# Apoptotic genes as potential markers of metastatic phenotype in human osteosarcoma cell lines

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**Abstract.** Metastasis is the most frequent cause of death among patients with osteosarcoma. We have previously demonstrated in independent experiments that the forced expression of L/B/K ALP and CD99 in U-2 OS osteosarcoma cell lines markedly reduces the metastatic ability of these cancer cells. This behavior makes these cell lines a useful model to assess the intersection of multiple and independent gene expression signatures concerning the biological problem of dissemination. With the aim to characterize a common transcriptional profile reflecting the essential features of metastatic behavior, we employed cDNA microarrays to compare the gene expression profiles of L/B/K ALP- and CD99-transfected osteosarcoma clones showing low metastatic ability with those of osteosarcoma cell lines showing contrasting behavior. Changes in gene expression were validated by real-time PCR and immunohistochemistry in independent samples. In our study we identified several differentially expressed genes (GADD45α, VCP, DHX9, survivin, α-catulin, ARPC1B) related to growth arrest and apoptosis. Most of these genes are functionally related with the nuclear factor (NF)-κB cell survival pathway that appeared to be inhibited in the less malignant osteosarcoma cells. Hence, we propose the inhibition of the NF-κB pathway as a rational strategy for effective management of human osteosarcoma.

## Introduction

Metastasis is the dissemination of cancer cells from the primary tumor to a distant organ and represents the most frequent cause of death for patients with cancer (1). Osteosarcoma (OS) is a malignant primitive bone tumor, usually developing in children and adolescents, with a high tendency to metastasize (2). Metastases in osteosarcoma patients spread through peripheral blood very early and colonize primarily the lung, and later other skeleton districts (3). Since disseminated hidden micrometastases are present in 80-90% of OS patients at the time of diagnosis, the identification of markers of invasiveness and metastasis forms a target of paramount importance in planning the treatment of osteosarcoma lesions and enhancing the prognosis.

In this study we performed a statistical analysis of multiple gene expression datasets concerning the common biological problem of metastasis. Our comprehensive analysis includes the gene expression datasets derived from two experimental models previously obtained from the U-2 OS osteosarcoma cell lines characterized by a common reversal of malignancy and lack of metastatic phenotype. In particular, we have demonstrated in independent experiments that the forced expression of L/B/K ALP and likewise CD99 in U-2 OS osteosarcoma cell lines markedly reduces the invasiveness and metastatic ability of these cancer cells (4,5). Alkaline phosphatases (ALPs) are a family of cell surface glycoproteins that catalyze the hydrolysis of phospho-monoesters and release inorganic phosphate. Liver-bone-kidney (L/B/K) ALP, one of the four major isoenzymes belonging to this family, participates in bone mineralization. The precise biochemical function of bone ALP activity is still unknown, but in U-2 OS osteosarcoma cell lines transfected with the full-length L/B/K ALP gene a reduction in tumorigenic and metastatic ability has been observed associated with high levels of cellular activity of L/B/K ALP (4). CD99 is a transmembrane glycoprotein (6) encoded by the MIC2 gene (7), which has been implicated in several cellular processes, such as cell adhesion, apoptosis and differentiation (8-12). Manara *et al* (5) have demonstrated that the forced expression of CD99 in two osteosarcoma cell lines significantly inhibits growth in anchorage independence as well as cell migration and leads to abrogation of tumorigenic and metastatic ability.

The fact that U-2 OS osteosarcoma cell lines transfected with both L/B/K ALP and CD99 show identical behavior (that is, marked reduction of metastatic ability), makes these

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Table I. *In vitro* and *in vivo* biological features associated with malignancy of U-2 OS transfected clones.

| Cell line | *In vitro* parameters[a] | | *In vivo* behaviour[b] | |
|---|---|---|---|---|
| | Soft-agar growth | Migration | Mice with tumor/total | Mice with metastases/total |
| U-2 OS | 742±102 | 215±40 | 7/10 (70%) | 16/19 (84%) |
| U-2/Empty | 574±68 | 218±40 | 3/5 (60%) | 9/10 (90%) |
| U-2/ALP23 | 723±16 | 183±20 | 3/5 (60%) | 10/10 (100%) |
| U-2/ALP28 | 109±7[c] | 26±5[c] | 0/5 (0%) | 3/7 (43%) |
| U-2/ALP40 | 118±17[c] | 92±6[c] | 0/5 (0%) | 4/14 (29%) |
| U-2/CD99wt57 | 67±17[c] | 90±18[c] | 0/5 (0%) | 0/10 (0%) |
| U-2/CD99wt 136 | 124±26[c] | 64±17[c] | 0/5 (0%) | 7/15 (47%) |

[a]Soft agar growth was measured by counting cell colonies 14 days after seeding of 10,000 cells in 0.33% agarose. Migration was assessed by counting the number of cells that migrated towards the filter to reach the lower chamber base of a Trans-well chamber. Cells ($10^5$) in IMDM plus 1% FBS were seeded in the upper compartment, whereas IMDM plus 10% FBS was placed in the lower compartment of the chamber as a chemoattractant. [b]Tumorigenicity was determined after subcutaneous injection of $30x10^6$ cells in female 4-5 week old Cr1:nu/nu (CD-1)BR athymic mice (Charles River Italia, Como). Experimental metastatic ability was evaluated after injection of $2x10^6$ cells into a tail lateral vein. For detailed methods see Manara *et al* (4,5). [c]$P<0.05$, Student's t-test.

cell lines a useful model to assess the intersection of multiple and independent gene expression signatures concerning the common biological problem of dissemination. In particular, in our study we employed cDNA microarray technology to compare the gene expression profiles of L/B/K ALP- and CD99-transfected osteosarcoma clones showing low metastatic ability with those of osteosarcoma cell lines showing contrasting behavior, the purpose being to characterize a common transcriptional profile reflecting the essential features of metastatic behavior.

## Materials and methods

*Cell lines*. The parental osteosarcoma cell line U-2 OS was obtained from the American Type Culture Collection (Manassas, VA). Cells overexpressing L/B/K ALP or CD99 were obtained by using the calcium-phosphate transfection method previously characterized (4,5). Transfectants were selected in 500 $\mu$g/ml neomycin (Sigma-Aldrich, St. Louis, MO) and maintained in a selective medium for a maximum of eight *in vitro* passages before *in vitro* and *in vivo* characterization. In particular, U-2/ALP28, and U-2/ALP40, showing high levels of L/B/K ALP expression and activity, and U-2/CD99 clones (U2-CD99wt57 and U2-CD99wt136), overexpressing CD99 at the cell surface, were chosen for their low level of malignancy. Table I summarizes the previously published (4,5) U-2 OS transfectant features associated with cancer malignancy relevant for this study. Cells transfected with the empty vector pcDNA3 (5) or clone U-2/ALP23, which shows very low levels of L/B/K ALP activity (4), were used as negative controls. Cells were routinely cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Paisley, Scotland), supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% inactivated fetal bovine serum (FBS) (Cambrex BioScience Verviers, Verviers, Belgium) and maintained at 37˚C in a humidified 5% $CO_2$ atmosphere.

*RNA isolation*. Total RNA was extracted using the TRIzol extraction kit (Invitrogen Life Technologies, Paisley, UK). The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S RNA bands were visualized under UV light. To perform microarray hybridization, two independent extractions of RNAs were performed from each cell clone.

*cDNA microarray hybridization*. Hybridizations were performed on Human 1 cDNA Microarray slides (Agilent Technologies, Inc., Palo Alto, CA) containing 16,281 cDNA sequences. Total RNA was used to obtain labeled cDNA, according to the manufacturer's instructions (Agilent Direct-Label cDNA synthesis kit protocol, Agilent Technologies). cDNA from osteosarcoma cell lines transfected with L/B/K ALP and with CD99 was labeled with Cyanine 5-dCTP (Cy5), while parental U-2/OS cDNA was labeled with Cyanine 3-dCTP (Cy3). In brief, 20 $\mu$g of total RNA was reverse-transcribed with a specific primer mixture using Moloney murine leukemia virus reverse transcriptase (Agilent Direct-Label cDNA synthesis kit) in the presence of Cy5 or Cy3 (Perkin-Elmer Life Sciences Inc., Boston, MA) at 42˚C for 1 h. RNaseI A (Agilent Technologies) was then added to degrade the RNA. Labeled cDNAs were purified with the QIAquick PCR purification kit (Qiagen, GmbH, Germany). Additional washes with 35% (w/v) guanidine hydrocloride were performed to ensure efficient removal of the unincorporated dye labeled nucleotides. Equivalent amounts of Cy5- and Cy3-cDNA were combined, vacuum dried, re-suspended in deposition hybridization buffer (Agilent Technologies) with human Cot-1 (Invitrogen) and deposition control targets (Qiagen) and hybridized to microarray slides for approximately 17 h, according to the manufacturer's instructions. The slides were washed with 0.5X SSC + 0.01% SDS for 10 min and with 0.06X SSC for 5 min.

*Microarray analysis*. Hybridized slides were scanned using the GenePix 4000A scanner (Axon Instruments, Foster City, CA) ensuring that the total number of saturated spots (saturation fluorescence value: 65,000) was <10. Images were analyzed using GENEPIX PRO 3.0 software (Axon Instruments).

GenePix Post-Processing Script (GP3) was used in order to filter and normalize raw microarray data from GenePix image analysis (13) (global normalization; percentile of mean signal across array for normalization factor: 90; threshold value: median background spot intensity + 3 standand deviation of background). Expression tables were assembled from GP3 output files and genes that proved to be filtered and preserved in 100% of the arrays were analyzed using the Significance Analysis of Microarray (SAM) program (14). SAM identifies genes with statistically significant changes in expression by performing a set of gene-specific t-tests. Each gene has a score (d) assigned on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Only genes showing a score of >2.5 or <-2.5 were considered, and among these, only genes up-regulated or down-regulated in at least 85% of the arrays were selected and employed for functional analysis.

*Gene ontology (GO).* We used FatiGO to assign the 253 differently expressed genes to non-mutually exclusive categories regarding biological processes. FatiGO is a web interface (http://fatigo.bioinfo.cipf.es) which carries out simple data mining using GO for DNA microarray data. The data mining consists in assigning the most characteristic GO term to each cluster. When we searched for the distribution of genes in a specific ontology (e.g. a biological process) we selected the ontology and also the GO term level (=3).

*Real-time PCR.* Total RNA (1 $\mu$g) was denatured at 65˚C for 10 min and then reverse-transcribed in a 100-$\mu$l reaction mixture containing 500 $\mu$M of each dNTP, 125 U of Multi-Scribe reverse transcriptase (Applied Biosystems, Foster City, CA), 40 U of RNase inhibitor (Applied Biosystems), 2.5 $\mu$M oligo d(T), 1X TaqMan RT Buffer, and 5 mM $MgCl_2$ at 48˚C for 40 min. Reactions performed in the absence of enzyme or RNA were used as negative controls. Gene-specific primers were designed using Primer Express software (Applied Biosystems): Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe as follows: forward 5' GAAGG TGAAGGTCGGAGTC 3', reverse 5' GAAGATGGTGATG GGATTTC 3'; probe: FAM-GAA 5' GCTTCCCGTTCTCA GCC 3'. Actin Related Protein 2/3 Complex, Subunit 1B, 41 kDa (ARPC1B): forward 5' TCAAGTGTCGGATCTTT TCAGC 3', reverse 5' AGTTCCCCAAAGGGCATCTT 3'. α-catulin [Catenin (cadherin-associated protein), α-like1, CTNNAL1]: forward 5' AGTGGGGAAGATGCAGTCC 3', reverse 5' CAAATGACAGCTAAACAGCAGC 3'. Survivin (Baculoviral IAP Repeat-Containing 5, BIRC5): forward 5' TGGCCCAGTGTTTCTTCTGC 3', reverse 5' CAACCGGA CGAATGCTTTTT 3'. Valosin-containing protein (VCP): forward 5' GTAACCTGGGAAGACATCGGG 3', reverse 5' GGAATTTGTCTGGGTGCTCC 3'. A SYBR-Green PCR Master mix (Applied Biosystems) was used with 1 ng of cDNA and with 100-400 nM of the primers. A negative control without any cDNA template was run with every assay. All PCR reactions were performed using an ABI PRISM 7900 sequence detection system (PE Applied Biosystems). The target gene mRNA was quantified by measuring the $C_T$ (threshold cycle) to determine the relative expression. Data were normalized to GAPDH. The relative expression of the different mRNAs was also normalized to U-2 OS mRNA, used as a calibrator and was expressed as: $2^{-\Delta\Delta CT}$, where $\Delta C_T = C_T$ target genes - $C_T$ GAPDH, and $\Delta\Delta CT = \Delta C_T$ sample - $\Delta C_T$ calibrator. All samples were resolved in a 2% agarose gel to confirm the PCR specificity.

*Analysis of apoptosis.* Detection and quantification of apoptotic cells was obtained by flow cytometric analysis (FACSCalibur, Becton Dickinson) of annexin-V-FITC-labelled cells. This test was performed according to the manufacturer's instructions (Medical and Biological Laboratories, Naka-ku Nagaya, Japan). PI incorporation was evaluated in combination with the fluorescent signal intensity to allow for discrimination of necrotic and apoptotic cells.

*Poly-HEMA assay.* Six-well plates were treated with poly-2 hydroxyethylmethacrylate (poly-HEMA) (Sigma) following the method of Folkman and Moscona (15). Briefly, wells were treated with a 1-ml solution of poly-HEMA diluted in 95% ethanol (12 mg/ml) and left to dry at room temperature. Cells (250,000/well) were seeded in IMDM 10% FBS and incubated at 37˚C in a humidified 5% $CO_2$ atmosphere. Viable and dead cells were counted after 24, 48, and 72 h. Detection and quantification of apoptotic cells as well as of cell cycle phases was obtained according to the procedures described above.

*Immunostainings on adherent fixed cells.* Cells were seeded in IMDM 10% FBS and grown on coverslips for 48 h before being fixed in 4% paraformaldehyde at room temperature and permeabilized with 0.15% Triton X-100 in PBS. All preparations were incubated with PBS containing 4% BSA to saturate non-specific binding. Immunofluorescence staining for cyclin D1 and NF-κB was performed with the primary cyclin D1 (H-295) MAb (Santa Cruz Biotechnolgy, Inc.) (diluted 1:10) or anti NF-κBp50 (H-119) MAb (Santa Cruz Biotechnolgy, Inc.) (diluted 1:20), respectively. FITC-conjugated phalloidin (5 U/ml) (Sigma) was applied for 30 h at room temperature to stain and visualize actin filaments.

*Wound-healing assay.* Monolayer wounds were made using a pipette tip on confluent cells. Cell migration was visualized, at regular intervals of time, at x100 magnification using an inverted microscope (Nikon Diaphot, Melville, NY), and photographed with a D70s Nikon digital camera (Nikon).

*Nuclei extraction and NF-κB Western blotting.* The cell pellet was resuspended in a buffer containing 10 mM Tris, pH 7.8, 1% NP-40, 10 mM 2-mercaptoethanol and protease inhibitors. Separation of nuclei was obtained by hypotonic shock and shearing; nuclei were obtained as pellet by a 300 x g centrifugation at 4˚C. Cytoplasmic fractions were clarified by centrifugation at 600 g. Each fraction was then resuspended in loading buffer, subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analysis. The expression of NF-κB in the nuclei was evaluated using anti-NF-κBp50 (H-119) MAb (Santa Cruz Biotechnolgy, Inc.) (diluted 1:100). The absence of cytoplasmic contamination in the nuclear fraction was checked by staining the cellular fractions with anti-GAPDH Mab (FL-335) (diluted 1:2000).

Table II. Up-regulated and down-regulated expressed genes in both L/B/K ALP and CD99 U2-OS transfected clones showing lack of metastatic ability compared with parental U2-OS cell line.

| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| Up-regulated genes | | | |
| KIF23 | Kinesin family member 23 | Hs.270845 | 5.872 |
| CRYAB | *Homo sapiens* crystallin, α B | Hs.408767 | 5.718 |
| OXA1L | Oxidase (cytochrome c) assembly 1-like | Hs.151134 | 3.853 |
| DUSP12 | Dual specificity phosphatase 12 | Hs.416216 | 3.545 |
| VGF | VGF nerve growth factor inducible | Hs.439672 | 3.491 |
| GNG11 | Guanine nucleotide binding protein (G protein), γ 11 | Hs.83381 | 3.401 |
| MTHFD2 | Methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 2, methenyltetrahydrofolate cyclohydrolase | Hs.469030 | 3.385 |
| RAD51C | RAD51 homolog C (*S. cerevisiae*) | Hs.412587 | 3.382 |
| BUB1 | Budding uninhibited by benzimidazoles 1 homolog (yeast) | Hs.469649 | 3.38 |
| ANXA1 | Annexin A1 | Hs.494173 | 3.376 |
| TAF13 | TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 18 kDa | Hs.502508 | 3.339 |
| PMAIP1 | Phorbol-12-myristate-13-acetate-induced protein 1 | Hs.96 | 3.32 |
| SLC38A2 | Solute carrier family 38, member 2 | Hs.221847 | 3.207 |
| ANAPC10 | Anaphase promoting complex subunit 10 | Hs.480876 | 3.162 |
| SLC22A11 | Solute carrier family 22 (organic anion/cation transporter), member 11 | Hs.220844 | 3.139 |
| MTX2 | metaxin 2 | Hs.470728 | 3.104 |
| SBDS | Shwachman-Bodian-Diamond syndrome | Hs.110445 | 3.08 |
| RCN2 | Reticulocalbin 2, EF-hand calcium binding domain | Hs.79088 | 3.079 |
| PBK | PDZ binding kinase | Hs.104741 | 3.059 |
| PCDHGC3 | protocadherin γ subfamily C, 3 gene, exon 1 | Hs.368160 | 3.023 |
| TJP2 | Tight junction protein 2 (zona occludens 2) | Hs.50382 | 3.006 |
| COPS4 | COP9 constitutive photomorphogenic homolog subunit 4 (Arabidopsis) | Hs.190384 | 2.974 |
| CTGF | Connective tissue growth factor | Hs.410037 | 2.93 |
| TRO | Trophinin | Hs.434971 | 2.899 |
| SNAPC1 | Small nuclear RNA activating complex, polypeptide 1, 43 kDa | Hs.179312 | 2.89 |
| TPT1 | Tumor protein, translationally-controlled 1 | Hs.374596 | 2.887 |
| RPL9 | Ribosomal protein L9 | Hs.513083 | 2.883 |
| NAT5 | N-acetyltransferase 5 (ARD1 homolog, *S. cerevisiae*) | Hs.368783 | 2.852 |
| EIF3S6 | Eukaryotic translation initiation factor 3, subunit 6 48 kDa | Hs.405590 | 2.848 |
| ADAM9 | A disintegrin and metalloproteinase domain 9 (meltrin γ) | Hs.591852 | 2.821 |
| DDEF1 | Development and differentiation enhancing factor 1 | Hs.106015 | 2.794 |
| RTN4 | Reticulon 4 | Hs.645283 | 2.785 |
| COX6C | Cytochrome c oxidase subunit VIc | Hs.351875 | 2.778 |
| PGRMC1 | Progesterone receptor membrane component 1 | Hs.90061 | 2.776 |
| GAD2 | Glutamate decarboxylase 2 (pancreatic islets and brain, 65 kDa) | Hs.231829 | 2.768 |
| TBCA | Tubulin-specific chaperone a | Hs.495912 | 2.766 |
| | Transcribed locus | Hs.598710 | 2.742 |
| WSB2 | WD repeat and SOCS box-containing 2 | Hs.506985 | 2.726 |
| PRNP | prion protein (p27-30) (Creutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia) | Hs.472010 | 2.706 |
| DDX18 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 18 | Hs.363492 | 2.702 |
| PHLDA2 | Pleckstrin homology-like domain, family A, member 2 | Hs.154036 | 2.702 |
| TM4SF8 | Tetraspanin 3 | Hs.5062 | 2.681 |
| HSBP1 | Heat shock factor binding protein 1 | Hs.250899 | 2.673 |
| SDHD | Succinate dehydrogenase complex, subunit D, integral membrane protein | Hs.356270 | 2.663 |
| RPS15A | Ribosomal protein S15a | Hs.370504 | 2.66 |
| NDUFB3 | NADH dehydrogenase (ubiquinone) 1 ß subcomplex, 3, 12 kDa | Hs.109760 | 2.649 |

Table II. Continued.

| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| PHF20 | PHD finger protein 20 | Hs.517044 | 2.636 |
| RPA3 | Replication protein A3, 14 kDa | Hs.487540 | 2.634 |
| PPP1R8 | Protein phosphatase 1, regulatory (inhibitor) subunit 8 | Hs.533474 | 2.634 |
| AP3B1 | Adaptor-related protein complex 3, ß 1 subunit | Hs.532091 | 2.632 |
| SF3B1 | Splicing factor 3b, subunit 1, 155 kDa | Hs.632554 | 2.626 |
| DNAJC3 | DnaJ (Hsp40) homolog, subfamily C, member 3 | Hs.591209 | 2.623 |
| UAP1 | UDP-N-acteylglucosamine pyrophosphorylase 1 | Hs.492859 | 2.62 |
| GADD45A | growth arrest and DNA-damage-inducible, α | Hs.80409 | 2.613 |
| ITM2B | Integral membrane protein 2B | Hs.652143 | 2.61 |
| RPS24 | Ribosomal protein S24 | Hs.356794 | 2.61 |
| RPL7 | Ribosomal protein L7 | Hs.571841 | 2.605 |
| PTPN2 | Protein tyrosine phosphatase, non-receptor type 2 | Hs.123352 | 2.594 |
| SSR1 | Signal sequence receptor, α (translocon-associated protein α) | Hs.114033 | 2.594 |
| COX7B | Cytochrome c oxidase subunit VIIb | Hs.522699 | 2.59 |
| CPOX | Coproporphyrinogen oxidase | Hs.476982 | 2.589 |
| PPP2CB | protein phosphatase 2 (formerly 2A), catalytic subunit, ß isoform | Hs.491440 | 2.588 |
| KIF5B | Kinesin family member 5B | Hs.591373 | 2.584 |
| NDUFAB1 | NADH dehydrogenase (ubiquinone) 1, α/ß subcomplex, 1, 8 kDa | Hs.189716 | 2.575 |
| STRAP | Serine/threonine kinase receptor associated protein | Hs.504895 | 2.572 |
| NUP107 | Nucleoporin 107 kDa | Hs.524574 | 2.564 |
| DHFR | Dihydrofolate reductase | Hs.83765 | 2.56 |
| MAD2L1 | MAD2 mitotic arrest deficient-like 1 (yeast) | Hs.591697 | 2.54 |
| ACTL6A | Actin-like 6A | Hs.432356 | 2.539 |
| HAT1 | Histone acetyltransferase 1 | Hs.632532 | 2.537 |
| ITGA6 | Integrin, α 6 | Hs.133397 | 2.531 |
| SNRPG | Small nuclear ribonucleoprotein polypeptide G | Hs.516076 | 2.526 |
| ZNF502 | zinc finger protein 502 | Hs.224843 | 2.522 |
| TIAL1 | TIA1 cytotoxic granule-associated RNA binding protein-like 1 | Hs.501203 | 2.521 |
| CTNNAL1 | Catenin (cadherin-associated protein), α-like 1 | Hs.58488 | 2.516 |
| Down-regulated genes | | | |
| NOLC1 | Nucleolar and Coiled-Body Phosphoprotein 1 | Hs.523238 | -6.736 |
| TPM2 | Tropomyosin 2 (ß) | Hs.300772 | -6.692 |
| EEF1D | Eukaryotic Translation Elongation Factor 1 δ (Guanine Nucleotide Exchange Protein) | Hs.333388 | -5.508 |
| TPM3 | Tropomyosin 3 | Hs.129512 | -5.457 |
| THOC4 | THO Complex 4 | Hs.534385 | -5.237 |
| HSPCB | Heat Shock 90 kDa Protein 1, ß | Hs.509736 | -5.066 |
| HNRPA2B1 | Heterogeneous Nuclear Ribonucleoprotein A2/B1 | Hs.487774 | -5.018 |
| LIG1 | Ligase I, DNA, ATP-Dependent | Hs.1770 | -4.922 |
| NDUFV1 | NADH Dehydrogenase (Ubiquinone) Flavoprotein 1, 51 kDa | Hs.7744 | -4.908 |
| TUBA1 | Tubulin, α 1 (Testis Specific) | Hs.75318 | -4.838 |
| ECHS1 | Enoyl Coenzyme A Hydratase, Short Chain, 1, Mitochondrial | Hs.76394 | -4.823 |
| HADHA | Hydroxyacyl-Coenzyme A Dehydrogenase/3-Ketoacyl-Coenzyme A Thiolase/Enoyl-Coenzyme A Hydratase (Trifunctional Protein), α subunit | Hs.516032 | -4.748 |
| LMAN2 | Lectin, Mannose-Binding 2 | Hs.75864 | -4.721 |
| TTN | Titin | Hs.134602 | -4.698 |
| GANAB | Glucosidase, α; Neutral AB | Hs.595071 | -4.589 |
| TUBB4Q | Tubulin, ß Polypeptide 4, Member Q | Hs.351544 | -4.58 |
| TSR2 | TSR2, 20S rRNA Accumulation, Homolog (S. Cerevisiae) | Hs.522662 | -4.502 |
| PTMA | Prothymosin, α (gene sequence 28) | Hs.459927 | -4.489 |
| DHX9 | DEAH (Asp-Glu-Ala-His) box polypeptide 9 | Hs.191518 | -4.485 |

Table II. Continued.

| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| SMARCA4 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | Hs.327527 | -4.473 |
| TUBAL3 | Tubulin, α-like 3 | Hs.163079 | -4.436 |
| VCP | Valosin-Containing Protein | Hs.529782 | -4.412 |
| SYNGR2 | Synaptogyrin 2 | Hs.464210 | -4.251 |
| SFN | Stratifin | Hs.523718 | -4.25 |
| EIF4H | Eukaryotic Translation Initiation Factor 4H | Hs.520943 | -4.217 |
| PCOLN3 | Procollagen (Type III) N-Endopeptidase | Hs.589427 | -4.216 |
| ETFB | Electron-transfer-flavoprotein, ß polypeptide | Hs.74047 | -4.097 |
| HLA-E | Major Histocompatibility Complex, Class I, E | Hs.118354 | -4.089 |
| STK24 | Serine/threonine kinase 24 (STE20 homolog, yeast) | Hs.508514 | -4.076 |
| SF3B2 | Splicing factor 3b, subunit 2, 145 kDa | Hs.406423 | -4.022 |
| SRF | Serum Response Factor (C-Fos Serum Response Element-Binding Transcription Factor) | Hs.520140 | -4.01 |
| FKBP2 | FK506 binding protein 2, 13 kDa | Hs.227729 | -4.009 |
| ENO2 | Enolase 2 (γ, neuronal) | Hs.511915 | -3.99 |
| CALR | Calreticulin | Hs.515162 | -3.919 |
| HNRPH3 | Heterogeneous Nuclear Ribonucleoprotein H3 (2H9) | Hs.652156 | -3.909 |
| NCL | Nucleolin | Hs.79110 | -3.908 |
| DKC1 | Dyskeratosis Congenita 1, Dyskerin | Hs.4747 | -3.891 |
| TUBA1 | Tubulin, α 1 | Hs.75318 | -3.889 |
| PIK4CB | Phosphatidylinositol 4-Kinase, Catalytic, ß polypeptide | Hs.632465 | -3.871 |
| CDC37 | CDC37 Cell Division Cycle 37 Homolog (*S. Cerevisiae*) | Hs.160958 | -3.868 |
| AMFR | Autocrine Motility Factor Receptor | Hs.295137 | -3.867 |
| CHST10 | Carbohydrate Sulfotransferase 10 | Hs.516370 | -3.82 |
| UBE2S | Ubiquitin-Conjugating Enzyme E2S | Hs.396393 | -3.775 |
| DYNC1H1 | Dynein, Cytoplasmic 1, Heavy Chain 1 | Hs.569312 | -3.701 |
| PYCR1 | Pyrroline-5-Carboxylate Reductase 1 | Hs.458332 | -3.69 |
| EML4 | Echinoderm Microtubule Associated Protein Like 4 | Hs.593614 | -3.663 |
| SLC25A6 | Solute Carrier Family 25 (Mitochondrial Carrier; Adenine Nucleotide Translocator), member 6 | Hs.350927 | -3.646 |
| IFRD2 | Interferon-Related Developmental Regulator 2 | Hs.315177 | -3.63 |
| FEN1 | Flap Structure-Specific Endonuclease 1 | Hs.409065 | -3.629 |
| CDC20 | CDC20 Cell Division Cycle 20 Homolog (*S. Cerevisiae*) | Hs.524947 | -3.577 |
| GNB2 | Guanine Nucleotide Binding Protein (G Protein), ß polypeptide 2 | Hs.185172 | -3.564 |
| B4GALT6 | UDP-Gal:ßglcnac ß 1,4- Galactosyltransferase, polypeptide 6 | Hs.591063 | -3.557 |
| PKM2 | Pyruvate Kinase, muscle | Hs.534770 | -3.551 |
| LRPAP1 | Low Density Lipoprotein Receptor-Related Protein Associated Protein 1 | Hs.533136 | -3.547 |
| TK1 | Thymidine Kinase 1, Soluble | Hs.515122 | -3.537 |
| MCAM | Melanoma Cell Adhesion Molecule | Hs.599039 | -3.532 |
| NDUFB7 | NADH Dehydrogenase (Ubiquinone) 1 ß subcomplex, 7, 18 kDa | Hs.532853 | -3.53 |
| ACTG1 | Actin, γ 1 | Hs.514581 | -3.53 |
| U2AF1 | U2(RNU2) Small Nuclear RNA Auxiliary Factor 1 | Hs.365116 | -3.502 |
| PEBP1 | Phosphatidylethanolamine Binding Protein 1 | Hs.652392 | -3.5 |
| RBM4 | RNA Binding Motif Protein 4 | Hs.533712 | -3.49 |
| MLC1SA | Myosin, Light Chain 6B, Alkali, smooth muscle and non-muscle | Hs.632731 | -3.481 |
| DHX38 | DEAH (Asp-Glu-Ala-His) Box Polypeptide 38 | Hs.570079 | -3.472 |
| TUBB2B | Tubulin, ß 2B | Hs.300701 | -3.468 |
| ARF1 | ADP-Ribosylation Factor 1 | Hs.286221 | -3.466 |
| TWF2 | Twinfilin, Actin-Binding Protein, Homolog 2 (*Drosophila*) | Hs.436439 | -3.449 |
| AMY2A | Amylase, α 2A; Pancreatic | Hs.484588 | -3.438 |
| CCT3 | Chaperonin Containing TCP1, Subunit 3 (γ) | Hs.491494 | -3.433 |
| GSR | Glutathione Reductase | Hs.271510 | -3.421 |

Table II. Continued.

| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| SIAHBP1 | Fuse-Binding Protein-Interacting Repressor | Hs.521924 | -3.415 |
| ACTA2 | Actin, α 2, smooth muscle, aorta | Hs.500483 | -3.413 |
| SNX17 | Sorting Nexin 17 | Hs.278569 | -3.399 |
| ACTG2 | Actin, γ 2, Smooth Muscle, Enteric | Hs.516105 | -3.397 |
| HSP90AB1 | Heat Shock Protein 90 kDa α (Cytosolic), Class B Member 1 | Hs.649923 | -3.389 |
| ZNF43 | Zinc Finger Protein 43 | Hs.534365 | -3.383 |
| PAM | Peptidylglycine α-Amidating Monooxygenase | Hs.369430 | -3.378 |
| EIF3S9 | Eukaryotic Translation Initiation factor 3, Subunit 9 η, 116 kDa | Hs.371001 | -3.368 |
| TUBA3 | Tubulin, α 3 | Hs.524390 | -3.36 |
| RORA | RAR-Related Orphan Receptor A | Hs.569497 | -3.351 |
| TK1 | Thymidine Kinase 1, Soluble | Hs.515122 | -3.349 |
| EIF4E2 | Eukaryotic Translation Initiation Factor 4E Member 2 | Hs.292026 | -3.285 |
| NAT10 | N-Acetyltransferase 10 | Hs.577281 | -3.272 |
| PCGF2 | Polycomb Group Ring Finger 2 | Hs.371617 | -3.259 |
| KIAA0174 | KIAA0174 | Hs.232194 | -3.249 |
| BIRC5 | Baculoviral IAP Repeat-Containing 5 (Survivin) | Hs.645371 | -3.234 |
| SLC35C2 | Solute Carrier Family 35, Member C2 | Hs.593344 | -3.221 |
| EFTUD2 | Elongation Factor TU GTP Binding Domain Containing 2 | Hs.151787 | -3.213 |
| PABPC4 | Poly(A) Binding Protein, Cytoplasmic 4 (Inducible form) | Hs.169900 | -3.2 |
| CSTF2 | Cleavage Stimulation Factor, 3' Pre-RNA, Subunit 2, 64 kDa | Hs.132370 | -3.196 |
| TPI1 | Triosephosphate Isomerase 1 | Hs.524219 | -3.196 |
| ASNA1 | ArsA Arsenite Transporter, ATP-Binding, Homolog 1 (Bacterial) | Hs.465985 | -3.185 |
| GLG1 | Golgi Apparatus Protein 1 | Hs.201712 | -3.182 |
| MLLT7 | Myeloid/Lymphoid or Mixed-Lineage Leukemia (Trithorax Homolog, *Drosophila*); translocated to, 7 | Hs.584654 | -3.166 |
| DDX39 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 | Hs.311609 | -3.165 |
| BAK1 | Bcl2-Antagonist/Killer 1 | Hs.485139 | -3.156 |
| VAT1 | Vesicle Amine Transport Protein 1 Homolog (T Californica) | Hs.514199 | -3.155 |
| EIF3S2 | Eukaryotic Translation Initiation Factor 3, subunit 2 ß, 36 kDa | Hs.530096 | -3.147 |
| P4HB | Procollagen-Proline, 2-Oxoglutarate 4-Dioxygenase (Proline 4-Hydroxylase), ß polypeptide | Hs.464336 | -3.141 |
| LOC729708 | LOC729708: Similar to Triosephosphate Isomerase (TIM) (Triose-Phosphate Isomerase) | Hs.567941 | -3.125 |
| HIST1H3D | Histone Cluster 1, H3d | Hs.532144 | -3.12 |
| PSMB6 | Proteasome (Prosome, Macropain) subunit, ß type, 6 | Hs.77060 | -3.114 |
| EXOC3 | Exocyst Complex Component 3 | Hs.646923 | -3.114 |
| GNL1 | Guanine Nucleotide Binding Protein-Like 1 | Hs.83147 | -3.093 |
| TOMM40 | Translocase of Outer Mitochondrial Membrane 40 Homolog (Yeast) | Hs.110675 | -3.09 |
| MAPKAPK3 | Mitogen-Activated Protein Kinase-Activated Protein Kinase 3 | Hs.234521 | -3.087 |
| RPN1 | Ribophorin I | Hs.518244 | -3.075 |
| SSRP1 | Structure Specific Recognition Protein 1 | Hs.523680 | -3.074 |
| PRNPIP | Prion Protein Interacting Protein | Hs.132497 | -3.062 |
| AP2M1 | Adaptor-Related Protein Complex 2, MU 1 Subunit | Hs.518460 | -3.059 |
| UPP1 | Uridine Phosphorylase 1 | Hs.488240 | -3.059 |
| LY6E | Lymphocyte Antigen 6 Complex, locus E | Hs.521903 | -3.056 |
| ARPC1B | Actin Related Protein 2/3 Complex, Subunit 1B, 41 kDa | Hs.489284 | -3.051 |
| FARSLA | Phenylalanine-tRNA Synthetase-Like, α Subunit | Hs.23111 | -3.044 |
| POLA2 | Polymerase (DNA directed), α 2 (70 kDa Subunit) | Hs.201897 | -3.043 |
| RFC5 | Replication Factor C (Activator 1) 5, 36.5 kDa | Hs.506989 | -3.042 |
| MLF2 | Myeloid Leukemia Factor 2 | Hs.524214 | -3.034 |
| AIP | Aryl Hydrocarbon Receptor Interacting Protein | Hs.412433 | -3.021 |
| MRPL28 | Mitochondrial Ribosomal Protein L28 | Hs.513230 | -3.015 |
| PPP2R4 | Protein Phosphatase 2A, Regulatory Subunit B' (PR 53) | Hs.400740 | -3.008 |

Table II. Continued.

| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| KEAP1 | Kelch-Like Ech-Associated Protein 1 | Hs.465870 | -2.985 |
| SNRPN | Small Nuclear Ribonucleoprotein Polypeptide N | Hs.564847 | -2.965 |
| CLIC1 | Chloride Intracellular Channel 1 | Hs.414565 | -2.965 |
| HNRPU | Heterogeneous Nuclear Ribonucleoprotein U (Scaffold Attachment Factor A) | Hs.166463 | -2.958 |
| FTL | Ferritin, Light Polypeptide | Hs.433670 | -2.957 |
| NDUFS2 | NADH Dehydrogenase (Ubiquinone) Fe-S Protein 2, 49 kDa (NADH-Coenzyme Q Reductase) | Hs.173611 | -2.946 |
| RBP1 | Retinol binding protein 1, cellular | Hs.529571 | -2.944 |
| KRT17 | Keratin 17 | Hs.2785 | -2.932 |
| PPP1CA | Protein Phosphatase 1, Catalytic Subunit, α isoform | Hs.183994 | -2.931 |
| HEXA | Hexosaminidase A (α polypeptide) | Hs.604479 | -2.924 |
| HSPA8 | Heat Shock 70 kDa Protein 8 | Hs.180414 | -2.917 |
| TRAP1 | TNF Receptor-Associated Protein 1 | Hs.30345 | -2.904 |
| ATP6V0D1 | ATPase, $H^+$ Transporting, Lysosomal 38 kDa, V0 Subunit D1 | Hs.106876 | -2.901 |
| TNIP1 | TNFAIP3 Interacting Protein 1 | Hs.543850 | -2.901 |
| MARCKSL1 | Marcks-Like 1 | Hs.75061 | -2.892 |
| BCAP31 | B-cell Receptor-Associated Protein 31 | Hs.522817 | -2.854 |
| CRIP2 | Cysteine-Rich Protein 2 | Hs.534309 | -2.854 |
| PRMT1 | Protein Arginine Methyltransferase 1 | Hs.20521 | -2.845 |
| UBTF | Upstream Binding Transcription Factor, RNA Polymerase I | Hs.89781 | -2.837 |
| PSMD13 | Proteasome (Prosome, Macropain) 26S Subunit, non-ATPase, 13 | Hs.134688 | -2.831 |
| KLHDC8B | Kelch Domain Containing 8B | Hs.13781 | -2.806 |
| CCT7 | Chaperonin Containing Tcp1, Subunit 7 (ETA) | Hs.368149 | -2.804 |
| LAMP1 | Lysosomal-Associated Membrane Protein 1 | Hs.494419 | -2.803 |
| HSPA1A | Heat Shock 70 kDa Protein 1A | Hs.520028 | -2.795 |
| TEC | TEC Protein Tyrosine Kinase | Hs.479670 | -2.78 |
| UROD | Uroporphyrinogen Decarboxylase | Hs.78601 | -2.777 |
| SEC13 | SEC13-Like 1 (*S. Cerevisiae*) | Hs.166924 | -2.768 |
| ZNF496 | Zinc Finger Protein 496 | Hs.168677 | -2.757 |
| FKBP4 | FK506 Binding Protein 4, 59 kDa | Hs.524183 | -2.732 |
| RAB21 | RAB21, Member Ras Oncogene Family | Hs.524590 | -2.727 |
| FADS1 | Fatty Acid Desaturase 1 | Hs.503546 | -2.724 |
| DNAJB1 | Dnaj (Hsp40) Homolog, Subfamily B, Member 1 | Hs.515210 | -2.71 |
| ITPK1 | Inositol 1,3,4-Triphosphate 5/6 Kinase | Hs.30812 | -2.703 |
| DDOST | Dolichyl-Diphosphooligosaccharide-Protein Glycosyltransferase | Hs.523145 | -2.702 |
| UBADC1 | Ubiquitin Associated Domain Containing 1 | Hs.9194 | -2.702 |
| SLC29A1 | Solute Carrier Family 29 (Nucleoside Transporters), member 1 | Hs.25450 | -2.694 |
| KIAA0859 | KIAA0859 | Hs.647726 | -2.692 |
| IGFBP6 | Insulin-Like Growth Factor Binding Protein 6 | Hs.274313 | -2.691 |
| PSMC3 | Proteasome (Prosome, Macropain) 26S Subunit, ATPase, 3 | Hs.250758 | -2.682 |
| KRT5 | Keratin 5 (Epidermolysis Bullosa Simplex, Dowling-Meara/Kobner/Weber-Cockayne Types) | Hs.433845 | -2.682 |
| EIF2S3 | Eukaryotic Translation Initiation Factor 2, subunit 3 γ, 52 kDa | Hs.539684 | -2.653 |
| FOSL2 | FOS-Like Antigen 2 | Hs.220971 | -2.645 |
| CLDN3 | Claudin 3 | Hs.647023 | -2.642 |
| COPZ1 | Coatomer Protein Complex, Subunit ζ 1 | Hs.505652 | -2.636 |
| GUK1 | Guanylate Kinase 1 | Hs.376933 | -2.609 |
| AP2S1 | Adaptor-Related Protein Complex 2, σ 1 subunit | Hs.119591 | -2.585 |
| SMARCE1 | Swi/Snf Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily E, Member 1 | Hs.547509 | -2.584 |
| FPGS | Folylpolyglutamate Synthase | Hs.335084 | -2.58 |
| HLA-B | Major Histocompatibility Complex, Class I, B | Hs.77961 | -2.578 |
| ID1 | Inhibitor Of DNA Binding 1, Dominant Negative Helix-Loop-Helix Protein | Hs.504609 | -2.576 |

Table II. Continued.

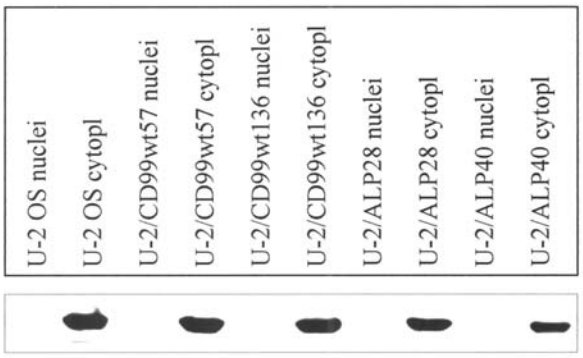
| Gene symbol | Gene name | Unigene | Score (d) |
|---|---|---|---|
| MYBPH | Myosin Binding Protein H | Hs.927 | -2.546 |
| GPAA1 | Glycosylphosphatidylinositol Anchor Attachment Protein 1 Homolog (Yeast) | Hs.627962 | -2.542 |
| CLTB | Clathrin, Light Polypeptide (Lcb) | Hs.484241 | -2.537 |
| UBC | Ubiquitin C | Hs.520348 | -2.53 |
| DGCR6L | Digeorge Syndrome Critical Region Gene 6-Like | Hs.410965 | -2.526 |
| SLC25A4 | Solute Carrier Family 25 (Mitochondrial Carrier; Adenine Nucleotide Translocator), member 4 | Hs.246506 | -2.505 |
| KIAA0664 | KIAA0664 | Hs.22616 | -2.505 |
| EIF3S4 | Eukaryotic Translation Initiation Factor 3, subunit 4 δ, 44 kDa | Hs.529059 | -2.503 |
| FUS | Fusion [Involved IN T(12;16) in malignant liposarcoma] | Hs.513522 | -2.502 |

Table III. Notable differentially expressed genes involved in biological processes strictly implicated in metastasis.

| Gene symbol | Gene name | Unigene | Biological process | Mean Log ratio | SE |
|---|---|---|---|---|---|
| VCP | Valosin-containing protein | Hs.529782 | Cell communication/ cell death | -1.6052 | 0.1691 |
| DHX9 | DEAH (Asp-Glu-Ala-His) box polypeptide 9 | Hs.191518 | Cell death | -0.9132 | 0.1557 |
| BIRC5 | Survivin (Baculoviral IAP Repeat-Containing 5) | Hs.645371 | Cell death | -0.5898 | 0.1485 |
| ARPC1B | Actin Related Protein 2/3 Complex, Subunit 1B, 41 kDa | Hs.489284 | Locomotion | -0.9834 | 0.1716 |
| CTNNAL1 | α-catulin [Catenin (cadherin-associated protein) α-like 1] | Hs.58488 | Cell death/ cell adhesion | 0.7292 | 0.3332 |
| GADD45A | Growth arrest and DNA-damage-inducible, α | Hs.80409 | Cell growth arrest/cell death | 0.734 | 0.2455 |
| AMFR | Autocrine Motility Factor Receptor | Hs.295137 | Cell communication/ locomotion | -0.686 | 0.2492 |
| MCAM | Melanoma Cell Adhesion Molecule | Hs.599039 | Cell adhesion | -1.0358 | 0.1748 |

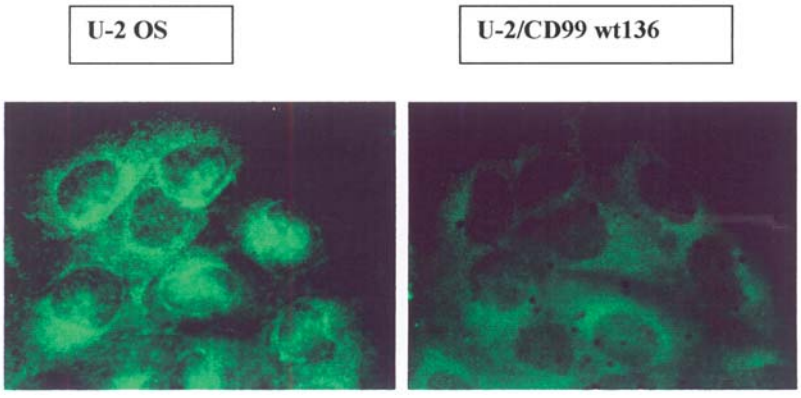
## Results

The gene expression profiles of both L/B/K ALP- and CD99-transfected clones were compared to those of the U-2/OS osteosarcoma cell line. Filtering and normalization of raw microarray data were performed using GP3. A total of 2506 cDNAs with GP3 quality-passed values were present in 100% of the arrays and were included in the final analysis by the SAM program. Four hundred and eighty sequences showed significantly different levels of expression in both L/B/K ALP- and CD99-transfected osteosarcoma clones, compared to U-2 OS. Only genes that were up-regulated or down-regulated in at least 85% of the arrays and showing a highly significant change of expression (score of >2.5 or <-2.5) were considered (Table II). Of these genes, 75 were up-regulated whereas 178 were down-regulated in transfected osteosarcoma clones. We then assigned the different expressed genes to non-mutually exclusive functional categories regarding biological processes, using FatiGO. The selected genes covered a broad range of functional activities, including regulation of cellular growth, signal transduction, transport, cell death, cell differentiation, cell adhesion, and cell motility. Among all genes selected, great relevance was attached to the genes belonging to two functional categories, namely cell growth arrest/cell death and cell motility, since these are intimately involved in tumor progression and invasiveness (Table III). In particular, we identified several differentially expressed genes involved in the NF-κB cell survival pathway, a signaling pathway directly implicated in cancer cell survival and apoptosis, including the gene encoding Valosin-containing protein (VCP), DEAH (Asp-Glu-Ala-His) box polypeptide 9, (DHX9), Survivin (BIRC5) and Growth arrest and DNA-damage-inducible, α (GADD45α). GADD45α, which belongs to a family that encodes three nuclear proteins related with growth arrest and inducible by DNA damage proteins (16,17), is up-regulated in both the transfected osteosarcoma cell lines, showing lack of metastatic ability. Since it is known that NF-κB-mediated repression of GADD45α is sufficient for survival of various cancer cell types (18), it is conceivable that the up-regulation of GADD45α, may be dependent on inhibition of the NF-κB




A


B




C

Figure 1. Protein nuclear fraction was extracted and GAPDH staining used to exclude cytoplasmic contamination. As expected GAPDH was evident only in the cytoplasmic but not in the nuclear fraction lysates (A). NF-κB expression was observed in the nuclear fractions of U-2 OS parental cells and transfectants. A lower level of NF-κB expression was observed in the less malignant variants both by Western blotting (B) and by immunofluorescence in adherent cells (C). Digital images were taken in identical conditions, at the same time and using the same image analysis software (Quips-XL genetic workstation, Abbot Visys).

signaling pathway. Indeed, we observed a decrease in the expression levels of NF-κB in the nucleus of U-2OS clones compared to parental cells (Fig. 1), which reflects a decreased activity of the nuclear factor. NF-κB is localized mainly in the cytoplasm in an inactive form bound to an inhibitory protein termed IkB (19-21). NF-κB activation occurs via phosphorylation of IkB, which is essential for the release of active NF-κB and its nuclear translocation, where it binds with specific DNA motifs in the promoter regions of target genes and activates their transcription. In keeping with a reduced NF-κB activity, in less malignant osteosarcoma clones we observed down-regulation of both VCP and DHX9, two activators of NK-κB pathways, and of Survivin, which is one of the downstream genes of the NF-κB pathway (Table III). The microarray analysis suggests that inhibition of NF-κB pathway signalling is an important mechanism to restore apoptosis/mitosis checkpoints. Quantitative PCR confirmed the significant decrease in the expression level of VCP and Survivin in all of the transfected clones (Fig. 2A). In accordance with this, we observed a decrease in the expression of cyclin D1 (Fig. 2B) and an increase in the apoptotic rate of U-2 OS transfected clones (Fig. 3A) that was particularly evident when cells were prevented from adhering (Fig. 3B). This is interesting since it is well known that non-transformed, anchorage-dependent cells undergo anoikis (i.e. detachment-induced apoptosis) when deprived of contact with the extracellular matrix. In keeping with the lower level of malignancy, U-2/CD99 and U-2/ALP cell survival was severely reduced

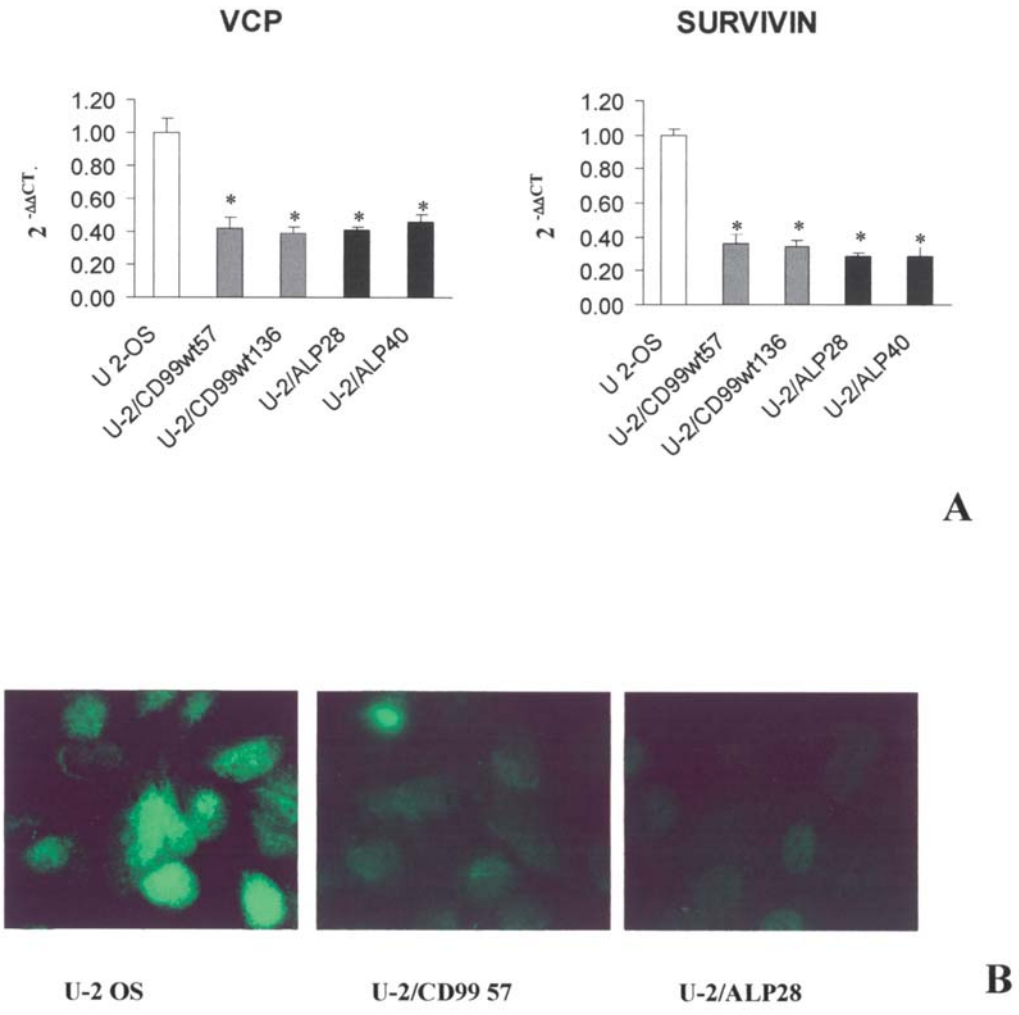



Figure 2. (A) Relative expression of VCP and Survivin mRNA in U-2 OS parental and derived cells. The relative target gene mRNA expression of U-2 OS parental cells was used as a calibrator ($2^{-\Delta\Delta CT}=1$). Data are expressed as mean values ± SE. (B) Immunostaining of cyclin D1 on adherent cells. U-2 OS transfectants expressed low levels of cyclin D1 in agreement with their lower malignant potential.

compared to parental cells when cells were cultured in a condition in which adherence of cells is prevented (15).

The decrease in Cyclin D1 expression may also be the consequence of the up-regulation of α-catulin. α-catulin is a member of the vinculin superfamily, whose mRNA expression appears to be differentially regulated following growth arrest of cells, suggesting that it may play a role in growth regulation (22). An antiproliferative role has been in fact demonstrated for α-catulin based on attenuation of cyclin D1 transcription through inhibition of Ras-mediated signals to the cyclin D1 promoter (23). Thus its overexpression in U-2 OS transfected clones, also confirmed by real-time PCR data (Fig. 4A), is in line with the reduction of cyclin D1 expression and the lower level of malignancy of transfected clones. In addition, α-catulin can associate with Rho pathway signaling *in vivo*, serving to localize Rho mediators in a multimolecular signaling complex linked to the cytoskeleton (23,24). Acting as a cytoskeletal linker protein, up-regulation of α-catulin in U-2 OS transfected clones may be associated with the higher level of actin cytoskeleton organization observed in these cells (Fig. 4B), negatively regulating actin disassembly and focal adhesion dynamics that characterize a motile phenotype. Indeed, U-2/CD99 and U-2/ALP cells overexpressing α-catulin exhibit features of strong adhesion, as characterized by abundant actin-stress fibers and focal adhesions (Fig. 4B), a pattern which has long been associated with cell differentiation and a stationary phenotype. Therefore, besides its antiproliferative role through decrease in cyclin D1 expression, α-catulin may functionally participate in the suppression of transfected U-2OS cell motility (Fig. 5A). From a molecular point of view, the dynamic assembly and disassembly of actin filaments and the formation of larger scale filament structures, crucial aspects of actin's function, are under scrupulous control by >100 actin-binding proteins. The actin-related protein-2/3 (ARP2/3) complex was the first to be identified of an important set of actin regulators that initiate formation of new actin filaments (25). Although the precise mechanisms of the ARP2/3 complex are still poorly defined, these molecules are involved in the dynamic actin disassembly during cell migration. In our transfected osteosarcoma cell lines there was a down-regulation of the ARPC1B gene (Fig. 5B), which encodes p41, a subunit of the human Arp2/3 protein complex. Therefore under-expression of ARPC1B may contribute to inhibiting disruption of actin-containing stress fibers.

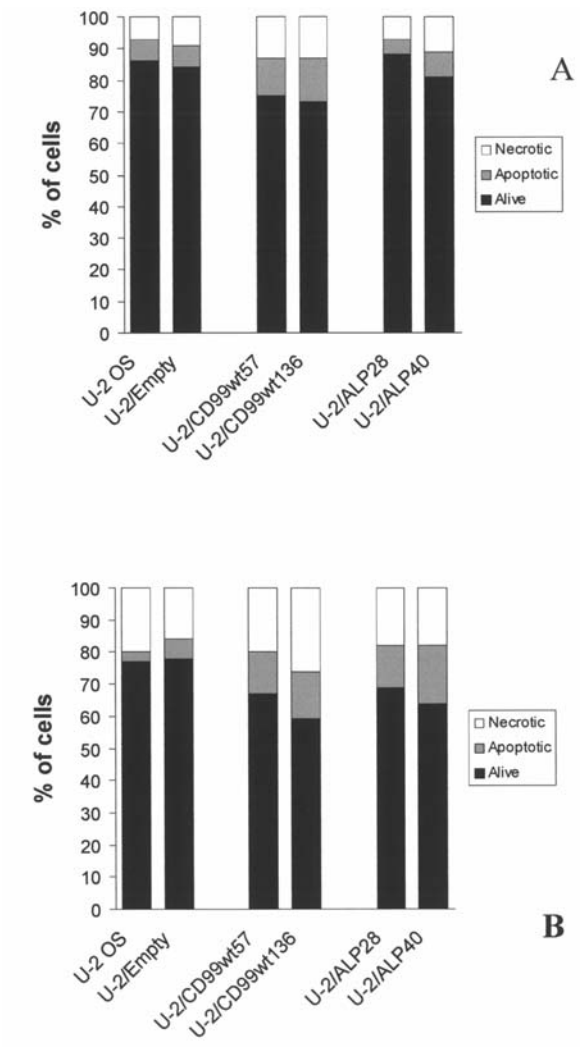



Figure 3. Cytofluorometric analysis of apoptotic U-2 OS cells and derivates by annexin-V and propidium iodide after 48 h in standard, monolayer, culture conditions (A) or on polyHEMA-coated dishes (B). Cells (250,000) were seeded on untreated or polyHEMA-coated dishes in IMDM 10% FBS and stained for annexin-V after 48 h.

## Discussion

The dissemination of osteosarcoma cells from the primary tumor to the lung is the most frequent cause of death among patients with osteosarcoma. In this study, with a view to identifying genes implicated in the spread of osteosarcoma, we employed osteosarcoma cell lines showing a reduction in metastatic ability as a result of L/B/K ALP-transfection and (likewise) CD99-transfection. Our data show that the reversal of the malignant phenotype observed in both differently transfected clones, may be due mainly to the modified expression of a set of genes related to growth arrest and apoptosis.

Recent studies have demonstrated that the forced expression of both CD99 and L/B/K ALP in U-2 OS osteosarcoma cell lines leads to an identical behavior by the differently transfected cancer cells, that is the abrogation of their invasiveness and metastatic ability (4,5). By comparing the gene expression profiles of transfected clones showing a lack of metastatic ability with those of non-transfected osteosarcoma cell lines showing contrasting characteristics, we were able to identify 253 genes that were up-regulated or down-regulated in at least 85% of the arrays to a highly significant level. These genes are particular intriguing because, being regulated in a similar manner in the majority of transfected clones, they delineate a common transcriptional profile of the metastatic phenotype. These genes can be ascribed to a broad range of functional categories including regulation of cellular growth, signal transduction, transport, cell death, cell differentiation, cell adhesion, and cell motility. Two of the most modulated categories, apart from those related to metabolism, are known to be particularly involved in tumor progression and invasiveness, namely cell growth arrest/cell death and cell motility. Although the molecular mechanisms of metastasis are poorly understood, the resistance to apoptosis seems to be not only one of the required selective advantages that a cell has to acquire in order to form a tumor, but also a crucial process in regulating metastasis. In fact, the induction of programmed cell death is important for the development of the primary tumor because it limits the time available for the accumulation of tumorigenic alterations. However a number of recent experimental observations indicate that crucial apoptotic modulators such as NF-κB, BAX, BCL2, DAPK and survivin are deregulated in metastatic cancer cells (26,27), providing support for the hypothesis that cancer cell survival and programmed cell death escape represent an advantage in metastatic behavior.

In our study we have pointed to regulation of the NF-kB cell survival pathway as a major molecular determinant of the metastasis process in osteosarcoma. In particular, we identified several differentially expressed genes involved in the NF-κB cell survival pathway, including GADD45α, VCP, DHX9 and survivin.

GADD45α is up-regulated in both transfected osteosarcoma cell lines showing a lack of metastatic ability. The GADD45 gene family encodes nuclear proteins (GADD45α, ß, γ) that interact with various cell-cycle-related proteins (16,17). It has been demonstrated that NF-κB-mediated repression of GADD45α is essential for various cancer cell types to escape programmed cell death (28). It is conceivable that the reversal of the malignant phenotype observed in our transfected osteosarcoma cell lines could equally be due in part to the up-regulation of GADD45α, which acts as a tumor suppressor gene.

VCP inhibits apoptosis via degradation of NF-κB inhibitor (29,30). It has been ascertained that VCP gene expression is associated with the metastatic ability of many human tumors such as hepatocellular carcinoma (31), gastric carcinoma (32), pancreatic ductal adenocarcinoma (33) and murine osteosarcoma cell lines (30). Again, DHX9 is involved as a transcriptional co-activator in NF-κB-mediated gene expression and its depletion reduces the NF-κB-dependent transactivation (34). In the present study both VCP and DHX9 are down-regulated in U-2 OS transfected osteosarcoma cell lines showing a lack of metastatic ability. In addition, survivin, a member of the inhibitor of apoptosis protein (IAP) family, is likewise down-regulated in our transfected osteosarcoma cell lines. Survivin is one of the downstream genes of the NF-κB pathway (35) and is overexpressed in human cancer cells (36). Furthermore it acts as a regulator of mitosis in the G2-M checkpoint representing an important interface between

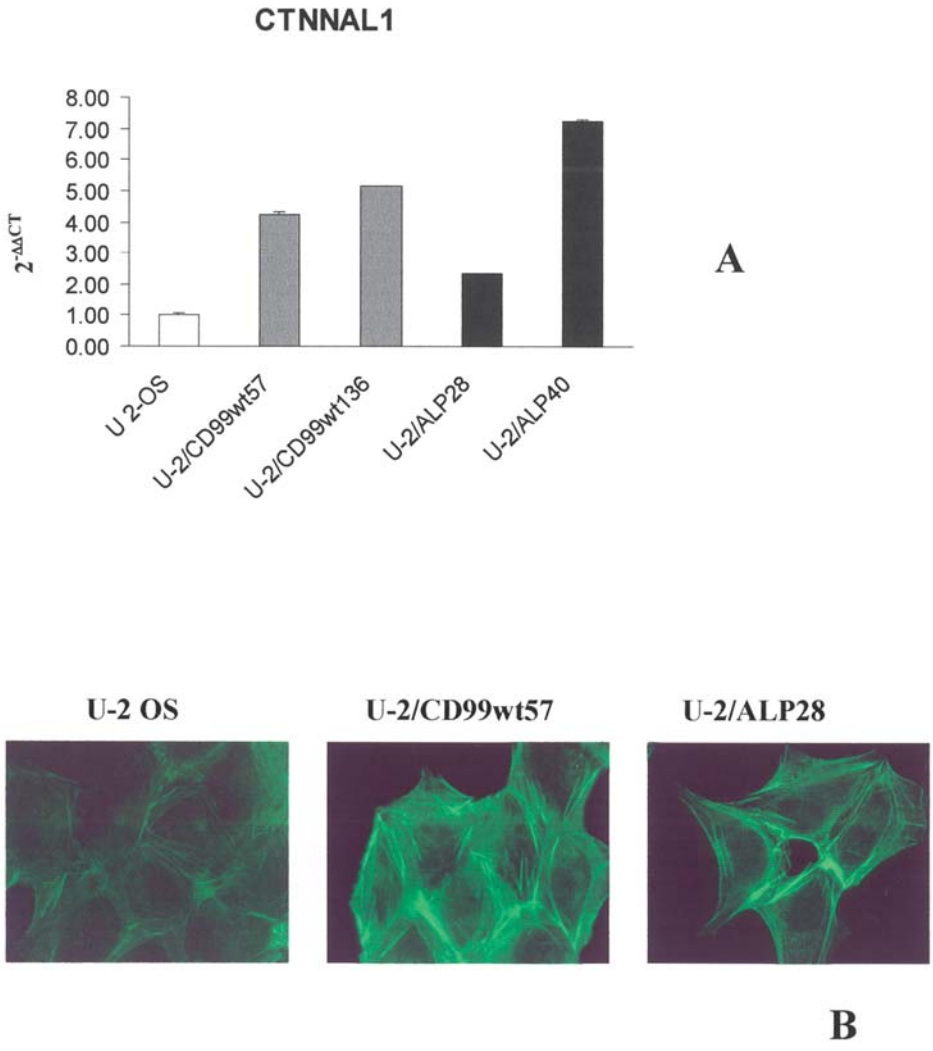



Figure 4. (A) Relative expression of CTNNAL1 mRNA in U-2 OS parental and derived cells. The relative target gene mRNA expression of U-2 OS parental cells was used as a calibrator ($2^{-\Delta\Delta C}$=1). Data are expressed as mean values ± SE. (B) Staining of actin filaments in U-2 OS cells. Cells transfected with CD99 or ALP show a higher level of actin expression and organization in keeping with a less migrating phenotype. Digital images were taken in identical conditions, at the same time and using the same image analysis software (Quips-XL genetic workstation, Abbott Visys).

cell-cycle progression and control of apoptosis (37). Survivin overexpression in cancer may obliterate this apoptotic checkpoint and allow the aberrant progression of transformed cells towards mitosis. The decrease in survivin, VCP, and DHX9 expression suggests that the observed lack of the malignant phenotype in transfected cell lines could be due to the restoration of apoptotic pathways, and especially sensitivity to anoikis, which was indeed observed in the clones with reduced malignancy when cells were experimentally prevented from adhering.

Interestingly, Mehlen and Puisieux (38) have convincingly reviewed the role of apoptosis as a multistep barrier that modulates the metastasis efficiency at three crucial levels: i) cell death, by anoikis and amorphosis, after the detachment of primary tumor cells from the extracellular matrix and neighboring cells; ii) the death of solitary cells in circulation through tumor immune surveillance or destruction by mechanical stresses; and iii) cell death during the phase of micrometastasis formation in a secondary organ. In line with the view that anoikis and amorphosis are crucial mechanisms during the metastatic process, the overexpression of α-catulin in our transfected osteosarcoma cell lines is particularly intriguing. α-catulin is a member of the vinculin superfamily, which includes proteins acting as mediators of cell-extracellular matrix adhesion, and seems to be one of the genes related to anoikis-sensitivity of tumor cells (39). α-catulin has an anti-proliferative role based on Ras-mediated inhibition of cyclin D1 promoter and is also involved in the regulation of actin polymerization, through its modulation of Rho signaling and interaction with ß-catenin (23). Thus, α-catulin up-regulation may explain the decrease in both cell growth and migration observed in U-2 OS transfected clones. Acting as a cytoskeletal linker protein, α-catulin may participate, together with the Arp 2/3 complex, in regulation of the dynamic assembly and disassembly of actin filaments and in the formation of larger scale filament structures. α-catulin may favor actin polymerization and strong cell adhesion, whereas the Arp 2/3 complex is known to regulate ß-actin polymerization at the leading edge and hence cell protrusion, and is thus functionally connected with a motile phenotype (40). In confirmation of this pattern, in our transfected osteosarcoma cell lines showing reduced invasive ability there proves to be an up-regulation

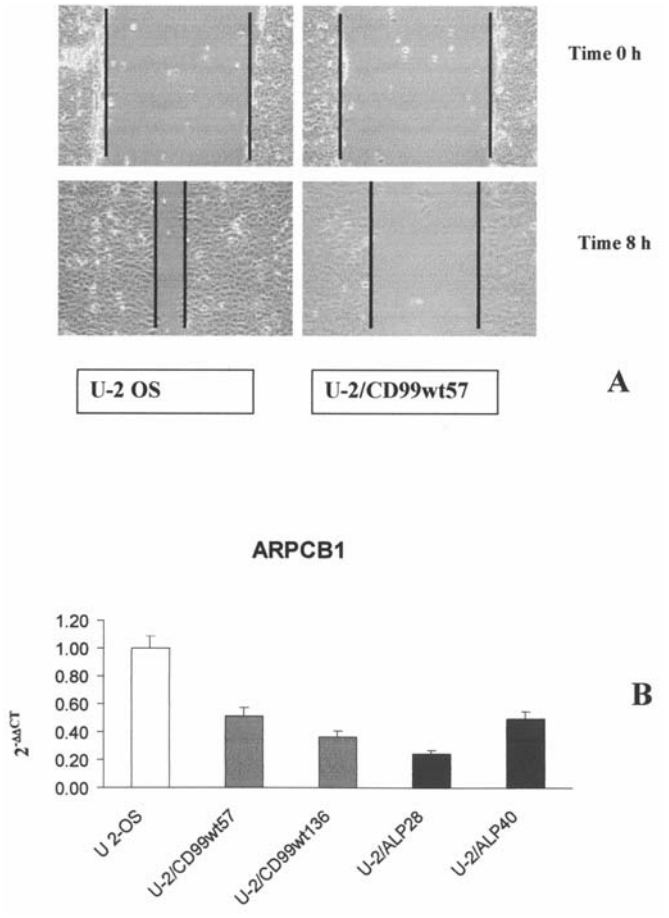



Figure 5. (A) Migration of U-2 OS cells overexpressing CD99. A wound healing assay is shown. Images were taken at time 0 and after 8 h from wounding. (B) Relative expression of ARPCB1 mRNA in U-2 OS parental and derived cells. The relative target gene mRNA expression of U-2 OS parental cells was used as a calibrator ($2^{-\Delta\Delta C}$=1). Data are expressed as mean values ± SE.

of α-catulin and down-regulation of ARPC1B. This gene encodes p41, one of seven subunits of the human Arp2/3 protein complex that is involved in assembling and maintaining the structure of the complex itself (41). Underexpression of ARPC1B presumably contributes to decreasing dynamic actin disassembly, a process that lies at the base of cancer cell migration. Moreover one cannot exclude the possibility that in our transfected cell lines a form of cell death might be induced by disruption of the motility processes involving the actin cytoskeleton.

In conclusion, our study highlights a set of few but significant genes involved in the mitosis progression/apoptosis switch which may play a pivotal role in the regulation of the metastasis process in the U-2 OS osteosarcoma cell line. It is of great interest that the expression profile of this gene set is common to two different experimental models, i.e. two differently transfected osteosarcoma cell lines (L/B/K ALP and CD99 clones), showing the same abrogation of metastatic ability. If changes in expression levels of a specific gene set can be correlated with acquiring or losing metastatic ability, such a gene set can be predictive of metastatic outcome. Thus, despite the fact that the precise involvement of the genes presented here needs to be better elucidated, we propose this set of genes as a tool to predict the clinical course of osteosarcoma. Of note, all these genes are functionally related with the NF-κB signaling pathway that appeared to be inhibited in the less malignant osteosarcoma cells, in keeping with a previous study showing that the inhibition of constitutively active NF-κB leads to reversion of malignancy in osteosarcoma (42). Hence, sustained inhibition of NF-κB may be a rational strategy for effective management of this disease.

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