***REVIEW***

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# DNA microarrays – future in oncology research and therapy

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**Abstract:** DNA microarrays are a modern and powerful tool for investigating biological processes through the widespread analysis of genes from a particular cell, tissue, or organism. The completion of the human genome project and the development of microarray technologies have opened new opportunities for progress in cancer research. DNA microarrays analysis became a useful tool for the identification of molecular markers, as well as prognostic and diagnostic potential in different human cancers. The medical implications of the microarray technology are described in many publications, most of them as the result of studies on leukemia, lymphoma and breast cancer. There are also initial articles on the use of microarrays in veterinary oncology. Microarray analysis can be categorized into 3 groups of studies: 1) transcriptomic profile of different tumour types to find characteristic genes for each tumour type, and the genes responsible for malignancy; 2) describing the disease and its clinical outcome; and 3) identifying therapeutic targets and new markers useful in cancer diagnosis and treatment.

**Key words:** DNA microarray, oncology, genomics, gene expression, canine mammary cancer

## INTRODUCTION

DNA microarrays are a modern and powerful tool for investigating biological processes through the widespread analysis of genes from a particular cell, tissue, or organism. In this system, multiple probes (DNA or oligonucleotides) are spotted onto glass slides. Each spot has a unique sequence which is different from the others in the array, and hybridize only to its complementary strand. The chip can contain from a few thousand to more than 1,000,000 spots. The principles of the technique rely on the specific hybridization between the probes on the chips and the DNA/RNA sample, and further comparing the intensity of the light emission of control and test samples [1, 2]. Generally, DNA microarrays are used for the large-scale study of gene expression levels, called 'transcriptional profiling'. This method may be used to find genes that are differentially expressed in examined tissues compared to the control, in the hope of finding new cancer markers, markers of clinical outcome, and targets for anti-cancer therapy [3].

During pathological conditions, the cellular processes are changed and dynamically regulated through specific changes in gene expression. The expression data can be used as a correlate of a particular cell phenotype. Such defined and specific profiles of gene expression are a kind of molecular signature, which could be useful to identify certain clinical conditions, classes, or phases of diseases. This novel method is the basis for the recent generation of diagnosis tests developed in the last decade. It could facilitate premature and precise identification of different diseases, such as cancer, and provide the basis for a more efficient therapy. In addition, molecular characterization may provide valuable prognostic information, since tumours that are morphologically similar, may have different molecular characteristics associated with the clinical outcome of the disease. Microarrays are also a modern tool for learning about cancer biology, and may be used for cancer classification to precisely sort cancer into various subgroups, which is not possible with other methods [4].

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**Microarray analysis of human tumour.** The completion of the human genome project and the development of microarray technologies have opened new opportunities for progress in cancer research. DNA microarrays analysis became a useful tool for the identification of molecular markers, as well as having prognostic and diagnostic potential in different human cancers. The medical implications of microarray technology have been described in many publications in recent years. There are great expectations from this new technology in oncology and pathology and their consequence in clinical studies.

In general, microarray analysis can be categorized into 3 groups of studies:

1) defining transcriptomic profile of different tumour types that identify characteristic genes for each tumour type, and the genes responsible for malignancy;
2) describing disease and clinical outcome;
3) identifying therapeutic targets and new markers, that are useful in cancer diagnosis and treatment [3].

**Transcriptomic profile of different tumours as a way to new classification.** In the past 30 years, most researchers focused on identifying the predictive and prognostic value of different molecules that are active in neoplasm. Those clinical studies were made possible by the development of new methods, such as Northern blotting, Western blotting, hybridization *in situ,* and PCR. The general aim of each study was to find and explore one molecule responsible for the oncogenesis. Unfortunately, the association of new markers and the clinical outcome was very often not clear and weak [5]. In recent years, almost 1,000 articles have been written about new prognostic molecules. Their role is very often

incompletely understood, or the important molecules interact in great biological pathways, the understanding of which is a key point for finding groups of cancer markers. The promise of microarray technology is in assessing the activity of large number of genes. The complex genes expression profile may be a source of information about characteristic pathways in oncogenesis.

**Leukemia.** The goal of one of the earliest large-scale microarray gene expression profiling was to identifying disease subsets to distinguish acute myelogenous leukemias (AML) from acute lymphocytic leukemias (ALL) [3]. This study was made with 38 bone marrow samples collected from patients with acute leukemia, and led to the identification of 1,100 genes correlated with the AML-ALL class. Further studies correctly categorized 29 of 34 unknown acute leukemias samples into correct categories based on the expression profile. In this study, ALL subtypes were correctly identified as originating from the B-cell line or T-cell line, which demonstrates the potential of this approach for tumour classification. AML and ALL are derived from distinct cellular precursors with different genes expression that distinguish these two cancers. More related tumours might be difficult to distinguish using this method. Such a study requires prior biologic knowledge of AML and ALL to make sense of the observed clusters. Armstrong et al. [6] attempted to establish the nature of mixed-lineage leukemia, a leukemia subset with an unfavourable prognosis, defined by a chromosomal translocation involving the mixed-lineage leukemia gene. The use of microarrays allows finding molecular markers (such as tyrosine kinase receptor FLT3), the expression of which differed in mixed lineage leukemia to ALL and AML. The obtained results suggest new strategies for molecularly targeted treatment in this type of cancer.

**Lymphoma.** Alizadeh et al. [7] used gene expression profiling to identify subclasses of a diffuse large B-cell lymphomas (DLBCL). These studies largely segregated normal, DLBCL, follicular lymphoma (FL), and lymphocytic leukemia (CLL) samples from each other based on their gene expression profiles.

Furthermore, analysis of the gene expression profiles revealed 2 subtypes within DLBCL cases [3]. These results suggest that lymphomas, currently diagnosed as DLBCL, may actually represent 2 different diseases. Further studies (Kaplan-Meier analysis) showed that patients with the 2 DLBCL subtypes had statistically significant differences in overall survival [3]. These studies demonstrate the role of the DNA microarray tool to distinguish unrecognized disease subtypes of distinct origin and clinical outcomes.

**Melanoma.** Bittner et al. [8] studied malignant melanoma with no molecularly defined subsets. Cluster analysis defined 2 putative subsets, and was able to define marker genes that were differentially expressed between these 2 subsets. One of these genes has a higher expression in more aggressive uveal melanoma cell line. This suggests that primary cancers might be less or more invasive depending on the subset to which they belong. They demonstrated that belonging to each subset was associated with different tissue invasion potential. This study demonstrates that microarray research is possible in the absence of prior knowledge, and that such findings can be validated using cancer cell line models.

**Breast cancer.** Breast cancer management is always an actual subject of clinical studies due to its extensive heterogeneity. In the literature there are many results of microarrays studies to identify breast cancer profile on the basis of gene expression. In the recent studies, many classifications of breast cancer were established. Perou et al. [9] studied genes expression in breast tumours before and after doxorubicin treatment. The tumours were segregated into 4 classes, based upon their expression patterns, with low or absent ER expression in each one of them:

1) An ERBB2 group expressing high levels of ERBB2 gene and other genes involved in the same pathway;
2) 'Normal-like' breast cluster expressing many genes characteristic of normal breast epithelium;
3) 'Basal-cell-like' cluster expressing genes characteristic of basal breast cells, in particular with respect to keratin 5 and 17;
4) 'Luminal-cell-cluster' characteristised by a relatively high expression of many genes, including ER, LIV-1 protein, GATA-binding protein 3, prolactin receptor and carnitine palmitoyl-transferase II.

This was the first large-scale microarray study, the results of which were the basis for new cancer classification. In a similar study, basal, luminal and mesenchymal subtypes of human breast cancer cell lines were identified [10].

Sorlie et al. [11] continued this study on larger number of samples and found that this gene expression classification can be used as a prognostic marker with respect to relapse-free survival in a group of patients undergoing uniform therapy. The results were confirmed by further studies, analyzing breast cancer patients with known clinical outcome, despite differences in patient population, treatment modules and technology employed.

In another study, the authors explored the expression profiling of 7 spontaneous and 15 hereditary breast adenocarcinomas with mutations in either *BRCA1* or *BRCA2*. They identified many differentially expressed genes between *BRCA1*-mutated and *BRCA2*-mutated tumours, and used these genes to accurately categorize these samples [12].

The most recent study examined the gene expression profile of primary tumours and their metastases [13]. Results of this study showed the existence of five major different molecular subtypes in breast cancer. They have a different ability to metastasize to distant organs, and have similar biological features and pathways to their distant metastatic site. The authors identified many differentially expressed genes. Several of them were characteristic to the subtype and the preferentially metastased site. Genes involved in WNT signaling were up-regulated in the basal and brain-specific relapse subtype, and down-regulated in the luminal and bone-specific relapse subtype.

**Prostate cancer.** Examination of expression profiling can be a tool for differentiating prostate cancer from benign hyperplasia (BPH) [14]. In one study, the clear differences between normal benign prostate tissue, BPH, localized prostate cancer, and metastatic prostate cancer based on RNA expression profiles, were established [15].

**Ovarian cancer.** Microarray experiments were performed with samples of normal ovaries, benign ovarian tumour and stage I and III of ovarian cancer [16]. Comparing benign ovarian tumour (pre-cancerous stage) to later stages of ovarian

cancer facilitated the identifaction of 46 genes overly expressed in early-staged cancer. The authors identified over-expressed genes at all stages of ovarian tumour/cancer. The over-expressed genes might be alternative candidates for further studies with developing early diagnostic markers. The authors of a different study [17] found a few differences in the gene signature of stage III primary ovarian adenocarcinomas and their corresponding omental metastases. Various studies have shown that metastatic expression profiles of primary tumors are predictive of subsequent metastasis. The authors found that in the samples of stage III ovarian adenocarcinomas, a number of predictive genes are over-expressed in primary, similarly or higher than in omental metastases. This supports the thesis that most tumour cells in advanced primary ovarian lesions have acquired the genetic signature enabling invasion and metastasis.

**Cervical cancer.** Cervical cells obtained with cytobrushes from women with high risk human papillomavirus-positive squamous cell carcinoma and papillomavirus-negative women revealed a different gene expression pattern, and allowed to distinguish between normal cervical epithelia and a squamous carcinoma [18]. Chung et al. [19] examined genes involved in radiation response of the cervical cancer (comparing radiation-sensitive and radiation-resistant squamous cell carcinoma). This study showed that ICAM3 is the main up-regulated factor in radiation-resistant tumours. In another study [20], the TACSTD1 and CEACAM5 were identified as poor prognostic factors in adenocarcinoma and adenosquamous cell carcinoma patients.

**Colon cancer.** Cancerous and noncancerous colon tissues using DNA microarrrays was profiled [21]. A global hierarchical study was able to distinguish between clinically relevant subgroups, normal *versus* cancer tissues, and metastatic *versus* nonmetastatic tumours. Analyses facilitated the identification of groups of differently expressed genes between normal tissue and tumour tissue, tumours associated with lymph node invasion or not, and tumours from the right or left colon. A similar examination identified a group of genes that divided patients with significantly different 5-year survival. Highly expressed genes were associated with various cellular processes. In other study, colorectal primary cancers and their corresponding metastases were analyzed. In nearly half of the experiments, a specific group of genes was identified which significantly differed between the metastasis and primary tumour [22].

**Microarrays as a prognostic tool to clinical outcome.** It is difficult to predict if chemotherapy will be effective for individual tumour cases. It is probable that in the future DNA microarrays will offer the possibility to use the knowledge about tumour expression profiles to predict chemosensitivity. The knowledge of specific target-genes expression and the knowledge of genes products involved in specific cellular pathways is a great hope and has a great future in anti-cancer therapy.

To demonstrate the utility of using pretreatment gene expression profiling to determine prognosis, Alizadeh et al. [7] using DNA microarrays, pursued a systematic characterization of gene expression in B-cell malignancies. They showed differences in gene expression among the tumours of DLBCL patients, especially involved in tumour proliferation rate and differentiation of the state of the tumour. Patients with germinal centre B-like DLBCL had a significantly better life-expectancy than those with activated B-like DLBCL. This work was the first study to demonstrate expression-based correlation of outcome and life-expectancy. Another study [23] demonstrated that the use of microarray enables to successfully distinguish adenocarcinomas originating from different tissues (the study was conducted with colon, lung, and ovary adenocarcinomas). The analysis of a comprehensive gene expression profile of different primary adenocarcinomas using microarrays was similar to histopathologic assessment of primary sites of 152 of the 154 cases.

These findings confirm that expression profiles could be useful in the clinical management of patients with metastatic cancer of unknown origin.

**Microarrays as a promising tool in cancer therapy.** A major goal is the use of expression profiles experiments to reflect the laboratory studies in clinical evaluation of chemotherapy drugs. New drugs are traditionally examined in clinically defined cancer types with no correlation with mechanisms of transformation [4]. However, a common problem is the low response rate seen in early clinical studies. The great hope in oncology and pharmacology is the knowledge of gene expression profiling with clinical studies of new agents. The next step of microarray studies should be the identification of correlation between gene expression and drug responsiveness and resistance in individual cases.

The latest studies show that radiation-induced gene expression profile provides a database for the analysis of cellular radio-response, and is useful to identify differences in the regulation of tumour and normal cell radiosensitivity [24]. Other studies describe drug-induced gene expressions. These markers might be used to prospectively identify populations of patients likely to respond to the agent.

**Microarrays in veterinary oncology.** There are no reports of similar studies using canine tumours. With the completion of the canine genome sequencing, a powerful research resource has been made available. Dr Jan A. Mol from Utrecht University in The Netherlands developed a dog-specific DNA microarray and used it to characterize canine mammary cell lines [25]. This study was the first microarray assay of a dog mammary tumour cell line, and successfully identified deregulated pathways pertaining to cell line phenotype similar to published human breast cancer data sets. Rao et al. [25] characterized 3 well-established canine mammary tumour cell lines originating from totally different types of primary tumours with distinct biological behaviours (osteosarcoma, benign mixed tumour and adenocarcinoma). Comparing the pathway profiles of the canine mammary cell line gene set, the human breast cancer tissue gene set and the human breast cancer cell line gene set, they identified 4 of the 5 major signaling pathways as common across the datasets. This study identified Wnt signaling, integrin signaling, cell cycle, alternative complement cascade and cytokine/Rho-GTPase signaling, as the main pathways differentially regulated in canine mammary gland tumour cell lines. These characterized canine mammary tumour cell lines with their unique gene expression and pathway profiles are valuable tools to prioritize biological pathways for a detailed study. In the light of the high incidence of mammary cancer in dogs, the cell lines isolated from mammary tumours are a valuable tool for developing

and testing new pathway-specific cancer therapeutics. These results are also the basis for further characterization of canine mammary carcinomas.

The aim of the next study was to identify the genes responsible for the high growth rate and anti-apoptotic potential in canine mammary cancer cells [26]. The comparison of 2 cell lines: simple carcinoma (CMT-U27) and spindle cell tumour (CMT-U309), revealed that the growth rate was significantly higher in the CMT-U27 cell line, manifested with 2-fold shorter cell cycle (53.4 h) and higher G2M/G1+S ratio than in CMT-U309 cell line. The anti-apoptotic potential was also significantly higher in the CMT-U27 cell line, manifested with a higher expression of Bcl-2 (the main anti- apoptotic protein), as well as lower spontaneous and camptothecin-induced apoptosis [27]. Comparison of transcriptomes revealed 29 genes involved in cell proliferation, adhesion and apoptosis, the expression of which was significantly different in the examined cell lines. Among the overexpressed genes in CMT-U27, 14 genes were associated with the regulation of cell proliferation, 3 genes involved in cell adhesion, and 2 genes associated with apoptosis. 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).

Our results raise an intriguing possibility: that high growth rate and high anti-apoptotic potential in simple mammary carcinoma CMT-U27 cells is associated with enhanced expression of genes involved in the $Ca^{2+}$ signaling pathway and the growth hormone cellular pathway, as previously indicated in breast cancer cells. On the other hand, the low-proliferative and pro-apoptotic phenotype of spindle cell

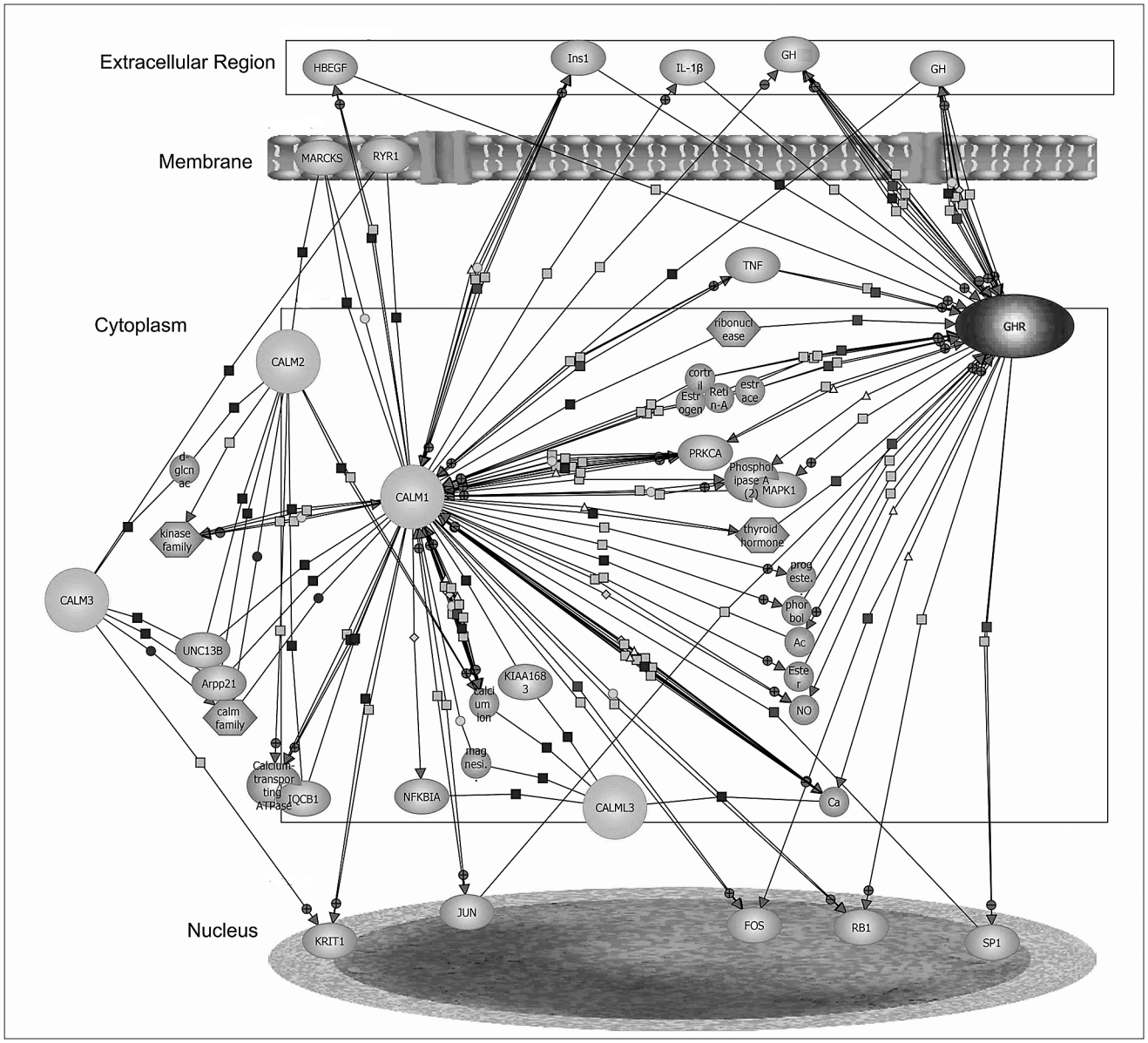


**Figure 1** Involvement of GHR and calmodulins in cellular pathways of mammary cancer cells with a high proliferative and anti-apoptotic potential. It has been shown interactions between growth hormone receptor (GHR) and calcium intracellular receptors – calmodulins 1, 2 and 3 (CALM1, CALM2, CALM3), other calmodulin family proteins, different calcium transporters and kinases (PRKCA – protein kinase C α, MAPK – mitogen activated protein kinase). This pathway interacts with hormones (GH – growth hormone, insulin, thyroid hormones, estrogen, progeresteone), cytokines (IL-1β, TNF-α) and transcription factors (NF-κB, JUN, FOS, SP1).
Arrows with symbol (+) – indicate the stimulation process, arrows with symbol (-) – indicate the inhibition process.
Additional symbols: HBEGF: heparin-binding EGF-like growth factor; MARCKS: myristoylated alanine-rich protein kinase C substrate; RYR1: ryanodine receptor 1; UNC13B: unc-13 homolog B; ARPP-21: cyclic AMP-regulated phosphoprotein; IQCB1: IQ motif containing B1; KRIT1: ankyrin repeat containing.
The scheme of the network was elaborated using Pathway Architect software (Stratagene, An Agilent Technologies Company, USA).

tumour CMT-U309 cells is more dependent on TGFβ1 and neuroregulin 1 pathways and adhesion-related molecules: integrin β1 and tetraspanin6. To confirm the microarray results, Western blot analysis was performed, which showed a significantly higher level of calmodulin [1-3] proteins in rapidly proliferative simple carcinoma CMT-U27 cells in comparison with spindle-cell tumour CMT-U309 cell line (unpublished). The possible interactions between the GH signaling pathway and calmodulins in highly proliferative canine simple carcinoma cells are shown on Fig. 1. The links in their pathways should be considered as a putative targets in cancer therapy.

Cell lines historically served as the primary experimental model system for exploration of tumour cell biology and pharmacology; however, their ability to accurately reflect the phenotype and genotype of the parental histology remains questionable, given the prevalence of documented cell line-specific cytogenetic changes. This is the reason for our next study [27] to compare genes expression in canine mammary tumour tissue to cell lines derived from those tumours using cDNA microarrays. Mammary tumours of 2 different origins were used: epithelial – adenocarcinoma and mesenchymal-chondrosarcoma and their primary cell lines isolated in our laboratory. It was found that cell culture gene expression profiles closely resembled those of their corresponding *in vivo* tumour. In adenocarcinoma and chondrosarcoma, only 6.0% and 2.7% of genes, respectively, showed significant expression difference. The most significant differences between tumour tissue and cellular transcriptome concerned genes involved in protein metabolism and modification, signal transduction and nucleotide, nucleoside and nucleic acid metabolism. These findings are similar to previously described studies performed on different types of tissue and cell lines where the differences between genes expression were not significant. Jessen et al. [28] and Perkins et al. [29] described that the similarity of liver genes expression in cell culture and tissue was 80% and 88%, respectively. Ertel et al. [30] showed a similarity in the expression of the genes in cancer tissue and derived cell culture. These studies with tumour tissue and the tumour cell lines of their origin confirmed that cell cultures can reflect *in vivo* tissue and can be used for investigation *in vitro* genomic alteration in cancers. Analyzing the list of up/down-regulated genes in cell culture, in comparison to adenocarcinoma tissue, we found genes involved in the same cellular pathways, e.g. Wnt and p53. These findings are related to the previously described studies, showing that Wnt genes are over-expressed in canine mammary adenocarcinoma cell line and in canine mammary gland tumours [25]. Apart from Wnt and p53 pathways, we found genes involved in Alzheimer disease, which is a quite typical pathway observed in human breast cancer. Alzheimer's disease signaling in the human breast cancer gene set was identified among the top 5 pathways.

Rao et al. [31] investigated the altered gene expressions in progestin-induced canine mammary hyperplasia (CMH) and in spontaneous canine mammary tumours (CMC). Gene expression profiles of CMH and CMC were compared to canine mammary tissue from healthy control dogs. This study identified altered expression of genes involved in important biological processes, such as tumour development and progression. Gene expression profile of canine mammary hyperplasia was dominated by altered expression of genes involved in cell proliferation and cell adhesion/motility, which are the main biological process required for the cell homeostasis. Deregulation of these processes may lead to cancer. Among 13 differentially expressed genes related with cell proliferation, all up-regulated genes were stimulators of cell proliferation or inhibitors of apoptosis, and the down-regulated genes were growth inhibitors or positive regulators. Pathway analysis of the canine mammary cancer profile identified a significant number of genes involved in integrin signaling, followed by genes involved in inflammation-mediated by chemokine and cytokines, and Wnt signaling. These results are comparable to the earlier observation in canine mammary cell lines, and in human breast cancer and cell line pathway profiles. CMH gene expression profiles indicate a strong progestin-induced cell proliferation. Alterations in the expression pattern of transcription factors, genes involved in maintaining DNA integrity and cell motility, may also indicate early stages of malignant transformation. It is important to state that benign hyperplasia is an important risk factor for breast cancer. The CMC gene expression profile indicated a more pronounced expression of genes involved in malignant transformation (cytoskeletal components/ECM/cell motility), in addition to amplified expression of many proliferation stimulating genes. The gene expression data of CMH shows the tumourigenic effects of progestins in canine mammary gland upon prolonged exposure.

## CONCLUSIONS

Studying the biological role of genes is the main goal of functional genomics. In medicine, this type of knowledge is the basis for an understanding of mammalian diseases. Related advances in technology, such as DNA microarrays, allow genome large-scale analysis of gene expression in an organism. Cancer expression studies have examined a relatively small number of clinical samples because microarray technology is a relatively new method, and there has not been sufficient time to reproduce many findings in this innovative field. Apart from its influence on the basic research field, microarray technology is an important factor for initiating the expansion of the biotechnological and pharmaceutical industries, with the promise of improving cancer diagnostics and treatment.

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