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BASIC RESEARCH

Transcriptional Profiling Identifies the Signaling Axes of IGF and Transforming Growth Factor-β as Involved in the Pathogenesis of Osteosarcoma

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Abstract

Background Osteosarcoma is the most common primary bone tumor in adolescents associated with skeletal development. The molecular pathogenesis of osteosarcoma has not been completely determined, although many molecular alterations have been found in human osteosarcomas and cell lines.

Questions/purposes We questioned whether (1) we could identify gene expression in osteosarcoma specimens that differs from normal osteoblasts and mesenchymal stem cells and (2) this would provide clues to the molecular pathogenesis of osteosarcoma?

Methods The whole-genome transcriptional profiles of osteosarcomas, including two primary biopsy specimens, two cell lines, two xenografts derived from patient specimens, and one from normal osteoblasts and from mesenchymal stem cells, respectively, were quantitatively measured using serial analysis of gene expression. A statistical enrichment was performed, which selects the common genes altered in each of the osteosarcomas compared with each of the normal counterparts independently.

Results Sixty (92%) of 65 total genes that were at least twofold downregulated in osteosarcoma compared with osteoblasts and mesenchymal stem cells, could be classified in four categories: (1) seven genes in the insulin–like growth factor (IGF) signaling axis, including three of the IGF-binding proteins (IGFBP) and three of the IGFBP-related proteins (IGFBPrP); (2) eight genes in the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signaling cascade; (3) 39 genes encoding cytoskeleton and extracellular matrix proteins that are

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This work was performed at The Children's Hospital of Montefiore, Bronx, NY, USA.

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regulated by TGF-β/BMPs; and (4) six genes involved in cell cycle regulation, including tumor suppressors TP63 and p21.

Conclusions Based on these transcriptional profiles, a coordinated theme of clustered gene deregulation in osteosarcoma has emerged. Cell proliferation driven by the IGF axes during bone growth is unrestrained owing to downregulation of IGFBPs and cell cycle regulators. Tumor cells may be maintained in an undifferentiated state secondary to impaired TGF-β/BMP signaling. This well-preserved pattern suggests that the alterations in the signaling axes of IGF-1 and TGF-β, in concert with cell cycle regulators, may be an important pathogenic basis of osteosarcoma.

Clinic Relevance This study provides a possible molecular basis of pathogenesis of osteosarcoma. This may help to develop new therapeutic targets and strategy for this disease. Preclinical and subsequently clinical testing of inhibitors of the IGF-1 and TGF pathways would be warranted.

Introduction

Osteosarcoma is a high-grade primary bone malignancy most commonly seen in children and young adults [18, 19]. The survival of patients with osteosarcoma has not improved during the past three decades since the advent of adjuvant chemotherapy, despite multiple clinical trials of intensified regimens or newer agents. Better understanding of the disease is needed. Although the etiology of osteosarcoma is not well understood [8], several factors suggest a correlation between skeletal development and initiation of osteosarcoma. First, the peak incidence of osteosarcoma coincides with a period of rapid bone growth. In addition, an earlier peak age in girls corresponds to the earlier age of their growth spurt, also suggesting a relationship with skeletal development. Moreover, most osteosarcomas occur near the major growing joints such as the distal femur, proximal tibia, and proximal humerus, which contribute to the majority of longitudinal bone growth. Other aspects of pathogenesis may be inferred from the genetic predisposition syndromes such as Li-Fraumeni syndrome (TP53 mutation; patients with this condition are at increased risk multiple cancers) and hereditary bilateral retinoblastoma (Rb mutation) [10, 11]. It appears that loss of function of tumor suppressor genes played a role in the tumorigenesis in osteosarcoma.

Bone growth is spatially and temporally controlled by systemic endocrine and local growth factors [38]. In response to growth hormone, the multipotent mesenchymal stem cells (MSCs) are recruited to the growth plate and differentiate into proliferating chondroblasts. Chondroblasts undergo hypertrophy and produce cartilage matrix underneath the growth plate. Bone marrow-associated MSCs differentiate into osteoblasts and replace cartilage with osteoid in concert with hematopoietic-derived osteoclasts. Osteoid then is mineralized with calcium and phosphate to form bone [4]. The majority of terminal osteoblasts undergo programmed cell death (apoptosis) with a few becoming osteocytes incorporated in the Haversian system. The transforming growth factor-β (TGF-β) superfamily members, especially the bone morphogenetic proteins (BMPs), are important regulators in this process, as is well documented in animal models and in vitro studies [4, 5]. The biologic action mediated by TGF-β signaling is tightly regulated at multiple levels [29]. However, the molecular basis for the relationship between bone growth and occurrence of osteosarcoma is not well understood.

Bone growth is promoted by insulin-like growth factors (IGF) systematically and locally [33]. IGF-1, the most abundant growth factor in bone, is the mediator of anabolic effects of growth hormone. It mostly is synthesized in the liver and also is secreted by MSCs and osteoblasts in an autocrine fashion. It also stimulates proliferation. In animal models, IGF level has been correlated with size and weight at birth [2, 17] and bone growth [36]. The IGF-signaling axis also includes six high-affinity IGF binding proteins (IGFBPs) and five low-affinity IGFBP-related proteins (IGFBPrPs). IGF signaling is tempered by its binding proteins in a negative feedback loop. It is among the few peptide hormones known to have binding proteins, which is usually characteristic of lipid-soluble hormones. Moreover, IGF-1, along with IGFBPs, reaches a peak level at puberty in response to growth hormone and decreases with age, in parallel with the incidence of osteosarcoma [28]. The mitogenic IGF-1 was reported to be involved in tumorigenesis in several tumor models [16]. In osteosarcoma, IGF-1 levels are associated with metastatic behavior of tumors in animal models [25]. The relationship between IGF signaling and osteosarcoma pathogenesis, however, is not entirely clear.

To better understand the molecular basis of osteosarcoma, we used a comprehensive approach to compare the transcriptional profile of primary tumor cells and cell lines with their normal cellular counterparts. In this study, serial analysis of gene expression (SAGE) was used to directly measure the transcriptome without any prior

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selection of genes for inclusion [34, 39]. Moreover, the absolute quantity of each transcript obtained for each sample with this approach allows a straightforward comparison to public databases to facilitate the exchange of information regarding rare diseases such as osteosarcoma [13]. We asked the following questions: (1) Can we identify gene expression in osteosarcoma specimens that differs from normal osteoblasts and MSCs; and (2) will this provide clues to the molecular pathogenesis of osteosarcoma?

Materials and Methods

Experimental Design

To compare the gene expression profile of osteosarcoma and its normal counterparts, we constructed SAGE libraries from two osteosarcoma biopsy specimens, two cell lines, and two xenografts derived from patient specimens–one from normal osteoblasts and one from MSCs (Fig. 1).

Osteosarcoma Specimens

The osteosarcoma standard cell line SaOS-2 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). In SaOS-2, both alleles of p53 are deleted. All other specimens were derived from patients with osteosarcoma who were treated at Memorial Sloan-Kettering Cancer Center. Specimen OS256 was obtained by biopsy from a 14-year-old female patient with osteosarcoma of the proximal tibia. Specimen OS259 was obtained by biopsy from a 13-year-old male patient with osteosarcoma of the distal femur. The osteosarcoma cell line, OS160CL, was derived from a lung metastatic lesion resected from an 11-year-old female patient with osteosarcoma of the proximal femur using described methods [27]. The OS63CL was derived from the primary surgical specimen resected from a 31-year-old male patient with osteosarcoma of the proximal humerus. Histologic diagnosis was confirmed by a pathologist (HD, AH). Cells were maintained as a monolayer in modified Eagle's-α media (α –MEM) supplemented with 10% fetal calf serum (Life Technologies, Bethesda, MD, USA), 100 units/mL penicillin, and 3 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Human MSCs (provided by Pamela Robey PhD, National Institutes of Health, Bethesda, MD, USA) were further induced toward osteoblastic differentiation with 50 µmol/L ascorbic acid and 10 mmol/L of β-glycerolphosphate for 2 weeks [27] before total RNA was extracted. The osteoblastic differentiation was confirmed by detection of osteoblast-specific

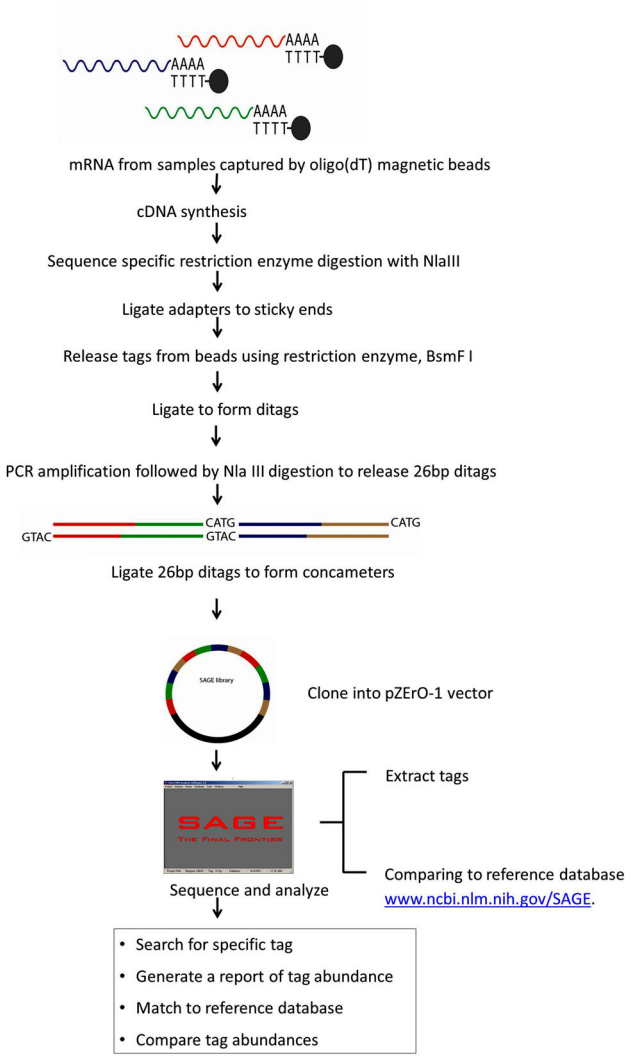

Fig. 1 A schematic for SAGE library construction is shown.

markers including alkaline phosphatase, osteocalcin, and collagen type Iα by reverse transcription–polymerase chain reaction (RT-PCR) as described previously [26]. All the cultured cells were checked monthly and shown to be free of mycoplasma contamination. The MSCs, MSC89R, were selected for SAGE analysis based on their high colony-forming efficiency and multilineage differentiation potential [7]. Briefly, the mononuclear cell fraction was recovered from a 10-mL aspirate of the iliac crest obtained from a healthy donor using a Ficoll (Ficoll-Paque™; Pharmacia, Piscataway, NJ, USA) gradient, and then cultured in α-MEM supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 20% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA, USA) lot selected for rapid growth of the cells. After 24 hours the nonadherent cells were removed and the adherent layer cultured until it reached 50% to 70% confluence.

Cells subsequently were passed twice and then collected for SAGE analysis or alternatively plated in 100-mm dishes at 10 cell/cm^2, cultured for 10 to 14 days, and a single cell-derived colony (clone forming-unit fibroblast 35 [CFUF35]) was isolated using cloning cylinders for analysis by MicroSAGE [32].

Osteosarcoma Xenografts

Tumor cells from patient-derived cell lines OS160CL and OS63CL were used to establish xenografts in mice as previously described according to a protocol approved by the Memorial Hospital Institutional Animal Care and Use Committee [37]. Xenografts were resected when the size was approximately 1.5 cm in diameter. Specimens were histologically confirmed to be osteosarcoma by a pathologist (HD, AH) using standard hematoxylin and eosin staining and were named M160xeno and M63xeno corresponding numerically with their respective parental cell lines.

Construction of SAGE Libraries

Total RNA was extracted with an RNeasy® mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. SAGE libraries of normal human osteoblasts, two primary osteosarcoma tumor specimens, two osteosarcoma xenografts (M160xeno, M63xeno), and two cultured osteosarcoma cell lines (SaOS-2, OS160CL) were constructed with an iSAGE™ Kit from Invitrogen™ (Carlsbad, CA, USA) according to the manufacturer's instructions. Sequencing reactions were performed using a BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA), and data were generated with an ABI 3100 sequencer (Applied Biosystems®). To yield tags, the raw data were processed with the SAGE2000 Version 4.5 software (Invitrogen) provided by the kit manufacturer. The SAGE library of mesenchymal stem cells, MSC89R, using the same methodology as described by Tremain et al. [32]. The SAGEmap database, downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SAGE), was used as a reference for tag UniGene identification [13]. Two additional SAGE libraries were included for analysis, including a single cell-derived MSC SAGE library named CFUF35 using micro-SAGE methodology with 16,407 tags in total [32] and a MSC SAGE library using the standard SAGE methodology with 202,962 tags in total [30].

Quantitative Real-time RT-PCR

Real-time fluorescent quantitative RT-PCR was used to validate a fraction of genes identified by SAGE to be differentially expressed using predesigned primers and probes (Applied Biosystems®) including inhibin β A, Assay ID: Hs00170103_m1; Smad4, Hs00232068_m1; CTGF, Hs00170014_m1; IGFBP7 (Mac25), Hs00266026_m1; p63, Hs00186613_m1; IGFBP6, Hs00181853_m1; IGFBP4, Hs00181767_m1; IGFBP3, Hs00181211_m1. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for template loading. Thermal cycling was performed with a 7500-fast real-time PCR system (Applied Biosystems®) using a 2 × TaqMan® Fast Universal PCR Master Mix provided by the manufacturer [37]. The relative quantity was calculated based on standard $2^{-\Delta T}$ method as described previously [37]. In total, eight SAGE libraries were constructed, including six of osteosarcomas, one of MSCs (MSC89R), and one of osteoblasts. At least 30,000 tags were obtained for each library to ensure sufficient coverage for the majority of the transcripts. The osteoblast SAGE library was constructed after 2 weeks of osteoblastic induction of the MSCs. Osteoblastic differentiation was observed by increased mRNA expression of alkaline phosphatase and type Iα

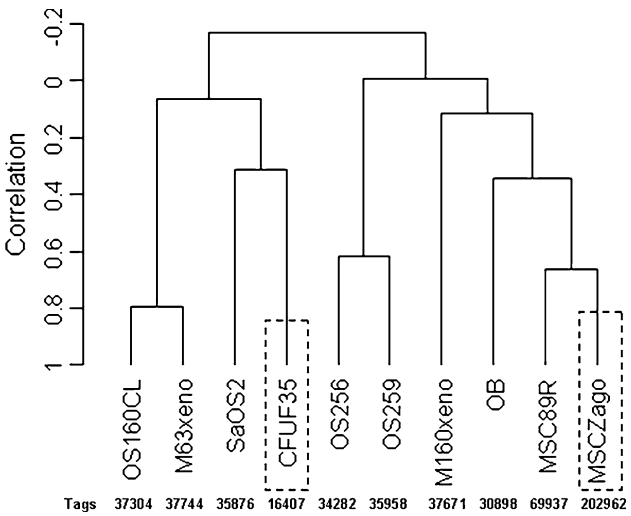

Fig. 2 Eight SAGE libraries obtained in this study, along with CFUF35, a single cell-derived MSC SAGE library established using MicroSAGE technique [32] (dashed boxes) and another human MSC SAGE library (MSCZago) using standard methodology (dashed boxes) [30] were normalized to tags/50,000 in total and were analyzed using BRB-Array Tools Version 3.6. A dendrogram was drawn to reveal the relationship between the SAGE libraries. The total numbers of tags obtained for each of the libraries before normalization are shown.

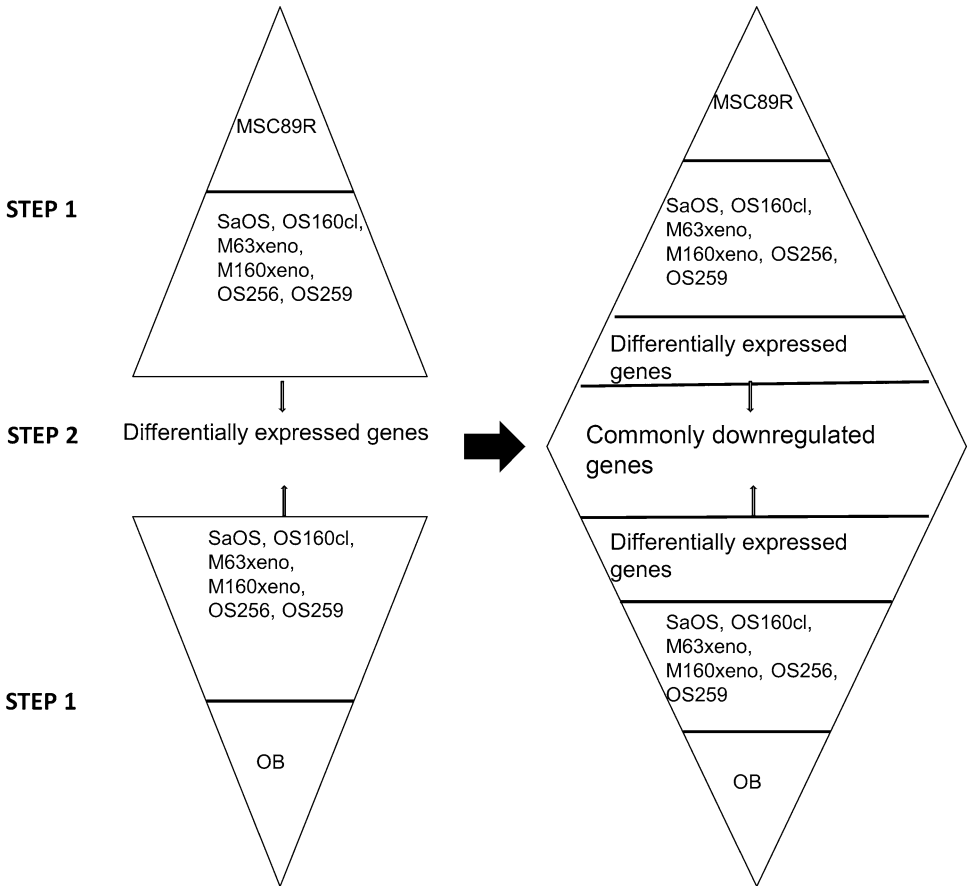

Fig. 3 A two-step enrichment was adapted in the statistical analyses to identify the common transcriptional alteration in all osteosarcomas and to filter out factors contributed by the individual samples possibly owing to the microenvironment. In Step 1, each osteosarcoma SAGE library was compared with that of osteoblasts and MSC89R independently. Genes (tags), which were differentially present between tumor and normal, are pooled together. A second step identifies genes, which are commonly downregulated in osteosarcoma compared with normal osteoblasts (OB) and MSC89R.

collagen by RT-PCR (data not shown). It was further confirmed by a steep increase in the number of tags representing collagen type Iα in the SAGE library of osteoblasts and by lack of cartilage markers such as type II and type X collagen, which were present in the SAGE library of MSC89R. A clustering analysis was performed using BRB-Array Tools Version 3.6 (Biometrics Research Branch, Division of Cancer Treatment & Diagnosis, National Cancer Institute, Bethesda, MD, USA), including eight SAGE libraries established in this study and two additional libraries of MSCs [30, 32] (Fig. 2). Considering the wide range of variation in the six osteosarcomas profiled in this study, a two-step enrichment was adapted in the statistical analyses to identify the common transcriptional alterations in all osteosarcomas and to filter out factors contributed by the individual samples possibly attributable to the growth environment (Fig. 3). A software package, the FosterTool, was designed and developed by one of us (WZ) to facilitate the statistical analyses of the data obtained by SAGE and is available for open access at http://fosterfoundation.com. In the first step, each SAGE library of osteosarcoma was pairwise compared with that of MSC89R and of osteoblast, respectively. In the second step, only gene tags significantly differentially expressed in every osteosarcoma versus osteoblast and MSC89R simultaneously were pooled as true characteristic genes of osteosarcoma. Gene tags were grouped according to their functions as described in the database (http://www.ncbi.nlm.nih.gov).

Statistical Analyses

All statistical analyses were performed by statisticians (YZ, WZ) at the Department of Applied Mathematics and Statistics (State University of New York at Stony Brook, Stony Brook, NY, USA). A computer program was developed (NN) at the Department of Computer Science (State University of New York at Stony Brook) to facilitate the parallel comparison between the SAGE libraries. All statistical analyses were two-tailed and a probability less

Table 1. The differentially expressed SAGE tags*

Gene	SAGE tag	MSC	OB	SaOS	M160xeno	OS160cl	M63xeno	OS256	OS259	Unigene ID	Description
IGF	TGGGTGAGCC	23	14	2	0	0	0	4	6	520898	CTSB Cathepsin B
	CATATCATTA	309	354	1	1	0	0	13	21	119206	IGFBP7 insulin-like growth factor binding protein 7
	TTTGCACCTT	254	274	0	5	25	0	34	9	410037	CTGF Connective tissue growth factor
	AGTGTCTGTG	56	20	0	0	0	0	9	1	8867	CYR61 Cysteine-rich, angiogenic inducer, 61
	ACTGAGGAAA	53	9	0	0	0	0	2	3	450230	IGFBP3 Insulin-like growth factor binding protein 3
	ATGTCTTTTC	90	47	9	3	0	0	10	4	1516	IGFBP4 insulin-like growth factor binding protein 4
	GGCCCCTCAC	42	26	0	1	0	0	5	0	274313	IGFBP6 Insulin-like growth factor binding protein 6
TGF-β	GTGGAATAAA	53	26	0	0	0	0	5	2	512776	LTBP2 Latent transforming growth factor β binding protein 2
	CAGTCAATAT	22	21	0	0	0	0	0	0	28792	INHBA Inhibin, beta A
	AGGTCTTCAA	34	30	0	0	0	0	2	1	164226	THBS1 Thrombospondin 1
	CCGTGACTCT	60	81	0	4	0	0	11	6	269512	FSTL1 Follistatin-like 1
	TCCTGTAAAG	60	18	0	0	0	0	7	0	74034	CAV1 Caveolin 1, caveolae protein, 22 kDa
	GGCTGTACCC	48	40	2	6	0	0	0	2	108080	CSRP1 Cysteine and glycine-rich protein 1
	AATCTGTAAC	25	13	0	0	0	0	0	0	40098	GREM1 Gremlin 1 homolog, cysteine knot superfamily
	GTGTGTTTGT	563	505	1	34	2	0	30	32	369397	TGFBI Transforming growth factor, beta-induced, 68 kDa

* Original tag number before normalization; SAGE = serial analysis of gene expression; IGF = insulin growth factor; TGF-β = transforming growth factor-β.

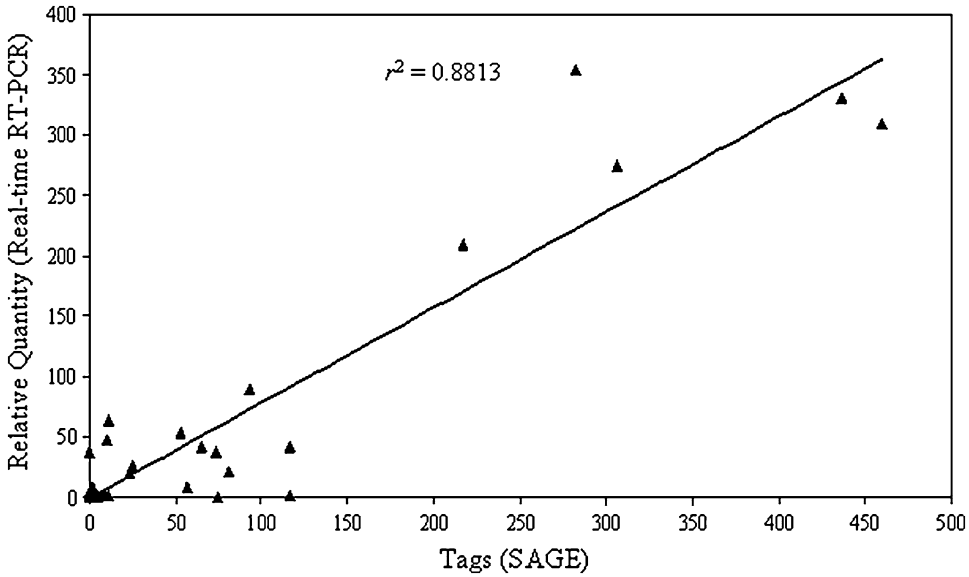

Fig. 4 The quantities of eight gene tags measured directly by SAGE are shown in the x-axis after normalization (tags/50,000 tags). The fold difference of mRNA expressions as measured by TaqMan® real-time RT-PCR is shown in the y-axis using predesigned primers and probes (Applied Biosystems). The genes were validated included inhibin β A, CTGF, IGFBP7 (IGFBPrP1), p63, IGFBP6, IGFBP4, and IGFBP3. The correlation coefficient between these two methods is calculated as $r^2 = 0.8813$.

than 0.05 was regarded as statistically significant. The clustering analysis was performed using BRB-Array tools Version 3.6.

Results

Four categories of genes accounted for the dominant majority of the genes that were downregulated in osteosarcoma SAGE libraries (92%; 60 of 65) compared with their normal counterparts. The first group of seven genes identified by SAGE includes those involved in the IGF signaling (Table 1). Three IGFBPs and three IGFBPrPs, seen abundantly in MSCs and osteoblasts, were minimally present in any of the six osteosarcoma SAGE libraries despite IGF-1 levels that were similar in MSC89R (129/50,000 tags), osteoblasts (66/50,000 tags), and osteosarcomas (mean, 67/50,000 tags). The differential expression was validated by TaqMan® real-time RT-PCR in a subset of the genes (Fig. 4), including IGFBP3, IGFBP4, IGFBP6, IGFBPrP1 (IGFBP7), and IGFBPrP3. The fold difference (Fig. 4) measured by SAGE (normalized to tags per 50,000 total tags in each library) and detected by real-time RT-PCR in these eight genes was highly comparable ($r^2 = 0.8813$).The second group of genes found to be downregulated in osteosarcoma includes eight genes involved in TGF- β signaling (Table 1). The identified genes are involved in regulating TGF-β signaling at various levels, including ligand (inhibin β), ligand release (TGF-β latent binding protein), ligand activation (thrombospondin 1), receptor internalization, and signal transduction (caveolin 1). Alterations also are present in extracellular antagonists (follistatin-like 1, Gremlin1, and CSRP1), including members of the IGFBPrPs (CTGF, IGFBP7, and CYR61) in the IGF-1 axis, which are important regulators of TGF-β signaling [1, 5, 14]. The third group tags identified to be downregulated in osteosarcoma encompass a group of genes largely regulated by TGF-β signaling (Table 2). These include genes involved in the extracellular matrix (ECM) formation (Type IV collagens, Type VI collagen, vimentin), ECM remodeling (SERPINs, TIMPs, LOX), and many constituents of the cytoskeleton [15].

Six genes involved in cell cycle regulation and apoptosis were downregulated in osteosarcoma (Table 3), including p63 and p21, both of which also potentially are involved in TGF-β signaling [21]. Additional genes (tags) with unknown functions that also were downregulated are shown (Table 3). In contrast to numerous genes downregulated in osteosarcoma, there was no gene identified as being consistently overexpressed in osteosarcomas at the high stringency statistical criteria applied in this analysis.

Discussion

Osteosarcoma is the most common primary bone tumor in children and young adults. Unfortunately, the prognosis of the patients with this disease has not improved during the past three decades, therefore better understanding of this

Table 2. Differentially expressed SAGE tags* involved in ECM and cytoskeleton in osteosarcoma as compared with its normal counterparts

Gene	SAGE tag	MSC	OB	SaOS	M160xeno	OS160cl	M63xeno	OS256	OS259	Unigene ID	Description
ECM	GACCGCAGGA	60	140	0	0	0	0	1	5	17441	COL4A1 Collagen, type IV, alpha 1
	GTGCTAAGCG	27	30	0	1	0	0	1	2	420269	COL6A2 Collagen, type VI, alpha 2
	GGAAGCTAAG	149	49	0	0	0	0	5	8	136348	POSTN Periostin, osteoblast specific factor
	GCCCCCAATA	521	234	36	80	5	0	51	61	445351	LGALS1 Lectin, galactoside-binding, soluble, 1 (galectin 1)
	TCCAAATCGA	260	57	0	6	0	0	11	6	533317	VIM Vimentin
	GCCTGTCCCT	45	22	0	0	0	0	7	3	821	BGN Biglycan
	GCCATAAAAT	20	21	0	0	0	0	7	1	1908	PRG1 Proteoglycan 1, secretory granule
ECM	TAAAAATGTT	138	116	0	0	0	0	2	1	414795	SERPINE1 (nexin, plasminogen activator inhibitor type 1), member 1
Remodeling	GGTTATTTTG	29	18	0	0	0	0	0	0	414795	SERPINE1 (nexin, plasminogen activator inhibitor type 1), member 1
	GCTGACGTCA	42	84	0	0	0	0	2	0	414795	SERPINE1 Serine (nexin, plasminogen activator inhibitor type 1), member 1
	TATTCACTAA	26	24	0	1	0	0	1	2	38449	SERPINE2 Serine (nexin, plasminogen activator inhibitor type 1), member 2
	AGCCTTTGTT	133	72	5	17	2	0	13	39	241579	SERPINH1 (heat shock protein 47), member 1, (collagen binding protein 1)
	GAGAGTGTCT	34	28	1	4	0	0	3	1	522632	TIMP1Tissue inhibitor of metalloproteinase 1
	TGTCATCACA	55	125	0	18	1	1	6	7	116479	LOXL2 Lysyl oxidase-like 2
	TATGTATTTC	40	24	0	4	0	0	0	0	102267	LOX Lysyl oxidase
	TCTTGTGCAT	72	48	9	15	1	0	3	6	2795	LDHA Lactate dehydrogenase A
Cytoskeleton	TGCTAAAAAA	35	16	0	1	0	0	4	3	474751	MYH9 Myosin, heavy polypeptide 9, non-muscle
	CCCTTAGCTT	37	17	5	0	0	0	1	2	190086	MRCL3 Myosin regulatory light chain MRCL3
	GGAGTGTGCT	151	69	0	22	1	0	6	5	504687	MYL9 Myosin, light polypeptide 9, regulatory
	GTGCTGAATG	203	60	26	26	0	1	11	13	505705	Myosin, light polypeptide 6,
	TTAAAGATTT	131	41	1	0	0	0	1	1	133892	TPM1 Tropomyosin 1 (alpha)
	GACCAGGCCC	110	73	0	2	0	0	11	0	300772	TPM2 Tropomyosin 2 (beta)
	AAAATATTTT	36	10	0	0	0	0	3	0	509765	Actinin, alpha 1
	AAGATCAAGA	34	39	0	1	7	4	0	0	1288	ACTA1 Actin, alpha 1, skeletal muscle

Table 2. continued

Gene	SAGE tag	MSC	OB	SaOS	M160xeno	OS160cl	M63xeno	OS256	OS259	Unigene ID	Description
	CTAGCCTCAC	271	62	37	6	0	0	14	5	514581	ACTG1 Actin, gamma 1
	GCTTTATTTG	209	64	5	4	0	0	15	8	456859	ACTB Actin, beta
	TTCTGTGAAT	64	54	2	3	2	6	5	6	490203	CALD1 Caldesmon 1
	ATAGTAGCTT	28	17	0	2	0	0	8	9	118400	FSCN1 Fascin homolog 1, actin-bundling protein
	TGCAATATGC	43	38	0	3	0	0	4	1	146447	FBN1 Fibrillin 1 (Marfan syndrome)
	CAGTACTGTA	21	27	0	0	0	0	0	6	252418	ELN Elastin
	GCCCAAGGAC	90	29	8	7	0	0	2	0	195464	FLNA Filamin A, alpha (actin binding protein 280)
	CTGCCAAGTT	101	50	1	2	0	0	6	4	413036	ZYX Zyxin
	ACAGGCTACG	151	110	1	0	0	0	3	0	4065186	Transgelin
	GTCTGGGGCT	105	71	11	20	1	0	17	15	104650	TAGLN2 Transgelin 2
	TGCCTCTGCG	84	55	3	32	0	0	1	7	5098268	CD151 CD151 antigen
	TCTGTGACCT	20	12	0	0	0	0	0	0	501928	MICAL2 Microtubule-associated monoxygenase, calponin and LIM domain containing 2
	CTCATCAGCT	20	11	0	0	0	0	0	0	370581	CAP1 CAP, adenylate cyclase-associated protein 1
	GCTTGGATCT	51	61	0	5	1	0	13	6	250723	TMAP1 Transmembrane anchor protein 1
	ATCTTGTTAC	427	185	0	4	0	0	38	14	203717	FN1 Fibronectin 1

* Original tag number before normalization; ECM = extracellular matrix; SAGE = serial analysis of gene expression.

disease is needed. In theory, osteosarcoma could arise from osteoblasts or the more primitive MSCs [22]. Our study provides a possible molecular basis of the disease by comparing the transcriptional profile of osteosarcoma with that of its potential normal counterparts, osteoblasts and MSCs.

There are limitations to our study. We used libraries from primary materials, cell lines, and xenografts. Although a primary tumor specimen from a biopsy may be the optimal material, these are available only in small amounts because of clinical standards of care and prioritization of tissue for diagnostic purposes. Patient specimens obtained after preoperative chemotherapy usually contain at least partly necrotic tissue. Cultured cell lines and xenograft tissues are more reproducible and can be expanded, but transcriptome differences from the primary material are likely [23]. We therefore used a more stringent criterion for statistical analysis, only genes that were commonly deregulated in all osteosarcoma SAGE libraries were selected. No genes were identified to be consistently overexpressed in osteosarcoma across all the specimens included in this study. This might reflect the variance of the materials we used, including biopsy specimens, cell lines, and xenografts. This also might represent the extreme genetic instability and complexity of the genome in this disease [24].

Our data suggest that dysregulation of the IGF signaling axis might represent an essential step in the tumorigenesis of osteosarcoma. IGF-1 is the mediator of the anabolic effects of growth hormone during rapid bone growth, and IGF-1 levels peak during puberty [28]. IGF-1 level is associated with body weight and bone growth in animal models [2, 17, 36]. Sutter et al. [31] reported that a single IGF-1 allele was a major determinant of the size of dogs. Canine osteosarcoma is most commonly seen in large breeds [20]. The IGFBPs and IGFBPrPs generally are believed to be inhibitory to the mitogenic and antiapoptotic effects of IGF-1, and their expression usually is stimulated by IGF-1 [9]. It appears that this autoregulatory mechanism is disrupted in osteosarcoma. Simultaneous loss of all the IGFBPs and IGFBPrPs in osteosarcoma compared with normal bone cells is striking. This was similarly found in a study using comparable methods [12]. Cells may obtain self-sufficiency by maintaining IGF-1 activity through downregulation of the IGFBPs. This might provide new targets for therapy for this disease [12].

In our study, disruption of TGF-β in osteosarcoma is suggested downregulation of genes of this superfamily at multiple levels (Table 1) and their target genes (Table 2). Even genes such as vimentin, which is widely regarded as a marker for a mesenchymal cellular origin by pathologists, was identified to be downregulated in osteosarcoma in this

Table 3. The differentially expressed SAGE tags* involved in cell cycle regulation and others in osteosarcoma compared with its normal counterparts

Gene	SAGE tag	MSC	OB	SaOS	M160xeno	OS160cl	M63xeno	OS256	OS259	Unigene identification number	Description
Cell cycle	GACCAGCAGA	21	20	0	0	0	2	7	6	137569	TP73L Tumor protein p73-like
	CTGAGAGCTG	26	17	1	0	0	0	0	0	369201	GAS6 Growth arrest-specific 6
	TGTCCTGGTT	23	48	9	1	2	4	6	4	370771	CDKN1A cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	GGTTGGCAGG	39	190	0	3	0	0	5	15	3745	MFGE8 Milk fat globule-EGF factor 8 protein
	CTTGATTCCC	28	16	0	2	0	0	1	5	518374	QSCN6 Quiescin Q6
	AAAGTCTAGA	76	33	6	2	0	0	1	4	523852	CCND1 Cyclin D1 (PRAD1: parathyroid adenomatosis 1)
Ca^{++} regulation	CTTCCAGCTA	52	29	1	3	0	0	8	4	511605	ANXA2 annexin A2
	CCCCCTGGAT	56	58	10	4	1	1	4	2	275243	S100A6 S100 calcium binding protein A6 (calcyclin)
	AGCAGATCAG	47	33	14	5	0	0	12	12	143873	S100A10 S100 calcium binding protein A10 (annexin II ligand, calpactin I)
Not classified	GCTGGGAGGG	47	23	0	11	1	0	1	4	325650	EHD2 EH-domain containing 2
	TTCTTGTTTT	21	13	0	1	0	0	3	3	472010	PRNP Prion protein (p 27–30)

* Original tag number before normalization is shown; SAGE = serial analysis of gene expression.

study. The TGF-β superfamily is involved in virtually every aspect of cellular activity. Osteoblast differentiation is regulated by the TGF-β signaling pathway, including the BMPs [5]. Mesenchymal cells are distinct from epithelial cells in their mechanisms of cell-cell communication. Instead of direct cell-cell contact through various junctions in epithelial cells, mesenchymal cells exist in a large amount of ECM mostly produced by themselves. The ECM and the cell-matrix interactions are important for cell proliferation, survival, and migration [6]. Loss of TGF-β signaling is a hallmark of tumorigenesis in many cancers of epithelial origin [29]. Further study on TGF-β signaling in this disease may shed light on the understanding of the disease.

Some genes consistently downregulated in osteosarcoma, however, were found to be upregulated during the osteoblastic induction from MSCs, including the IGFBPs and genes in the TGF-β/BMP signaling cascade, in agreement with previous studies [1, 2, 5, 9, 14, 28]. This suggests that osteosarcoma perhaps is more primitive than MSCs, the progenitor of osteoblasts, or may suggest a trend of dedifferentiation in osteosarcoma. The question of cell of origin of osteosarcoma is still debated [3, 22, 35]. Our study provides comprehensive evidence that links osteosarcoma with skeletal development. The dysregulated genes identified in osteosarcoma, such as components in IGF and TGF-β/BMP signaling, are major players regulating normal bone growth as suggested in an animal model [35]. These findings suggest that future therapeutic strategies might be directed toward promoting cell differentiation instead of (or in addition to) more-conventional chemotherapeutic approaches. Mutations on p53 and Rb genes were found in osteosarcoma. We did not find the gene expression levels to be different among the osteosarcoma specimens versus normal tissues in this study. This suggests the loss of function of the tumor suppressors is probably the mechanism, instead of expression. Six genes involved in cell cycle regulation and apoptosis were downregulated in osteosarcoma (Table 3), including p63 and p21. Further study is needed to clarify the role of these genes in osteosarcoma.

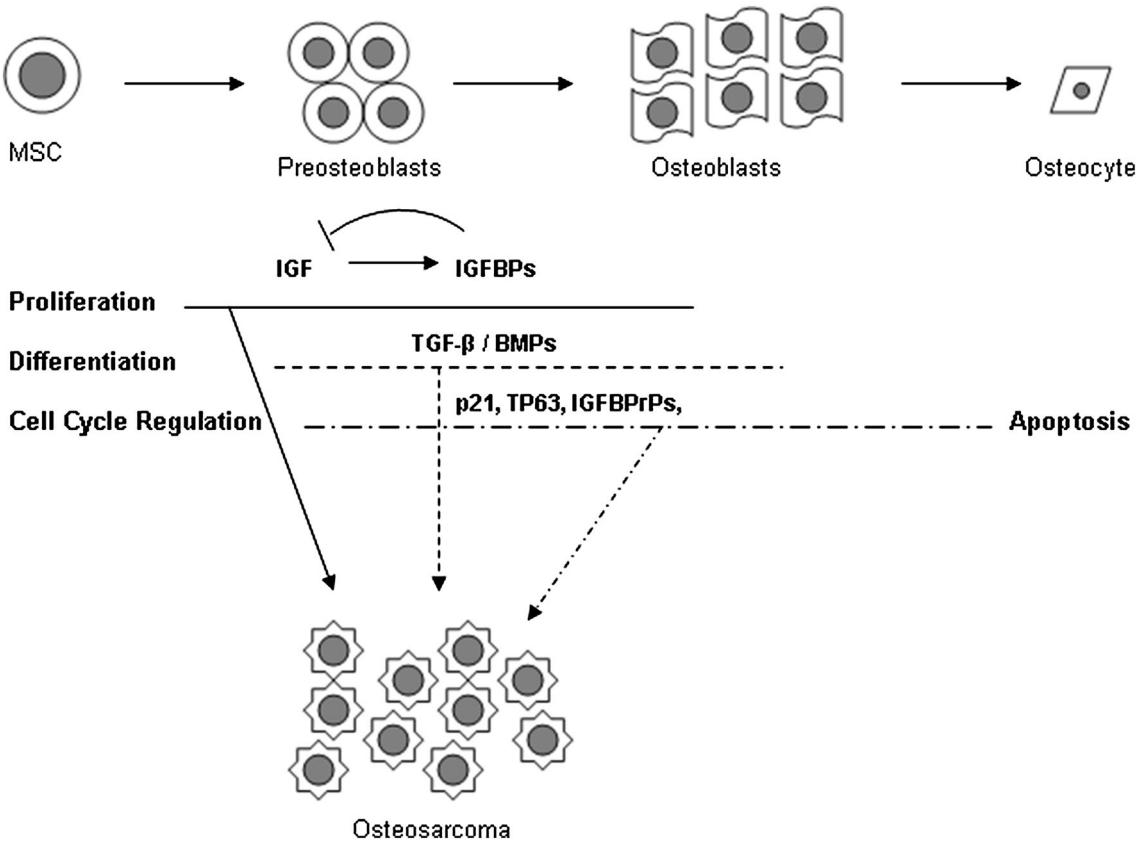

Fig. 5 During osteogenesis, MSCs are activated by the growth hormone (GH)-IGF axis to enter a proliferation phase and proceed to osteoblastic differentiation regulated by the TGF-β/BMPs. After matrix production, the majority of mature osteoblasts undergo apoptosis with only a few becoming osteocytes. Thus, sequential molecular events may contribute to the pathogenesis of osteosarcoma: (1) downregulation of IGFBPs free IGF-1 to stimulate unopposed cell proliferation; (2) TGF-β/BMPs signaling disrupts differentiation; and (3) alteration of apoptosis and cell cycle regulation.

For the first time, a highly clustered transcriptional profile, which is well preserved in osteosarcomas despite being derived from different materials grown in various conditions, was revealed by SAGE. This well-coordinated expression pattern suggests that profound alterations in the signaling axes of IGF-1 and TGF-β, in concert with cell cycle regulators, may be involved in the pathogenesis of osteosarcoma (Fig. 5). This provides a basis for further investigation to better understand the disease and to identify these pathways as potential new therapeutic targets. This study suggests the therapeutic value of inhibiting the IGF-1 and TGF pathways. Direct inhibitors of the IGF-1 signaling pathway exist in the form of IGF-1R antibodies. Although directly targeting the TGF pathway is not possible, R-spondin is one example of many drugs that target the TGF pathway and could be tested in patients with osteosarcoma. Preclinical testing of inhibitors of the IGF-1 and TGF pathways seems warranted, and with additional promising preclinical data, trials of these inhibitors in patients with osteosarcoma would be warranted.

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