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*Original article*

# Gene expression pattern in canine mammary osteosarcoma

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## Abstract

Canine mammary sarcomas are usually very aggressive and easily metastasize. Unfortunately the biology of this type of tumor is not well known because they are a very rare type of tumors.

The aim of this study was to find differences in gene expression patterns in canine mammary osteosarcomas (malignant) versus osteomas (benign) using DNA microarrays. Our microarray experiment showed that 11 genes were up-regulated in osteosarcoma in comparison to osteoma whereas 36 genes were down-regulated. Among the up-regulated genes were: PDK1, EXT1, and EIF4H which are involved in AKT/PI3K and GLI/Hedgehog pathways. These genes play an important role in cell biology (cancer cell proliferation) and may be essential in osteosarcoma formation and development. Analyzing the down-regulated genes, the most interesting seemed to be HSPB8 and SEPP1. HSPB8 is a small heat shock protein that plays an important role in cell cycle regulation, apoptosis, and breast carcinogenesis. Also SEPP1 may play a role in carcinogenesis, as its down-regulation may induce oxidative stress possibly resulting in carcinogenesis.

The preliminary results of the present study indicate that the up-regulation of three genes EXT1, EIF4H, and PDK1 may play an essential role in osteosarcoma formation, development and proliferation. In our opinion the cross-talk between GLI/Hedgehog and PI3K/AKT pathways may be a key factor to increase tumor proliferation and malignancy.

**Key words**: canine mammary osteosarcoma, gene expression, transcriptome, microarray

## Introduction

Osteosarcoma is the most frequently diagnosed and reported canine bone tumor (Loukopoulos et al. 2006) and it is considered as one of the most malignant and aggressive tumors. On the other hand, mammary neoplasms of mesenchymal origin (e.g. osteosarcomas, fibrosarcomas, chondrosarcomas) are rare types of tumors in the bitch (only about 5-8%) and extremely rare in humans (just a few case reports are

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available), which limits possibilities of their study and precise characterization (Hellmen et al. 1993, Sobczak-Filipiak et al. 2002). The origin of mesenchymal tumors in the mammary gland, especially the origin of cartilage and bone tissue is questioned. Various cells have been suggested as the cells of origin, including myoepithelium (Monteros et al. 2005) and pluripotent stem cells (Gartner et al. 1999, Hellmen et al. 2000). These alternatives were also considered in human breast cancer research (Leibl and Moinfar 2006, Polyak 2007).

Canine mammary sarcomas are usually very aggressive and easily metastasize. The biology of the carcinomas and sarcomas shows important differences not only in behavior but also in morphology (Wensman et al. 2009). That is why there is a big necessity to gain knowledge about their physiology.

The completely new possibilities to investigate biological processes of cancer cells arose due to DNA microarrays. They are used in a large-scale studies of gene expression, called: "transcriptional profiling". Up-to-date there are just few papers describing gene expression profiles in canine mammary cancer (Pawłowski et al. 2009, Król et al. 2009, Rao et al. 2009, Król et al. 2010a,b) but to the best of our knowledge, only one describes transcriptional profile of canine mammary tumors of mesenchymal origin (Wensman et al. 2009). The authors found that sarcomas expressed a higher number of homeobox and BMP genes than carcinomas, indicating that sarcomas probably have a larger fraction of tumor stem cells than carcinomas. These findings support the theory that mesenchymal tumors arise from stem cells.

The aim of this study was to find gene expression patterns in canine mammary osteosarcomas using DNA microarrays. Because in the previous paper (Wensman et al. 2009) the gene expression in various sarcomas versus carcinomas was assessed, now our experimental model was focused on genes and their cellular pathways that discriminate malignant canine mammary osteosarcoma from benign osteoma. The preliminary results of this study indicate that there is up-regulation of 11 genes and down-regulation of 36 genes in osteosarcoma versus osteoma. Few of them suspects to be especially related to cancer cell proliferation and formation of malignant osteosarcoma.

## Materials and Methods

### Tumor samples

Tumor samples of two canine mammary osteosarcomas and two osteomas (as a control) used in the microarray experiment were obtained from patients subjected to surgery. One osteosarcoma sample was obtained from 8-years-old dachshund female (no neoplastic cells found in lymph nodes), the tumor was diagnosed 8 months before the surgery. The other osteosarcoma sample was obtained from 10-years-old german shepherd female (neoplastic cells found in lymph nodes) 10 months after the diagnosis. One osteoma sample was obtained from mixed-breed female at the age of 6 years (no neoplastic cells found in lymph nodes), the mastectomy was performed 5 months after the diagnosis, whereas the other osteoma sample was obtained from 15-years-old yorkshire terrier (no neoplastic cells found in lymph nodes) 2 months after the diagnosis. The half of each tumor sample was snap-frozen in liquid nitrogen and stored at -80ºC and remaining half of the sample was embedded in paraffin and dedicated for histopathological examination. Four mm sections from paraffin blocks were fixed on glass slides, stained with hematoxylin – eosin and examined according to the WHO classification by certified pathologists (Fig. 1). Pictures were taken using Olympus microscop BX60.

### RNA Isolation, validation, amplification, reverse transcription, labeling and hybridization

Total RNA from frozen tumors was isolated using RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of RNA was examined using Bioanalyzer (Agilent Technologies, USA). The Quick Amp Labeling Kit (Agilent, USA) was used to amplify and label target RNA to generate complementary RNA (cRNA) for oligo microarrays used in gene expression profiling and other downstream analyses. Each sample was examined in dye-swap. The hybridization was performed with canine-specific AMADID Release GE 4x44K microarrays (Agilent, USA) using Gene Expression Hybridization Kit (Agilent, USA) according to the manufacture's protocol.

### Signal detection, quantification and analysis

Acquisition and analysis of hybridization intensities were performed using DNA microarray scanner (Agilent, USA). The Agilent DNA microarray scanner is a 48-slide scanning system enabled by SureScan High-Resolution Technology capable of scanning with 2, 3, 5 or 10 micron resolution that can read 1 x 3" glass slide microarrays and seamlessly extract features from them using Agilent's Feature Extraction Software with normalization and robust statistical analyses. Results were statistically analyzed using Future Extraction and Gene Spring software (Agilent, USA). The unpaired t-test with Benjamin-Hochberg FDR < 5% (false discovery rate) correction was applied

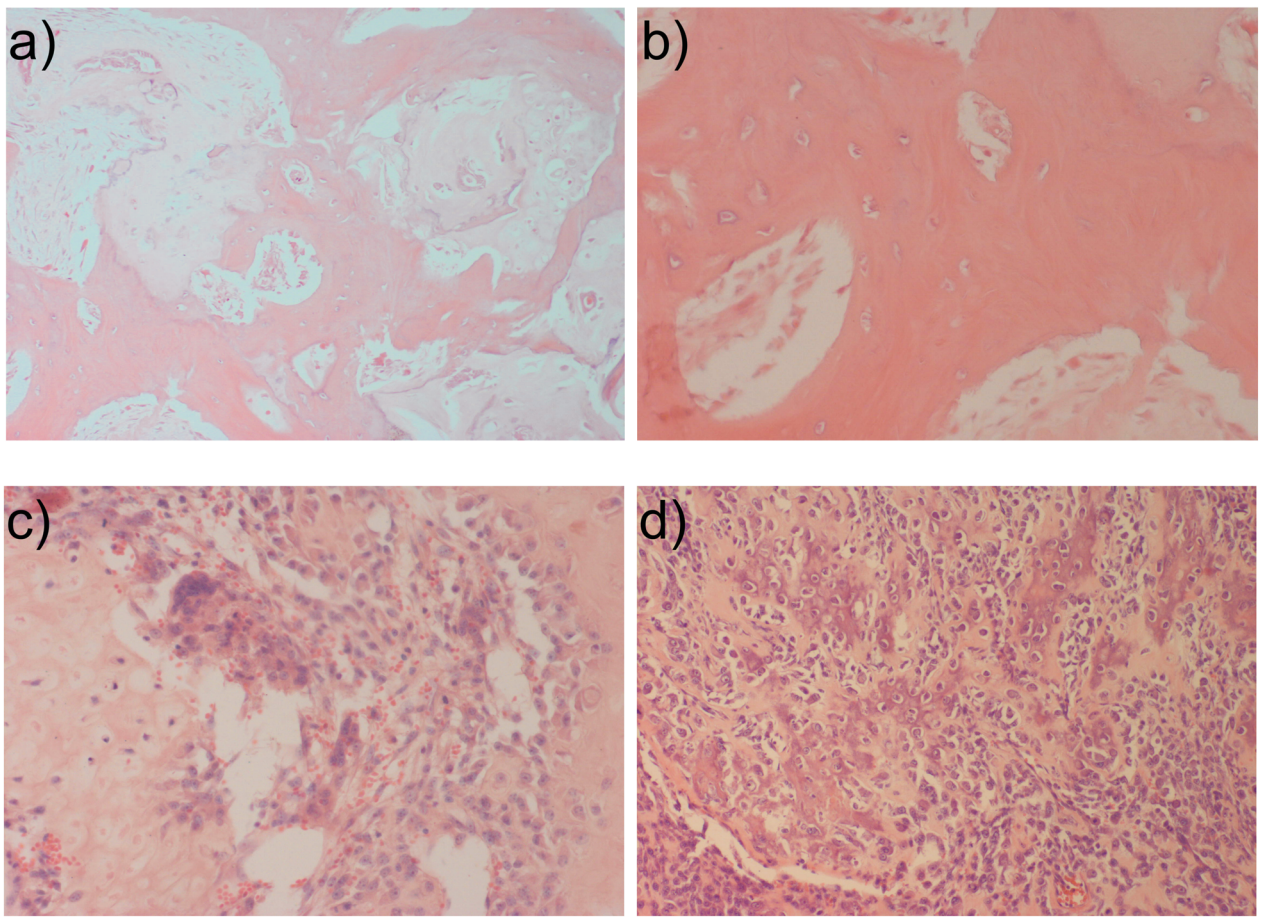


Fig. 1. The pictures of canine mammary osteomas (a and b) and osteosarcomas (c and d) that were subjected to the microarray analysis. The paraffin sections were stained with hematoxylin – eosin and examined according to the WHO classification by certified pathologists. The pictures were taken using Olympus BX60 microscope (x200).

Table 1. Primers used in this study. The primers were designed using PRIMER3 sequences. HPRT and RPS19 genes were used as non-regulated reference genes for normalization of target gene expression (Brinkhof et al. 2006, Etschmann et al. 2006).

| Target gene | Forward Primer | Reverse primer | Optimum annealing temp. (°C) | Optimum annealing time (sec) |
|---|---|---|---|---|
| *hprt* | AGCTTGCTGGTGAAAAGGAC | TTATAGTCAAGGGCATATCC | 59 | 5 |
| *rps19* | CCTTCCTCAAAAAGTCTGGG | GTTCTCATCGTAGGGAGCAAG | 61 | 10 |
| *rad54l2* | GTGTTCAGCCAGAGCCTTTC | ATAGCCAGGTGGTGAGGTTG | 62 | 10 |
| *ext1* | TGCTGGTATTCAAGGGGAAG | ACAGCGAGAATCCTTGTGCT | 57 | 12 |
| *eif4h* | CTCCAACTGAAGCCTCGAAC | AGGCTGTGGTCTCGAGCTAA | 59 | 10 |
| *pdk1* | ATGGAACACCATGCTGACAA | ATGGGCAATCCATAACCAAA | 57 | 10 |
| *sf3b* | GAGAACAACGGTCTCGCTTC | GTGTCAGACAGGTCCCCAGT | 58 | 10 |
| *hspb8* | GTGGCATCGTTTCCAAGAAT | ATGGTGAGTAAGGGGGAACC | 57 | 6 |
| *camk2g* | CTCGGAACTGAGGGACTGAG | CATCAGCTTTGTAGCGGACA | 60 | 10 |

(with $p$ value cut-off < 0.05). For the further analysis we chose only genes which expression changed at least 1.5 fold in each of examined slides. Gene function was identified using the Gene Spring, NCBI database, PANTHER pathway analysis software (Mi et al. 2005) and Pathway Studio software (Agilent, USA).

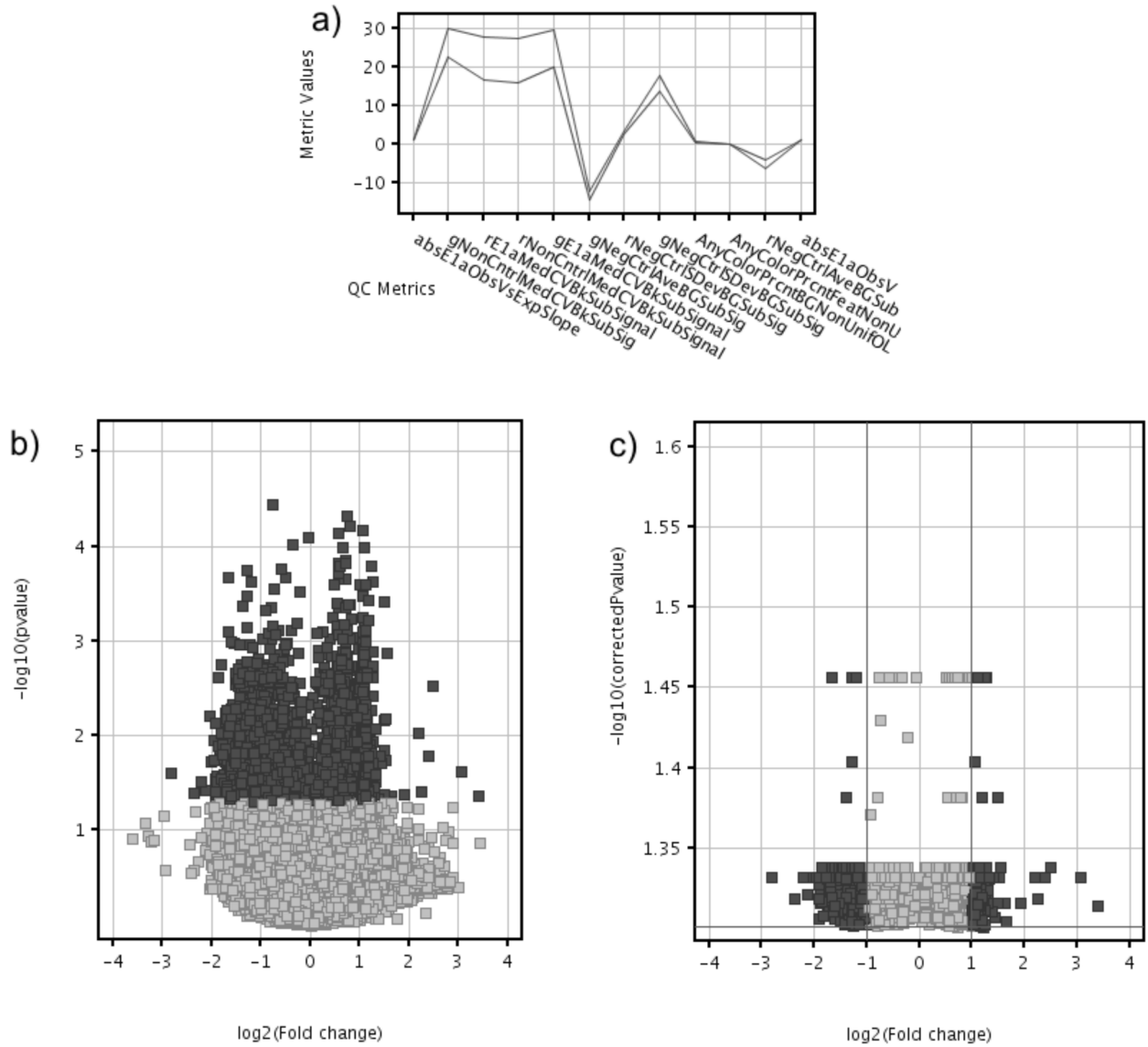


Fig. 2. Gene Spring (Agilent, USA) diagrams of: a) quality control gene expression in both microarray experiments (in dye-swaps) shows highly repeatable results; b) all genes expression in both microarray experiments (in dye-swaps); c) genes that differed significantly at p value < 0.05 with fold cut-off = 1.5 (unpaired t-test and Benjamin-Hochberg FDR < 5% correction). To the further analysis the genes marked as dark grey spots were taken.

### Real-Time qPCR

Primers were designed using PRIMER3 software (free on-line access). Primers sequences are listed in Table 1. HPRT and RPS19 genes were used as non-regulated reference genes for normalization of target gene expression (Brinkhof et al. 2006, Etschmann et al. 2006). Quantitative RT-PCR was performed using fluorogenic SYBR Green and the Sequence Detection System Fast 7500 (Applied Biosystems). Data analysis was carried out using the 7500 Fast System SDS Software Version 1.4.0.25 (Applied Biosystems). Relative transcript abundance of a gene is expressed in ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). The fold change in expression of the target gene was estimated as a $2^{\Delta\Delta Ct}$. Estimated fold change for each gene was compared with the same estimation from the microarray analysis (ANOVA and Tukey test; Graph Pad Prism 3.0, USA).

## Results

### Global gene expression of the canine mammary osteosarcoma

The microarray-based transcriptional profile of the canine mammary osteosarcoma was compared to the canine mammary osteoma used as a reference. For each comparison 2 separate dye-swap experi-

Table 2. List of genes regulated (up- and down-) in canine mammary osteosarcoma versus osteoma. The list of genes was generated by Gene Springe (Agilent, USA) software ($p < 0.05$ in t-test with Benjamin-Hochberg FDR < 5% correction). Fold changes results are means of dye-swap experiments.

| Gene symbol | Gene name | Systematic symbol | Fold change |
|---|---|---|---|
| ENSCAFT00000034824 | NADH-ubiquinone oxidoreductase chain 2 | ENSCAFT00000034824 | ↑3.38 |
| EIF4H | Eukaryotic translation initiation factor 4H | XM_844533 | ↑3.13 |
| LOC476315 | enolase | XM_853327 | ↑3.07 |
| RAD54L2 | RAD54-like 2 | XM_855524 | ↑3.00 |
| NDUFS6 | NADH dehydrogenase | XM_535802 | ↑2.97 |
| PDK1 | 3-phosphoinositide-dependent protein kinase | XM_534032 | ↑2.53 |
| TUFM | Tu translation elongation factor | XM_536924 | ↑1.88 |
| THOC6 | THO complex 6 homolog | XM_536996 | ↑1.79 |
| EXT1 | exostoses (multiple) 1 | XM_539145 | ↑1.63 |
| BCHE | butyrylcholinesterase | ENSCAFT00000023011 | ↓31.53 |
| LALBA | lactalbumin, alpha | NM_001003129 | ↓24.90 |
| LUM | lumican | XM_539716 | ↓11.76 |
| SFRP2 | secreted frizzled-related protein 2 | NM_001002987 | ↓11.61 |
| ABI3BP | ABI family, member 3 (NESH) binding protein | XM_535721 | ↓11.53 |
| STEAP2 | six transmembrane epithelial antigen of the prostate 2 | XM_539408 | ↓10.99 |
| BNC2 | basonuclin 2 | XM_848855 | ↓9.47 |
| CTSC | cathepsin C | AF060171 | ↓8.40 |
| RAB38 | RAB38, member RAS oncogene family | XM_845119 | ↓7.43 |
| SEMA3C | sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C | XM_533139 | ↓7.38 |
| LOC476890 | small VCP/p97-interacting protein | XM_534092 | ↓6.99 |
| HSPB8 | heat shock 22kDa protein 8 | ENSCAFT00000015840 | ↓6.28 |
| JPH1 | junctophilin 1 | XM_844878 | ↓6.06 |
| ENSCAFT00000022876 | G-protein-coupled receptor GPR34 | ENSCAFT00000022876 | ↓5.91 |
| FGL2 | fibrinogen-like 2 | XM_533109 | ↓5.86 |
| XDH | xanthine dehydrogenase | XM_857565 | ↓5.55 |
| SF3B3 | splicing factor 3b, subunit 3 | XM_536791 | ?5.52 |
| SGTB | small glutamine-rich tetratricopeptide repeat (TPR)-containing, beta | XM_535258 | ↓3.85 |
| SEPP1 | selenoprotein P, plasma, 1 | NM_001115118 | ↓3.52 |
| TNFAIP8 | tumor necrosis factor, alpha-induced protein 8 | XM_538548 | ?3.43 |
| SPARCL1 | secreted protein, acidic, cysteine-rich (osteonectin) – like 1 | XM_844952 | ↓2.94 |
| ZNF704 | zinc finger protein 704 | XM_848443 | ↓2.91 |
| PSPH | phosphoserine phosphatase | XM_843195 | ↓2.54 |
| FUCA2 | fucosidase, alpha-L- 2, plasma | XM_541133 | ↓2.21 |
| CAMK2G | calcium/calmodulin-dependent protein kinase II gamma | XM_858286 | ↓2.18 |
| PRKAG1 | protein kinase, AMP-activated, gamma 1 non-catalytic subunit | XM_543685 | ↓1.64 |

ments were performed using canine-specific AMADID Release GE 4x44K microarrays analysis. This study showed 47 statistically significant ($p < 0.05$; Fold change = 1.5) differently regulated genes (Fig. 2). Further analysis (Table 2) showed 11 up-regulated genes and 36 down-regulated genes in osteosarcoma versus osteoma.

### Function of identified genes

PANTHER software analysis of identified up-regulated genes showed that they were mainly involved in molecular functions as: nucleic acid binding (EIF4H, TUFM, RAD54L2), kinase (PDK1), oxidoreductase (NDUFS6), and transferase (EXT1). Most of the up-regulated genes were involved in the following biological processes: protein metabolism and modification (EIF4H, TUFM, PDK1, EXT1) cell motility and structure (PDK1), signal transduction (PDK1), and developmental processes (PDK1). Up-regulated genes also took part in nucleoside, nucleotide and nucleic acid metabolism (RAD54L2), carbohydrate metabolism (EXT1) and electron transport (NDUFS6).

Analyzing molecular functions of down-regulated genes we noticed that most of them played roles of kinases (CAMK2G), hydrolases (BCHE), proteases (CTSC), phosphatases (PSPH), chaperons (HSPB8, SGTB), and synthases (LALBA). Some of them were involved in such processes as: nucleic acid binding (SFN3B3, BNC2) and extracellular matrix remodeling (LUM, SEPP1, FGL2). Products of some genes were: receptors (LUM), regulatory molecules (RAB38, PRKAG1), transcription factors (ZNF704, BNC2), and signaling molecules (SFRP2, SEMA3C, FGL2).

### Up- and down-regulated cellular pathways in osteosarcoma

Analyzing osteosarcoma cellular up-regulated pathways we mainly focused on a few of them: insulin/IGF, Wnt, interleukin, p53, AKT, PI3K and Ras signaling pathways. In our opinion, the most intriguing pathways which are under control of down-regulated genes were: angiogenesis, AMPK, and ionotropic glutamate receptor. The inflammation mediated by chemokine and cytokine signaling pathways in canine mammary osteosarcoma was regulated by PDK1 (up-regulated) and CAMK2G (down-regulated).

### Real-time qPCR gene expression

For the purpose of microarray data validation, we have randomly selected 7 genes: EXT1, EIF4H, PDK1, RAD54L2, CAMK2G, SF3B3, and HSPB8. Real-time qPCR results showed similar trends in gene expression changes as we observed in microarray studies (Fig. 3). The selected key genes were similarly expressed in both examined osteosarcomas.

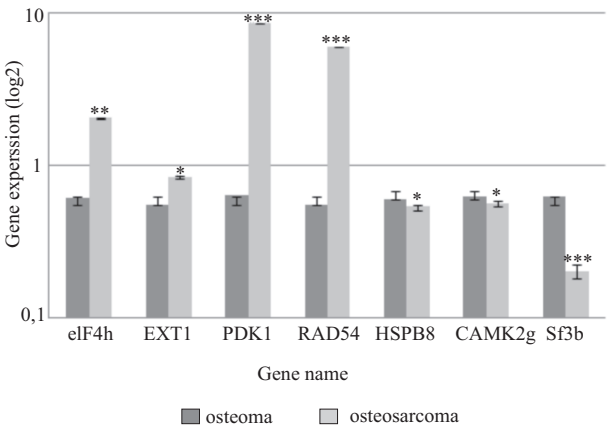


Fig. 3. The real-time qPCR expression of examined genes (n = 3). Estimated fold change ($log_2$) for each gene was compared between osteosarcomas and osteomas (ANOVA and Tukey test; Graph Pad Prism 3.0, USA). The $p$ value < 0.05 was regarded as significant and marked as *, $p < 0.01$ was regarded as highly significant and marked as **, $p < 0.001$ was regarded as highly significant and marked ***.

## Discussion

So far only several papers describing transcriptional studies in canine mammary gland tumors and cell lines were published (Rao et al. 2008, Król et al. 2009, Pawłowski et al. 2009, Rao et al. 2009, Król et al. 2010a,b). Up-to-date only one paper of the Swedish group from Uppsala University was focused on gene expression in mammary tumor tissue of mesenchymal origin versus canine mammary carcinoma (Wensman et al. 2009).

The aim of the present study was to find genes that differentiated osteosarcomas from benign osteomas. Our microarray experiment showed 11 up-regulated and 36 down-regulated genes in osteosarcoma in comparison to osteoma (Fig. 2). Among up-regulated genes, the most interesting appeared to be: PDK1, EXT1, and EIF4H (Fig. 4).

PDK-1 is a serine/threonine kinase that is activated in response to insulin and growth factor treatment by a mechanism involving phosphoinositide-3 kinase (PI3-K) (Mora et al. 2004). PDK-1 phosphorylates and activates AKT which is involved in cell growth and survival and is activated in many cancers, including canine osteosarcoma tissue and cell lines (Alessi et al. 1997, Stokoe et al. 1997, Le Good et al. 1998, Balendran et al. 2000, Levine el al. 2002). PDK-1 can transform normal human cells into tumor cells (its gene is over-expressed in the majority of hu-

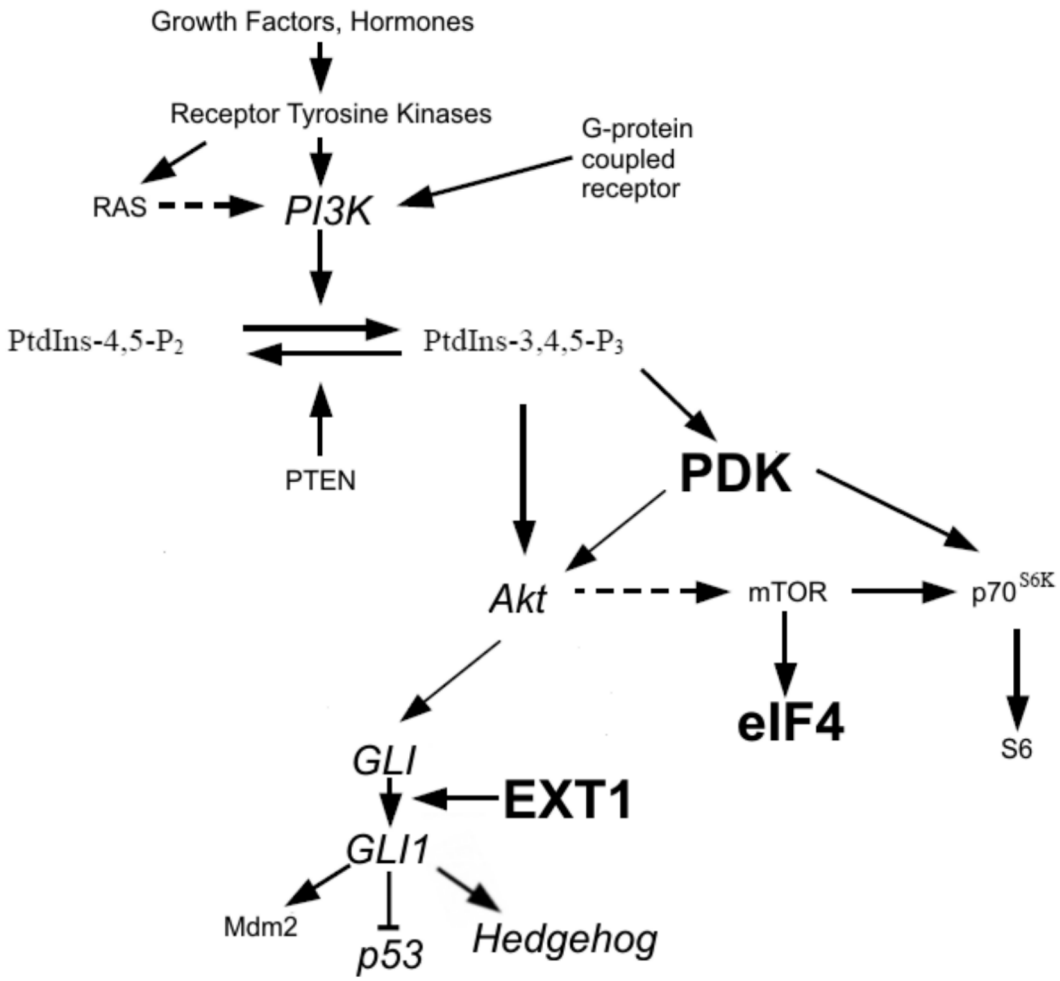


Fig. 4. The involvement of PDK, EXT1 and eIF4 genes in PI3K/AKT and GLI/Hedgehog cellular pathways. The short names of three identified in microarray study genes were bolded, whereas the most important genes/names in the pathway were written in italic. The arrows indicate the activation, interrupted arrows indicate probably mechanism of action, whereas bar-headed line indicates inhibition.

The explanation of abbreviations:

Akt – protein kinase B; eIF4 – eukaryotic initiation factor 4; EXT1 – exostosin 1; GLI – GLI transcription factor; GLI1 – GLI transcription factor 1 (activator); Hedgehog – Hedgehog signaling pathway; Mdm2 – murine double minute oncogene; mTOR – mammalian target of rapamycin; p53 – protein 53; $p70^{S6K}$ – ribosomal protein S6 kinase; PDK – serine/threonine kinase; PI3K – Phosphatidylinositol 3-kinase; PtdIns-3,4,5-$P_3$ – Phosphatidylinositol 3,4,5-trisphosphate; PtdIns-4,5-$P_2$ – Phosphatidylinositol 4,5-bisphosphate; PTEN – phosphatase and tensin homolog deleted on chromosome ten; RAS – RAS protein; S6 – S6 kinase.

Based on Vogelstein B and Kinzler KW (2004); Paez JG and Sellers WE (2010); Stecca B et al. (2010). The graph made in Corel Photo Paint 3.0 (by Corel Draw, USA).

man breast cancers) and may be involved in invasion and metastatic processes (Zeng et al. 2002, Xie et al. 2003, 2006, Maurer et al. 2009). Maurer and co-workers (2009) showed that alteration of PDK1 is a critical component of oncogenic PI3K signaling in breast cancer. Constitutive activation of PI3-K/PDK-1/AKT signaling mediates the survival signals and confers resistance to apoptosis induced by anticancer cytotoxic agents in human cancer cells (Page et al. 2000, Nesterov et al. 2001, Clark et al. 2002). In some investigators opinions PDK-1/AKT signaling inhibition represent promising new therapeutic target in osteosarcoma treatment (Levine et al. 2002, Cen et al. 2007). PDK-1 activates GLI/Hedgehog pathway which is also activated by other gene up-regulated in osteosarcoma: EXT1 (Paez at al. 2003) (Fig. 4). EXT1 (exostosis-1) gene encodes a glycosyltransferase involved in HS (heparan sulfate) chain elongation, promotes GPG translation in PI3K pathway and GLI1 activation in GLI/Hedgehog pathway (Stecca et al. 2007, 2010). EXT1 abnormalities are usually associated with hereditary multiple exos-

toses but, what is particularly interesting, EXT1 is also listed as a major cancer-predisposing gene in hereditary bone cancer (Vogelstein and Kinzler, 2004) via GLI proteins in Hedgehog pathway. The Hedgehog-GLI signaling pathway is very important in animal development and tumorigenesis (Sanchez et al. 2005). This pathway is activated in following tumors: basal skin cell carcinomas, medulloblastomas, prostate, pancreatic and lung cancers (Sanchez et al. 2005). Enhanced activation of GLI-Hedgehog pathways is also documented in several metastatic and high-grade prostate tumors (Sanchez et al. 2005).

An extremely interesting fact is that we previously found up-regulation of genes involved in both pathways: Insulin/PI3K/AKT and Hedgehog in canine mammary cancer cell lines with high proliferative potential (Król et al. 2010 b). So, we assume that the cross-link and activation of these two pathways may play a very important role in neoplasm formation and proliferation. Similar results were also described in various cancer cells (Stecca et al. 2007, for review, see Stecca et al. 2010).

Additionally, the third up-regulated gene EIF4H, seems to be also an important element of the osteosarcoma "molecular portrait" (Fig. 4). EIF4H (Eukaryotic initiation factor) is the prototypic member of the DEAD box family of ATP-dependent RNA helicases (EIF4s). These proteins are involved in almost all aspects of RNA metabolism, such as: transcription, ribosomal biogenesis, pre-mRNA splicing, RNA export, translation, and RNA degradation (Rogers et al. 2001). There are few reports describing some EIF4s involvement in tumorigenesis, cell cycle and proliferation. So far its function in cancer development is not fully understood. EIF4H may be a very interesting factor for further investigation because of its important role in translation (Rogers et al. 2001, Bordeleau et al. 2006). EIF4s control the translation of various malignancy-associated mRNAs which are involved for example in polyamine synthesis, cell cycle progression and proliferation, activation of antiapoptotic factors, angiogenesis, autocrine growth stimulation, cell survival, invasion and communication with the extracellular environment (Mamane et al. 2004). The finding that EIF4s are over-expressed in several human cancers makes them a main target for anticancer therapies. The previously listed in this manuscript signal transduction pathways (PI3K/AKT and GLI/Hedgehog) play also a critical role in regulating the mRNA translation and cellular transformation. EIF4 expression is indirectly activated by AKT via mTOR (Fig. 4) that places it in the same pathway as the two described previously genes. Finding the close correlations between these three genes and the links between PI3K/AKT and GLI/Hedgehog pathways may give very interesting results.

The function of other identified genes still remains unknown.

Among the down-regulated genes in osteosarcoma the most interesting seem to be HSPB8 and SEPP1. Unfortunately we did not find any common pathways for the identified down-regulated genes.

HSPB8 is a small heat shock protein that plays important roles in cell cycle regulation, apoptosis, and breast carcinogenesis – this is mainly associated with cyclin D1 and ER positive tumors (Trent et al. 2007, Madak-Erdogan et al. 2008). Trent et al. (2007) showed that knockdown of HSPB8 increases radiation sensitivity *in vitro* and *in vivo*. This is an interesting finding, because radiation therapy is used in treatment of canine patients with osteosarcoma when surgery is not possible.

The down-regulation of SEPP1 expression level was found in tumor lung tissue (Gresner et al. 2009), prostate cancer (Cooper et al. 2008) and also in canine mammary carcinoma and hyperplasia versus normal tissue (Rao et al. 2009). It seems possible, that the down-regulation of SEPP1 expression may lead to induction of oxidative stress which may enhance the possibility of carcinogenesis.

The preliminary results of the present study indicate that the up-regulation of three genes EXT1, EIF4H, and PDK1 may play an essential role in osteosarcoma formation and development (Fig. 4). In our opinion the cross-talk between Hedgehog-GLI and PI3K-AKT pathways may be a key factor for tumor high proliferation potential and malignancy but it requires further investigation also at the protein level.

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