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TRANSCRIPTOMIC PROFILE OF TWO CANINE MAMMARY CANCER CELL LINES WITH DIFFERENT PROLIFERATIVE AND ANTI-APOPTOTIC POTENTIAL

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The aim of the study was to identify the genes responsible for the high growth rate and anti-apoptotic potential in selected canine mammary cancer cells. cDNA canine microarrays were used to compare the transcriptome in simple carcinoma CMT-U27 and spindle-cell tumor CMT-U309 cell lines. In CMT-U27 cell line the growth rate (shorter cell cycle), anti-apoptotic potential (higher expression of Bcl-2) was higher and spontaneous and induced apoptosis was lower. Comparison of transcriptomes revealed 333 genes which expression differed similarly. We focused on genes involved in cell proliferation, adhesion and apoptosis, and selected 29 of them. The high growth rate and anti-apoptotic potential in CMT-U27 cells was associated with enhanced expression of genes (at the level of transcripts) involved in Ca^{2+} signaling pathway (Calmodulin 1, 2, 3 and SPSB2) and growth hormone cellular pathway. The low-proliferative and pro-apoptotic phenotype of CMT-U309 cells was more dependent on TGFβ, neuregulin 1 pathways and adhesion-related molecules.

Keywords: *canine mammary cancer, gene expression profile, cDNA microarray, cell line gene expression*

INTRODUCTION

Mammary cancer is the most common malignant neoplasm in bitch (1). The annual incidence in dogs is 198/100 000 what is three times higher than in woman, when canine and human incidences were adjusted to the same population distribution. Middle-aged female dogs (about 7 years) are mainly affected by mammary tumors. Mammary tumors have complex morphology and they may be classified as epithelial, mixed and mesenchymal tumors. Based on biological and histological criteria it can be estimated that approximately 30 percent of the mammary tumors are malignant (2). Carcinomas constitute 40-45% of them.

There is only few detailed data about etiology, pathogenesis and molecular foundations of mammary tumor malignancy in dog. In bitches a considerable protective effect against mammary carcinoma is offered by early ovariectomy. Injectable progestins regularly used in some countries to prevent estrus in the bitch, was found to considerably enhance the risk of benign, but not malignant mammary tumors (3, 4). In further studies it was shown that progestins induce the expression of GH in focal areas of the canine mammary gland. Animal experiments and human studies have suggested that a high-fat diet and associated obesity increase the risk of breast cancer. In canine mammary tumors similar types of genetic alterations as in humans are found, like oncogene

amplification, inactivation of tumor suppressor genes, mutations, translocations, *etc*. Some genes are known to increase cellular transformation (TGF-alpha) or growth by overexpression (EGF, c-ErbB2) or by mutation (p53). However the biology of this disease remains incompletely understood (5, 6).

Three primary methods are still used to classify mammary tumors: histogenetic, histological descriptive and prognostic (7), however they may not be objective enough. The cellular and molecular heterogeneity of mammary gland tumors with addition to large number of genes responsible for controlling cell cycle, growth, proliferation, differentiation and death, emphasize the requirement of studying multiple genetic alterations. Correlation of expression patterns of thousands of genes in tumors or tumor cell lines with specific features of phenotypic variation, might constitute the basis for an improved classification of canine mammary malignancies. That knowledge may help understand the biological pathways involved in mammary gland cancer (8).

This study was undertaken in order to identify the genes responsible for the high growth rate and anti-apoptotic potential in canine mammary cancer cells. For this purpose we compared the transcriptomic profile using cDNA microarray in two cell lines. One cell line was isolated from spontaneous primary canine mammary spindle-cell tumor (CMT-U309) and the second one was isolated from canine mammary carcinoma (CMT-U27).

MATERIALS AND METHODS

Media and reagents

Phosphate buffer saline, pH 7.4 (PBS), penicillin-streptomycin, fungizone and fetal bovine serum (FBS), were obtained from Gibco BRL (Gaithersburg, MD, USA), Acridine orange and DAPI were obtained from Invitrogen (USA). Antibodies against Bcl-2 were supplied by Dako Cytomation (Danemark), antibodies anti 89 kDa PARP were supplied by BD Biosciences (San Jose, CA, USA). RPMI 1640 medium, 7-amino-actinomycin D (7AAD), camptothecin (CPT), Vinblastine sulfate salt, Ribonuclease A and all the other reagents were obtained from Sigma Chemicals (St. Louis, MO, USA).

Glassware and plastics

Sterile conical flasks, 4 and 8-chamber culture slides (Lab-Tek), 24-chamber coulture plates, sterile cell scrapers and sterile disposable pipettes were purchased from Nunc Inc. (Naperville, IL, USA).

Cell culture

The canine mammary tumor cell lines CMT-U27 and CMT-U309 were used in this study (9, 10). Both cell lines were isolated and established at Uppsala University in Sweden. CMT-U27 cell line was isolated from simple carcinoma tumor, CMT-U309 cell line was isolated from spindle cell tumor.

Cells were cultured in a medium (RPMI-1640 enriched with 10% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 iU/ml), fungizone (2.5 mg/ml), in an atmosphere of 5% CO_2/95% humidified air at 37°C and routinely subcultured every second day.

Immunofluorescence staining for cytometry

Exponentially growing cells were seeded on Lab-Tek 4-chamber culture slides (NUNC Inc., USA) and cultured for 24 h. The medium was then removed and replaced medium containing 0.3 µg/ml CPT for 1, 6, 9 and 12 h. Cells cultured in 10% FBS medium without CPT were used as a control (four replicates were performed).

Cells were fixed in 0.25% formaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70% methanol and incubated at 4°C for 30 min. Finally, the methanol was aspirated and samples were stored at –80°C until staining. At the time of analysis, cells were washed twice with PBS and incubated in the dark for 30 min with antibodies (anti-Bcl2) diluted 1:100 with PBS. After incubation the cells were washed twice with PBS and finally incubated with 5 µg/ml solution of 7-aminoactinomycin D (7AAD) (Sigma, USA) in PBS containing 2% FCS, 0.1% sodium azide and 0.3% saponin (Sigma) for 10 min in the dark to counterstain the DNA. Finally, coverslips were mounted on microscope slides using ICN mounting medium (ICN Biomedicals Inc, Aurora, OH, USA).

Cytometry

The slides were examined using an Olympus Scan^R screening station (Olympus Optical Co., Hamburg, Germany), the modular microscope-based imaging platform designed for fully automated image acquisition and data analysis of biological samples and analysis software (Olympus Scan^R software for screening applications). Digital images were processed using Adobe Photoshop software.

The results were analyzed using Microsoft_Excel 2003 software (Microsoft Corporation, Redmond, WA, USA) and Prism version 3.00 software (GraphPad Software, San Diego, CA, USA).

Confocal microscopy

The cells exposed to CPT were evaluated morphologically after labeling with FITC -conjugated anti 89 kDa PARP antibody and 7-AAD for DNA.

The slides were examined using an FV 500 confocal microscope (Olympus Optical Co., Hamburg, Germany). The cells were examined using the Fluoview program (Olympus Optical Co., Hamburg, Germany).

DNA ploidy analysis

The DNA ploidy analysis has been described in details (11). The canine mammary tumor cells were stained with DAPI. As external DNA standard were used lymphocytes obtained from healthy dog. The whole blood was treated with RBC solution (A&A Biotechnology, Poland) for hemolysis. The leukocytes sample was prepared in separate tube and run in parallel with the sample. 10^5 cell nuclei were analyzed in each sample with FACSC Vantage flow cytometer (Becton Dickinson Sunnyvale, CA). The DNA index (DI) is a value given to express the amount of DNA content relative to normal and is calculated by the following formula (1):

DI = Mean or modal channel No. of DNA aneuploid G_0G_1 peak / Mean or modal channel No. of DNA diploid G_0G_1 peak

A DNA diploid population (G_0/G_1) is given a DI of 1.00 ±10% by definition. The greater than the 2N amount of DNA are termed "DNA hyperdiploid", the fewer than the 2N amount of DNA are termed "DNA hypodiploid" or "DNA near diploid" if the results are close to 2N value.

Growth rate

Exponentially growing cells were seeded on 24-chamber culture plates (NUNC Inc., USA) and cultured for 24 h. The medium was then removed and replaced medium containing 0.05 µg/ml of Vinblastine (Sigma, USA). Cells were incubated for 1, 2, 3, 4 and 5 hrs (four replicates were performed). After removal with trypsin-EDTA cells were stained with Acridine orange (Invitrogen, USA) after Ribonuclease A (Sigma, USA). Mitotic Index was counted with FACS Vantage flow cytometer (Becton Dickinson Sunnyvale, CA) (11). The graph was obtained by plotting the logarithm of the Mitotic Index (1 + Mitotic Index) versus hours of cells incubation in Vinblastine. Doubling time (T_d) was calculated by the following formula:

T_d = log2 / a, where "a" is the graph variable: y = ax + b.

Microarray analysis

1. RNA Isolation and Quality Assessment

CMT-U27 and CMT-U309 cells were cultured until 90% confluence. The medium was next removed and replaced with PBS. Cells were scraped and total RNA from cells suspension samples was isolated using Total RNA kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water. Quantity of isolated RNA was measured using NanoDrop (NanoDrop Technologies, USA). The samples with adequate amount of RNA were treated with DNase I to eliminate DNA contamination. The samples were subsequently purified using RNeasy MiniElute Cleanup Kit (Qiagen, Germany). Finally RNA samples test were analyzed on a BioAnalyzer (Agilent, USA) to measure final RNA quality and integrity. Three replicates were performed (three different passages).

2. Probes Labelling

Total RNA (10 µg) was reverse-transcribed using SuperScript Plus Indirect cDNA Labelling kit (Invitrogen, USA) according to the manufacturer's protocol. Single strand cDNA was labeled with Alexa 555 or Alexa 647 dyes (Invitrogen, USA). Efficiency of dyes incorporation was measured using NanoDrop (NanoDrop Technologies, USA).

3. Hybridization

Before hybridization, dog-specific cDNA microarray slides (printed at Utrecht University, The Netherlands) with 21 000 genes were prepared according recommended protocol (12). Hybridization was performed using automatic hybridization station HybArray12 (PerkinElmer, USA). Slides were fixed in hybridization chambers and, after o-ring conditioning probes, were added. Hybridization of slides was performed using 18 h step-down hybridization protocol provided by producer. After hybridization slides were automatically washed.

4. Hybridization signal detection, quantification and analysis

Acquisition and analysis of hybridization intensities were performed using microarray scanner ScanArray HT and ScanExpress software (PerkinElmer, USA). Different types of values were obtained for quantification of the dot intensity. Due to experimental variations in specific activity of cDNA target preparations or exposure time that might alter

the signal intensity, data from different hybridizations were automatically normalized (LOWESS method) (13) by ScanExpress software. The average ratio of each spot on three slides was calculated using ScanExpress software. Genes that are 2-fold changed in three slides with value difference between the slides less than 5% were examined. Gene function was identified using the NCBI database and PANTHER pathway analysis software (www.pantherdb.org).

Statistical analysis

Results were statistically analyzed using Two way ANOVA Bonferroni multiple range tests with Prism version 3.00 software (GraphPad Software, San Diego, CA, USA); $P \leq 0.05$ was regarded as significant and $P \leq 0.01$ as highly significant.

RESULTS

Analysis of cell cycle and apoptosis in CMT-U27 and CMT-U309 cells

Cytometric analysis revealed highly significant differences in the proliferation rate between examined cell lines. The cell cycle length of CMT-U27 line and CMT-U309 was 53.4 and 103.8 hours respectively. The density of CMT-U27 cells was 14 times higher than CMT-U309 cells after 36 h incubation in the optimal conditions to growth (*Fig. 1*). The cell cycle evaluation in CMT-U27 cell line showed an average: 64%, 15% and 20% of cells in G1, S and G2M phase, respectively. In CMT-U309 the corresponding percentages of cells were: 61%, 17% and 16%, respectively. The G2M/G1+S ratio was higher in CMT-U27 (0.25) than CMT-U309 (0.20) cell line. The number of cells undergoing spontaneous apoptosis was significantly higher in CMT-U309 (6%; SEM=0.34% of 4 experiments) than in CMT-U27 (1%; SEM=0.125% of 4 experiments) cell line. Scan^R screening station used in this study permitted for simultaneous biochemical (cellular DNA content) and morphological analysis of cell cycle (*Fig. 1*). The cell galleries showed morphological features typical for cells being in particular phases of cell cycle as well as cells from sub-G1 region identified as apoptotic cells. The most characteristic features of apoptosis were: condensation of the chromatin and fragmentation of the nucleus and formation of apoptotic bodies. These features were similar for both examined cell lines (*Fig. 1*).

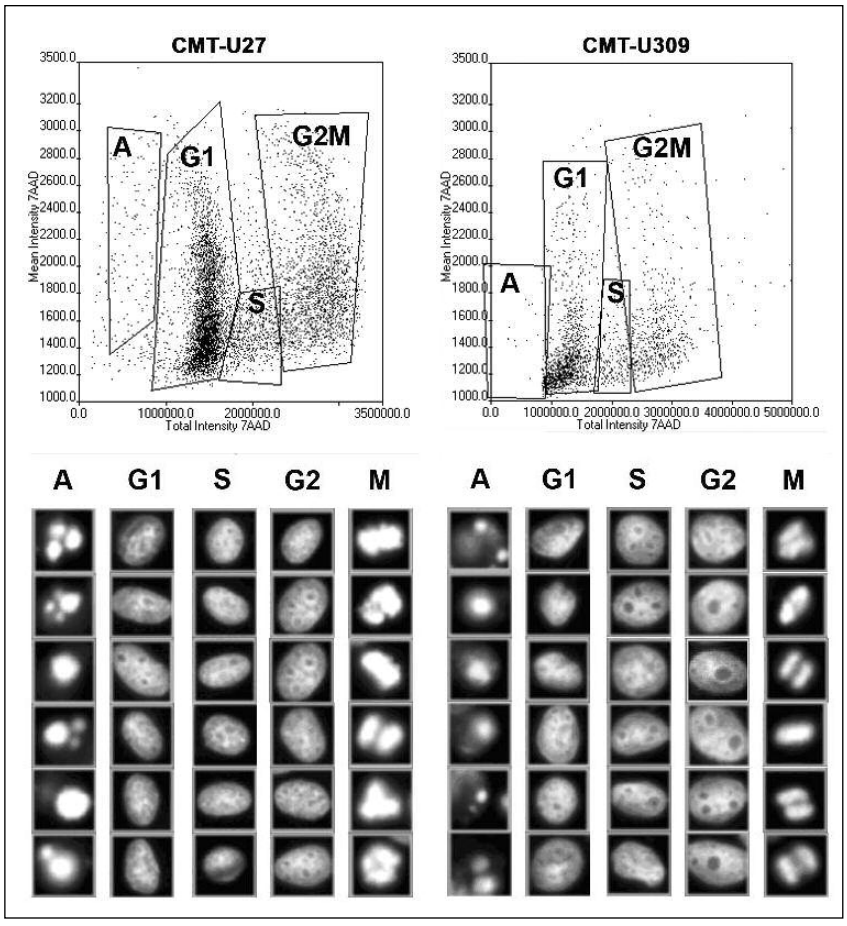

Fig. 1. Comparison of cytograms and galleries of cells between CMT-U27 and CMT-U309 cell lines cultured in optimal conditions to growth.

Examination of ploidy (*Fig. 2*) revealed, that CMT-U27 cells are diploid (2N) and CMT-U309 cells are "DNA near diploid" (2N). The mean G1 peak values for leukocytes DNA, CMT-U27 and CMT-U309 was 200.72, 206.79 and 174.27, respectively. The DI was 1.03 and 0.87 for CMT-U27 and CMT-U309 cell line, respectively. The CV values were <5%. CMT-U309 cells ploidy was previous described (10) but the study was made with different passage number.

Expression of Bcl-2 and susceptibility to apoptogenic stimulus in CMT-U27 and CMT-U309 cells

It is commonly accepted that Bcl-2 is the main anti-apoptotic protein, and its over–expression in cancer cells ensures their resistance to chemotherapy. We compared the Bcl-2 content in two examined mammary cancer cell lines, using anti-Bcl-2 antibody conjugated with FITC and counting in Scan^R screening station. Bcl-2 histograms clearly show a higher Bcl-2 – related fluorescence in CMT-U27 than in CMT-U309 cells (*Fig. 3*). The galleries of cells relocated from the histograms confirmed more intensive Bcl-2 – related green fluorescence in CMT-U27 than in CMT-U309 cells (*Fig. 3*).

To examine cell susceptibility to apoptotic stimulus we applied the test of cell cultures exposure to camptothecin (CPT), an inhibitor of DNA topoisomerase I, used as an anticancer drug. The number of apoptotic cells before administration CPT was significantly higher in CMT-U309 than CMT-U27, which confirms results presented on *Fig.1* (*Fig. 4a*). CPT induced progressive, significant increase in apoptotic cell number in CMT-U27 cells, reaching 7% after 12 h of cell culture exposure to the drug. The number of apoptotic cells in CMT-U309 line increased to 6 h of culture exposure to CPT, and significantly decreased thereafter (*Fig. 4a*). We assume that this effect was artificial and resulted from detachment of apoptotic cells and washing them out from the slides in the course of staining procedure, this experiment was repeated and the result of apoptotic cells detachment was seen in every study.

The induction of apoptosis by CPT was confirmed by confocal images of cells stained with FITC - conjugated anti 89 kDa PARP antibody. In the both examined cell lines apoptotic cells contained a higher concentration of 89 kDa fragment of PARP (the product of caspase 3 activity), which was manifested with the intense green fluorescence (*Fig. 4b*). CPT increased the number of cells in S phase of cell cycle which corresponds with literature data showing elongation of this phase by this drug (14, 15) (*Fig. 5a*, *b*). Simultaneous tendency to decrease of cell number in G1 phase suggests that apoptosis concerns first of all cells from this phase.

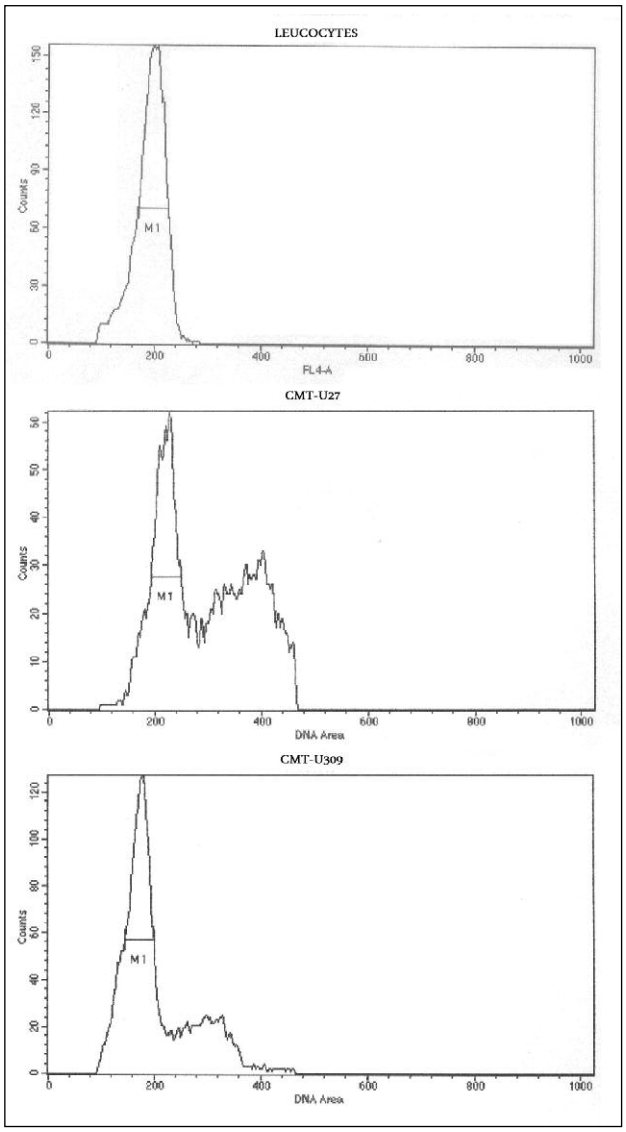

Fig. 2. Comparison of leukocytes (a), CMT-U27 cells (b) and CMT-U309 cells (c) histograms used to establish DNA ploidy of examined cell lines. Cells were cultured in optimal conditions to growth. Region M1 corresponds to G1 peak. Histograms are representative of four separate experiments.

Comparison of transcriptomic profile in CMT-U27 and CMT-U309 cell line

To compare the transcriptome of the two cell lines we performed cDNA microarray analysis in 3 separate experiments on cultures growing under optimal conditions. In the course of the experiment we selected 333 genes which expression differed similarly in three pairs of compared cultures. We focused on genes involved in the regulation of cell proliferation, adhesion and apoptosis and then selected 29 genes (*Table 1*). 19 genes exhibited higher expression in

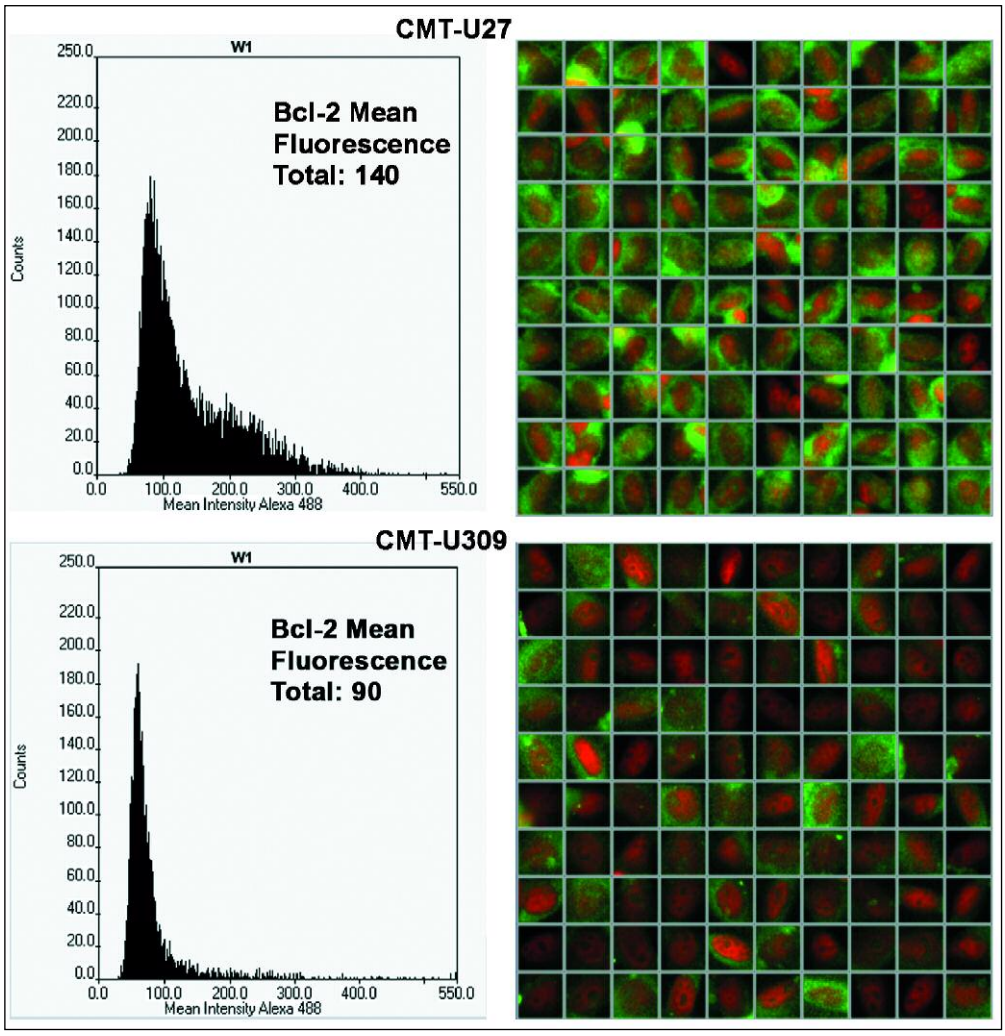

Fig. 3. Comparison of histograms and galleries of CMT-U27 and CMT-U309 cells labeled with FITC – conjugated anti Bcl2 antibody (green fluorescence). DNA was stained with 7-AAD (red fluorescence). Cells were cultured in optimal conditions to growth. Histograms and galleries are representative of four separate experiments.

CMT-U27 cells with a fold change between 2.02 and 6.15, whereas 10 genes were expressed more in CMT-U309 cells with a fold change between 2.01 and 8.60. Among the overexpressed genes in CMT-U27 14 genes were associated with regulation of cell proliferation: TOB1, ZFAND6, RBAK, MORN2, NR6A1, MACF1, EIF4EBP3, SPSB2, CALM1, CALM2, CALM3, CLK1, GHR, RPS6KA2; 3 genes involved in cell adhesion: ARHGAP26, LIN7A, PCOLCE2; and 2 genes associated with apoptosis: SH3GLB1, TM2D1 (*Table 1*). In the case of CMT-U309 cells a higher expression was observed in genes engaged mainly in adhesion and signaling pathways of growth factors (9 genes): TSPAN6, GDAP1, ACVR2B, SMAD1, NRG1, ITGβ1, KIAA1212, TMEFF1, DDEFL1; and 1 gene involved in apoptotic degradation: DNASE2B (*Table 1*).

DISCUSSION

This is the first report revealing the transcriptomic profile in canine cancer cells dependent on the prolifetation rate and ability to trigger apoptosis. It was shown by the comparison of the transcriptome (*Table 1*) with the length and profile of the cell cycle (*Figs. 1, 2*), expression of Bcl-2 – the main antiapoptotic protein (*Fig. 3*) and cancer cell response to apoptotic stimulus (*Figs. 4, 5*). Generally, simple carcinoma CMT-U27 cells showed a higher proliferative and antiapoptotic potential than spindle cell tumor CMT-U309 cells, which was manifested with shorted cell cycle length, a higher G2M:G1 ratio and growth rate (*Fig. 1*), a higher expression of Bcl-2 (*Fig. 3*) and a lower spontaneous apoptosis (*Fig. 4*). Above significant differences in phenotype features of examined cell lines were dependent on expression of genes involved in proliferation, signal transduction, adhesion and apoptosis identified by DNA microararray (*Table 1*).

As was shown in *Fig. 6*, where identified genes (*Table 1*) were placed on the network of cellular pathways (Pathway Architect software; Stratagene USA), a high growth rate in CMT-U27 was associated with enhanced expression of genes involved in Ca^{2+} signaling pathway e.g. calmodulin

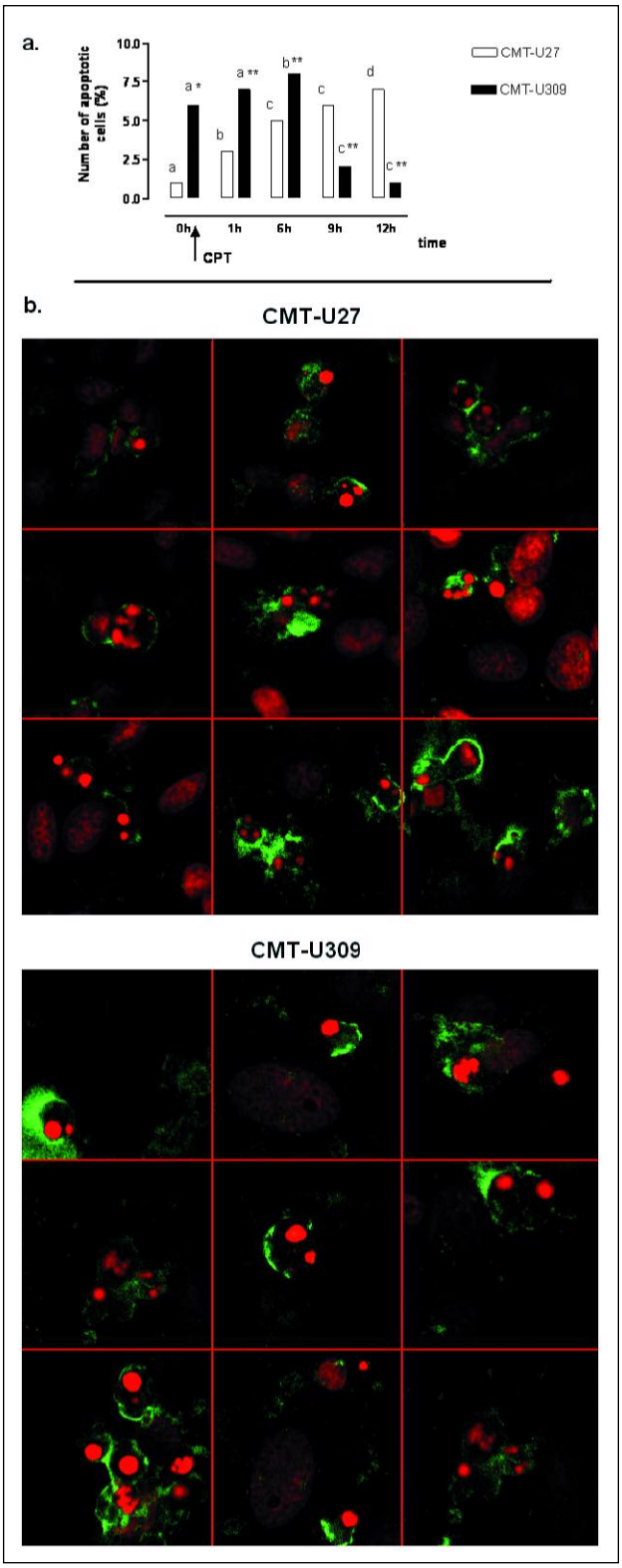

Fig. 4. The number of apoptotic cells (%) in CMT-U27 and CMT-U309 cell cultures before and 1, 6, 9 and 12 hours after CPT (0.3 µg/ml) administration. (a) Means for the same cell line described with different letter differ significantly ($p \leq 0.05$). Significant ($p \leq 0.05$) and highly significant ($p \leq 0.001$) differences between means for cell lines at the same time of exposure to CPT are superscribed with: * and **, respectively (n=4). (b) Confocal images of apoptotic cells labeled with 7AAD (DNA – red fluorescence) and FITC – conjugated anti – 89 kDa PARP antibody (green fluorescence).

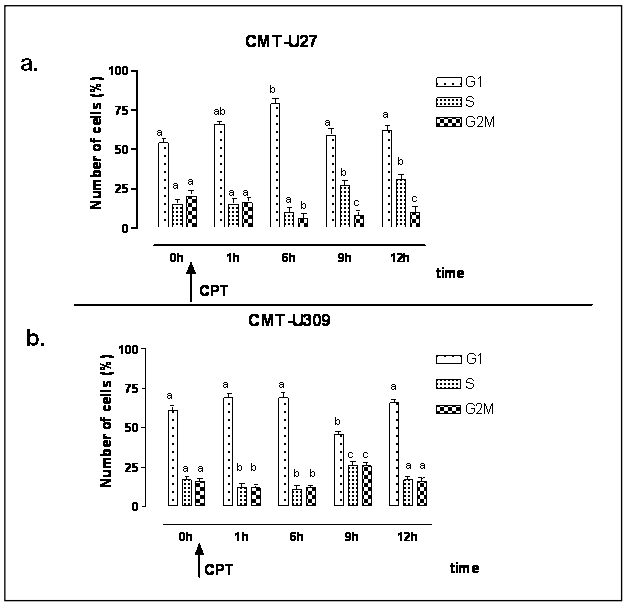

Fig. 5. The number of cells (%) in G1, S and G2M phase of cell cycle before and 1, 6, 9 and 12 hours after CPT (0.3 µg/ml) administration in CMT-U27 (a) and CMT-U309 (b) cell cultures. Means for the same phase of the cell cycle described with different letters differ significantly ($p \leq 0.05$), n=4.

1, 2 and 3, Sp1A/ryanodine receptor domain and SOCS box containing 2 (SPSB2) as well as signals coming from growth hormone receptor. These findings are supported by the similar results of previous studies with breast cancer (16) and mammary gland tumor in mouse.

Calcium is universally required for cell growth and proliferation. The main cellular (internal) receptor for Ca^{2+} is calmodulin. It is well known that calcium and its receptor (calmodulin) are required for cell cycle regulation, but their pathway of cellular action is not clearly defined. Potential targets include the calcium/calmodulin-dependent kinases (CaM-K) and CREB1. It also activates the MAPK cascade. Rodriguez-Mora *et al.* (16) determined the role of the CaM-Ks on cell proliferation and progress through the cell cycle in breast cancer MCF-7 cells. By culturing human breast cancer cells in the presence of the CaM-K inhibitor (KN-93), they indicated that CaM-KK and CaM-KI are required for G1 phase progression in the cell cycle and consequently cell proliferation. It has been shown that the Ca21-binding protein CaM plays an important role in assembly of the ERzERE complex what is basic to E2-regulated gene expression in breast cancer cells (17). Disregulation of Ca21 homeostasis and modulation of expression of the class of genes encoding Calcium binding proteins such as CaM are associated with breast cancer cell proliferation and apoptosis. Synergistic inhibition of growth by CaM inhibitors and antiestrogens has been also reported.

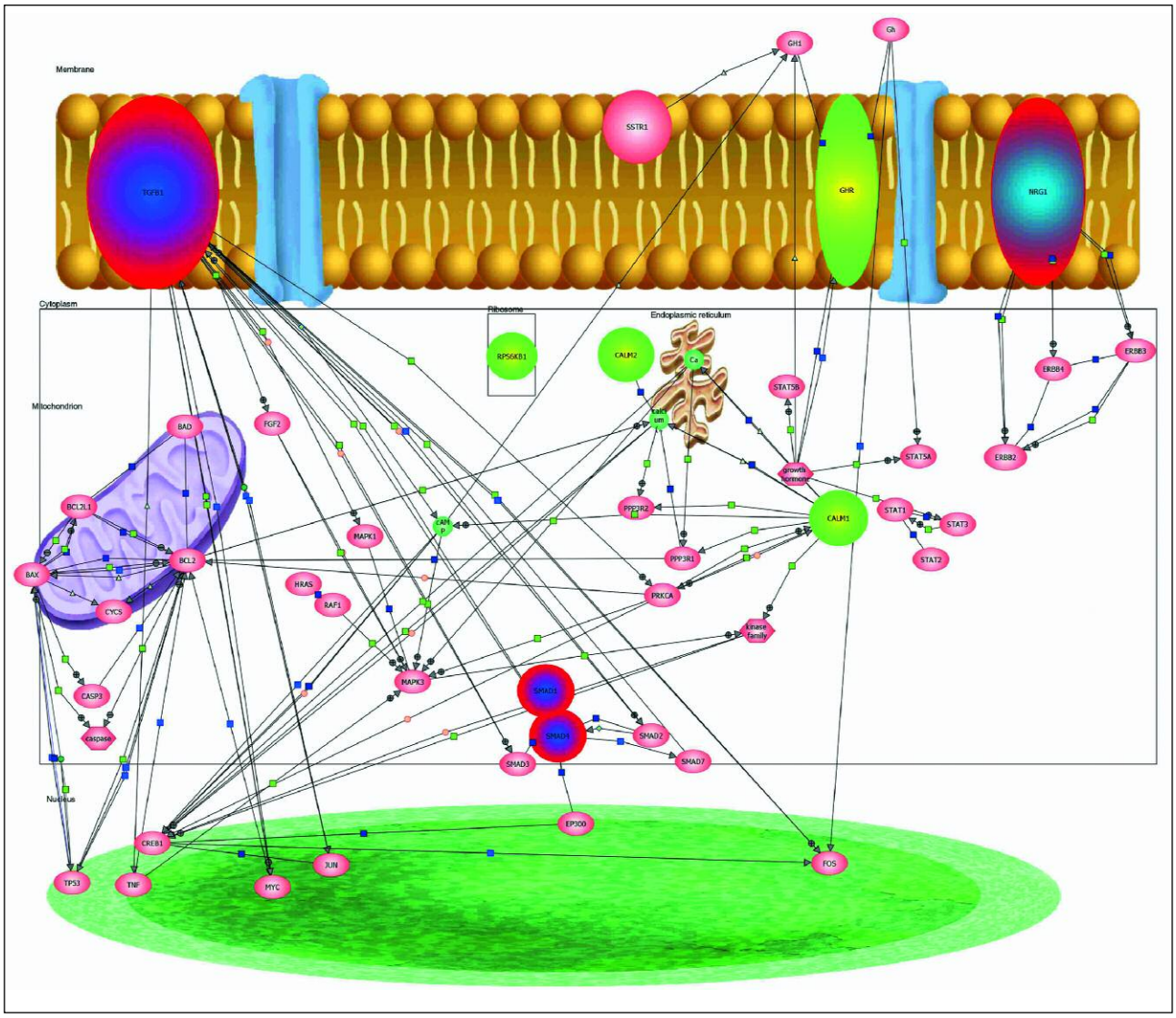

Fig. 6. Involvement of identified genes in cellular pathways. The scheme of the network was elaborated using Pathway Architect software (Stratagene, An Agilent Technologies Company, USA). The CMT-U27 and CMT-U309 transcripts were marked on the picture as green – yellow color, and blue – red, respectively.

GH expression in mammary tumors has been related to the increase and spreading of cell proliferation (18). It activates proteins of the STAT family (STAT 1, 2, 3, 5A and 5B). Using the MCF-7 human breast cancer cell line, it has been demonstrated autocrine GH-stimulated mammary carcinoma cell proliferation and decreased apoptosis rate (19). It has been suggested that forced expression of the hGH gene in immortalized human mammary epithelial cells increased proliferation, decreased apoptosis and resulted in oncogenic transformation (20). Moreover, autocrine hGH disrupted normal mammary architecture with luminal filling and deregulated proliferation in three-dimensional epithelial cell culture. Autocrine hGH utilized homeobox A1 to govern the transcriptional program required for autocrine hGH–stimulated oncogenic transformation of human mammary epithelial cells, including transcriptional up-regulation of c-Myc, cyclin D1, and Bcl-2 (21). Expression of *hGH* gene is associated with metastatic mammary carcinoma cells, suggesting functional contribution of autocrine hGH on metastatic process. Mukhina *et al*. (21) have demonstrated that autocrine hGH dramatically enhances spreading of mammary carcinoma cells on a collagen substrate. These observations suggest that autocrine production of hGH may have influence on the conversion of human mammary epithelial cells to a mesenchymal phenotype. It was demonstrated that in mammary carcinoma cells with epithelial morphology, autocrine production of hGH promotes mesenchymal cellular morphology, increasing cell migration and invasive behavior both *in vitro* and *in vivo*.

Table 1. Genes involved in the regulation of cell proliferation, adhesion and apoptosis which expression was higher (+) or lower (-) in CMT-U27 than in CMT-U309 canine cancer cells. The results are presented as means of fold in three separated experiments. Only these genes are shown, which expression differed similarly between examined cell lines in 3 separate experiments.

No.	Transcript ID	Gene name, Gene Symbol	Ontology	Fold
1.	NM_015071	Rho GTPase activating protein 26; ARHGAP26	G-protein modulator; Cell adhesion-mediated signaling	+ 6.15
2.	NM_005749	Transducer of ERBB2, 1; TOB1	Other miscellaneous function protein; Receptor protein serine/threonine kinase signaling pathway; MAPKKK cascade; JNK cascade; Cell cycle control; Cell proliferation and differentiation	+ 5.75
3.	NM_019006	Zinc finger, AN1-type domain 6; ZFAND6	Other signaling molecule; Other miscellaneous function protein; Signal transduction	+ 5.74
4.	NM_021163	RB-associated KRAB zinc finger; RBAK	KRAB box transcription factor; mRNA transcription regulation; Cell proliferation and differentiation	+ 4.46
5.	NM_194270	MORN repeat containing 2; MORN2	Other kinase; G-protein mediated signaling	+ 4.43
6.	NM_004664	Lin-7 homolog A (C. elegans); LIN7A	Cell adhesion molecule; Other cell junction protein, Cell adhesion; Other neuronal activity; Cell structure	+ 3.84
7.	NM_001489 NM_033334	Nuclear receptor subfamily 6, group A, member 1; NR6A1	Nuclear hormone receptor; Transcription factor; Nucleic acid binding; mRNA transcription regulation; Other intracellular signaling cascade; Spermatogenesis and motility; Cell proliferation and differentiation	+ 3.28
8.	NM_012090 NM_033044	Microtubule-actin crosslinking factor 1; MACF1	Other select calcium binding proteins; Cell cycle control	+ 3.21
9.	NM_003732	Eukaryotic translation initiation factor 4E binding protein 3; EIF4EBP3	Translation factor, Protein biosynthesis; Translational regulation; Cell proliferation and differentiation; Tumor suppressor	+ 3.06
10.	NM_032641	SplA/ryanodine receptor domain and SOCS box containing 2; SPSB2	Molecular function unclassified; Cell surface receptor mediated signal transduction; Intracellular signaling cascade; Homeostasis	+ 2.35
11.	NM_006888	Calmodulin 1 (phosphorylase kinase, delta); CALM1	Calmodulin related protein, Calcium mediated signaling; Cell cycle; Cell proliferation and differentiation; B cell activation->Calmodulin; Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction->Calmodulin; T cell activation->Calmodulin	+ 2.29
12.	NM_001743	Calmodulin 2 (phosphorylase kinase, delta); CALM2	Calmodulin related protein; Calcium mediated signaling; Cell cycle; Cell proliferation and differentiation; B cell activation->Calmodulin;;Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction->Calmodulin; T cell activation->Calmodulin	+ 2.29
13.	NM_005184	Calmodulin 3 (phosphorylase kinase, delta); CALM3	Calmodulin related protein Calcium mediated signaling; Cell cycle; Cell proliferation and differentiation; B cell activation->Calmodulin; Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction->Calmodulin; T cell activation->Calmodulin	+ 2.29
14.	NM_016009	SH3-domain GRB2-like endophilin B1; SH3GLB1	Molecular function unclassified; Neuronal activities; Apoptosis	+ 2.27
15.	NM_004071	CDC-like kinase 1; CLK1	Non-receptor serine/threonine protein kinase; Protein phosphorylation	+ 2.20
16.	NM_032027	TM2 domain containing 1; TM2D1	Other miscellaneous function protein; Neuronal activities; Induction of apoptosis	+ 2.15
17.	NM_013363	Procollagen C-endopeptidase enhancer 2; PCOLCE2	Other enzyme regulator; Other extracellular matrix; Cell adhesion	+ 2.07
18.	NM_000163	Growth hormone receptor; GHR	Other receptor; Cell surface receptor mediated signal transduction; Intracellular signaling cascade; Developmental processes	+ 2.04
19.	NM_021135 NM_001006932	Ribosomal protein S6 kinase, 90kDa, polypeptide 2; RPS6KA2	Non-receptor serine/threonine protein kinase, Protein phosphorylation; MAPKKK cascade; Neurogenesis; Skeletal development; Cell cycle control	+ 2.02
20.	NM_017707	Development and differentiation enhancing factor-like 1; DDEFL1	Nucleic acid binding; Other G-protein modulator; G-protein mediated signaling; Cell adhesion; Cell structure and motility	- 2.01
21.	NM_003692	Transmembrane protein with EGF-like and two follistatin-like domains 1; TMEFF1	Membrane-bound signaling molecule, Oncogenesis	- 2.14
22.	NM_018084	KIAA1212; KIAA1212	Kinase modulator Other receptor mediated signaling pathway; Other intracellular signaling cascade	- 2.66
23.	NM_033669 NM_033666 NM_133376 NM_033667 NM_033668 NM_002211	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12); ITGB1	Other receptor; Cell adhesion molecule, Cell adhesion-mediated signaling; Extracellular matrix protein-mediated signaling; Cell adhesion; Blood clotting; Cell motility	- 3.00
24.	NM_013960 NM_013957 NM_013956 NM_013964 NM_013961 NM_013958 NM_004495 NM_013959 NM_013962	Neuregulin 1; NRG1	Growth factor, Receptor protein tyrosine kinase signaling pathway; Ligand-mediated signaling; Neurogenesis; Cell proliferation and differentiation	- 3.10
25.	NM_001003688 NM_005900	SMAD family member 1; SMAD1	Other transcription factor, mRNA transcription regulation; Receptor protein serine/threonine kinase signaling pathway; Other intracellular signaling cascade; Ligand-mediated signaling; Developmental processes; Oncogenesis	- 3.13
26.	NM_001106	Activin A receptor, type IIB; ACVR2B	TGF-beta receptor; Serine/threonine protein kinase receptor; Protein kinase; Protein phosphorylation; Cytokine and chemokine mediated signaling pathway; Receptor protein serine/threonine kinase signaling pathway	- 4.23
27.	NM_018972 NM_001040875	Ganglioside-induced differentiation-associated protein 1; GDAP1	Other signaling molecule, Other intracellular signaling cascade; Other developmental process	- 4.35
28.	NM_003270	Tetraspanin 6; TSPAN6	Other cell adhesion molecule, Cell adhesion-mediated signaling; Cell adhesion; Other neuronal activity	- 5.09
29.	NM_021233 NM_058248	Deoxyribonuclease II beta; DNASE2B	Endodeoxyribonuclease; DNA degradation; Apoptotic processes	- 8.60

Conversely, the phenotype of slower proliferative CMT-U309 cells was more dependent on TGFβ and neuroregulin 1 pathways as well as function of adhesion – related molecules *e.g.* integrin beta 1 receptor and tertraspanin 6.

Transforming growth factor beta (TGF-β) engages a large family of signaling molecules, i.e.: the type I receptor (TβRI) and the type II receptor (TβRII), SMAD family transcriptional regulators (SMAD 1, 2, 3, 4, 7) and other molecules (22). It activates the MAPK cascade, CREB 1 and TNF. TGF-β is a multifunctional cytokine involved in several cellular processes (23, 24). It plays an essential role in cellular processes, from early embryonic development, cell growth, differentiation, motility, to the apoptosis. In nontransformed epithelial cells, the most studied TGF-β response is growth inhibition, but in many tumors of epithelial origin, cells become resistant to TGF-β-mediated growth inhibition. Our earlier studies proved antiproliferative and apoptotic properties of TGF-β1 in mouse (25, 26) and bovine (27-29) mammary epithelial cells. For this reason TGF-β1 is regarded as an important regulator of mammary gland remodeling (30). Effects of TGF-β1 are opposite in normal and neoplastic mammary epithelium. Transgenic mice that overexpress active TGF-β1 in mammary epithelium exhibit hypoplastic mammary glands that are resistant to oncogene- or carcinogen-induced mammary cancers (31, 32). Once tumors develop, TGF-β1 enhances tumor progression to a highly invasive spindle cell phenotype (33). Ha-Ras–induced mammary tumor cells secretes high levels of TGF-β and displays highly invasive characteristics *in vitro* and *in vivo* studies (34).

Neuregulins (NRGs) play a significant role in regulating cellular proliferation and differentiation *in vivo*. Four receptors tyrosine kinases of the ErbB family play essential roles in several physiological processes and have also been implicated in tumor generation and/or progression. NRG activates ErbB2, ErbB3, and ErbB4 receptors. It has been shown that the Erk5 mitogen-activated protein kinase (MAPK) pathway participates in NRG signal transduction (35). In MCF7 cells, NRG activated Erk5 in a time- and dose-dependent fashion. The action of NRG on Erk5 was dependent on the kinase activity of ErbB receptors but was independent of Ras. These results suggest that Erk5 may play a role in the regulation of cell proliferation by NRG receptors and indicate that constitutively active NRG receptors may induce proliferative responses in cancer cells through this MAPK pathway. It has been also shown that NRG affects the proliferation and differentiation of cultured mammary cells (36). NRG stimulates or inhibits proliferation of human mammary tumor cells which frequently overexpress erbB family receptors, while it stimulates proliferation and milk protein synthesis in mouse mammary epithelial cells.

One class of cell surface receptors that plays an important role in mediating cell-extracellular matrix (ECM) interactions is β1 integrin, a major contributor for growth factor receptor signaling. β1 integrins belong to a family of transmembrane receptors that transmit biomechanical cues that critically mediate cell-ECM interactions (37, 38).

β1 integrin is overexpressed in human breast carcinomas and has been shown to play a basic role in growth, apoptosis, invasion, and metastasis. In addition to its role in cancer progression, β1 integrin signaling plays a significant role in mediating resistance to cytotoxic chemotherapies by enhancing cell survival in hematologic malignancies, lung, and breast cancers (39-42). Inhibition of β1 integrin has also been shown to abrogate the formation of metastasis in gastric and breast cancer models (43-45). It has been shown that β1 integrin inhibition results in a significant loss of cancer cells, decrease in proliferation and increase in apoptosis, and a global change in the composition of residual colonies (46). In contrast, nonmalignant cells that formed tissue-like structures remained resistant. Moreover, these cancer cell–specific antiproliferative and pro-apoptotic effects were confirmed *in vivo* with no discernible toxicity to animals (47). It indicates that β1 integrin is a promising therapeutic target, and that it can be used to effectively distinguish malignant and normal tissue response to therapy.

Tetraspanins, or tetraspan proteins, are a large family of ubiquitously expressed membrane proteins that are implicated in a number of basic biological phenomena, including cell proliferation, cell migration, and tumor cell invasion. Although the biochemical function(s) of transpan proteins remains undefined, it had been proposed that the proteins may have an important role in the assembly of signaling complexes that also include other transmembrane proteins (48).

Our results of genes expression at the level of transcripts raise the intriguing possibility that a high growth rate and high anti-apoptotic potential in simple mammary carcinoma CMT-U27 cells is associated with enhanced expression of genes involved in Ca^{2+} signaling pathway and growth hormone cellular pathway as it was previously indicated in breast cancer cells. On the other hand the low–proliferative and pro-apoptotic phenotype of spindle tumor CMT-U309 cells is more dependent on TGFβ, neuroregulin 1 pathways and adhesion – related molecules integrin beta 1 and tertraspanin 6.

Our future study is directed towards:

1. extension of this kind of work to other canine mammary tumor cell lines isolated in our laboratory or others, which are different in proliferation rate and resistance to apoptosis;
2. examination of silencing effect of particular identified genes to show their effect on growth and apoptosis in canine mammary cancer cells.

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REFERENCES

1. Dorn CR, Taylor DON, Frye FL, Hibbard HH. Survey of animal neoplasms in Alemeda and Contra Costa Counties, California I. Methodology and description of cases. *J Natl Cancer Inst* 1968; 40: 295-305.
2. El Etreby MF, Muller-Peddinghaus R, Bhargava AC, Fath El bar MR, Graf KJ, Trautwein G. The role of the pituitary gland in spontaneous canine mammary carcinogenesis. *Vet Pathol* 1980; 17: 2-16.
3. McVean DW, Monlux AW, Anderson PS, Silverberg SL, Roszel JF. Frequency of canine and feline tumors in a defined population. *Vet Pathol* 1978; 15: 700-715.
4. Rijnberk A, Kooistra HS, Mol JA. Endocrine diseases in dogs and cats: similarities and differences with endocrine diseases in humans. *Growth Hormon IGF Res* 2003; 13: 158-164.
5. Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG. Multistep carcinogenesis of breast cancer and tumor heterogeneity. *J Mol Med* 1997; 75: 429-439.
6. Ingvarsson S. Molecular genetics of breast cancer progression. *Semin Cancer Biol* 1999; 9: 277-288.
7. Misdorp W, Else W, Hellmen E, Lipscomb TP. Histological classification of the mammary tumors of the dog and the cat. *World Health Organization International Histological Classification of Tumors of Domestic Animals*, AFIP, Washington, DC. 1999, 2nd series, vol. 7, pp 1-59.
8. Sorlie T, Perou CM, Tibshirani R, *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; 98: 10869-10874.
9. Hellmen E. Characterization of four in vitro established canine mammary carcinoma and one atypical benign mixed tumor cell lines. *In Vitro Cell Dev Biol* 1992; 5: 309-319.
10. Hellmen E, Moller M, Blankenstein MA, Andersson L, Westermark B. Expression of different phenotypes in cell lines from canine mammary spindle-cell tumours and osteosarcomas indicating a pluripotent mammary stem cell origin. *Breast Cancer Res Treat* 2000; 61: 197-210.
11. Darzynkiewicz Z, Robinson JP, Crissman HA. Methods In Cell Biology. Flow Cytometry 1994; 41: Part A.
12. Rao NAS, Wolferen van ME, Ham van den R *et al*. cDNA mocroarray profiles of canine mammary tumor cell lines reveal deregulated pathways pertaining to their phenotype. *Animal Genetics* 2008; 39: 333-345.
13. Oshlack A, Emsile D, Corcoran LM, Smyth GK. Normalization of boutique two-color microarrays with a high proportion of differentially expressed probes. *Genome Biol* 2007; 8: R2.
14. Park DS, Morris EJ, Greene LA, Geller HM. G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis. *J Neurosci* 1997; 17: 1256-1270.
15. Bhonde MR, Hanski ML, Notter M *et al.* Equivalent effect of DNA damage-induced apoptotic cell death or long-term cell cycle arrest on colon carcinoma cell proliferation and tumour growth. Oncogene 2006; 25: 165-175.
16. Rodriguez-Mora OG, LaHair MM, McCubrey JA, Franklin RA. Calcium/calmodulin-dependent kinase I and calcium/calmodulin-dependent kinase kinase participate in the control of cell cycle progression in MCF-7 human breast cancer cells. *Cancer Res*, 2005; 65: 5408-5416.
17. Biswas KB, Reddy PV, Pickard M, Makkad B, Pettit N, Pardee AB. Calmodulin is essential for estrogen receptor interaction with its motif and activation of responsive promoter. *J Biol Chem* 1998; 273: 33817-33824.
18. Mol JA, Garderen van E, Selman PJ, Wolfswinkel J, Rijnberk A, Rutteman GE. Growth hormone mRNA in mammary gland tumors in dogs and cats. *J Clin Invest* 1995; 95: 2028-2034.
19. Gil-Puig C, Blanco M, Garcia-Caballero T, Segura C, Perez-Fernardez R. Pit-1/GHF-1 and GH expression in the MCF-7 human breast adenocarcinoma cell line. *J Endocrinol* 2002; 173: 161-167.
20. Zhu T, Starling-Emerald B, Zhang X *et al*. Oncogenic transformation of human mammary epithelial cells by autocrine human growth hormone. *Cancer Res* 2005; 65: 317-324.
21. Mukhina S, Mertani HC, Guo K, Lee KO, Gluckman PD, Lobie PE. Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone. *Proc Natl Acad Sci USA* 2004; 101: 15166-15171.
22. Yue J, Mulder KM. Transforming growth factor-beta signal transduction in epithelial cells. *Pharmacol Ther* 2001; 91: 1-34.
23. Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998; 67: 753–791.
24. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001; 29: 117–129.
25. Kolek O, Gajkowska B, Godlewski MM, Motyl T. Molecular mechanism of TGF-β1-induced apoptosis in HC11 mouse mammary epithelial cells (MEC). *Cell Mol Biol* 2001; 47: 197-208.
26. Kolek O, Gajkowska B, Godlewski MM, Motyl T. Co-localization of apoptosis-regulating proteins in mouse mammary epithelial HC11 cells exposed to TGF-β1. *Eur J Cell Biol* 2003; 82: 303-312.
27. Kolek O, Gajkowska B, Godlewski MM, Motyl T. Antiproliferative and apoptotic effect of TGF-β1 in bovine mammary epithelial BME-UV1 cells. *Comp Biochem Physiol C* 2003; 134: 417-430.
28. Gajewska M, Motyl T. IGF binding protein mediate TGF-β1-induced apoptosis in bovine mammary epithelial BME-UV1 cells. *Comp Biochem Physiol C* 2004; 139: 65-75.
29. Gajewska M, Gajkowska B, Motyl T. Apoptosis and autophagy induced by TGF-β1 in bovine mammary

epithelial BME-UV1 cells. *J Physiol Pharmacol* 2005; 56: 143-157.
30. Zarzynska J, Gajkowska B, Wojewodzka U, Dymnicki E, Motyl, T. Apoptosis and autophagy in involuting bovine mammary gland is accompanied by up-regulation of TGF-β_1 and suppression of somatotropic pathway. *Polish J Vet Sci* 2007; 10: 1-9.
31. Jhappan C, Geiser AG, Kordon EC *et al*. Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J* 1993; 12 : 1835-1845.
32. Pierce DF, Gorska AE, Chytil A *et al*. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci USA* 1995; 92: 4254–4258.
33. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res*. 2000; 2: 125–132.
34. Muraoka RS, Dumont N, Ritter CA *et al*. Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002; 109: 1551-1559.
35. Esparis-Ogando A, Diaz-Rodriguez E, Montero JC, Yuste L, Crespo P, Pandiella A. Erk5 Participates in Neuregulin Signal Transduction and Is Constitutively Active in Breast Cancer Cells Overexpressing ErbB2. *Mol Cell Biol* 2002; 22: 270-285.
36. Riese DJ, Raaij van TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neuregulins is governed by complex interactions of the ErbB receptor family. *Mol Cell Biol*, 1995; 10: 5770-5776.
37. Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999; 285: 1028-1032.
38. White DE, Kurpios NA, Zuo D *et al*. Targeted disruption of h1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell* 2004; 6: 159-170.
39. Shaw LM. Integrin function in breast carcinoma progression. *J Mammary Gland Biol Neoplasia* 1999; 4: 367-376.
40. Zutter MM, Krigman HR, Santoro SA. Altered integrin expression in adenocarcinoma of the breast. Analysis by in situ hybridization. *Am J Pathol* 1993; 142: 1439-1448.
41. Aoudjit F, Vuori K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* 2001; 20: 4995-5004.
42. Lewis JM, Truong TN, Schwartz MA. Integrins regulate the apoptotic response to DNA damage through modulation of p53. *Proc Natl Acad Sci USA* 2002; 99: 3627-3632.
43. Sethi T, Rintoul RC, Moore SM *et al*. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. *Nat Med* 1999; 5: 662-668.
44. Kawamura T, Endo Y, Yonemura Y *et al*. Significance of integrin a2/h1 in peritoneal dissemination of a human gastric cancer xenograft model. *Int J Oncol* 2001; 18: 809-815.
45. Fujita S, Watanabe M, Kubota T *et al*. Alteration of expression in integrin h1-subunit correlates with invasion and metastasis in colorectal cancer. *Cancer Lett* 1995; 91: 145-149.
46. Elliott BE, Ekblom P, Pross H *et al*. Anti-h1 integrin IgG inhibits pulmonary macrometastasis and the size of micrometastases from a murine mammary carcinoma.*Cell Adhes Commun* 1994; 1: 319-332.
47. Park CC, Zhang H, Pallavicini M *et al.* B1 Integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Res* 2006; 66: 1525-1535.
48. Berditchevski F, Odintsova E. Characterization of integrin–tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J Cell Biol* 1999; 146: 477-492.

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