Clinical and molecular features of high-grade osteosarcoma

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Profiling of high-grade central osteosarcoma and its putative progenitor cells identifies tumorigenic pathways

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ABSTRACT

Background

Osteosarcoma is the most prevalent primary malignant bone tumor of children and young adults, with poor survival in 40%. In order to identify signaling pathways involved in tumorigenesis we compared gene expression in osteosarcoma versus its presumed normal counterparts.

Methods

Genome wide expression profiles were generated from 25 high grade central osteosarcoma pre-chemotherapy biopsies, 5 osteoblastomas, 5 MSC populations and these same MSCs differentiated to osteoblasts. Genes that were differentially expressed between were analyzed in the context of the pathways in which they function using the GenMAPP program.

Results

MSCs, osteoblasts and osteosarcomas clustered separately and thousands of differentially expressed genes were identified. Most significantly altered pathways are involved with cell cycle regulation and DNA replication. Several upstream components of the Wnt signaling pathway are down regulated in osteosarcoma. Two genes involved in degradation of β-catenin protein, the key effectors of Wnt signaling, Axin and GSK3-β show decreased expression, suggesting that Wnt signaling is no longer under control of the regular signals. Comparing benign osteoblastomas with osteosarcomas identified cell cycle regulation as the most prominently changed pathway.

Conclusion

These results show that up-regulation of the cell cycle and down-regulation of Wnt signaling play an important role in osteosarcoma genesis. Gene expression differences between highly malignant osteosarcoma and benign osteoblastoma involve cell cycle regulation.

INTRODUCTION

Osteosarcoma is the most common primary bone malignancy, with a yearly incidence of approximately 6 per million children and 2 per million adults (1). The peak incidence occurs in late puberty, with 50% of the patients being between 10-20 years, and 60% younger than 25 years. Osteosarcoma in patients over 40 years of age is in a substantial number of cases generally considered secondary, such as after exposure to irradiation, or it arises in areas of pre-existing Paget's disease of bone (2). It might thus be considered as different disease than osteosarcoma in young patients.
Several histological subtypes are distinguished, of which conventional high-grade central or intramedullary osteosarcoma is the most common (75%) (3). The etiology of high-grade central osteosarcoma in young patients is elusive. No benign-, or malignant precursor lesions are known. These tumours recapitulate osteogenesis, compliant with their capacity to produce osteoid, alkaline phosphatase, osteocalcin, osterix and bone sialoprotein.
The outcome for patients with high-grade osteosarcoma has improved substantially since the introduction of multimodal chemotherapy, with present overall survival rates, ranging 65-75%. However, this improvement has reached a plateau despite several trials opting for intensifying dose or applying alternative chemotherapy schedules. Increasing the dose of chemotherapy prior to surgery only improved response rate, but not survival (4, 5). In order to treat patients that are refractory to chemotherapy or those that relapse alternative targets for therapy are required which can be identified through knowledge on molecular biological characteristics of the tumor.
Molecular studies on osteosarcoma are greatly hampered by the enormous genetic instability, that obscures the identification of genetic loci involved in osteosarcoma genesis (6), furthermore by the lack of benign precursors and no certainty on the normal counterpart or the progenitor cells. Osteoblastoma is a benign bone tumor occurring at the same site, but this tumor has never been reported to progress to osteosarcoma. A potential cell-of-origin of osteosarcomas is the mesenchymal stem cell (MSC), the precursor of osteoblasts as has been suggested in mouse models (7). Genome wide expression profiling to identify genes that are involved in response to chemotherapy and survival of osteosarcoma have been reported (8-10). Respectively 104, 44 and 60 differentially expressed genes were reported when comparing good and poor responders to chemotherapy. Remarkably these lists of genes do not overlap by one single gene.

Here we report on a genome wide expression profiling study on a homogeneous series of high-grade central osteosarcomas of patients younger than 40 years of age. Using strict criteria to correct for multiple testing we were not able to identify genes that were significantly different when comparing good and poor responders. Comparing the osteosarcoma expression profiles with the putative progenitor cells of osteosarcoma, i.e. mesenchymal stemcells (MSCs) and the same MSCs differentiated into osteoblasts resulted in the identification of large sets of genes that show very significant differential expression. These genes could be grouped

according to signal transduction pathways in which they function, thereby identifying possible culprit molecular events responsible for osteosarcoma genesis.

MATERIALS & METHODS

Patient material and Mesenchymal stem cells

Patients and their clinical data are listed in Table 1. All patients were treated at Leiden University Medical Center (LUMC). For osteosarcoma patients the difference in response to chemotherapy was stratified as good or poor response, using the Huvos criteria (11). Good response was defined if less than 10% of the tumor cells are viable after pre-operative chemotherapy, poor response if more than 10% of the tumor cells are viable. This response rate has been shown to be the best predictive marker for prognosis (12). Chemotherapy protocols include both pre- and postoperative treatment and were comparable (4). Osteoblastoma patients were treated with surgery only. Difference in survival of osteosarcoma was stratified as good if patients were still alive after 5 years follow-up, whereas poor survivors were patients who died from their disease within this time window. Disease course for osteoblastoma patients was usually without remission, except recurrence in one patient.

RNA was extracted from frozen biopsies, which were obtained before pre-operative chemotherapy was administered. For osteosarcoma core biopsies with at least 70% tumor cells and with non-necrotic tissue were used in this study. For osteoblastoma the resected tumors were used for RNA extraction.

We used human bone-marrow-derived mesenchymal stem cells and osteoblasts derived from the same cells upon osteogenic differentiation. Cells were either isolated from bone marrow samples as previously described (13). MSC1, MSC2 and FMSC1 were obtained from the department of Hematology, Leiden University Medical Center, Leiden, The Netherlands. 220L and 240R were purchased from Tulane University, New Orleans. All cells used were derived from adult patients, except for FMSC1, which was derived from fetal bone marrow and were obtained according to the ethical guidelines of the national organization of scientific societies (FEDERA). All cells were characterized either at passage 2 or passage 3 via FACS analysis as previously described (14). The phenotypes were uniform among all the different cells tested and in agreement with those reported for MSCs: i.e. CD90, CD105, CD166, HLA-A, B,C positive (>95%) and CD34, CD 45, CD31,CD80, HLA-DR negative (<5%). Furthermore all the cells were tested for their ability to be committed, under the proper conditions, towards adipogenesis, chondrogenesis and osteogenesis, as previously described (14). All cells that were induced to osteogenic differentiation showed diffuse positive staining for alkaline phosphatase activity and alizarin red positive calcium depositions, as previously described (14).

All tissue samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue in The Netherlands", Dutch Federation of Medical Scientific Societies, http://www.federa.org).

Expression array analysis

RNA was extracted from frozen tissue sections of 20 μm as described previously (15). Generation of cRNA and labeling was performed according to the Affymetrix protocol, briefly, 10 μg RNA was used to generate double-stranded cDNA by an oligo-dT primer and a T7-RNA polymerase promotor. Reverse transcription and subsequent amplification and labeling were done in accordance with protocols recommended by Affymetrix using the BioArray HighYield RNA Transcript Labeling kit (ENZO Life Sciences, Farmingdale, NY). Every step of the reverse transcription and labeling procedure is monitored by gel electrophoresis and spectrophotometry.

Labeled RNA is hybridized with Hu133A GeneChip Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol (http://www.affymetrix.com/support/technical/manuals.affx) and scanned on a Affymetrix GeneChip scanner.

Quality of the hybridization is assessed by calculating the ratio of the 5' and 3' features for the reference genes GAPDH and actin. When this ratio is greater than 2, this is a measure of poor quality and the chip is discarded.

All expression array data are available at the BJC online supplementary material website.

Data analysis

GeneChip data were normalized using GC-RMA, an algorithm provided by the Bioconductor project (http://www.bioconductor.org/) which looks only at perfect match values (16). The algorithm runs under statistical language R and was shown to give less false positive variance in technical duplicates and has a greater sensitivity and specificity (17) as was recently confirmed in our laboratory (18).

The Spotfire decision site for functional genomics was used to perform unsupervised hierarchical clustering on all genes with a variance of at least 0.5.

In order to select genes that can be used as classifiers for histological response on pre-operative treatment and survival, Limma (linear models for microarray data) package of Bioconductor (http://www.bioconductor.org) was applied to the data set. Limma is a moderated t-statistic that detects differentially expressed genes between groups, given the natural variance within these groups, corrected for the false discovery rate due to multiple testing (19).

For pathway analysis, the array data were mined with GO-Elite, a tool to identify pathways that are most significantly changed between groups (http://www.genmapp.org/go_elite/go_elite.html and PMID: 15961447). To visualize gene expression data in biological pathways GenMAPP was used (20).

Quantitative reverse transcriptase PCR was performed as described previously (21). Primers for control genes and Wnt5a have been submitted to the Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/).

TABLE 1.
Clinical data

Sample ID	Chip no.	Type	Age	Gender	Subtype[1]	Adj. CT[2]	Chemo Response	Overall Survival	metastasis
L1370	IB10	osteosarcoma	14	male	HG Conv.	PIA	Good	good	lung
L1372	IB12	osteosarcoma	10	male	HG Conv.	AP	Good	good	0
L1382	IB14	osteosarcoma	16	male	Tel.	PIA	Poor	poor	lung
L1385	IB16	osteosarcoma	13	female	Tel.	MA	Poor	poor	lung
L1016	IB19	osteosarcoma	4	male	HG Conv.	AP	Poor	good	0
L2620	IB21	osteosarcoma	16	male	HG Conv.	AP	Poor	poor	lung+bone
L1375	IB22	osteosarcoma	8	male	HG Conv.	AP	Poor	good	local
L428	IB32	osteosarcoma	16	male	HG Conv.	AP	Poor	good	lung
L436	IB33	osteosarcoma	18	male	HG Conv.	MA	Poor	good	0
L432	IB34	osteosarcoma	17	male	HG Conv.	AP	Poor	poor	lung
L361	IB35	osteosarcoma	16	female	HG Conv.	AP	Poor	good	0
L1368	IB36	osteosarcoma	10	female	HG Conv.	PIA	Good	good	0
L1376	IB37	osteosarcoma	9	female	HG Conv.	AP	Good	good	0
L1386	IB38	osteosarcoma	12	female	HG Conv.	AP	Poor	poor	lung
L2702	IB39	osteosarcoma	16	male	HG Conv.	AP	Good	poor	lung
L2302	IB40	osteosarcoma	19	female	HG Conv.	AP	Poor	good	0
L2296	IB41	osteosarcoma	16	male	HG Conv.	AP	Good	poor	lung+else
L2295	IB42	osteosarcoma	40	female	HG Conv.	AP	Poor	good	0
L2611	IB43	osteosarcoma	20	female	HG Conv.	AP	Good	good	0
L2300	IB44	osteosarcoma	13	male	HG Conv.	AP	Good	good	0
L2294	IB45	osteosarcoma	17	female	HG Conv.	AP	Poor	good	0
L2290	IB46	osteosarcoma	36	male	HG Conv.	AP	Poor	poor	local
L2301	IB47	osteosarcoma	25	male	HG Conv.	AP	Poor	poor	lung+else
L2281	IB48	osteosarcoma	17	male	HG Conv.	AP	Poor	poor	lung
L2289	IB54	osteosarcoma	11	male	HG Conv.	AP	Poor	good	0
L578	IB55	osteoblastoma	22	male				relapse	
L579	IB56⋆	osteoblastoma	22	male				relapse	
L580	IB57	osteoblastoma	13	male				remission	
L581	IB58	osteoblastoma	16	male				remission	
L601	IB59	osteoblastoma	44	male				remission	

FMSC-OB-diff	IB49	osteoblasts
MSC1-OB-diff	IB50	osteoblasts
220-OB-diff	IB51	osteoblasts
240-OB-diff	IB52	osteoblasts
MSC2-OB-diff	IB53	osteoblasts
MSC1	IB54	MSC
MSC2	IB61	MSC
C220R	IB62	MSC
C240R	IB63	MSC
FMSC	IB64	MSC

[1] HG = high grade, [2] Adj. CT = adjuvant chemotherapy; PIA = cisplatinum, ifosfamide and adriamycin; AP = adriamycin and cisplatinum; MA = methotrexate and adriamycin; MSC = mesenchymal stem cell; HG conv = high grade conventional, Tel. = Telangiectatic
* IB 56 is the recurrence from IB 55

RESULTS

Comparing expression profiles of osteosarcomas

For 25 pre-operative biopsies from high-grade central osteosarcomas we obtained good quality genome wide expression data. One sample was repeated twice and three were repeated once to test for technical reproducibility. All four samples were most similar to their duplicates as demonstrated by hierarchical clustering, since replicates always clustered together (data not shown). For further analyses we used only one of the replicates. The entire file containing all expression profiling data can be found in supplementary Table 1.

Hierarchical clustering of all osteosarcoma profiles did not result in separation into groups, implying no big differences between possible clinical subsets. Previous publications reported that there are significantly differentially expressed genes when comparing osteosarcomas from patients with good versus poor response to chemotherapy (8, 9). However we could not identify any significantly expressed gene when comparing good and poor responders when applying a moderated T-statistic, that corrects for multiple testing as described in the methods section.

For all patients at least 5 year of follow up data was available. Poor survivors are defined as having less than 5 year survival as compared to good survivors with more than 5 year. The same T-statistic was used for the classification in good and poor survival, however no significantly differentially expressed genes were acknowledged and thereby no prognostic markers identified.

Genes differentially expressed due to comparing cultured cells and primary tissue
In order to identify biological processes involved in osteosarcoma genesis the expression profiles of the 25 osteosarcomas were compared with profiles of the presumed progenitors of this tumor, i.e bone marrow derived mesenchymal stem cells (MSC) (n = 5) and osteoblasts derived from these MSCs (13). Furthermore profiles of five osteoblastomas were included, which are not considered as benign precursors, since these tumors have never been reported to progress to osteosarcoma. Hierarchical clustering clearly distinguished the four groups into separate clusters (Fig. 1). The t-test in Limma assigned many significant differentially expressed genes when doing pair-wise comparisons (table 2).
The GO-Elite program selected the pathways that are most significantly different when comparing groups. GO-Elite ranks pathways with excess of differentially expressed genes. One of the most significant pathways when comparing MSCs with osteosarcoma was the MHC class II receptor activity pathway, which was upregulated in osteosarcoma. It is difficult to understand how the increase of such a pathway could contribute to mesenchymal transformation. We hypothesized that some of the genes identified by the T-test are merely different because cultured cells (MSCs) are compared with primary tissue. The genes that are most likely to belong to this category are those that show similar expression in the cultured MSCs and osteoblasts as well as in primary osteosarcoma and osteoblastoma, but significant difference between the group of cultured cells and the primary tissues. To identify these genes Venn diagrams were made of all differentially expressed genes for all comparisons using the limma package from Bioconductor (http://www.bioconductor.org). A final Venn diagram (Fig. 2) identified 492 genes that are likely to be different because of comparing cultured cells with primary tissue. The overlapping category in Fig 2 consists of all genes that are significantly different when cultured cells are compared with tissue, for both the highly malignant osteosarcomas as well as the benign osteoblastomas. The procedure to construct the VENN diagrams is explained in the legend of Fig 2. GennMAPP analysis was performed on the entire dataset, with the 'culture-tissue' category marked as leading parameter in the expression dataset, marked purple. The group of eight genes in the MHC classII receptor pathway that had a p-value of less than 0.05 appears to consist of seven genes that were assigned to the purple-colored 'culture-tissue' category (Fig 3). This suggests that the approach to filter out the genes that may be the result of comparing cultured cells and tissue is a valid one. However, this approach has its limitations because separate genes can not be validated with a gold standard, nor can be excluded that there are genes in this set that are similarly differentially expressed between MSCs in vivo versus both osteoblastomas and osteosarcomas.

FIGURE 1.
Hierarchical clustering

Hierarchical clustering of expression profiling data clearly shows separate clusters for osteosarcomas, osteoblastomas, MSCs and the same MSSCs differentiated to osteoblasts

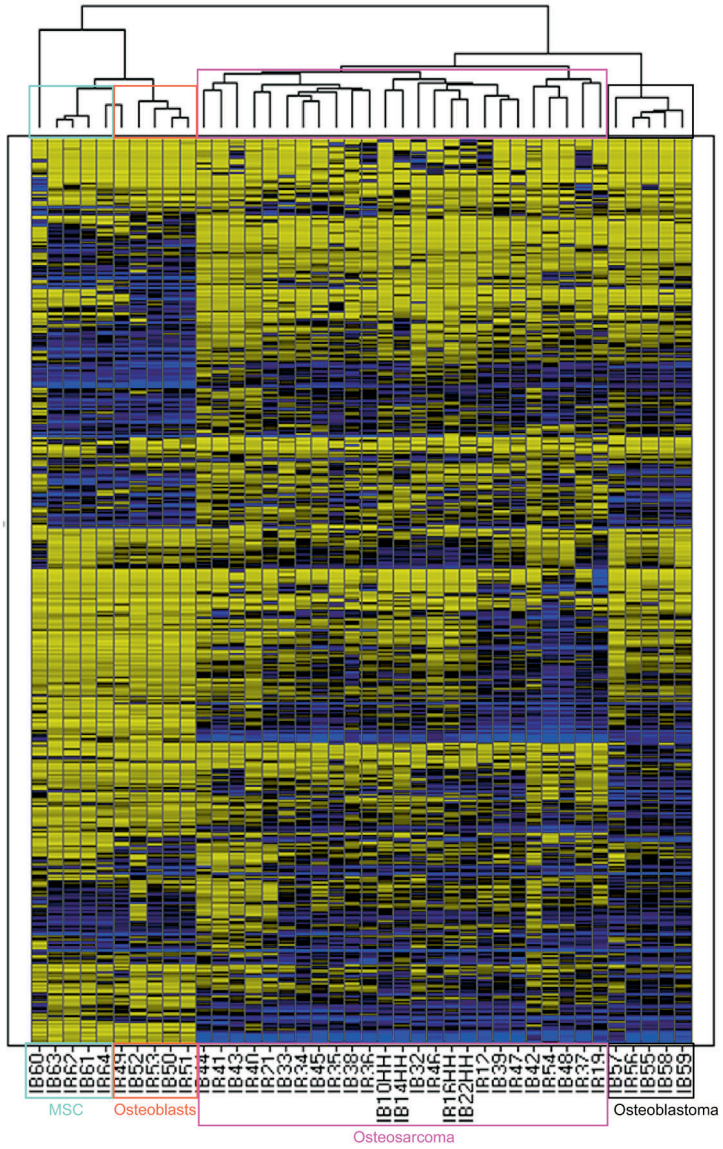


FIGURE 2.
Venn diagram of the 'culture-tissue' gene subset

The circles from these VENN diagram represent the differentially expressed genes when comparing two groups of arrays. The overlap between two circles contains the genes that are the same in both comparisons. OS = osteosarcomas; OB = osteoblastomas; MS = mesenchymal stem cells; DO = MSCs differentiated to osteoblasts. The lower VENN diagram displays the overlap of the 492 differentially expressed genes when comparing expression profiles from primary tissue (OS, osteosarcoma and OB, osteoblastoma) with cultured cells (MS MSCs and DO, differentiated to osteoblasts). The circle OSMS_OBMS contains all genes differentially expressed when comparing osteosarcoma and MSC that overlap with the differentially expressed genes when comparing osteoblastoma and MSC. OSDO_DOOB is the same as OSMS_OBMS, but for MSCs differentiated to osteoblasts.

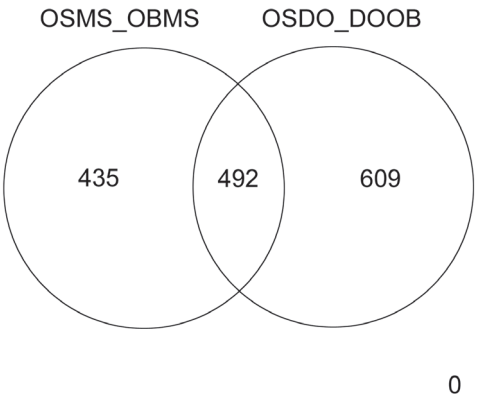


FIGURE 3.
MHC class II normal versus tissue cultrure related

MHC classII receptor activity pathway with genes that are differentially expressed between osteosarcoma and MSCs colored. Green is upregulated in osteosarcoma, purple indicates that a gene belongs to the 492 genes of the culture-tissue set. The left panel was analyzed without taking this set into account, the right set with the 'culture-tissue' gene set as the first parameter

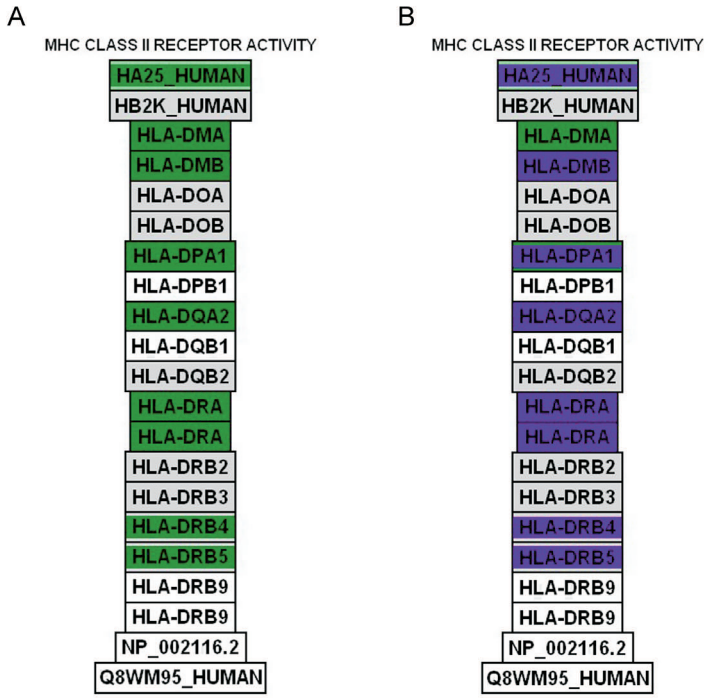


TABLE 2.
Group comparisons and nr of significant genes identified with Benjamini-Hochberg adjusted p-value

Comparison	total p<0.05	up	Down	avg of 100x 5 OS	Presumed process
OS vs MSC	2973	1159	1814	2456	genes that are altered in osteosarcoma (OS) progression from MSC
OS vs DO	3041	1144	1897	2586	genes that are altered in OS progression from differentiated osteoblasts (DO)
OS vs OB	882	225	657	937	genes involved in malignancy of OS compared to benign osteoblastoma (OB)
DO vs MSC	369	175	194		genes involved in MSC differentiation to osteoblasts
OB vs MSC	1245	606	639		genes involved in osteoblastoma progression from MSC
OB vs DO	1573	770	803		genes involved in osteoblastoma progression from osteoblasts

Comparing osteosarcoma with its presumed progenitors

The 25 osteosarcomas as a single group compared with five cultures of undifferentiated mesenchymal stem cells. This resulted in a substantial number of 3300 differentially expressed genes (corrected p-value< 0.01), of which 1302 genes are higher expressed in MSCs than in osteosarcomas and 1998 lower. We furthermore compared osteosarcomas with the same MSC cultures differentiated to osteoblasts. This resulted in 3335 differentially expressed genes ($p<0.01$). Table 2 summarizes the results of all comparisons made. There is a large overlap of 1006 genes in the osteosarcoma versus MSC and the osteosarcoma versus differentiated osteoblasts (DO). One gene that was significantly over-expressed in osteosarcoma was Wnt5a. This gene, involved in non-β-catenin Wnt signaling (22) has been tested with quantitative RT-PCR on the same series of RNA that has been used on the microarrays as an alternative method to verify the array-data. Correlation between qPCR and arraydata was good, i.e. 92% (Fig 4).

Given the high number of significantly differentially expressed genes we did not consider it relevant to make a shortlist of the most significant genes. Instead the program GO-Elite was used to identify pathways with a high number of differentially expressed genes and GENMAPP was used to look specifically at pathways that are known to be involved in normal osteoblast differentiation. For the GO-Elite analysis we removed the 492 'culture-tissue' artifact genes from the significant list.

FIGURE 4.
q-RT-PCR for Wnt5a

Comparison q-RT-PCR and array data for Wnt5a data shows 92% correlation

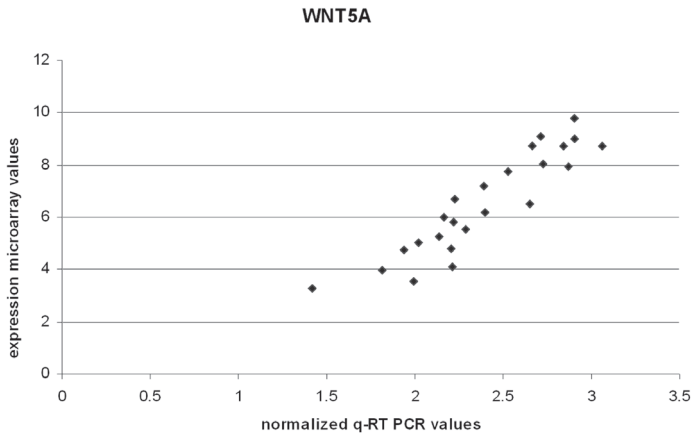


Table 3 lists pathways that contain most differentially expressed genes when comparing MSCs and osteosarcoma. Pathways in this table have an adjusted p-value smaller than 0.05 upon strict statistical criteria i.e. Benjamini Hochberg (23). The significant pathways are associated with DNA replication and mitosis, of which several genes involved in positive regulation are upregulated in osteosarcoma, such as CCNB when compared to MSC. None of the significant genes in these pathways are identified as 'culture-tissue artifacts'.

In order to further mine the data we looked at specific pathways that are known or suspected to be involved in osteosarcoma genesis. Inactivation of the p53 pathway has been reported in osteosarcoma (24) and this is indeed confirmed when comparing expression profiles from osteosarcoma with its presumed progenitor, MSCs and osteoblasts. Fig 5 shows the p53 mediated apoptotic pathway with genes that are downregulated in osteosarcoma ($p<0.05$) in green. Downregulation of p53 mediated signaling is reflected by downregulation of the specific downstream gene BBC3/PUMA.

The Wnt pathway has been shown to play an important role in osteoblast differentiation (25) and therefore here we visualized this pathway with the GenMAPP application using the expression data. Wnt signaling seems downregulated when comparing MSCs or differentiated osteoblasts with osteosarcomas. Fig 6 shows the Wnt pathway when comparing osteosarcoma and MSCs. The picture is similar when comparing with osteoblasts, although less prominent. Both upstream, the Wnt receptors FZD2 and -7 and LRP5 as downstream CCND1 and AXIN are downregulated.

TABLE 3.
Differentially expressed significant pathways

Pathway	Z_score
Comparison OS vs MSC	
macromolecule localization	5.99
mitotic cell cycle checkpoint	5.00
DNA replication	4.57
condensed chromosome, centromeric region	4.04
Comparison OS vs DO	
negative regulation of S phase of mitotic cell cycle	5.34
Comparison OS vs OB	
cell cycle	7.09
spindle	6.34
IgG binding	5.69
cell division	5.43
condensed chromosome\, centromeric region	5.36
proteinaceous extracellular matrix	5.08
chromosome segregation	4.94
DNA replication	4.80
Comparison DO vs MSC	
cadmium ion binding	11.28
trans\-1\,2\-dihydrobenzene\-1\,2\-diol dehydrogenase activity	7.39
acute\-phase response	5.57
steroid biosynthetic process	5.14
sterol metabolic process	5.12
copper ion binding	4.45
Adipogenesis	4.67
Comparison OB vs MSC	
developmental process	7.53
Cholesterol Biosynthesis	7.36
proteinaceous extracellular matrix	5.27
cytokine and chemokine mediated signaling pathway	4.48
Comparison DO vs OB	
negative regulation of transcription\, DNA\-dependent	5.58
amine oxidase activity	4.99
urogenital system development	4.94

Z-score = corrected score as determined by GO-elite. OS = osteosarcoma; MSC = mesenchymal stem cell, DO = differentiated osteoblasts, OB = osteoblastoma

Osteosarcoma versus osteoblastoma

Expression profiles of osteosarcoma were compared with those of five osteoblastomas, a benign bone tumour occurring at a similar site, in the long bones, and in a similar age group as osteosarcoma. The large difference in disease course is reflected by a large set of significantly differentially expressed genes (n = 882) of which 657 are higher in osteoblastoma and 225 higher in osteosarcoma. Comparing osteoblastomas with MSCs/osteoblasts results in less differences (6%/7%) than with osteosarcomas (13%). This may imply that osteoblastomas are more similar to MSCs and osteoblasts than osteosarcoma, thereby reflecting the difference in malignancy. The pathways that are most significantly altered when comparing osteosarcoma and osteoblastoma are the cell cycle, with an upregulation in the malignant tumors and pathways associated with cell division, especially regulation of the mitotic spindle. The significant pathways are listed in Table 3. To determine whether the larger size of the osteosarcoma group (n = 25) underlies this difference in significant genes we repeated the comparisons with only 5 osteosarcomas. Calculations were repeated 100 times for different combinations of 5 osteosarcomas and the results were averaged. The results are shown in Table 2, in the column labeled 'avg of 100 x 5 OS'. This indeed resulted in a reduction of the number of significant genes, but the difference between osteosarcoma versus MSC or osteoblasts was still substantial, i.e. 11 % for MSC and osteoblasts, whereas the comparison for osteoblastoma was only 6 or 7%.

FIGURE 5.
p53 pathway upregulated in osteosarcoma

The apoptosis/p53 pathway components when comparing osteosarcoma with differentiated osteoblasts with genes upregulated in osteosarcoma in green (dark green when p-value <0.01, light green when p<0.05) and genes downregulated in red (red, p-value<0.01, pink p<0.05).

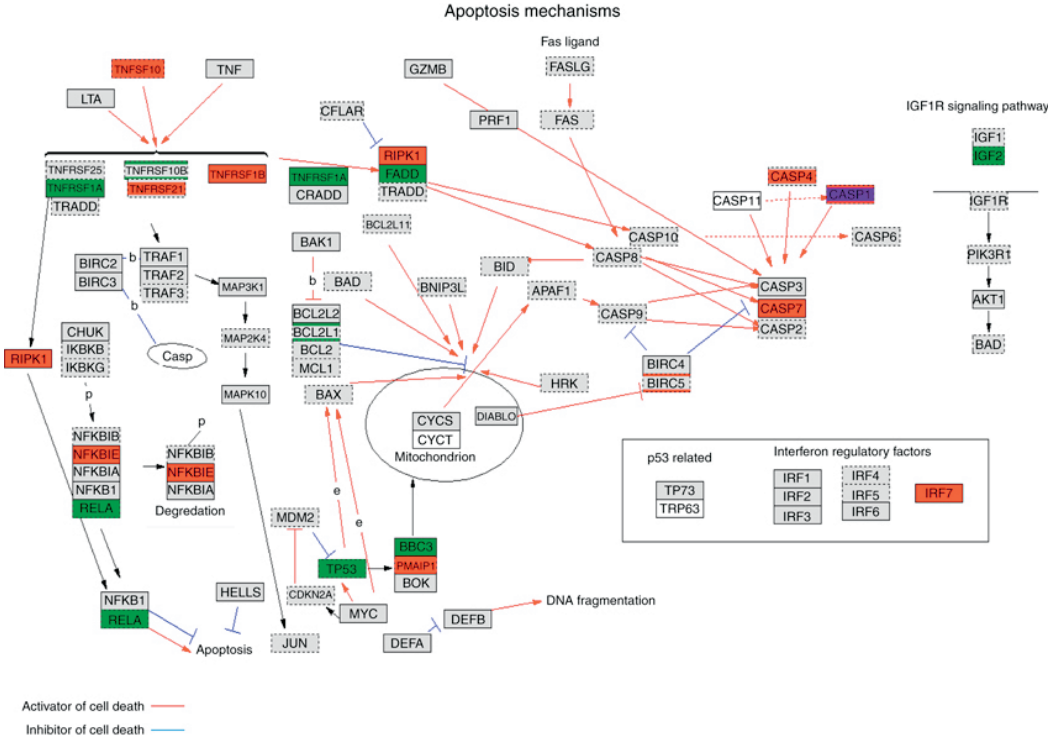


FIGURE 6.
Wnt signalling pathway downregulated in osteosarcoma

The Wnt signalling pathway when comparing osteosarcoma and MSCs, legend is the same as Fig. 5

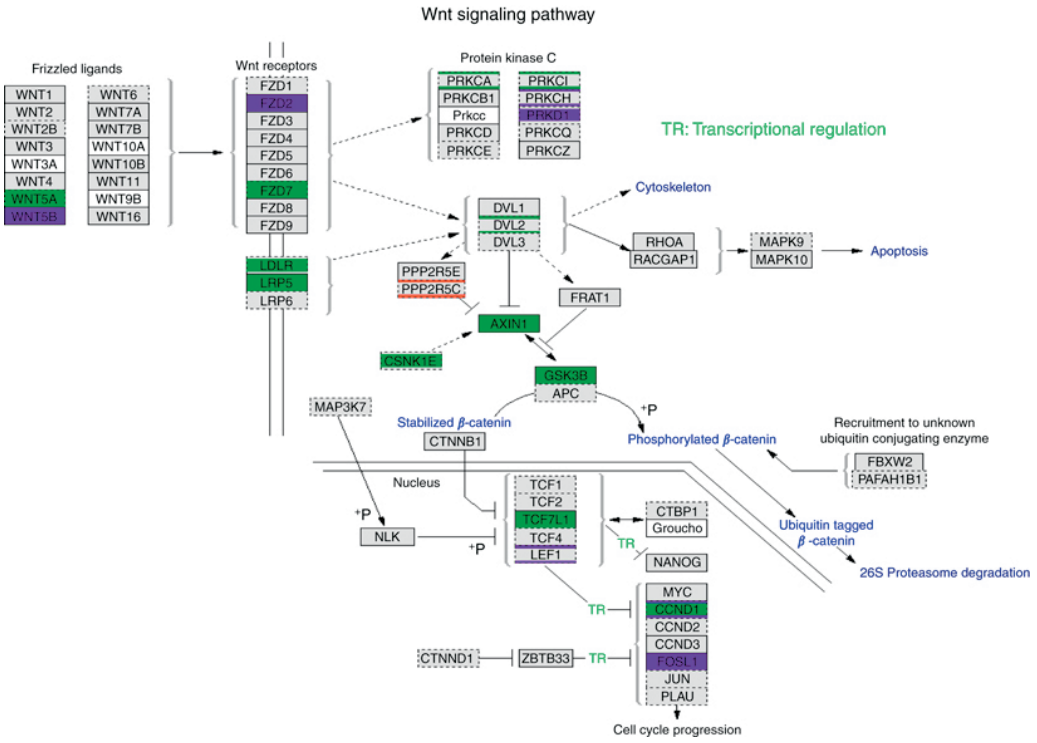
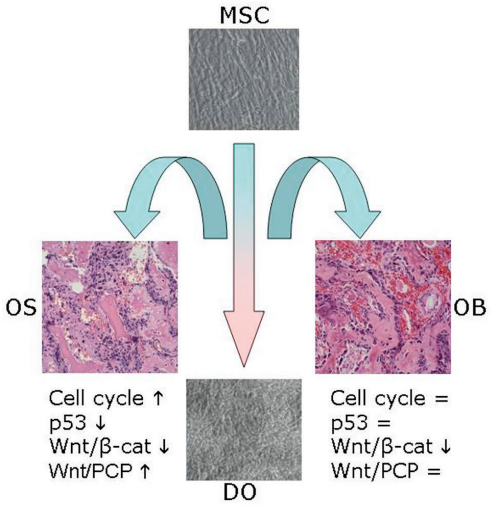


FIGURE 7.
Model for osteosarcoma genesis

Proposed model for osteosarcoma genesis. Osteosarcoma and osteoblastoma originate from mesenchcymal stemcells that are differentiatiting to osteoblasts. Increase in cell cycle activity and overactivity of Wnt/Planar cell polarity signalling and P53 function contribute to malignancy



DISCUSSION

Previous studies on genome wide expression profiling of osteosarcoma have reported lists of genes that were found to be differentially expressed when comparing tumors with a poor histological response to chemotherapy and those with a good response (8-10). Our study, comparing pre-chemotherapy biopsies from 8 good responders with those of 17 poor responding patients did not result in a single significantly differentially expressed gene. Size and homogeneity of the patient cohort, type of expression profiling platform, and statistical analysis may all account for this lack of significant genes. However, patient cohorts did not differ a lot in size, i.e. respectively 30, 28 and 13 cases, compared to 25 in our study, so size appears to be a highly unlikely explanation for this difference. A long follow-up was available for our patient cohort for comparing for outcome of disease, however this did not result in the identification of significantly differentially expressed genes.

Several meta-analysis studies on gene expression profiling provide a clarification for the lack of consistent results between different studies. Ein-Dor et al. report that there are many genes associated with different clinical behavior, but the differences in expression are quite

small and vary with different patient cohorts (26, 27). They conclude that a significant set of genes for predicting survival requires thousands of patient samples. For a relatively rare tumor like osteosarcoma this is obviously not achievable, especially given the variation in clinical presentation and treatment of this tumor.

To identify possible biological characteristics of osteosarcoma, by comparing osteosarcoma expression profiles with profiles from their presumed progenitors, i.e. mesenchymal stem cells (MSCs) and osteoblasts derived from these MSCs by *in vitro* differentiation resulted in a large set of 3300 differentially expressed genes. This result validates our statistical analysis, thereby justifying the negative results obtained with the comparison within the osteosarcoma profiles. However, this set of genes is definitely contaminated with a subset that is the result of the different source of the primary tumor tissues and the *in vitro* cultured MSCs and osteoblasts. Identification of common differentially expressed genes in osteosarcoma and benign osteoblastoma (most probably derived from the same progenitor cells, but with a complete different clinical behavior) as compared to the cultured MSCs and osteoblasts identified pathways that could most probably be attributed to the different sources of RNA. A subset of the 492 genes identified as commonly different in osteoblastoma and osteosarcoma when compared to cultured MSCs and osteoblasts could be assigned to specific pathways, thereby marking these as possible 'culture-tissue artifacts'. Especially the most significant pathway identified by GenMAPP analysis, i.e. up-regulation of the MHC class II pathway in both osteosarcoma and osteoblastoma is the most obvious example, most probably caused by infiltrating cells that contaminate the tumor tissue as has been described (28).

Pathways characterized by an excess of differentially expressed genes between MSCs and osteosarcomas, but lacking the possible 'culture-tissue artifacts' are most likely involved in malignant transformation. The GO-Elite application (http://www.genmapp.org/go_elite/go_elite.html) generates a non-redundant list of significant signal transduction pathways from the Gene Ontology (GO) project from a gene list with specific criteria. The criteria in this study included genes with a significant difference in mRNA expression between osteosarcoma and MSC or MSCs differentiated to osteoblasts. Criteria were strict and corrected for false discovery rate (FDR) due to multiple testing. Upon these restricted p-values the GO-Elite algorithm imposes another FDR correction. Table 3 lists the pathways that survive this double FDR.

The pathways that subsist the FDR correction are involved with cell cycle regulation, mitosis, DNA replication, the ususal suspects when comparing tumors with their progenitor cells. Osteosarcoma is especially characterized by high growth rate and numerous mitotic figures (29) and chemotherapy protocols are aimed at inhibition of the cell cycle. However, the current protocols are not effective in 40% of the cases (4) and this may be due to variable expression of certain cell cycle components.

Of special interest are developmental pathways which are known or suspected to play a role in osteosarcomagenesis. The Wnt signaling pathway shows downregulation when comparing MSCs or osteoblasts with osteosarcoma. Given the crucial role of this pathway in normal osteogenesis (25) and tumorigenesis in general this observation suggests a role for Wnt

signaling that differs from that in colorectal cancer, where upregulation of the pathway is considered as crucial for tumorigenesis (30). Indeed we have recently shown with a functional reporter assay that Wnt/β-catenin signaling seems to be absent in osteosarcoma cell lines (31). In addition we showed absence of nuclear β-catenin staining in primary osteosarcomas, indicative of inactive Wnt/β-catenin signaling. Also osteoblastoma showed a decrease of genes involved in Wnt/β-catenin signaling. The non-canonical Wnt5a ligand, which is involved in Wnt/planar cell polarity (32) was overexpressed in osteosarcoma cells. Both observations in osteosarcoma and osteoblastoma can be clarified from the fact that Wnt/β-catenin signaling is important for maintaining cells in the MSC state (33). Non-canonical Wnt signaling mediated by Wnt5a antagonizes this activity and promotes osteoblastogenesis of MSCs (34). Thus abnormal Wnt5a expression may be a key event in the malignant transformation in osteosarcoma. The findings of this study have led us to propose a model for osteosarcoma genesis, which is shown in Fig 7. Increase of Wnt signaling when comparing DO with MSCs is not observed. Wnt signaling changes during the process of differentiation and at different phases in osteoblastogenesis, different Wnt activities are observed.

The comparison between osteoblastoma and the same presumed progenitor cells MSCs and osteoblasts did not result in pathways associated with cell cycle regulation. The profiles of osteoblastomas have fulfilled a dual purpose in this study, they were instrumental in identifying differentially expressed genes that resulted from a difference in cell culture and primary tissue and they helped to recognize the cell cycle pathway as most important for malignant transformation of osteosarcoma.

From this analysis can be concluded that osteosarcoma differs from its presumed progenitor cells, MSCs and osteoblasts in terms of cell cycle regulation and developmental pathways. Benign osteoblastomas with the same progenitor cells but a much more favorable disease course are not characterized by an increase in cell cycle but by a decrease in components of canonical Wnt signaling.

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