NM_000213	*ITGB4*	+	↑	≈	↓
DG2-68i3	NM_002291	*LAMB1*	+	↓	↑	↓
DG11-131c5	NM_000227	*LAMA3*	+	↓↓	↑	↓
Cytoskeletal/Rho-GTPase signalling						
DG14-194i10	NM_001848	*COL6A1*	+	↑	≈	↓
DG11-93m20	NM_138455	*CTHRC1*	+	↑	≈	↓
DG11-175f19	NM_138455	*CTHRC1*	+	↑	≈	↓
DG2-24b7	NM_002576	*PAK1*	+	↑	≈	↓
DG32-41p15	NM_005965	*MYLK*	+	↑	↓	↓
DG2-105g11	NM_030808	*MYH10*	+	≈	↓	↑
DG2-28b10	NM_053024	*PFN2*	+	≈	↓	↑

RT-PCR quantification for the randomly selected set of candidate genes (Fig. 4).

Pathway analysis

Annotated differentially expressed gene lists of all three cell lines were analysed using the PANTHER pathway analysis tool (Table 2). In CMT-U229, many key cell cycle-activating cyclins (*CCND1*, *CCNG1* and *CCNI*) were overexpressed and cell cycle inhibitor (*CDKN1A*) was underexpressed. Interestingly, *RGC32*, a complement-signalling-induced gene believed to have a role in the cell cycle, and *BF*, a gene involved in the assembly of the membrane attack complex of the complement cascade, are also upregulated in this cell line. Genes involved in cytokine/Rho-GTPase signalling (*SOCS5*, *PAK1* and *MYLK*) and integrins *ITGA6* and *ITGB4* are also overexpressed in CMT-U229. Cell line P114 had high expression of many activators and direct targets of the Wnt pathway (*CSNK1A1*, *CTNNB1*, *CCND1*, *DDR1* and *OPN*) and lower levels of inhibitors of the Wnt pathway (*DKK3*, *SFRP1*). P114 also overexpressed the cell cycle inhibitor *CDKN1A*, laminins (*LAMA3* and *LAMB1*) and fibronectin (*FN1*). CMT-U335 overexpressed many genes involved in integrin signalling such as collagens (*COL12A1*, *COL1A2* and *COL4A1*), *OGN*, *RAP1B*, *VCL*, *ITGB5* and *MAPK14*.

To gain additional insight, we compared the pathway profiles with those of the human gene sets (Sorlie *et al.* 2003; Charafe-Jauffret *et al.* 2006). Sorlie *et al.* (2003) identified five distinct molecular subtypes of human breast carcinomas using the expression pattern of an intrinsic gene set (≈500 genes). Charafe-Jauffret *et al.* (2006) identified differentially expressed genes (≈1400 genes) between the basal, luminal and mesenchymal subtypes of human breast cancer cell lines. Direct comparison of these human gene sets with canine cell line data was impossible because of a lack of representation of these genes on the canine microarray. Instead, the CMT gene set of all differentially expressed genes, the human breast cancer cell line gene set and the breast carcinoma intrinsic gene set were subjected to the PANTHER™ pathway analysis. Even though the majority of the genes in each gene set were not classified under any signalling network, pathway analysis identified a striking similarity in the pathway profiles of these three gene sets. Four pathways (integrin, Wnt, angiogenesis and cytokine/chemokine signalling) were among the top five pathways commonly identified in all three gene sets (Table 3). Parkinson's disease, Alzheimer's disease and PDGF signalling pathways were differentially represented among the three gene sets (Table 3).

Table 3 Major (top five) deregulated cellular signalling pathways in CMTs, human breast cancer cell lines (BCL) and human breast carcinomas (BC). A list of all differentially expressed genes in CMTs, and previously published BCL (Charafe-Jauffret *et al.* 2006) and BC (Sorlie *et al.* 2003) gene lists were independently compared to a common reference gene list available in the PANTHER pathway database for their over-representation in cellular signalling pathways: *P*-value = random overlapping *P*-value; No. of genes/% genes = number/percentage of genes in each gene list.

PANTHER major pathways hits ($P < 0.001$)	No. genes	% genes	*P*-value
Canine mammary cell line gene list			
Integrin signalling pathway	17	7.70	0.00034
Wnt signalling pathway	15	7.30	0.00057
Parkinson's disease	11	5.30	0.00049
Chemokine and cytokine signalling pathway	8	3.80	0.00031
Angiogenesis	6	2.90	0.00005
Human breast cell line gene set			
Integrin signalling pathway	29	5.20	0.00034
Angiogenesis	27	4.80	0.00005
Wnt signalling pathway	23	4.10	0.00057
Chemokine and cytokine signalling pathway	23	4.10	0.00031
PDGF signalling pathway	18	3.20	0.00047
Human breast cancer gene set			
Wnt signalling pathway	15	6.50	0.00057
Alzheimer's disease-presenilin pathway	12	5.20	0.00004
Angiogenesis	10	4.30	0.00005
Integrin signalling pathway	8	3.50	0.00034
Chemokine and cytokine signalling pathway	8	3.50	0.00031

Discussion

With the advent of microarray technology, there have been many reports on the molecular characterization of human breast tumours and cancer cell lines. Five different subtypes (luminal-A, luminal-B, basal, ERBB2-amplified and normal-like) of human breast tumours have been identified using an intrinsic gene set as classifier (Sorlie *et al.* 2003). In a similar study, basal, luminal and mesenchymal subtypes of human breast cancer cell lines were identified (Charafe-Jauffret *et al.* 2006). To the best of our knowledge, this study is the first microarray study to use dog mammary tumour cell lines. In this study we characterized three well-established CMTs originating from totally different types of primary tumours with distinct biological behaviours. Two of these three cell lines carry missense point mutations in the DNA-binding domain of tumour suppressor p53 (CMT-U335 and CMT-U229), whereas P114 carries a wild-type p53 (Van Leeuwen *et al.* 1996). Genomic instability (amplification/deletion) is a frequently observed phenomenon in tumours and tumour cell lines that may influence their gene expression profile. However, we did not observe a significant number of differentially expressed genes being localized to a single chromosomal area in CMTs, indicating low chances of karyotypic instability-induced bias in the CMT gene expression profiles. A comparative genomic hybridization (CGH) analysis using a recently developed

canine CGH array (Thomas *et al.* 2007) in combination with the gene expression profile may have, however, a synergistic effect on the understanding of the development of deregulated pathways in canine mammary tumours.

Cell line CMT-U229

The CMT-U229 cell line originated from a canine atypical benign mixed tumour. Although rare in human breast cancer, they are common in dogs. Benign mixed tumours usually contain epithelial, mesenchymal and myoepithelial cell types. Although benign, they are known to carry clonal karyotypic abnormalities probably indicating potential malignancy (Bartnitzke *et al.* 1992). In a proliferation assay CMT-U229 showed the fastest growth rate compared to CMT-U335 and P114. This high growth rate and previously reported high immunoreactivity to epithelial markers and low reactivity to mesenchymal marker vimentin (Hellmen 1992) indicate that this cell line might have evolved from a small population of malignant epithelial cells of the parent tumour. Alternatively it might have acquired the malignancy *in vitro*, which is supported by its ability to form anaplastic tumours when inoculated into nude mice (Hellmen 1992).

Pathway analysis of the gene expression data indicated an enrichment of candidate genes involved in the immune system-mediated signalling (*B2M*, *CLU*, *BF*, *RGC32* and *SOCS5*) in CMT-U229 (Table 2). The major histocompatibility complex-1 (MHC-1) is of central importance in regulating immune response against tumour cells. Increased expression of *beta-2-microglobulin* (*B2M*), a component of MHC-1, by tumour cells has significance with regard to escape from immune surveillance and cell survival (Abdul & Hoosein 2000; Scheffer *et al.* 2002). Clusterin (CLU) is an important glycoprotein that biologically binds to the MHC-1 complex rendering it inactive and making tumour cells resistant to cytolysis mediated by MHC-1 (Koch-Brandt & Morgans 1996). CLU is also reported to inhibit the BAX family of genes mediating apoptosis, making tumour cells resistant to apoptosis (Shannan *et al.* 2006), and it is found to be overexpressed in many solid tumours including human breast carcinomas (Redondo *et al.* 2000). Factor B (BF) is a component of the alternative complement pathway of immune signalling and its exact role in tumourigenesis is yet to be investigated. *Response gene to complement 32* (*RGC32*) is known to be involved in complement pathway-mediated cell cycle progression and is reported to be overexpressed in many solid tumours (Badea *et al.* 2002; Fosbrink *et al.* 2005). *Suppressor of cytokine signalling* (*SOCS5*) is found be overexpressed in many solid tumours and cell lines and is attributed to their resistance to pro-inflammatory cytokines (Evans *et al.* 2007). Overexpression of the above-discussed genes in CMT-U229 may partially contribute to its high growth rate by making it resistant to apoptosis or by stimulating the cell cycle.

Cell cycle aberrations are the most common phenomena observed in tumourigenesis. Cell cycle activating cyclins (*CCND1*, *CCNG1* and *CCNI*) and the cell cycle inhibitor *CDKN1A* ($p21^{waf/cip}$) are upregulated in CMT-U229, indicating a deregulated cell cycle, which could be another cause for its high growth rate. Apart from cell cycle genes, oncogenic integrins, namely the *α-6* and *β-4 integrins* (*ITGA6* and *ITGB4*), are overexpressed in CMT-U229. The role of *ITGB4* and *ITGA6* is also highlighted in increased cell survival, anchorage-independence and invasion potential of many types of solid tumours, including breast tumours (Lipscomb & Mercurio 2005). Overexpression of these integrins in CMT-U229 is in contrast to its relatively low invasion potential and anchorage-independent growth ability; however, higher expression of *ITGB4* and *ITGA6* in CMT-U229 may also contribute to its high growth rate by promoting cell survival. CMT-U229, with high expression of many genes involved in cell cycle, immune-related (cytokine, complement cascade) and cytoskeletal Rho-GTPase signalling, could serve as an interesting model to further investigate the role of these signalling pathways in canine mammary carcinogenesis.

Cell line P114

Cell line P114 was isolated from a highly malignant canine primary anaplastic carcinoma and has been reported to carry wild-type p53 (Van Leeuwen *et al.* 1996). The proliferation assay revealed a relatively fast doubling time (22 h) for this cell line (Fig. 1). In addition, the *in vitro* invasion and soft agar colony formation assays indicated that P114 was the most tumourigenic of the three cell lines. The histogenesis of the anaplastic carcinomas is not very clear, but they are believed to have an epithelial origin (Benjamin *et al.* 1999) and indeed high expression of *CK18* and *CK8* transcripts (epithelial markers) in P114 compared to non-epithelial CMT-U335 was evident from our microarray data. This suggests that the cell line P114 may have retained key characteristics of its tumour of origin. Additionally, P114 has also showed differentiation ability when cultured in Matrigel matrix by demonstrating branching morphogenesis (Fig. 2).

Gene expression profiles revealed a clear set of genes that are differentially expressed in P114 compared to the other two cell lines. Many genes involved in the Wnt-signalling pathway were identified when these differentially expressed genes were subjected to the pathway analysis. Wnt signalling is driven by secreted Wnt ligands and their frizzled receptors and is very important in early embryonic development and adult tissue maintenance (Logan & Nusse 2004). In many tissues aberrant activation of Wnt signalling is associated with malignancy (Brennan & Brown 2004). Mutations in key regulators of Wnt signalling, namely *APC*, *beta-catenin* and *TCF*, are the main causes of active Wnt signalling in colorectal cancer (reviewed in

Barker & Clevers 2006). Similar mutations are uncommon or not yet reported in the tumourigenesis of breast and other tissues. Among the breast cancer studies, there are many reports of high nuclear beta-catenin immunoreactivity, which is a hallmark of active Wnt signalling (Lin *et al.* 2000; Brennan & Brown 2004). In this study, P114 seems to have a highly active Wnt pathway. This observation is supported by the overexpression of many activators or positive regulators of Wnt signalling (*CTNNB1*, *CSNK1A1*, *DDR1*, *CHD1L*, *OPN* and *CCND1*) and downregulation of many Wnt-signalling repressors (*DKK3*, *SFRP1*). An activated canonical Wnt pathway mediated by beta-catenin/TCF activity in P114 was further confirmed using a TCF-luciferase reporter assay (N. A. S. Rao, A. Gracanin & J. A. Mol, in preparation). Active beta-catenin has been reported to regulate cadherin-mediated cell–cell adhesion as well as cell proliferation (Orford *et al.* 1999). Loss of expression of Wnt antagonists like *SFRP1* and *DKK3* has been identified in many types of cancers and is known to be associated with aggressiveness (Hoang *et al.* 2004; Lo *et al.* 2006; Veeck *et al.* 2006). P114 indeed showed potent anchorage-independent growth ability and was the most invasive cell line in our study based on the Transwell invasion assay. Constitutively active Wnt signalling could downregulate E-cadherin and make cells more invasive, although this may not be the cause of high tumourigenicity of P114 because E-cadherin expression was not significantly lower in P114. Another possible explanation could be through the Wnt-signalling target gene *osteopontin* (*OPN*) (El-Tanani *et al.* 2004), which is also overexpressed in P114. *OPN* can make tumours highly tumourigenic by regulating the expression of *urokinase plasminogen activator* (*uPA*) through phosphatidylinositol 3-kinase/akt signalling (Das *et al.* 2004). *OPN* is also overexpressed in P114, and there is also a higher expression of *uPA* mRNA in P114 compared to the other two cell lines (N.A.S. Rao, A. Gracanin & J.A. Mol, unpublished data).

Wnt signalling has a major role in breast cancer, as evidenced by observed high nuclear beta-catenin immunoreactivity in 60% of analysed human breast tumours (Lin *et al.* 2000) and loss or reduced expression of Wnt antagonist SFRP1 in 80% of human breast carcinomas (Ugolini *et al.* 2001). Therefore, the highly tumourigenic P114 cell line with its active Wnt pathway could be a valuable model to study the role of the Wnt pathway in mammary tumourigenesis and progression.

Cell line CMT-U335

Cell line CMT-U335 was isolated from a rarely occurring (<1%) primary canine mammary osteosarcoma (Benjamin *et al.* 1999), an aggressive tumour that expresses bone components and extracellular matrix (ECM) proteins including vimentin and displays weak or no staining for epithelial cytokeratins (Sando *et al.* 2006). The gene expression profile of CMT-U335, the slowest growing cell line of the three cell lines, revealed a large number of differentially expressed genes compared to P114 and CMT-U229 (Table S1), indicating that it is more distinct from the other two cell lines. Enrichment of the gene expression data by pathway analysis identified many overexpression genes involved in integrin signalling. Genes responsible for active Wnt signalling and the cell cycle were expressed at lower levels in CMT-U335, which may explain its slower growth rate.

The ECM, through its cell-surface integrin receptors, exerts enormous control over the behaviour of normal as well as cancerous cells. Specific combinations of ECM and integrin interactions control cell motility, shape, growth and survival (Guo & Giancotti 2004). The highly expressed *integrin-β-5* (*ITGB5*) in CMT-U335 may have stimulated autocrine TGF-β signalling, leading to the deposition of ECM proteins (Asano *et al.* 2006). This hypothesis is further supported by the strong expression of collagens (*COL1A2*, *COL4A1* and *COL12A1*) in this cell line. *COL1A2* is normally expressed in supportive or connective tissue and plays a key role in osteogenesis (Molyneux *et al.* 1993). Overexpression of *COL1A2* or other ECM proteins involved in actin cytoskeleton assembly and cell–cell or cell–matrix interaction (*VCL*, *ARPC5*) indicates possible aberrant integrin signalling in this cell line. Additionally, epithelial cytokeratin transcripts (*CK8*/*CK18*) were expressed at a lower level and osteogenic transcripts (*COL1A2*, *OGN*) were expressed at a higher level, indicating the preserved mesenchymal characteristics of its primary tumour. CMT-U335 formed fewer colonies when grown in soft agar and invaded at a slower rate through Matrigel-coated Transwells. In addition, CMT-U335, representative of a rarely occurring primary tumour that overexpresses many integrin signalling genes, would be an interesting mammary osteosarcoma model for *in vitro* studies.

Cross-species comparison of pathway profiles

A cross-species comparison can be a powerful biological sieve to select important oncogenic pathways that may have evolutionary significance. Comparing the pathway profiles of the canine mammary cell line gene set, the human breast cancer intrinsic gene set and the human breast cancer cell line gene set identified four of the five major signalling pathways as common across the datasets. These results stress the importance of Wnt, integrin, angiogenesis and cytokine/chemokine signalling in mammary cancer of dogs and humans. Wnt signalling is already known for its role in human mammary development and carcinogenesis (reviewed in Brennan & Brown 2004). Angiogenesis plays a central role in both local tumour growth and distant metastasis, and its importance in breast cancer development and progression has been documented by many

authors (Schneider & Miller 2005). Cytokine and chemokine signalling have been implicated in breast cancer, but the signalling network as a whole is not well understood in relation to the aetiology of breast cancer. The importance of integrin signalling in breast cancer has been established by many authors (Asano *et al.* 2006; Carraway & Sweeney 2006). Parkinson's disease in the canine gene set and Alzheimer's disease signalling in the human breast cancer gene set were identified among the top five pathways. Because these pathways are less likely to be associated with breast cancer and were not common across the three gene sets, they can be considered as of trivial significance for mammary carcinogenesis.

In conclusion, this study identified Wnt signalling, integrin signalling, cell cycle, alternative complement cascade and cytokine/Rho-GTPase signalling as the main pathways regulated differentially in CMTs. A cross-species comparison of pathway profiles identified four of the five major deregulated signalling pathways that are common in human and canine data sets, indicating molecular similarity of the disease across species. Further studies correlating an individual cell line phenotype with its gene expression signature may facilitate in annotating large groups of differentially expressed, unannotated genes. These characterized CMTs with their unique gene expression and pathway profiles are valuable tools to prioritize biological pathways for a detailed study. In light of the high incidence of mammary cancer in dogs, these cell lines are a valuable tool to develop and to test new pathway-specific cancer therapeutics. These results are also the basis for further characterization of canine mammary carcinomas.

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Competing interests

All authors declare that they have no competing interests.

References

Abdul M. & Hoosein N. (2000) Changes in beta-2 microglobulin expression in prostate cancer. *Urologic Oncology* **5,** 168–72.

Asano Y., Ihn H., Jinnin M., Mimura Y. & Tamaki K. (2006) Involvement of alphavbeta5 integrin in the establishment of autocrine TGF-beta signaling in dermal fibroblasts derived from localized scleroderma. *Journal of Investigative Dermatology* **126,** 1761–9.

Badea T., Niculescu F., Soane L., Fosbrink M., Sorana H., Rus V., Shin M.L. & Rus H. (2002) RGC-32 increases p34CDC2 kinase activity and entry of aortic smooth muscle cells into S-phase. *Journal of Biological Chemistry* **277,** 502–8.

Barker N. & Clevers H. (2006) Mining the Wnt pathway for cancer therapeutics. *Nature Reviews Drug Discovery* **5,** 997–1014.

Bartnitzke S., Motzko H., Rosenhagen C. & Bullerdiek J. (1992) Benign mixed tumor of canine mammary gland showing an r(X) and trisomy 5 as the only clonal abnormalities. *Cancer Genetics and Cytogenetics* **62,** 29–31.

Benjamin S.A., Lee A.C. & Saunders W.J. (1999) Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles. *Veterinary Pathology* **36,** 423–36.

Bhatti S.F., Rao N.A., Okkens A.C. *et al.* (2007) Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domestic Animal Endocrinology* **33,** 294–312.

Brennan K.R. & Brown A.M. (2004) Wnt proteins in mammary development and cancer. *Journal of Mammary Gland Biology and Neoplasia* **9,** 119–31.

Brinkhof B., Spee B., Rothuizen J. & Penning L.C. (2006) Development and evaluation of canine reference genes for accurate quantification of gene expression. *Analytical Biochemistry* **356,** 36–43.

Carraway K.L. III & Sweeney C. (2006) Co-opted integrin signaling in ErbB2-induced mammary tumor progression. *Cancer Cell* **10,** 93–5.

Charafe-Jauffret E., Ginestier C., Monville F. *et al.* (2006) Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* **25,** 2273–84.

Das R., Mahabeleshwar G.H. & Kundu G.C. (2004) Osteopontin induces AP-1-mediated secretion of urokinase-type plasminogen activator through c-Src-dependent epidermal growth factor receptor transactivation in breast cancer cells. *Journal of Biological Chemistry* **279,** 11051–64.

El-Tanani M., Platt-Higgins A., Rudland P.S. & Campbell F.C. (2004) Ets gene *PEA3* cooperates with beta-catenin-Lef-1 and c-Jun in regulation of *osteopontin* transcription. *Journal of Biological Chemistry* **279,** 20794–806.

Evans M.K., Yu C.R., Lohani A., Mahdi R.M., Liu X., Trzeciak A.R. & Egwuagu C.E. (2007) Expression of *SOCS1* and *SOCS3* genes is differentially regulated in breast cancer cells in response to proinflammatory cytokine and growth factor signals. *Oncogene* **26,** 1941–8.

Fosbrink M., Cudrici C., Niculescu F., Badea T.C., David S., Shamsuddin A., Shin M.L. & Rus H. (2005) Overexpression of *RGC-32* in colon cancer and other tumors. *Experimental and Molecular Pathology* **78,** 116–22.

Galbiati F., Volonte D., Engelman J.A., Watanabe G., Burk R., Pestell R.G. & Lisanti M.P. (1998) Targeted downregulation of *caveolin-1* is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO Journal* **17,** 6633–48.

Guo W. & Giancotti F.G. (2004) Integrin signalling during tumour progression. *Nature Reviews Molecular Cell Biology* **5,** 816–26.

Hellmen E. (1992) Characterization of four *in vitro* established canine mammary carcinoma and one atypical benign mixed tumor cell lines. *In Vitro Cellular & Developmental Biology* **28A,** 309–19.

Hoang B.H., Kubo T., Healey J.H., Yang R., Nathan S.S., Kolb E.A., Mazza B., Meyers P.A. & Gorlick R. (2004) Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway. *Cancer Research* **64,** 2734–9.

Koch-Brandt C. & Morgans C. (1996) Clusterin: a role in cell survival in the face of apoptosis? *Progress in Molecular and Subcellular Biology* **16,** 130–49.

Lin S.Y., Xia W., Wang J.C., Kwong K.Y., Spohn B., Wen Y., Pestell R.G. & Hung M.C. (2000) Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 4262–6.

Lipscomb E.A. & Mercurio A.M. (2005) Mobilization and activation of a signaling competent alpha6beta4integrin underlies its contribution to carcinoma progression. *Cancer and Metastasis Reviews* **24,** 413–23.

Lo P.K., Mehrotra J., D'Costa A., Fackler M.J., Garrett-Mayer E., Argani P. & Sukumar S. (2006) Epigenetic suppression of *secreted frizzled related protein 1* (*SFRP1*) expression in human breast cancer. *Cancer Biology & Therapy* **5,** 281–6.

Logan C.Y. & Nusse R. (2004) The Wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology* **20,** 781–810.

MacEwen E.G. (1990) Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. *Cancer and Metastasis Reviews* **9,** 125–36.

Mi H., Lazareva-Ulitsky B., Loo R. *et al.* (2005) The PANTHER database of protein families, subfamilies, functions and pathways. *Nucleic Acids Research* **33,** D284–8.

Misdorp W. (1991) Progestagens and mammary tumours in dogs and cats. *Acta Endocrinologica* **125**(Suppl. 1), 27–31.

Mol J.A., van Garderen E., Rutteman G.R. & Rijnberk A. (1996) New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans. *Journal of Steroid Biochemistry and Molecular Biology* **57,** 67–71.

Molyneux K., Starman B.J., Byers P.H. & Dalgleish R. (1993) A single amino acid deletion in the alpha 2(I) chain of type I collagen produces osteogenesis imperfecta type III. *Human Genetics* **90,** 621–8.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65,** 55–63.

Orford K., Orford C.C. & Byers S.W. (1999) Exogenous expression of beta-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. *Journal of Cell Biology* **146,** 855–68.

Parkin D.M., Bray F., Ferlay J. & Pisani P. (2005) Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians* **55,** 74–108.

van de Peppel J., Kemmeren P., van Bakel H., Radonjic M., van Leenen D. & Holstege F.C. (2003) Monitoring global messenger RNA changes in externally controlled microarray experiments. *EMBO Reports* **4,** 387–93.

Perou C.M., Sorlie T., Eisen M.B. *et al.* (2000) Molecular portraits of human breast tumours. *Nature* **406,** 747–52.

Pfaffl M.W., Horgan G.W. & Dempfle L. (2002) RELATIVE EXPRESSION SOFTWARE TOOL (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30,** e36.

Redondo M., Villar E., Torres-Munoz J., Tellez T., Morell M. & Petito C.K. (2000) Overexpression of clusterin in human breast carcinoma. *American Journal of Pathology* **157,** 393–9.

Roepman P., Wessels L.F., Kettelarij N. *et al.* (2005) An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nature Genetics* **37,** 182–6.

Russo I.H. & Russo J. (1998) Role of hormones in mammary cancer initiation and progression. *Journal of Mammary Gland Biology and Neoplasia* **3,** 49–61.

Sando N., Oka K., Moriya T., Saito H., Nagakura S., Mori N., Suzuki T., Ueki H. & Ohtani H. (2006) Osteosarcoma arising in the breast. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **114,** 581–7.

Scheffer G.L., de Jong M.C., Monks A., Flens M.J., Hose C.D., Izquierdo M.A., Shoemaker R.H. & Scheper R.J. (2002) Increased expression of beta 2-microglobulin in multidrug-resistant tumour cells. *British Journal of Cancer* **86,** 1943–50.

Schneider B.P. & Miller K.D. (2005) Angiogenesis of breast cancer. *Journal of Clinical Oncology* **23,** 1782–90.

Schneider R., Dorn C.R. & Taylor D.O. (1969) Factors influencing canine mammary cancer development and postsurgical survival. *Journal of the National Cancer Institute* **43,** 1249–61.

Selman P.J., Mol J.A., Rutteman G.R., van Garderen E. & Rijnberk A. (1994) Progestin-induced growth hormone excess in the dog originates in the mammary gland. *Endocrinology* **134,** 287–92.

Shannan B., Seifert M., Boothman D.A., Tilgen W. & Reichrath J. (2006) Clusterin and DNA repair: a new function in cancer for a key player in apoptosis and cell cycle control. *Journal of Molecular Histology* **37,** 183–8.

Sorlie T., Perou C.M., Tibshirani R. *et al.* (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 10869–74.

Sorlie T., Tibshirani R., Parker J. *et al.* (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 8418–23.

Thomas R., Duke S.E., Bloom S.K. *et al.* (2007) A cytogenetically characterized, genome-anchored 10-Mb BAC set and CGH array for the domestic dog. *Journal of Heredity* **98,** 474–84.

Trusolino L., Cavassa S., Angelini P., Ando M., Bertotti A., Comoglio P.M. & Boccaccio C. (2000) HGF/scatter factor selectively promotes cell invasion by increasing integrin avidity. *FASEB Journal* **14,** 1629–40.

Tusher V.G., Tibshirani R. & Chu G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 5116–21.

Ugolini F., Charafe-Jauffret E., Bardou V.J. *et al.* (2001) WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene *SFRP1* in most invasive carcinomas except of the medullary type. *Oncogene* **20,** 5810–7.

Van Leeuwen I.S., Hellmen E., Cornelisse C.J., Van den Burgh B. & Rutteman G.R. (1996) P53 mutations in mammary tumor cell lines and corresponding tumor tissues in the dog. *Anticancer Research* **16,** 3737–44.

Veeck J., Niederacher D., An H. *et al.* (2006) Aberrant methylation of the Wnt antagonist *SFRP1* in breast cancer is associated with unfavourable prognosis. *Oncogene* **25,** 3479–88.

Yang Y.H., Dudoit S., Luu P., Lin D.M., Peng V., Ngai J. & Speed T.P. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* **30**, e15.

Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2008.01733.x

Table S1 Statistically significant differentially expressed genes in canine mammary cell lines when the false discovery rate was set below 5% (SAM generated).

Table S2 List of unique genes in CMTs generated by SAM analysis at a false discovery rate set below 5%.

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