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ORIGINAL PAPER

Microarray analysis identifies distinct gene expression profiles associated with histological subtype in human osteosarcoma

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Abstract Osteosarcoma is the most common primary malignant bone tumour. Currently osteosarcoma classification is based on histological appearance. It was the aim of this study to use a more systematic approach to osteosarcoma classification based on gene expression analysis and to identify subtype specific differentially expressed genes. We analysed the global gene expression profiles of ten osteosarcoma samples using Affymetrix U133A arrays (five osteoblastic and five non-osteoblastic osteosarcoma patients). Differential gene expression analysis yielded 75 genes up-regulated and 97 genes down-regulated in osteoblastic versus non-osteoblastic osteosarcoma samples, respectively. These included genes involved in cell growth, chemotherapy resistance, angiogenesis, steroid- and neuropeptide hormone receptor activity, acute-phase response and serotonin receptor activity and members of the Wnt/ß-catenin pathway and many others. Furthermore, we validated the highly differential expression of six genes including angiopoietin 1, IGFBP3, ferredoxin 1, BMP, decorin, and fibulin 1 in osteoblastic osteosarcoma relative to non-osteoblastic osteosarcoma. Our results show the utility of gene expression analysis to study osteosarcoma subtypes, and we identified several genes that may play a role as potential therapeutic targets in the future.

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Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents. The introduction of multiagent chemotherapy followed by surgical resection and postoperative chemotherapy has improved the long-term survival of patients with osteosarcoma from only 20% to nearly 70% during the last 30 years [1]. However, there is still a large number of patients whose tumours respond poorly to chemotherapy and who are at high risk for local recurrence and metastasis. These patients do not benefit from the improvements [2] achieved so far and still die early. The ability to identify a high-risk group among osteosarcoma patients would be of major importance in the development of new and risk-adapted strategies.

Osteosarcoma is classified as a malignant mesenchymal neoplasm in which the tumour produces defective, immature bone (osteoid). Despite this simple definition, the clinical behaviour of osteosarcoma is highly heterogeneous in many aspects.

Some osteosarcoma patients can be cured by local therapy without any further adjuvant therapy, whereas others are resistant to chemotherapeutic drugs and present with widespread distant metastasis at the time of diagnosis. The histomorphological findings of each tumour also show a great variety of characteristics.

The predominant cell type in most osteosarcoma is osteoblastic, while others show more fibroblastic–fibrohistocytic and chondroblastic features. Furthermore, osteosarcoma is one of the most frequent tumours associated with other malignancies or hereditary syndromes such as Li-Fraumeni-, Werner-, or Rothmund-Thomson syndrome. This pronounced heterogeneity raises the question whether osteosarcoma is a single entity at all.

The biological and clinical significance of osteosarcoma subtypes are controversial in literature because data based upon large enough controlled randomised trials recognising osteosarcoma subtypes as separate entities are lacking.

Currently most osteosarcomata are categorised on the basis of morphological and histological criteria as common, chondroblastic, small cell, teleangiectatic, fibroblastic, osteoclast rich, anaplastic, and others.

The prognostic relevance of histological subtypes of osteosarcoma has received little attention and remains a controversial issue [3–6]. Previous studies have shown that the histological subtype of osteosarcoma is a predictive factor for response to chemotherapy [7, 8] and correlates with disease-free [9, 10] and overall survival [3]. Furthermore, a non-common subtype of osteosarcoma raises the possibility of an individual belonging to a family with hereditary cancer syndrome, reflecting a possible genetic background for malignancy [11]. So far the treatment options for most patients with osteosarcoma are not different between either of these histological subtypes. There is an urgent need to identify markers that distinguish subtypes of osteosarcoma and which may have therapeutic and prognostic implications.

The development of advanced technologies, including serial analysis of gene expression has provided the means to identify global gene expression patterns for a large number of tumour and normal tissue samples. These approaches have been used to characterise genes whose altered expression is important in the development and behaviour of subtypes of tumours. Furthermore, gene expression array profile with bioinformatics analysis can be used to identify the molecular signature of an individual patient's tumour. Subsequent pathway analysis of the resulting gene lists can reveal distinct signalling events which might account for the biological properties attributed to each tumour type.

The aim of this study was to present a comprehensive genomic analysis of osteosarcoma and to better characterise the molecular expression profiles of different sub-types of osteosarcoma.

We applied a microarray-based gene expression profiling approach on ten OS samples to identify molecular signatures that distinguish osteosarcoma subtypes. Elucidation of such molecular expression signatures may be useful in predicting the clinical behaviour of osteosarcoma as well as identifying candidate cellular pathways that can be targets for future therapeutic approaches.

Materials and methods

Patients and total RNA isolation

The study included tissue specimens from ten patients who underwent open biopsy for definite diagnosis of osteosarcoma and before receiving preoperative chemotherapy. All tumour samples were classified by two experienced pathologists. Total RNA was extracted from frozen tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity, and integrity of RNA samples were determined by UV absorbance at 260 nm and electrophoresis [12].

cRNA synthesis and gene expression profiling

Total RNA from ten osteosarcoma samples was isolated as described above. Total RNA was repurified with RNeasy MinElute kit per manufacturer's instructions (Qiagen, Valencia, CA). The total RNA (5 µg) was then used for GeneChip analysis. Preparation of cRNA, hybridisation to human U133A GeneChips (Affymetrix, Santa Clara, CA, USA) and scanning of the arrays were carried out according to manufacturer's protocols (https://www.affymetrix.com) as previously published [13].

Bioinformatic analysis

RMA signal extraction, normalisation and filtering was performed as described by Bioconductor (http://www.bioconductor.org) [14]. A non-specific filter was applied prior to hypothesis testing in order to remove genes of low informational content. The filtering criteria for the exemplary data sets required the expression level to be higher than 100 in more than 20% of the samples and the interquartile range (IQR) across the samples on the log base 2 scale to be at least 0.5. To identify genes differentially expressed between the two conditions, we performed a statistical comparison using the limma package implemented in the Bioconductor suite (www.bioconductor.org), which estimates the fold change between predefined sample groups by fitting a linear model and using an empirical Bayes method to moderate the standard errors of the estimated log-fold changes for each probe set. A multiple testing correction based on the false discovery rate (FDR) was performed to produce adjusted p-values

[15]. Identification of significantly enriched pathways and gene groups was performed using the methods outlined in a previous publication [16]. For the purpose of visualisation, genes were clustered using a hierarchical cluster algorithm with average linkage and Spearmans rank correlation distance, as provided by the software EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/). Results were visualised with the help of heatmaps and dendrograms. The heatmaps show colour-coded expression levels (*red* high expression, *black* medium expression, and *green* low expression) as seen in Fig. 1.

Real-time PCR

RNA was extracted using Tri-Reagent (Sigma) according to the manufacturers' protocols. cDNA was synthesised as previously described and PCR amplification was performed as previously described. Primer pairs were selected to span exon boundry sequences to avoid signal detection from human genomic DNA and were purchased from Applied Biosystems. Primer assays used were: Hs00919202_m1 (angiopoietin 1), Hs00400446_m1 (IGFBP3), Hs01070066_g1 (ferredoxin 1), Hs01002399_m1 (BMP), Hs01072200_m1 (decorin), and Hs00972625_m1 (fibulin 1). Human B2M (beta-2-microglobulin, NM_004048.2, Applied Biosystems) was used as endogenous control.

For each PCR, 6 µl cDNA (diluted 1:3 in nuclease-free water), 25 µl Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM forward primer, 900 nM reverse primer, 200 nM probe and nuclease-free water were added to a final volume of 50 µl. Amplification and detection were carried out in a DNA Thermal Cycler 480 (Perkin-Elmer). Cycling conditions were as follows: depending on the primers, 25–35 cycles were carried out at 94°C for 1 min, 68°C for 2 min, 72°C for 2 min, with an extension of 5 s with each subsequent cycle.

Results

Identification of differentially expressed genes between osteoblastic and non-osteoblastic osteosarcoma based on gene-expression profiles

Considering the difficulties in determining the histological subtype in osteosarcoma biopsies, we examined genes

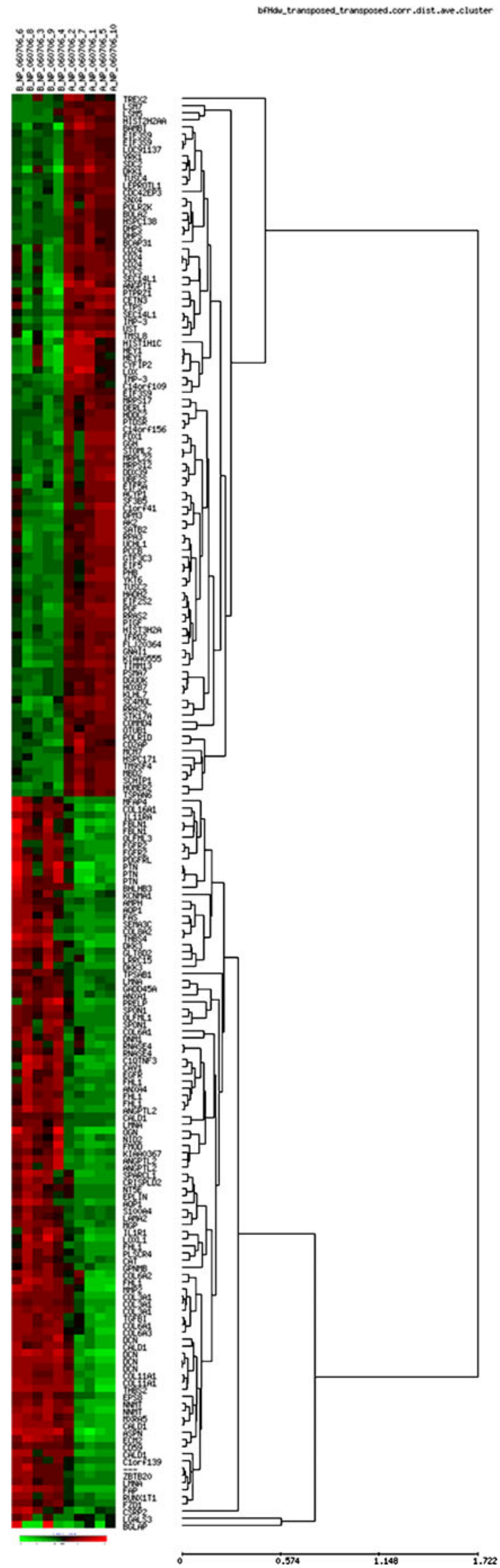

Fig. 1 Heat map and supervised hierarchical clustering of genes that distinguish osteoblastic from non-osteoblastic osteosarcoma patients. Each row represents a gene, and patient samples are depicted in columns. *Red* indicates genes that are expressed at higher levels. *Green* indicates genes that are expressed at lower levels compared with mean expression

whose expression differed between the histological subclasses. Two patient groups were compared: group A which included exclusively osteoblastic osteosarcoma samples (five cases) and group B including non-osteoblastic osteosarcoma samples (five cases).

We used Affymetrix Gene Chip arrays containing more than 20,000 genes to generate gene expression profiles for ten osteosarcoma samples. We detected 172 genes differentially expressed between the five osteoblastic and five non-osteoblastic osteosarcoma samples (Table 1). Of these, 75 were significantly up-regulated and 97 significantly down-regulated in osteoblastic versus non-osteoblastic osteosarcoma.

Several genes involved in growth, maturation and signalling (TMSL8, ANGPT1, PGF, IMP-3, DKK1, BAMBI, and RRAS2) were expressed in higher levels in osteoblastic osteosarcoma. Genes involved in regulation of gene expression (histone 1, histone 2, histone 3, centrin, and C1orf41) were also expressed in increased levels. Furthermore, several genes implicated in cell cycle and metabolism (SEC14L1, UBE2S, Ferredoxin 1, GGH, Cytochrome c, EIF5A, and prohibitin) and cell–cell interaction/kinase activation (lysyl oxidase, CTP synthase, CD24, CD2AP, adenylate kinase 2, SNX4, syndecan 2, ACYP1 and UCHL1) had increased expression in osteoblastic osteosarcoma compared with non-osteoblastic osteosarcoma. Genes with >2-fold overexpression are presented in Table 1.

In contrast, 97 genes had reduced expression in osteoblastic osteosarcoma patients compared with non-osteoblastic osteosarcoma patients. There was an overrepresentation of members of genes involved in collagen synthesis (COL3A1, COL6A1, COL8A2, COL11A1, COL6A2, COL6A3, and COL16A1) and extracellular matrix (ECM2, MMP2, MGP, and SPON1). Table 2 lists the names and biological functions of genes expressed in reduced levels with a fold difference >2.

To determine whether particular functional categories of genes were highly enriched in one of the groups we identified gene ontology functional categories that were statistically significant among the list of differentially regulated genes. Genes with increased expression in osteoblastic osteosarcoma were linked to nucleobase- and polyamine metabolism and aerobic respiration. Genes expressed in reduced levels in osteoblastic osteosarcoma included genes involved in steroid- and neuropeptide hormone receptor activity, acute-phase response and serotonin receptor activity. Additional functional and pathway classification of the differentially expressed genes is shown in Fig. 2.

Real-time PCR validation of microarray data

To confirm the results obtained using microarrays, we performed real-time PCR on six selected genes. These genes included angiopoietin 1, IGFBP3, ferredoxin 1, BMP, decorin, and fibulin 1. We used RNA from the same ten tumour samples that were used for microarray analysis.

As shown in Fig. 3, RT-PCR analysis performed on five osteoblastic and five non-osteoblastic osteosarcoma demonstrated significant expression differences. This result indicates that the RT-PCR results are highly consistent with the microarray data.

Discussion

Microarray technology has provided the means for studying the molecular basis of tumours by examining thousands of genes simultaneously. Using whole genome expression profiling of osteosarcoma samples, we showed that conventional, osteoblastic osteosarcoma are clearly distinct from other osteosarcoma subtypes.

This is consistent with the distinct clinicopathological aspects of different osteosarcoma subtypes [10]. The subtype of osteosarcoma seems to be a predictive factor for response to chemotherapy [17] and tends to be associated with disease-free and overall survival [4, 8].

Although classification of osteosarcoma based on morphological appearance of the tumour is an important prognostic factor, histological subclassification can be difficult even among experienced pathologists. Therefore there is a need to develop new objective methods of osteosarcoma subclassification.

The results of our study using microarray expression signature suggest that osteosarcoma can be classified into two groups based on gene expression profiles, which showed a strong association with histomorphological subtype. Several genes involved in the formation of extracellular matrix showed a clearly distinct expression pattern. For example, the collagen types 3, 6, 11, and 16 were down-regulated in the osteoblastic osteosarcoma subgroup. This is in accordance with previous studies where the histological appearance of osteosarcoma specimens has been linked to differences in collagen expression [18]. Furthermore, our comparison of differentially expressed genes within these clusters identified several genes with important implications concerning the origin and clinical behaviour of osteosarcoma and genes that may be targeted for novel therapeutics.

Differential expression of genes encoding for growth factors and receptors

We found a significantly different expression of transforming growth factor, beta-induced (TGFBI) between osteoblastic and non-osteoblastic osteosarcoma. Transforming growth factor, beta-induced (TGFBI) is an extracellular matrix molecule initially cloned from human adenocarcinoma cells treated with TGF-β. Transforming growth

Table 1 Genes upregulated in osteoblastic versus non-osteoblastic osteosarcomas

Gene symbol	Mean (A)	Mean (B)	Fold change (A/B)	Gene title
TMSL8	3,403	612	5.56	Thymosin-like 8
PTPRZ1	1,680	430	3.90	Protein tyrosine phosphatase, receptor-type
LOX	4,386	790	5.55	Lysyl oxidase
ANGPT1	1,989	409	4.86	Angiopoietin 1
HIST1H1C	2,442	492	4.97	Histone 1, H1c
DKK1	2,386	606	3.94	Dickkopf homolog 1 (Xenopus laevis)
CYFIP2	3,521	978	3.60	Cytoplasmic FMR1 interacting protein 2
BAMBI	6,453	1,865	3.46	BMP and activin membrane-bound inhibitor homolog
HEY1	4,315	1,203	3.59	Hairy/enhancer-of-split related with YRPW motif 1
CTPS	1,314	453	2.90	CTP synthase
SEC14L1	391	123	3.17	SEC14-like 1 (S. cerevisiae)
IMP-3	327	102	3.20	IGF-II mRNA-binding protein 3
TIMM13	1,998	634	3.15	Translocase of inner mitochondrial membrane13 homolog
SC4MOL	1,214	428	2.84	Sterol-C4-methyl oxidase-like
C1orf41	748	241	3.11	Chromosome 1 open reading frame 41
RRAS2	767	256	3.00	Related RAS viral (r-ras) oncogene homolog 2
UCHL1	3,664	1,213	3.02	Ubiquitin carboxyl-terminal esterase L1
HOMER2	870	290	3.00	Homer homolog 2 (Drosophila)
UBE2S	2,351	821	2.86	Ubiquitin-conjugating enzyme E2S
RRAS2	291	103	2.83	Related RAS viral (r-ras) oncogene homolog 2
PGF	869	321	2.71	Placental growth factor
FDX1	2,028	705	2.88	Ferredoxin 1
IMP-3	253	87	2.90	IGF-II mRNA-binding protein 3
CETN3	631	243	2.59	Centrin, EF-hand protein, 3
GGH	1,231	437	2.82	Gamma-glutamyl hydrolase
GNAI1	423	161	2.63	Guanine nucleotide binding protein (G protein)
CD24	1,850	767	2.41	CD24 antigen
SATB2	180	71	2.54	SATB family member 2
TM9SF4	585	223	2.63	Transmembrane 9 superfamily protein member 4
CD2AP	406	145	2.79	CD2-associated protein
TREX2 /IP1	320	114	2.80	Three prime repair exonuclease 2
DDX39	1,150	453	2.54	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39
YKT6	362	143	2.52	SNARE protein Ykt6
CYCS	6,205	2,743	2.26	Cytochrome c, somatic
PIGF	453	186	2.44	Phosphatidylinositol glycan, class F
SCHIP1	562	239	2.36	Schwannomin interacting protein 1
EIF5A	1,766	765	2.31	Eukaryotic translation initiation factor 5A
AK2	1,083	469	2.31	Adenylate kinase 2
UST	430	197	2.18	Uronyl-2-sulfotransferase
RPA3	2,553	1,108	2.30	Replication protein A3, 14 kDa
HIST2H2AA	1,130	480	2.35	Histone 2, H2aa
LEPROTL1	643	285	2.25	Leptin receptor overlapping transcript-like 1
MRPL22	952	394	2.42	Mitochondrial ribosomal protein L22
POLR2K	1,483	628	2.36	Polymerase (RNA) II (DNA directed)
FLJ20364	366	163	2.25	Hypothetical protein FLJ20364
CDC42EP3	984	463	2.13	CDC42 effector protein (Rho GTPase binding) 3
SNX4	264	116	2.28	Sorting nexin 4
HIST3H2A	1,145	524	2.18	Histone 3, H2a
STK17A	692	318	2.18	Serine/threonine kinase 17a (apoptosis-inducing)

Table 1 (continued)

Gene symbol	Mean (A)	Mean (B)	Fold change (A/B)	Gene title
SDC2	4,912	2,350	2.09	Syndecan 2
MCM7	846	363	2.33	MCM7 minichromosome maintenance deficient 7
MRPS12	667	308	2.17	Mitochondrial ribosomal protein S12
PTDSR	347	159	2.19	Phosphatidylserine receptor
LSM5	1,092	522	2.09	LSM5 homolog, U6 small nuclear RNA associated
BCAP31	3,018	1,350	2.24	B-cell receptor-associated protein 31
DPM3	814	378	2.16	Dolichyl-phosphate mannosyltransferase polypeptide 3
C14orf156	1,924	893	2.15	Chromosome 14 open reading frame 156
ACYP1	342	166	2.06	Acylphosphatase 1, erythrocyte (common) type
PHB	1,214	566	2.15	Prohibitin
STOML2	1,086	496	2.19	Stomatin (EPB72)-like 2
MRPS17	887	426	2.08	Mitochondrial ribosomal protein S17
PCCB	681	338	2.01	Propionyl Coenzyme A carboxylase, beta polypeptide
C14orf109	342	160	2.14	Chromosome 14 open reading frame 109
EIF3S9	814	388	2.10	Eukaryotic translation initiation factor 3
KIAA0555	323	158	2.05	Jak and microtubule interacting protein 2
MBD2	1,114	549	2.03	Methyl-CpG binding domain protein 2
HDDC2	1,785	880	2.03	HD domain containing 2
VRK1	517	259	2.00	Vaccinia related kinase 1
DERL1	1,509	736	2.05	Der1-like domain family, member 1
DHPS	468	228	2.05	Deoxyhypusine synthase
BOLA2	1,132	553	2.05	BolA-like 2 (E. coli)
EIF5	821	411	2.00	Eukaryotic translation initiation factor 5
TSPAN6	613	304	2.02	Tetraspanin 6
HOXB7	724	357	2.02	Homeo box B7
TUSC2	534	266	2.01	Tumor suppressor candidate 2

Selected genes with increased expression in osteoblastic versus non-osteoblastic osteosarcoma samples

Genes are ranked in order of fold change and are listed with their gene symbol ID, mean expression osteoblastic osteosarcoma patients (mean A) and non-osteoblastic osteosarcoma patients (mean B), and with their gene description

factor-beta (TGF-ß) isoforms play an important role in the regulation of cell development and growth. Osteosarcoma expression of TGF-ß isoforms is related to tumour grade and disease progression [19], and it is a key molecule triggering the expression of extracellular matrix components that play an essential role in tumour cell behaviour [20]. Furthermore, we found a strong decrease of the expression of fibulin 1, a secreted glycoprotein, in osteoblastic osteosarcoma. The fibulins modulate cell morphology and growth and play a role in adhesion and invasion of sarcoma cells [21].

Moreover, the two osteosarcoma subgroups showed a different expression level of heparin binding growth factor 8, pleiotrophin (PTN). PTN modulates cell growth and proliferation of various tumours and has been linked to chemoresistance in osteosarcoma cells [22]. Another component that modulates proliferation, cell adhesion, and migration is Syndecan-2. The Syndecans are cell surface heparan sulphate proteoglycans that can induce apoptosis [23] and sensitise osteosarcoma cells to the cytototoxic effect of chemotherapeutics [24]. Furthermore, in our setting, the expression level of fibroblast growth factor receptor 2 (FGFR2) was significantly different in both osteosarcoma subgroups. Fibroblast growth factor receptor 2 (FGFR2) plays an essential role in bone morphogenesis, and inherited mutations of the FGFR2 gene result in skeletal dysplasias [25]. Loss of heterozygosity of FGFR2 has been found in high grade osteosarcoma [26], and rearrangement of FGFR2 was reported in rat osteosarcoma cells [27]. The clinical relevance of FGFR2 expression in human osteosarcoma is yet to be determined.

Expression of genes involved in chemotherapy resistance

The success of conventional chemotherapy in osteosarcoma has been limited by drug resistance mechanisms [9, 28].

Table 2 Genes down-regulated in osteoblastic versus non-osteoblastic osteosarcomas

Gene symbol	Mean (A)	Mean (B)	Fold change (A/B)	Gene title
AMPH	120	245	−2.03	Amphiphysin
LMNA	680	1,367	−2.01	Lamin A/C
ANXA1	2,275	4,603	−2.02	Annexin A1
S100A4	3,170	6,728	−2.12	S100 calcium binding protein A4
FZD1	231	484	−2.09	Frizzled homolog 1
C1orf139	234	503	−2.15	Chromosome 1 open reading frame 139
CD59	1,155	2,364	−2.05	CD59 antigen p18-20
CAT	976	2,187	−2.24	Catalase
OLFML1	341	728	−2.14	Olfactomedin-like 1
LMNA	1,019	2,242	−2.20	Lamin A/C
NT5E	130	290	−2.23	5′-nucleotidase, ecto (CD73)
GLT8D2	381	842	−2.21	Glycosyltransferase 8 domain containing 2
LAMA2	94	221	−2.35	Laminin, alpha 2
RNASE4	92	209	−2.28	Ribonuclease, RNase A family, 4
EPLIN	865	2,040	−2.36	Epithelial protein lost in neoplasm beta
GADD45A	251	556	−2.21	Growth arrest and DNA-damage-inducible, alpha
KCNMA1	378	925	−2.44	Potassium large conductance calcium-activated
LGALS3	2,364	5,638	−2.38	Lectin, galactoside-binding, soluble, 3 (galectin 3)
RUNX1T1	140	341	−2.43	Runt-related transcription factor 1; translocated to, 1
EPS8	393	918	−2.33	Epidermal growth factor receptor pathway substrate 8
GPNMB	2,309	5,634	−2.44	Glycoprotein (transmembrane) nmb
COL3A1	9,038	20,742	−2.30	Collagen, type III, alpha 1
SPON1	45	121	−2.70	Spondin 1, extracellular matrix protein
DNM1	175	456	−2.61	Dynamin 1
KIAA0367	172	457	−2.65	KIAA0367
IL11RA	125	341	−2.72	Interleukin 11 receptor, alpha
COL6A1	390	1,075	−2.76	Collagen, type VI, alpha 1
COL8A2	282	859	−3.04	Collagen, type VIII, alpha 2
LRRC15	1,522	4,094	−2.69	Leucine rich repeat containing 15
ZBTB20	407	1,149	−2.82	Zinc finger and BTB domain containing 20
SPON1	304	873	−2.87	Spondin 1, extracellular matrix protein
FAS	137	421	−3.08	Fas (TNF receptor superfamily, member 6)
CRISPLD2	563	1,553	−2.76	Cysteine-rich secretory protein LCCL
MGP	2,104	6,137	−2.92	Matrix Gla protein
PLSCR4	191	557	−2.92	Phospholipid scramblase 4
EGFR	111	361	−3.26	Epidermal growth factor receptor
NID2	233	736	−3.17	Nidogen 2 (osteonidogen)
RNASE4	112	358	−3.21	Ribonuclease, RNase A family, 4
IL1R1	293	914	−3.12	Interleukin 1 receptor, type I
DKK3	89	321	−3.61	Dickkopf homolog 3
MFAP4	143	590	−4.12	Microfibrillar-associated protein 4
CSRP2	589	2,004	−3.40	Cysteine and glycine-rich protein 2
ANGPTL2	114	411	−3.61	Angiopoietin-like 2
FHL1	157	557	−3.55	Four and a half LIM domains 1
COL3A1	6,245	18,135	−2.90	Collagen, type III, alpha 1
COL6A1	2,820	8,359	−2.96	Collagen, type VI, alpha 1
CALD1	417	1,254	−3.01	Caldesmon 1
BHLHB3	289	1,007	−3.48	Basic helix-loop-helix domain containing, class B, 3
SEMA3C	54	205	−3.77	Sema domain, immunoglobulin domain

Table 2 (continued)

Gene symbol	Mean (A)	Mean (B)	Fold change (A/B)	Gene title
CALD1	96	321	−3.35	Caldesmon 1
COL6A2	1,754	5,247	−2.99	Collagen, type VI, alpha 2
AQP1	638	2,785	−4.36	Aquaporin 1 (channel-forming integral protein)
DCN	3,851	13,005	−3.38	Decorin
PDGFRL	236	944	−4.01	Platelet-derived growth factor receptor-like
FGFR2	65	329	−5.10	Fibroblast growth factor receptor 2
NNMT	382	1,268	−3.32	Nicotinamide N-methyltransferase
ANXA4	221	856	−3.87	Annexin A4
MXRA5	831	2,852	−3.43	Matrix-remodelling associated 5
TGFBI	2,013	6,936	−3.45	Transforming growth factor, beta-induced, 68 kDa
OLFML3	717	2,831	−3.95	Olfactomedin-like 3
PRELP	467	2,287	−4.89	Proline/arginine-rich end leucine-rich repeat protein
COL6A3	3,161	10,536	−3.33	Collagen, type VI, alpha 3
COL11A1	3,111	9,944	−3.20	Collagen, type XI, alpha 1
SPARCL1	734	3,136	−4.28	SPARC-like 1 (mast9, hevin)
CALD1	1,283	4,535	−3.54	Caldesmon 1
CAV1	602	2,814	−4.68	Caveolin 1, caveolae protein, 22 kDa
DCN	2,351	10,377	−4.41	Decorin
FMOD	503	2,694	−5.36	Fibromodulin
FBLN1	271	1,686	−6.21	Fibulin 1
ANGPTL2	705	3,621	−5.14	Angiopoietin-like 2
C1QTNF3	259	1,428	−5.51	C1q and tumor necrosis factor related protein 3
DKK3	402	1,922	−4.78	Dickkopf homolog 3 (Xenopus laevis)
NNMT	545	1,979	−3.63	Nicotinamide N-methyltransferase
ANGPTL2	198	1,114	−5.61	Angiopoietin-like 2
THBS2	914	2,833	−3.10	Thrombospondin 2
MMP2	1,670	7,241	−4.34	Matrix metallopeptidase 2
THBS4	298	2,250	−7.54	Thrombospondin 4
COL16A1	253	1,509	−5.97	Collagen, type XVI, alpha 1
ECM2	187	954	−5.11	Extracellular matrix protein 2
FHL1	988	4,692	−4.75	Four and a half LIM domains 1
DCN	1,864	8,437	−4.53	Decorin
FAP	244	1,376	−5.65	Fibroblast activation protein, alpha
LOXL1	185	1,272	−6.87	Lysyl oxidase-like 1
FBLN1	183	1,777	−9.71	Fibulin 1
PTN	424	3,781	−8.92	Pleiotrophin (heparin binding growth factor 8)
DCN	380	2,379	−6.26	Decorin
ASPN	1,415	9,796	−6.92	Asporin (LRR class 1)
OGN	78	1,027	−13.20	Osteoglycin (osteoinductive factor, mimecan)
BGLAP	178	5,425	−30.54	Bone gamma-carboxyglutamate (gla)

Selected genes with lower expression in osteoblastic versus non-osteoblastic osteosarcoma samples

Genes are ranked in order of fold change and are listed with their gene symbol ID, mean expression osteoblastic osteosarcoma patients (mean A) and non-osteoblastic osteosarcoma patients (mean B), and with their gene description

Therefore one of the most important prognostic factors in osteosarcoma is the response to preoperative chemotherapy. The administration of more intensified chemotherapy to poor responders has failed to improve survival in this patient group in several clinical trials [17]. Therefore it has been suggested that there may be an immanent genetic difference between responsive and non-responsive tumours [29].

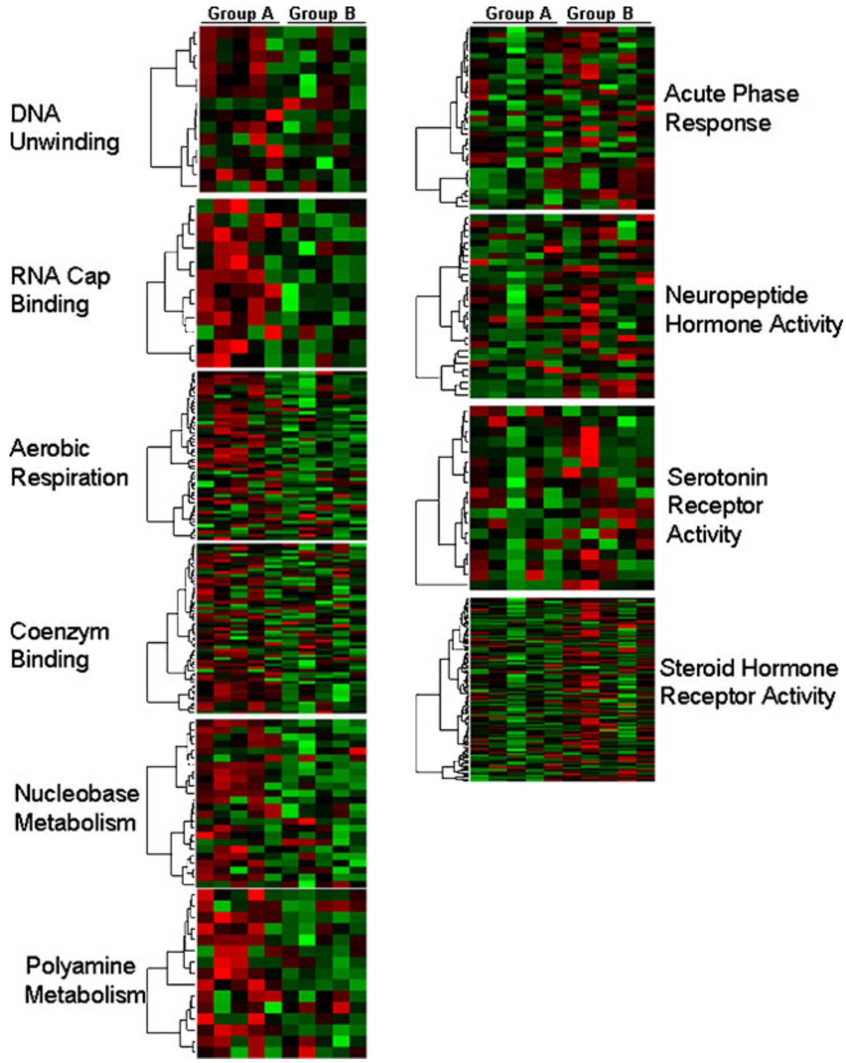

Fig. 2 Functional and pathway classification of the differentially expressed genes. Columns represent the gene expression levels of osteoblastic osteosarcoma patients (group A) and non-osteoblastic osteosarcoma patients (group B). Gene expression profile through pathway analysis demonstrates that genes involved in nucleobase- and polyamine metabolism and aerobic respiration were up-regulated in osteoblastic osteosarcoma patients (group A). Genes involved in steroid- and neuropeptide hormone receptor activity, acute-phase response and serotonin receptor activity were upregulated in non-osteoblastic osteosarcoma patients (group B). Each row represents a gene, and patient samples are depicted in columns. *Red* indicates genes that are expressed at higher levels. *Green* indicates genes that are expressed at lower l evels compared with mean expression

Interestingly we found a different expression of several genes related to drug resistance including prohibitin, Annexin1, Annexin 4 and gamma-glutamyl hydrolase (GGH) among the two osteosarcoma subgroups. Prohibitin is a potential tumour suppressor protein that plays an essential role in the modulation of drug-induced cell death and significantly reduced chemotherapy resistance in osteosarcoma cells [30]. The annexins are involved in bone resorption and formation and have been linked to drug resistance in osteosarcoma patients [29] and several human cancer cell lines [31]. One of the drugs most commonly used in systemic osteosarcoma therapy is Methotrexate (MTX). Overexpression of gamma-glutamyl hydrolase (GGH) decreases intracellular MTX and thereby impairs anti-tumour activity. Increased expression of GGH has also been shown to be associated with resistance to MTX in sarcoma cell lines [32].

Expression of genes involved in angiogenesis

Malignant proliferating cells depend on supply of nutrients and oxygen. Several genes whose expression is associated with the activation of angiogenesis were differentially expressed between the two subgroups. Among those were angiopoietin (Ang)-1 and Ang-2, decorin and Interleukin 1 receptor. Angiopoietins promote endothelial cell migration, proliferation and capillary formation and have been found to be critical mediators of angiogenesis in several tumours [33]. Differential expression of angiopoietins partially regulated by Interleukin 1 beta was also demonstrated in chondrosarcoma cells [34]. Furthermore, Decorin, an extracellular matrix protein, suppressed angiogenesis and tumour growth in osteosarcoma [35]. Decorin also inhibited cell motility and invasion and the occurrence of pulmonary metastasis in a murine osteosarcoma model [36].

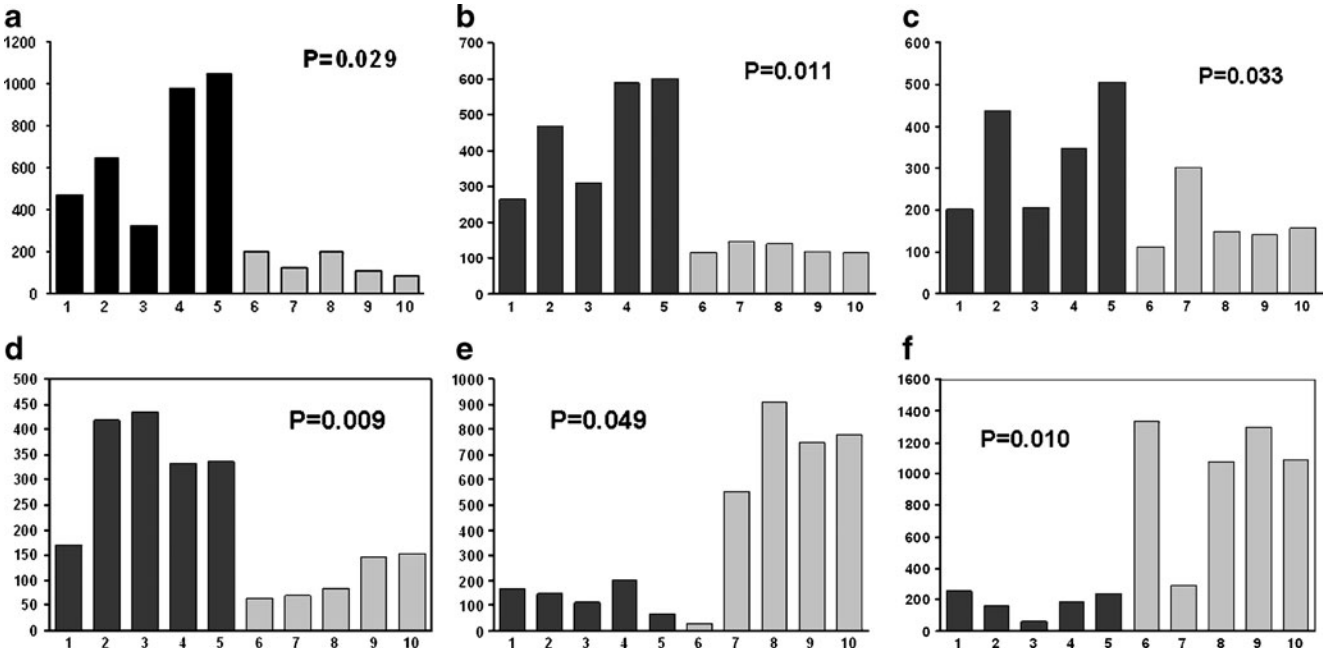

Fig. 3 Real-time (RT)-PCR for angiopoietin (**a**), IGFBP-3 (**b**), ferredoxin 1 (**c**), BMP (**d**), decorin (**e**), and fibulin 1 (**f**) was performed on total RNA extracted from tumour biopsies deriving from the malignant tissue of five patients with osteoblastic osteosarcoma and from five patients with non-osteoblastic osteosarcoma. Differences in gene expression levels between the two groups were analysed by Student's *t* test. Primers and cycling conditions for each of the amplified genes are described in Materials and Methods

Differential expression of members of the Wnt/ß-catenin pathway

The Wnt/ß-catenin signal transduction pathway promotes new bone formation acting as a positive regulator of osteoblasts. Over-expression of the Wnt pathway inhibitors, the Dickkopf (DKK) protein family members, have been associated with osteolytic metastatic bone disease in prostate carcinoma [37]. In osteosarcoma, Dickkopf (DKK) homolog 1 increased proliferation by activation of the cell cycle. Another member of the Dickkopf family, DKK 3 inhibited invasion and motility of osteosarcoma cells by modulating the Wnt/ß-catenin pathway and plays a possible role in the pathobiology and progression of osteosarcoma [38].

Low expression of Fas by osteoblastic osteosarcoma

The Fas receptor and its ligand belong to the tumour necrosis factor receptor family. Fas plays an important role in tumour cell apoptosis and tumorigenesis and in several clinical studies a decrease of Fas expression correlated with poor prognosis [39]. Furthermore, inhibition of Fas signalling promoted lung metastases growth in a murine osteosarcoma model and was considered as a potential therapeutic target for the treatment of osteosarcoma [40].

Conclusion

Using microarray-based differential expression and gene set analysis, we identified a distinct gene expression pattern of osteoblastic and non-osteoblastic osteosarcoma subgroups. The results of this analysis included genes and gene sets important to osteosarcoma pathogenesis and progression.

We are aware that our study relates to a small sample size; even so, the highly significant results distinguishing the two groups are remarkable. This study could be the basis for further investigations of osteosarcoma gene expression which may lead to the development of an important prognostic tool and the identification of potential targets for the development of new targeted therapy in the future.

Conflicts of interest statement None declared.

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