Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort

Ottaviano, L.; Schaefer, K.L.; Gajewski, M.; Huckenbeck, W.; Baldus, S.; Rogel, U.; ... ; Poremba, C.

Molecular Characterization of Commonly Used Cell Lines for Bone Tumor Research: A Trans-European EuroBoNet Effort

Laura Ottaviano,[1†] Karl-Ludwig Schaefer,[1*†] Melanie Gajewski,[1] Wolfgang Huckenbeck,[2] Stefan Baldus,[1] Uwe Rogel,[1] Carlos Mackintosh,[3] Enrique de Alava,[3] Ola Myklebost,[4] Stine H. Kresse,[4] Leonardo A. Meza-Zepeda,[4] Massimo Serra,[5] Anne-Marie Cleton-Jansen,[6] Pancras C. W. Hogendoorn,[6] Horst Buerger,[7] Thomas Aigner,[8] Helmut E. Gabbert,[1] and Christopher Poremba[1,9]

[1]Institute of Pathology, University Medical Center Duesseldorf, Duesseldorf, Germany
[2]Institute of Forensic Medicine, University Medical Center Duesseldorf, Duesseldorf, Germany
[3]Centro de Investigación del Cáncer-IBMCC, Universidad de Salamanca-CSIC, Salamanca, Spain
[4]Department of Tumor Biology, The Norwegian Radium Hospital, Rikshospitalet University Hospital, Oslo, Norway
[5]Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, Bologna, Italy
[6]Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[7]Institute of Pathology, University Medical Center Muenster, Muenster, Germany
[8]Institute of Pathology, University of Leipzig, Leipzig, Germany
[9]Center of Histopathology, Cytology and Molecular Diagnostics (CHCMD), Research Park Trier, Trier, Germany

Usage of cancer cell lines has repeatedly generated conflicting results provoked by differences among subclones or contamination with mycoplasm or other immortal mammalian cells. To overcome these limitations, we decided within the EuroBoNeT consortium to characterize a common set of cell lines including osteosarcomas (OS), Ewing sarcomas (ES), and chondrosarcomas (CS). DNA fingerprinting was used to guarantee the identity of all of the cell lines and to distinguish subclones of osteosarcoma cell line HOS. Screening for homozygous loss of 38 tumor suppressor genes by MLPA revealed deletion of *CDKN2A* as the most common event (15/36), strictly associated with absence of the CDKN2A (p16) protein. Ten cell lines showed missense mutations of the *TP53* gene while another set of nine cell lines showed mutations resulting in truncation of the TP53 protein. Cells harboring missense mutations expressed high levels of nuclear TP53, while cell lines with nonsense mutations showed weak/absent staining for TP53. *TP53*^{wt} cell lines usually expressed the protein in 2–10% of the cells. However, seven *TP53*^{wt} osteosarcomas were negative for both mRNA and protein expression. Our analyses shed light on the correlation between immunohistochemical and genetic data for *CDKN2A* and *TP53*, and confirm the importance of these signaling pathways. The characterization of a substantial number of cell lines represents an important step to supply research groups with proven models for further advanced studies on tumor biology and may help to make results from different laboratories more comparable.

INTRODUCTION

While sarcomas of bone represent almost 5–10% of malignancies in children and young adults (Skubitz and D'Adamo 2007; van den Berg et al., 2008), overall these tumors are quite rare accounting for only about 0.2% of all cancers (Greenlee et al., 2001). Therefore, research on bone tumors is inherently hampered by the reduced availability of a substantial number of cases making standardization and networking among different research groups an essential prerequisite. In addition, due to this limited amount of primary tumor specimens, inclusion of in vitro models in the research on bone tumors is of high importance. However, usage of established tumor cell lines is sometimes problematic, as many cell lines are erroneously classified, or contaminated by mycoplasm or other immortal cell lines (Garraway et al., 2005; Wang et al., 2006). Moreover, a systematic characterization of these tumor models at the genetic level is not always available.

Therefore, we set out within the EuroBoNeT consortium to characterize in detail a common

Additional Supporting Information may be found in the online version of this article.

†Laura Ottaviano and Karl-Ludwig Schaefer contributed equally to this study.

*Correspondence to: Karl-Ludwig Schaefer, PhD, Institute of Pathology, Heinrich-Heine-University Duesseldorf, Germany, Moorenstr. 5, 40225 Duesseldorf, Germany. E-mail: l.schaefer@med.uni-duesseldorf.de

Received 4 June 2009; Accepted 28 June 2009

DOI 10.1002/gcc.20717

Published online 29 September 2009 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Bone Tumor Cell Lines in EuroBoNET

Cell line	ATCC[a]	Localization[b]	Diagnosis[c]	EWSR1 rearrangement	Age[d]	Sex	Reference
HAL		Bone	OS	neg	16	M	Høifødt, Oslo[e]
HOS	CRL-1543	Bone	OS	neg	13	F	ATCC
HOS-143b	CRL-8303	Bone	OS	neg	13	F	ATCC
IOR/MOS		Bone	OS	neg	13	F	Benini et al., 1999
IOR/OS10		Bone	OS	neg	10	F	Benini et al., 1999
IOR/OS14		Bone	OS	neg	13	M	Benini et al., 1999
IOR/OS15		Bone	OS	neg	12	F	Benini et al., 1999
IOR/OS18		Bone	OS	neg	33	M	Benini et al., 1999
IOR/OS9		Bone	OS	neg	15	M	Benini et al., 1999
KPD		Bone	OS	neg	7	F	Bruland et al., 1988
MG-63	CRL-1427	Bone	OS	neg	14	M	ATCC
MHM		Bone	OS	neg	41	F	Kjønniksen et al., 1994
MNNG-HOS	CRL-1547	Bone	OS	neg	13	F	ATCC
OHS		Bone	OS	neg	14	M	Fodstad et al., 1986
OSA	CRL-2098	Bone	OS	neg	19	M	ATCC
Saos-2	HTB-85	Bone	OS	neg	11	F	ATCC
SARG		Bone	OS	neg	25	M	Benini et al., 1999
U2OS	HTB-96	Bone	OS	neg	15	F	ATCC
ZK-58		Bone	OS	neg	21	M	Schulz et al., 1993
CH2879		Chest wall	CS	neg	35	F	Gil-Benso et al., 2003
JJ		Unknown	CS	neg.	39	M	Jagasia, 1996
SW1353	HTB-94	Humerus	CS	neg	72	F	Ouyang, 1998
A673	CRL-1598	Muscle	ES	t(11;22)	15	F	ATCC
CADO-ES		Pleural effusion	ES	t(21;22)	19	F	van Valen 1998
CHP-100		Mediastinum	pPNET	t(11;22)	12	F	van Valen 1998
ET10		Muscle (para spinal)	ES	t(2;22)	2	F	Peter et al., 1997
EW3		Rib	ES	t(21;22)	10	M	Urano et al., 1998
RD-ES	HTB-166	Humerus	ES	t(11;22)	19	M	ATCC
RM-82		Femur	ES	t(21;22)	8	M	van Valen 1998
SK-ES1	HTB-86	Bone	ES	t(11;22)	18	M	ATCC
SK-N-MC	HTB-10	Supraorbital	pPNET	t(11;22)	14	F	ATCC
STA-ET1		Humerus	pPNET	t(11;22)	13	F	van Valen 1998
STA-ET2.1		Fibula	pPNET	t(11;22)	15	M	van Valen 1998
TC71		Humerus	ES	t(11;22)	22	M	van Valen 1998
VH-64		Pleural effusion	ES	t(11;22)	24	M	van Valen 1998
WE-68		Fibula	ES	t(11;22)	19	F	van Valen 1998

[a]Cell lines also available from ATCC are given with their respective identifier.
[b]Localisation of tumor from which the cell line was derived.
[c]OS osteosarcoma, CS chondrosarcoma, ES Ewing sarcoma, pPNET peripheral primitive neuroectodermal tumor.
[d]Age of patients at time of biopsy/surgical resection.
[e]Personal communication.

collection of cell lines including osteosarcomas, Ewing sarcomas, and chondrosarcomas. By standardizing the cell line panel, data generated from different groups within the network and beyond can more easily be compared and integrated.

To guarantee the identity of the 36 cell lines kept in the EuroBoNet cell line repository, DNA fingerprinting was performed and the results were compared to public databases. Since these cells are involved in a broad range of tumor progression studies both within the consortium as well as by other research groups, genomic data concerning key pathways like *TP53* mutations, *MDM2* amplification, and *CDKN2A/B* deletion status were also (re-) evaluated. In addition, 38 tumor suppressor genes were monitored for genomic deletion by MLPA analysis.

MATERIALS AND METHODS

Cell Lines

Cells were derived from ATCC or the different partner institutes as given in Table 1. All of the 10 Ewing sarcoma (ES) and the 4 peripheral primitive neuroectodermal tumor (pPNET) cell lines used in this study were grown on gelatin-coated culture flasks in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 1% Penicillin-Streptomycin (10,000 U ml^{-1} penicillin

and 10 mg ml^{-1} streptomycin, Invitrogen), 2 mM L-glutamine (Invitrogen) and 10% FCS (STA-ET10 20% FCS) under standard conditions. ES/pPNET cells were characterized for EWSR1 gene rearrangement (Table 1) as described elsewhere (Friedrichs et al., 2006).

The osteosarcoma (OS) and chondrosarcoma (CS) cell lines CH2879 and SW1358 were grown in uncoated culture flasks as described above, whereas CS cell line JJ was grown in DMEM/F12 (Invitrogen) supplemented with 1% Penicillin-Streptomycin (10,000 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin, Invitrogen), 50 μg ml^{-1} Ascorbate (Sigma Munich, Germany), 100 nM Hydrocortisone (Sigma) and 1% ITS (Sigma). Control for mycoplasm contamination was routinely done using a PCR-based commercially available detection kit according to the manufacturer's protocol (Venor(Gem, Minerva Biolabs, Berlin, Germany).

Preparation of Nucleic Acids

Genomic DNA was prepared according to the Puregene DNA extraction protocol (Biozym, Hess. Oldendorf, Germany) including proteinase-K digestion followed by treatment with RnaseA. RNA was isolated using TRIZOL (Sigma) according to the manufacturer's protocol.

TP53 Mutation Analysis

Standard cycle sequencing techniques were performed to determine *TP53* mutation status (Exons 5–8) using primer sequences already described (Schaefer et al., 2002). Amplification of Exon 4 was carried out employing primers Exon 4-Fwd (5′-GTCCTCTGACTGCTCTTTTCACCCATCTAC-3′) and Exon 4-Rev (5′-GGGATACGGCCAGGCATTGAAGTCTC-3′). The reverse primer was used for DNA sequence analysis. *TP53* cDNA amplification spanning the complete coding sequence was performed using primer 5′-GTGACACGCTTCCCTGGAT-3′ and 5′-ACACGCAAATTTCCTTCCAC-3′ related to exon 1-6, primer 5′-CCTCACCATCATCACACTGG-3′ and 5′-TTATGGCGGGAGGTAGACTG-3′ related to Exon 7–12. Both forward and reverse primers were used for DNA sequence analysis.

MDM2 Amplification Analysis

Amplification of the *MDM2* gene, located in the long arm of chromosome 12, was measured by quantitative real-time PCR using the LightCycler system (Roche Diagnostics, Mannheim, Germany) together with the 2xSYBR Green Master Mix (Qiagen, Hilden, Germany). Oligonucleotide primers used in this study were *MDM2*-Fwd (5′-AAGCCAAACTGGAAAACTCAACAC-3′) and *MDM2*-Rev (5′-CAGGAACATCAAAGCCCTCTTC-3′). Amplification of alpha-albumin (*AFM*, chromosome 4) using primers *Albumin*-Fwd (5′-TTTATTCACATCATTCTCTC-3′) and *Albumin*-Rev (5′- GAGTGAGATATGAGTTGAG-3′, was performed as an internal reference for relative quantification. For all primer pairs an initial denaturation/activation at 95°C for 15 min was followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 25 sec, and extension at 72°C for 20 sec.

Quantitative analysis was performed using the LightCycler Software (version 3.5). The specificity of the PCR products was determined by the LightCycler Software's melting curve analysis. All quantitative measurements were performed as two independent replicates.

Quantification of *TP53* mRNA

Gene expression was quantified on the LightCycler System (Roche Diagnostics) by qRT-PCR using QuantiTect SYBR Green PCR Kit (both Qiagen) as described elsewhere (Domagk et al., 2007). *TP53* mRNA was amplified using primers *p53*-exon7-fwd (CCTCACCATCATCACACTGG) and *p53*-exon8-rev (GGAGAGGAGCTGGTGTTGTT), quantification of *GAPD* mRNA (primers *GAPD*-fwd: GAGTCCACTGGCGTCTTCA and *GAPD*-rev: GGGGTGCTAAGCAGTTGGT) was used to calculate delta-Ct-values.

Cell Line Identity

DNA fingerprinting was performed by using genRES® MPX-2 and genRES® MPX-3 kits (serac, Bad Homburg, Germany) according to the manufacturers protocol. In brief, 1 ng of genomic DNA was amplified by multiplex PCR including 9 and 12 different STR systems respectively (Supporting Information Table 1). PCR products were analyzed on an ABI 310 capillary sequencer and typed by genotyper V3.1 software (ABI). Probabilities of identity were checked by use of pooled European allele frequencies as deposed in the "Human DNA PCR polymorphisms" database http://www.uni-duesseldorf.de/WWW/MedFak/Serology/dna.html).

TABLE 2. MLPA, IHC, and aCGH Analysis of *CDKN2A*

Cell line	MLPA	IHC[a]	aCGH
HAL	normal	100%	normal
HOS	homozygous deletion	neg	homozygous loss
HOS-143B	homozygous deletion	neg	homozygous loss
HOS-MNNG	homozygous deletion	neg	homozygous loss
IOR/MOS	normal	100%	normal
IOR/OS10	homozygous deletion	neg	homozygous loss
IOR/OS14	normal	100%	normal
IOR/OS15	homozygous deletion	neg	homozygous loss
IOR/OS18	homozygous deletion	neg	homozygous loss
IOR/OS9	homozygous deletion	neg	homozygous loss
IOR/SARG	hemizygous deletion	100%	hemizygous loss
KPD	normal	100%	hemizygous loss
MG-63	homozygous deletion	neg	homozygous loss
MHM	hemizygous deletion	100%	normal
OHS	normal	100%	gain
OSA	hemizygous deletion	100%	hemizygous loss
Saos2	normal	100%	normal
U2OS	hemizygous deletion	neg	normal
ZK-58	normal	100%	normal
AD062394	normal	100%	n.a
CH2879	homozygous deletion	neg	n.a.
JJ	homozygous deletion	neg	n.a
SW1353	normal	neg	n.a.
A673	homozygous deletion	neg	loss
CADO-ES	homozygous deletion	neg	microdeletion[b]
CHP100	hemizygous deletion	++ 60%	loss (aneuploid[c])
EW3	normal	+++ 20%	normal
RD-ES	hemizygous deletion	+ 40%	loss
RM82	normal	+++ 100%	normal
SK-ES	hemizygous deletion	neg	loss
SK-N-MC	normal	+++ 100%	normal
STA-ET1	homozygous deletion	neg	loss
STA-ET10	normal	neg	normal
STA-ET2.1	homozygous deletion	neg	loss
TC71	hemizygous deletion	neg	microdeletion
VH64	homozygous deletion	neg	loss
WE68	hemizygous deletion	neg	microdeletion

[a]Immunohistochemical staining for CDKN2A(p16) protein (+++ strong, ++ moderate, + weak, neg not detectable); percentage indicates number of positive cells.
[b]Only one or two probes deleted.
[c]Baseline in aCGH analysis not "0". n.a.: not analysed.

MLPA Analysis

For all of the cell lines Multiplex Ligation-dependent Probe Amplification (MLPA) was used for the detection of homozygous deletions in a set of tumor suppressor genes, in particular *CDKN2A*. For these purposes, MLPA kit ME001B tumor suppressor probe mix was purchased from MRC Holland, Amsterdam and the analyses were performed according to the supplied protocol. Details are described elsewhere (Schouten et al., 2002). In brief, 200 ng DNA were diluted in 5 µl TrisCl-EDTA (pH 7.5) and denatured at 98°C for 5 min before hybridization of the probes at 60°C for 16–18 hr. Then the hybridized two parts of each probe were ligated at 54°C for 15 min, followed by PCR amplification (40 cycles) with FAM-labeled primers, using standard conditions according to the MRC Holland protocol. PCR products were diluted 1:30 with H_2O and 1 µl was used in a mixture of formamide (HiDi formamide, Applied Biosystems, Darmstadt, Germany) and GeneScan^{TM} - 1000 ROX^{TM} size standard (Applied Biosystems). After denaturation at 94°C for 2 min, fragment analysis was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

For analysis of raw data the genotyping software GeneMarker®(Version 1.7, SoftGenetics LLC) was used. Peaks were identified and assigned to their corresponding gene through

TABLE 3. *TP53* and *MDM2* Status

Cell line	AA 72[a]	*TP53*[b]	TP53 IHC[c]	*TP53* mRNA[d]	17p13.1[e]	*MDM2* amp[f]	MDM2 IHC[g]
HAL	arg	wt	neg	low	normal	no	neg
HOS	arg	p.Arg156Pro	+++ 100%	high	gain	no	neg
HOS-143B	arg	p.Arg156Pro	+++ 100%	high	gain	no	neg
HOS-MNNG	arg	p.Arg156Pro	+++ 100%	high	gain	no	neg
IOR/MOS	arg	c.249_572del	+ 50%	high	normal	no	neg
IOR/OS10	arg	splicing ex9/10[h]	+ 50%	mod.	normal	no	neg
IOR/OS14	arg	Wt	neg	low	loss	no	neg
IOR/OS15	pro	Wt	+ 40%	low	gain	no	neg
IOR/OS18	n.a.	del[i]>EX3/EX4	neg	mod.	normal	no	neg
IOR/OS9	pro	wt	neg	low	normal	no	neg
IOR/SARG	arg	p.Tyr205X	neg	low	loss	low	neg
KPD	arg	wt	neg	low	normal	no	neg
MG-63	pro	wt	neg	low	normal	low	neg
MHM	arg	wt	++ 50%	mod.	normal	high	pos
OHS	arg	p.Glu286Lys	+++ 100%	high	gain	no	neg
OSA	arg	wt	++ 10%	high	normal	high	pos
Saos2	n.a.	del[2]>EX4-EX8	neg	low	gain	no	neg
U2OS	arg	wt	++ 10%	high	gain	no	neg
ZK-58	arg	wt	neg	low	normal	no	neg
CH2879	pro	wt	++ 5%	high	n.a.	n.a.	n.a.
JJ	pro	p.Gly199Val	+++ 100%	high	n.a.	n.a.	n.a.
SW1353	arg /pro	p.Val203Leu	++ 60%	mod.	n.a	n.a.	n.a.
A673	pro	c.551_552insCA	neg	mod.	normal	n.a.	n.a.
CADO-ES	arg	wt	+++ 5%	mod.	normal	n.a.	n.a.
CHP100	pro	wt	+++ 2%	mod.	normal	n.a.	n.a.
EW3	arg	c.852_858del	+++ 70%	mod.	normal	n.a.	n.a.
RD-ES	arg	p.Arg273Cys	+++ 100%	high	normal	n.a.	n.a.
RM82	arg	p.Arg273His	+++ 100%	high	normal	n.a.	n.a.
SK-ES	arg	p.Cys176Phe	+++ 3%	mod.	normal	n.a.	n.a.
SK-N-MC	arg	c.170_572del	neg	high	loss	n.a.	n.a.
STA-ET1	arg	wt	+++ 10%	high	normal	n.a.	n.a.
STA-ET10	arg/pro	wt	+++ 10%	high	normal	n.a.	n.a.
STA-ET2.1	arg	p.Cys277Tyr	+++100%	high	normal	n.a.	n.a.
TC71	arg	p.Arg213X	neg	mod.	normal	n.a.	n.a.
VH64	arg	wt	+++ 5%	mod.	normal	n.a.	n.a.
WE68	arg	wt	+++ 5%	mod.	normal	n.a.	n.a.

TP53 mutation analysis was based on NM_000546.4 as a reference sequence.
[a]Polymorphism for amino acid 72.
[b]Mutation status of *TP53* gene.
[c]Immunohistochemical staining for p53 protein (+++ strong, ++ moderate, + weak, neg not detectable).
[d]Expression of *TP53* by qRT-PCR as determined by calculation of ΔΔCt values.
[e]aCGH analysis of chromosomal band 17p13.1 harbouring the *TP53* gene.
[f]Amplification of MDM2 gene as determined as the ratio of MDM2/albumin by real-time PCR (no <4, 4<low<10, high>10).
[g]Staining for MDM2 protein; n.a.: not analysed.
[h]Mature mRNA contains sequences of intron 9; no changes in genomic DNA found.
[i]del: PCR recurrently negative for the denoted exon.

their different lengths and thereby differing migration relative to the 1000 ROX^{TM} size standard. Complete lack of one or more peaks indicates a homozygous deletion of the corresponding gene. Peak areas between 0.2 and 0.7-fold compared to seven healthy donors were regarded as hemizygous gene deletions in tumor cells.

Copy Number Analysis by Array Techniques

For validation purposes, DNA copy number changes in osteosarcoma cell lines were also identified using Affymetrix Genome-Wide Human SNP Array 6.0. Samples were processed following the supplier's protocol. Quality control, normalization and copy number analysis were performed in Genotyping Console v. 3.0.1 (Affymetrix, CA). Data were quality controlled using the contrast quality control (CQC) algorithm with a minimal call rate of $\geq$86%. Intensities were quantile normalized and DNA copy number analysis was performed using a GC waviness correction algorithm and a reference model of 270 HapMap samples (Kresse et al., unpublished results).

DNA copy number changes in Ewing Tumor samples were additionally determined using whole genome BAC-aCGH based on the Sanger 1-Mb clone set (kindly provided by Dr. K. Szuhai LUMC, The Netherlands), as described elsewhere (Fiegler et al., 2003; Knijnenburg et al., 2005).

Log_2 data were acquired using Axon 4000B scanner and GenePix software. Normalization was done with GenePix software using the mean of the median of ratios of all of the autosomal features in the array, excluding those removed by the quality flagging scripts. Gpr files were subsequently processed with Bioconductor packages (CRAN) incorporating scripts for removing SD >0.2 and GenePix flagged spots. DNA copy algorithm and Merge Levels scripts (both implemented in snap CGH package) were applied for segmentation of the data.

Cell Line Array (CLA)

Each cell line was cultured in 6–8 75 cm^2 cell culture flasks. Cells were washed with RPMI and scraped out with 1 ml 4% formalin. Cells were sedimented by brief centrifugation and pellets were treated for at least 48 hr in formalin. Before pellets were embedded in paraffin, the cells were dehydrated in ethanol (70, 90, 95, and 99%) and xylene. To produce a cell line array of all of the cell lines, punches of 2 mm in diameter of the embedded cells were arranged on one prepared paraffin block (Beecher Instruments, WI).

Immunohistochemistry (IHC)

IHC analysis of the cell line array was performed as already described (Alldinger et al., 2007).

For specific protein detection monoclonal antibodies targeting TP53 (clone Ab-6, MERCK/Calchembio, Darmstadt, Germany), CDKN2A(p16) (clone Ab-7, NeoMarkers/Dunn, Asbach, Germany), or MDM2 (clone Ab-1, Oncogene Science/Dianova, Hamburg, Germany) were used. Antibody binding was detected using the Elite Vectastain ABC kit together with the VIP peroxidase substrate kit for visualization (both Vector Laboratories., Peterborough, United Kingdom).

RESULTS

DNA Fingerprinting

To validate our cell lines and exclude cross-contamination, DNA fingerprint analysis of 14 polymorphic short tandem repeat (STR) sequences was performed for all of the 36 bone tumor cell lines.

Thirty-three profiles were obtained showing at least 11 (85%) different STRs (Supporting Information Table 1) confirming that no cross-contamination has occurred. Comparison of the remaining three cell lines MNNG/HOS, 143B (referred to as HOS-MNNG and HOS-143B, Tables 1–3), and their assumed ancestor cell line HOS revealed 10 and 9 identical STR markers, respectively, but also additional alleles for one of the cell lines in the pair-wise analysis (Supporting Information Table 1), documenting both a clear relatedness as well as genetic evolution.

For 10 of the 36 EuroBoNet cell lines, STR profile data are also provided by the American Type Culture Collection (ATCC) including the DNA markers *VWA*, *THO1*, *TPOX*, *D16S539* and *D5S818*. They completely agree with our results except for the following differences: MNNG-HOS (but not HOS and 143B) and SK-N-MC were referred to as heterozygous for *TPOX* according to ATCC. In our hands only one of the published alleles was present. The cell lines A673 and 143B show an additional weak signal for *D5S818*, 143B also for the marker *VWA*.

Screening for Loss of Tumor Suppressor Genes

All samples were screened for homozygous deletion at 38 tumor suppressor gene loci by MLPA. Complete deletion of *CDKN2A/B* (chromosome band 9p21) represented the most common genetic event in this set of bone tumor cell lines and was found in 8 (42%) OS, 2 CS, and 5 (36%) ES/pPNET cell lines (Table 2). Additional homozygous deletions were found for *PTEN* in the RM-82 ES cell line, and *TP73* in the ZK-58 OS cell line.

CDKN2A Analysis

Because of the high impact of deletions of *CDKN2A/B* in bone tumor cell lines, we refined MLPA analysis for this locus aiming to differentiate between "normal" and "hemizygous deletion." Correlating MLPA data to corresponding aCGH data (unpublished results) for band 9p21 revealed peak areas less than 0.7-fold compared

to seven healthy donors as the most appropriate cutoff value. According to this definition, hemizygous deletions of *CDKN2A/B* were found in additional 4 (21%) OS and 5 (36%) ES/pPNET samples (Table 2).

We further investigated the correlation between *CDKN2A/B* gene dosage and CDKN2A(p16) protein expression by immunohistochemistry. In none of the 14 homozygously deleted cell lines could staining for CDKN2A be observed, while 10 of the 12 cell lines showing no loss of *CDKN2A* stained positive ($P = 1.3 \times 10^{-5}$ χ^2 test). No correlation of gene dosage to protein expression was found within the group of the nine hemizygously deleted cell lines, of which four stained negative and five were positive (Figs. 1C and 1D).

TP53 Analysis

Mutations in the *TP53* gene were detected by standard genomic DNA and cDNA sequencing. Overall, 19 (53%) cell lines were characterized by a mutation in *TP53* (Table 3). Nine of the 19 osteosarcoma cell lines (47%, including HOS, HOS-MNNG and HOS-143B), two chondrosarcoma and eight (57%) ES/pPNET cell lines hold mutation in *TP53*.

In 10 of the mutant cell lines, a missense mutation changing just a single amino acid was detected while in the other nine mutant cell lines the mutations could be predicted to incur more extensive changes due to genomic loss of several exons, non-sense mutation, out of frame insertions/deletions, or splice site mutations.

Moderate to strong nuclear expression of TP53 protein in more than 50% of the cells was observed in 11 cell lines while intense staining in 2–10% of cells was found in another 11 cell lines. Fourteen cell lines showed just weak or even no nuclear staining.

We observed a significant difference between the TP53 staining characteristics of mutant cell lines compared to wild type cells: while most of the wild type cell lines (16/17) were characterized by 0–10% of the cells staining positive for TP53, nine of the 19 mutant cell lines showed moderate to strong nuclear staining in more than 50% of the cells ($P = 0.0015$, χ^2 test) (Figs. 1E–1H).

However, there was also a significant difference regarding the TP53 staining pattern between cell lines with missense mutation and cell lines affected by more extensive mutation: nine of the ten former cell lines showed moderate to strong staining in more than 50% of cells, whereas eight of the nine latter cell lines showed weak or even absent staining for TP53 ($P = 0.000085$, χ^2 test).

Finally, there was also a basic difference in the staining pattern of $TP53^{wt}$ OS cells compared to $TP53^{wt\ ES/pPNET}$ cells: while all of the six $TP53^{wt}$-ES cells showed moderate to strong expression of TP53 in 2-10% of cells, seven of the ten $TP53^{wt}$wt-OS cell lines were negative. By quantitative RT-PCR we found that these seven cell lines were characterized by very low levels of *TP53* mRNA. Levels of *TP53* mRNA in the three $TP53^{wt}$-OS cell lines staining positive for TP53 were in the same range as levels of *TP53* mRNA of the corresponding $TP53^{wt}$-ES cell lines.

Osteosarcoma cell lines were analyzed for amplification of the *MDM2* gene using the *albumin* gene on chromosome 4 for normalization. Low level gains of *MDM2* were observed for MG 63 and IOR SARG according to the ratio *MDM2/albumin* (5.8 and 3.4, respectively). Cell lines MHM and OSA presented with high level *MDM2* amplification (19.4 and 50.0, respectively). MHM and OSA were the only OS cell lines showing positive nuclear staining for MDM2 protein (Figs. 1A and 1B). Interestingly, cell lines MHM and OSA belong to the three $TP53^{wt}$-OS cell lines staining positive for TP53.

Overall, almost all cell lines were impaired for at least for one of the key genes *TP53* or *CDKN2A/B*. Only the ES cell line STA ET10 did not show either *TP53* mutation or transcriptional silencing, *MDM2* over expression, or *CDKN2A* loss or absence of CDKN2A(p16) protein.

DISCUSSION

Human in vitro cancer cell lines derived from patient samples are widely used to study molecular mechanisms of cancer-associated regulatory pathways as well as cellular characteristics of human tumors. However, interpretation of experimental data derived from these cell lines, which often were established decades before, is repeatedly hampered by the lack of precise clinical information on the originating tumor tissue. Another well known but frequently ignored problem using these in vitro models is the probably high rate of incorrect labeling or cross-contamination of these cells. This problem was already reviewed in the early eighties especially focusing on contamination by HeLa and nonhuman cells in other established human tumor cells (Nelson-Rees et al., 1981). Even more worrying is the fact

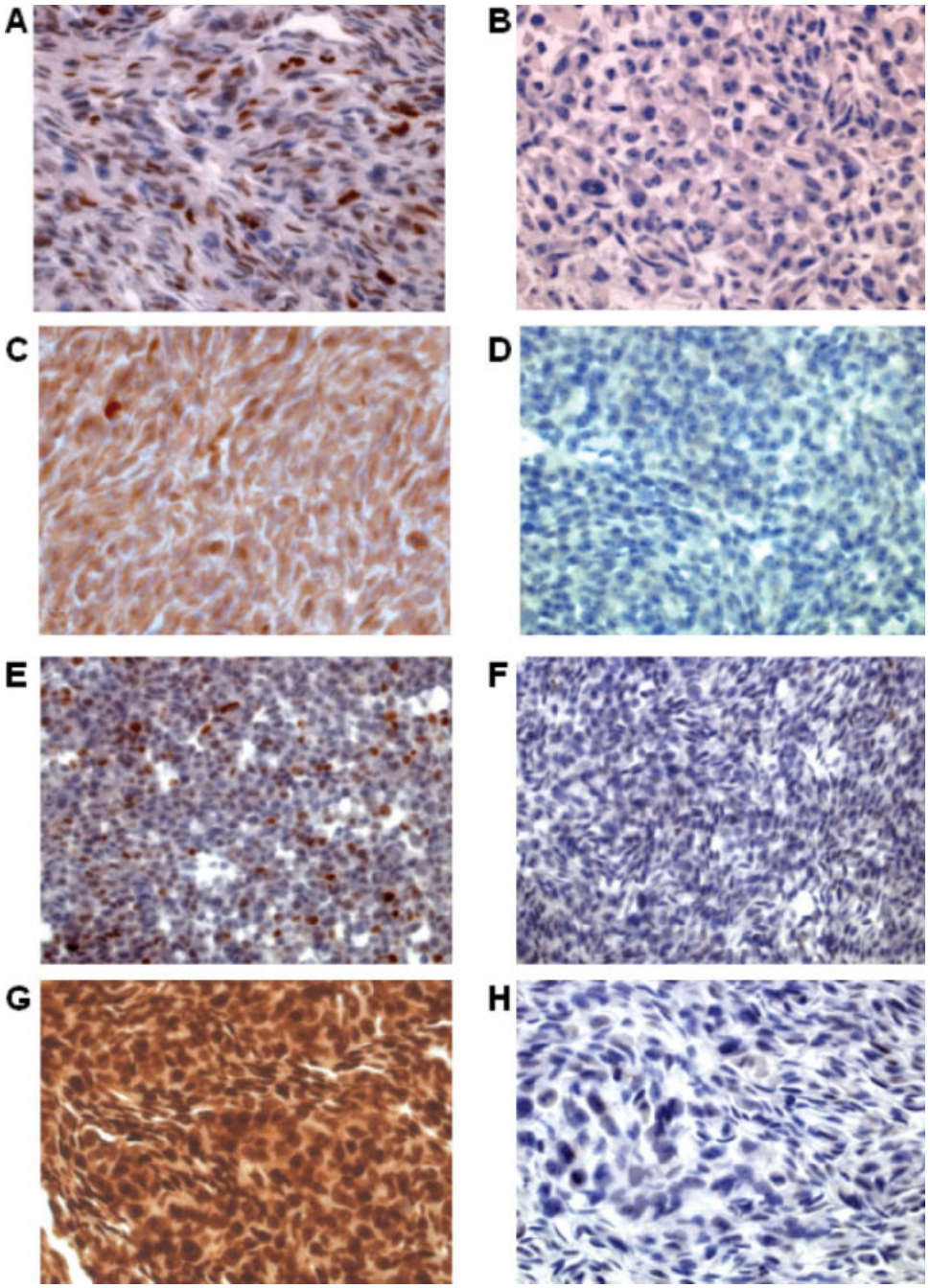

Figure 1. Paraffin-embedded bone tumor cell lines were stained for (A,B) MDM2; (C,D) CDKN2A; and (E–H) TP53 protein by immunohistochemistry. (A) Nuclear staining for MDM2 was observed in MHM osteosarcoma cells, which were characterized by *MDM2* gene amplifiation. (B) Osteosarcoma cell line IOR SARG showed neither *MDM2* gene amplification nor staining for MDM2 protein. (C) Positive staining for CDKN2A in pPNET CHP100 (hemizygous *CDKN2A* deletion). (D) CDKN2A was absent in ES cell line TC71 (hemizygous *CDKN2A* deletion). (E) 10% of -$TP53^{wt}$ ES cell line VH64 showed nuclear staining for TP53 protein. (F) Absence of TP53 protein in pPNET cell line SK-N-MC, which is deleted for TP53 Exon 2–4. (G) 100% of $TP53^{arg156pro}$ mutated OS cell line HOS show nuclear staining of TP53. (H) $TP53^{wt}$ cell line IOR OS 9, which is negative for both TP53 mRNA and protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that also nowadays erroneously tagged cell lines were found in the NCI60 panel, the reference set of tumor cell lines of the US National Cancer Institute (Garraway et al., 2005; Wang et al., 2006). Therefore the correct identification and exclusion of cross-contamination of tumor cell lines is crucial, especially when results from different institutions are to be compared.

Within the EuroBoNet consortium, a continuously expanding set of currently 36 bone sarcoma cell lines is used to investigate these rare tumor diseases on the genomic, transcriptomic, proteomic, and functional level. To generate a high degree of comparability of published results even for researchers working with the same cell lines from other sources, all these cell lines were collected in one central cell bank (Institute of Pathology, Duesseldorf), checked for identity and cross-contamination by DNA fingerprint analysis and then redistributed to the partners. We could confirm that all of the cell lines were different from each other and that no allele pattern gave rise to the suspicion of a mixture of different cell lines. For 10 of the cell lines, STR profile data are also available from the ATCC, confirming their supposed origin. Cell lines 143B and MNNG-HOS, which both are derived from HOS cells by either KRAS transformation or treatment with the carcinogenic nitrosamine MNNG (Rhim et al., 1977), respectively, showed a high degree of similarity but also some characteristic differences from the maternal cell line HOS. While some of these differences may be due to loss of heterozygosity in HOS-143B and HOS-MNNG (in STRs *TPOX* and *D21S11*), missing alleles in HOS compared to the daughter cell lines may indicate that the HOS clone used in this study has also experienced some genetic evolution. Another interesting finding is the occurrence of additional small alleles of the STRs *VWA* and *D5S818* in HOS-143B, which are absent in HOS, HOS-MNNG and also in the subclones deposited at the ATCC database. Since at least the tetranucleotide repeat *VWA* was already shown to be frequently involved in microsatellite instability in gastric cancer (Pelotti et al., 2007), we hypothesize that some of the additional alleles in HOS-143B are related to length alterations in their microsatellite sequences.

To further extend genomic characterization of the cell lines in a more functional context, MLPA analysis on 38 tumor suppressor gene loci was performed to detect homozygous deletion of these genes. Loss of *CDKN2A/B* was detected as the most common (40%) aberration in our set of tumor cell lines with comparable prevalence in osteosarcoma and Ewing sarcoma (42 and 36%, respectively). Homozygous loss of other tumor suppressor genes like *TP73* and *PTEN* were detected in only one each of the 36 cell lines. Even if other mechanisms like mutations or epigenetic silencing may also lead to functional impairment of tumor suppressor genes, homozygous deletion represents an irreversible feature of a respective cell line, and in this way these MLPA analyses are of permanent relevance for cell line categorization.

Regarding the *CDKN2A* status, conflicting results were observed in former genetic studies for some of the cell lines. At least in part, this is related to the different techniques used to study these chromosomal changes. One principle difference between FISH and MLPA on the one hand and array-based whole genome analysis on the other hand is based on the different algorithm these techniques use to define the status of a given genetic locus: While FISH and MLPA analyze each locus separately, array-based approaches also take into account the status of the adjacent SNP probes/cosmide clones of the specific locus to reduce false discovery rate. For small deletions which affect only one probe of the array this algorithm may lead to some loss of information.

For example, RD-ES was described as a cell line with no genetic aberration and moderate CDKN2a protein expression based on southern and Western blotting (Kovar et al., 1997), while FISH and aCGH analysis seemed to indicate homozygous deletion of *CDKN2A* (Savola et al., 2007). In our hands, RD-ES was proven to show hemizygous loss of *CDKN2A* by MLPA and aCGH together with reduced CDKN2A protein expression. Because loss of CDKN2A represents one of the rare molecular markers that were shown to predict adverse clinical outcome for both osteosarcoma as well as Ewing sarcoma (Maitra et al., 2001; Huang et al., 2005), reliable techniques should be used to test for this marker in clinical settings. In our study we observed a high degree of correlation between CDKN2A protein expression (by immunohistochemistry) and homozygous deletion of the *CDKN2A* gene (by MLPA or array techniques) documenting the robustness of these methods. However, while we found homozygous deletions for 42 and 36% of osteosarcoma and Ewing sarcoma samples, respectively, in primary clinical sarcoma samples, the prevalence of homozygous *CDKN2A* deletion is expected to be significantly lower (10–23% in OS, Savola et al., 2007, reviewed in Sandberg et al., 2003, 13–32% in ES, reviewed in Honoki et al., 2007). This observation may reflect a general bias in the composition of the cell line panel favoring more aggressive tumors to grow in vitro as it was already noticed for the prevalence of *TP53* mutation in primary Ewing's sarcoma

specimen and ES/pPNET cell lines (Kovar et al., 1993).

In ES/pPNET loss of TP53 function represents another accepted molecular marker that defines a subgroup of patients showing reduced overall survival due to poor response to chemotherapy (De Alava et al., 2000; Huang et al., 2005; Alldinger et al., 2007; Brownhill et al., 2007; Schaefer et al., 2008). In osteosarcoma, there is no such a clear relationship between *TP53* status and survival (Wunder et al., 2005; Pakos et al., 2004).

One explanation why the clinical impact of *TP53* status on OS obviously differs from that on ES/pPNET may be that the TP53 antagonist *MDM2* is amplified in a subset of OS (10–15%) but not in ES/pPNET (Kovar et al., 1993; Momand et al., 1998, Henriksen et al., 2003). For our set of OS cell lines we could confirm that *MDM2* amplification and *TP53* mutation are mutually exclusive, in agreement with observations in primary tumors specimens (Momand et al., 1998). Since most clinical studies on the prognostic value of loss of TP53 signaling did not include the simultaneous analysis of *MDM2*, the impairment of this key regulatory pathway is probably underestimated. In addition, many of the studies analyzing *TP53* as a potential prognostic marker are based on immunohistochemistry, assuming that overexpression of TP53 protein is associated with *TP53* mutation. However, in eight out of the 18 cell lines harboring a *TP53* mutation we found just a very weak or even no staining for TP53. More precisely, this was found if cells were characterized by nonsense or out-of-frame mutations, an observation that was already made in other tumor entities (Bodner et al., 1992; Meinhold-Heerlein et al., 2001). According to our data on bone tumor cell lines, immunohistochemistry by far underestimates the prevalence of *TP53* mutations which may also explain some of the published differences of the prognostic value of *TP53* status in bone tumors.

Finally, p53 protein is assumed to be regulated to a large extent by posttranscriptional mechanisms (Vogelstein et al., 2000) and almost no data are available addressing the transcriptional control of *TP53* (reviewed in Hall and McCluggage, 2006). However, Chandar et al., already reported that rearrangements of *TP53* were leading to the absence of *TP53* mRNA transcripts in the $TP53^{wt}$ OS cell line MG-63 (Chandar et al., 1992). In our study, seven of the ten $TP53^{wt}$ OS cell lines (including MG-63) were characterized by remarkably low levels of *TP53* mRNA transcripts, while showing only weak or even no staining for TP53 protein. This transcriptional downregulation was only found in OS cell lines, and not in any of the other seven $TP53^{wt}$ bone tumor cell lines. By array techniques we could exclude that gross deletions of chromosomal band 17p13.1 were responsible for the absence of *TP53* mRNA. Whether this transcriptional regulation is involved in an alternative mechanism of TP53 functional control in OS remains to be elucidated. At least for the different *TP53* mRNA isoforms evidence is increasing that usage of alternative transcriptional starting points as well as alternative splicing are important factors to control specific TP53 target genes in a tissue and disease specific manner (Bourdon, 2007).

According to current concepts on sarcoma biology, these tumors may be divided into a cytogenetically simple group often characterized by tumor-specific chromosomal translocations including Ewing's sarcoma, alveolar rhabdomyosarcoma, and synovial sarcoma and sarcomas with complex karyotypes like osteosarcoma, embryonal rhabdomyosarcoma, or chondrosarcoma (Helman and Meltzer, 2003). The latter group is characterized by a high degree of aneuploidy, complex chromosomal rearrangements and absence of recurrent reciprocal rearrangements. The underlying genetic mechanisms are supposed to include disturbance in cell-cycle and checkpoint genes like *TP53*, *RB1*, or *CDKN2A*, which may indicate that impairment of TP53 or RB1/CDKN2A pathways in osteosarcoma is more important for tumor development than tumor progression. This aspect of osteosarcoma development was also emphasized by Ternovoi et al., 2006, emphasizing that $TP53^{-/-}$ mice develop osteosarcoma but no other malignant bone tumors.

Taken together, our genetic and immunohistochemical characterization of a substantial number of sarcoma cell lines represents an important step to supply research groups inside and outside the EuroBoNet with proven preclinical models for further advanced studies on tumor biology.

ACKNOWLEDGMENTS

Departments 1, 3, 4, 5, 6, 7, and 8 are partners of the EuroBoNeT consortium, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumors (see www.EuroBoNet.eu).

REFERENCES

Alldinger I, Schaefer KL, Goedde D, Ottaviano L, Dirksen U, Ranft A, Juergens H, Gabbert HE, Knoefel WT, Poremba C. 2007. Microsatellite instability in Ewing tumor is not associated with loss of mismatch repair protein expression. J Cancer Res Clin Oncol 133:749–759.

Benini S, Baldini N, Manara MC, Chano T, Serra M, Rizzi S, Lollini PL, Picci P, Scotlandi K. 1999. Redundancy of autocrine loops in human osteosarcoma cells. Int J Cancer 80:581–588.

Bodner SM, Minna JD, Jensen SM, D'Amico D, Carbone D, Mitsudomi T, Fedorko J, Buchhagen DL, Nau MM, Gazdar AF. 1992. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. Oncogene 7:743–749.

Bourdon JC. 2007. p53 and its isoforms in cancer. Br J Cancer 97:277–282.

Brownhill SC, Taylor C, Burchill SA. 2007. Chromosome 9p21 gene copy number and prognostic significance of p16 in ESFT. Br J Cancer 96:1914–1923.

Bruland OS, Fodstad O, Stenwig AE, Pihl A. 1988. Expression and characteristics of a novel human osteosarcoma-associated cell surface antigen. Cancer Res 48:5302–5309.

Chandar N, Billig B, McMaster J, Novak J. 1992. Inactivation of p53 gene in human and murine osteosarcoma cells. Br J Cancer 65:208–214.

DeAlava E, Antonescu CR, Panizo A, Leung D, Meyers PA, Huvos AG, Pardo-Mindán FJ, Healey JH, Ladanyi M. 2000. Prognostic impact of P53 status in Ewing sarcoma. Cancer 89:783–792.

Domagk D, Schaefer KL, Eisenacher M, Braun Y, Wai DH, Schleicher C, Diallo-Danebrock R, Bojar H, Roeder G, Gabbert HE, Domschke W, Poremba C. 2007. Expression analysis of pancreatic cancer cell lines reveals association of enhanced gene transcription and genomic amplifications at the 8q22.1 and 8q24.22 loci. Oncol Rep 17:399–407.

Fiegler H, Carr P, Douglas EJ, Burford DC, Hunt S, Scott CE, Smith J, Vetrie D, Gorman P, Tomlinson IP, Carter NP. 2003. DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. Genes Chromosomes Cancer 36:361–374. Erratum in: Genes Chromosomes Cancer 37:223.

Friedrichs N, Kriegl L, Poremba C, Schaefer KL, Gabbert HE, Shimomura A, Paggen E, Merkelbach-Bruse S, Buettner R. 2006. Pitfalls in the detection of t(11;22) translocation by fluorescence in situ hybridization and RT-PCR: A single-blinded study. Diagn Mol Pathol 15:83–89.

Fodstad O, Brøgger A, Bruland O, Solheim OP, Nesland JM, Pihl A. 1986. Characteristics of a cell line established from a patient with multiple osteosarcoma, appearing 13 years after treatment for bilateral retinoblastoma. Int J Cancer 38:33–40.

Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, Beroukhim R, Milner DA, Granter SR, Du J, Lee C, Wagner SN, Li C, Golub TR, Rimm DL, Meyerson ML, Fisher DE, Sellers WR. 2005. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 436:117–122.

Gil-Benso R, Lopez-Gines C, López-Guerrero JA, Carda C, Callaghan RC, Navarro S, Ferrer J, Pellín A, Llombart-Bosch A. 2003. Establishment and characterization of a continuous human chondrosarcoma cell line, ch-2879: Comparative histologic and genetic studies with its tumor of origin. Lab Invest 83:877–887.

Greenlee RT, Hill-Harmon MB, Murray T, Thun M. 2001. Cancer statistics. CA Cancer J Clin 51:15–36.

Hall PA, McCluggage WG. 2006. Assessing p53 in clinical contexts: Unlearned lessons and new perspectives. J Pathol 208:1–6.

Helman LJ, Meltzer P. 2003. Mechanisms of sarcoma development. Nat Rev Cancer 3:685–694.

Henriksen J, Aagesen T, Maelandsmo GM, Lothe RA, Myklebost O, Forus A. 2003. Amplification and overexpression of *COPS3*potentially target TP53 for proteasome-mediated degradation. Oncogene 22:5358–5361.

Hensler PJ, Annab LA, Barrett JC, Pereira-Smith OM. 1994. A gene involved in control of human cellular senescence on human chromosome 1q. Mol Cell Biol 14:2291–2297.

Huang HY, Illei PB, Zhao Z, Mazumdar M, Huvos AG, Healey JH, Wexler LH, Gorlick R, Meyers P, Ladanyi M. 2005. Ewing sarcomas with p53 mutation or p16/p14ARF homozygous deletion: A highly lethal subset associated with poor chemoresponse. J Clin Oncol 23:548–558.

Honoki K, Stojanovski E, McEvoy M, Fujii H, Tsujiuchi T, Kido A, Takakura Y, Attia J. 2007. Prognostic significance of p16 INK4a alteration for Ewing sarcoma: A meta-analysis. Cancer 110:1351–1360.

Jagasia AA, Block JA, Qureshi A, Diaz MO, Nobori T, Gitelis S, Iyer AP. 1996. Chromosome 9 related aberrations and deletions of the CDKN2 and MTS2 putative tumor suppressor genes in human chondrosarcomas. Cancer Lett 105:91–103.

Kjønniksen I, Winderen M, Bruland O, Fodstad O. 1994. Validity and usefulness of human tumor models established by intratibial cell inoculation in nude rats. Cancer Res 54:1715–1719.

Knijnenburg J, Szuhai K, Giltay J, Molenaar L, Sloos W, Poot M, Tanke HJ, Rosenberg C. 2005. Insights from genomic microarrays into structural chromosome rearrangements. Am J Med Genet A 132A:36–40.

Kovar H, Auinger A, Jug G, Aryee D, Zoubek A, Salzer-Kuntschik M, Gadner H. 1993. Narrow spectrum of infrequent p53 mutations and absence of MDM2 amplification in Ewing tumours. Oncogene 8:2683–2690.

Kovar H, Jug G, Aryee DN, Zoubek A, Ambros P, Gruber B, Windhager R, Gadner H. 1997. Among genes involved in the RB dependent cell cycle regulatory cascade, the p16 tumor suppressor gene is frequently lost in the Ewing family of tumors. Oncogene 15:2225–2232.

Maitra A, Roberts H, Weinberg AG, Geradts J. 2001. Loss of p16(INK4a) expression correlates with decreased survival in pediatric osteosarcomas. Int J Cancer 95:34–38.

Meinhold-Heerlein I, Ninci E, Ikenberg H, Brandstetter T, Ihling C, Schwenk I, Straub A, Schmitt B, Bettendorf H, Iggo R, Bauknecht T. 2001. Evaluation of methods to detect p53 mutations in ovarian cancer. Oncology 60:176–188.

Momand J, Jung D, Wilczynski S, Niland J. 1998. The MDM2 gene amplification database. Nucleic Acids Res 26:3453–3459.

Nelson-Rees WA, Daniels DW, Flandermeyer RR. 1981. Cross-contamination of cells in culture. Science 212:446–452.

Ouyang P. 1998. An in vitro model to study mesenchymal-epithelial transformation. Biochem Biophys Res Commun 246:771–776.

Pakos EE, Kyzas PA, Ioannidis JP. 2004. Prognostic significance of TP53 tumor suppressor gene expression and mutations in human osteosarcoma: A meta-analysis. Clin Cancer Res 10:6208–6214.

Pelotti S, Ceccardi S, Alù M, Lugaresi F, Trane R, Falconi M, Bini C, Cicognani. 2007. Cancerous tissues in forensic genetic analysis. Genet Test 11:397–400.

Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H, Delattre O. 1997. A new member of the ETS family fused to EWS in Ewing tumors. Oncogene 14:1159–1164.

Rhim JS, Putman DL, Arnstein P, Huebner RJ, McAllister RM. 1977. Characterization of human cells transformed in vitro by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Int J Cancer 19:505–510.

Sandberg AA, Bridge JA. 2003. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and relatcd tumors. Cancer Genet Cytogenet 145:1–30.

Savola S, Nardi F, Scotlandi K, Picci P, Knuutila S. 2007. Microdeletions in 9p21.3 induce false negative results in CDKN2A FISH analysis of Ewing sarcoma. Cytogenet Genome Res 119:21–26.

Schaefer KL, Wai D, Poremba C, Diallo R, Boecker W, Dockhorn-Dworniczak B. 2002. Analysis of TP53 germline mutations in pediatric tumor patients using DNA microarray-based sequencing technology. Med Pediatr Oncol 38:247–253.

Schaefer KL, Eisenacher M, Braun Y, Brachwitz K, Wai DH, Dirksen U, Lanvers-Kaminsky C, Juergens H, Herrero D, Stegmaier S, Koscielniak E, Eggert A, Nathrath M, Gosheger G, Schneider DT, Bury C, Diallo-Danebrock R, Ottaviano L, Gabbert HE, Poremba C. 2008. Microarray analysis of Ewing's sarcoma family of tumours reveals characteristic gene expression signatures associated with metastasis and resistance to chemotherapy. Eur J Cancer 44:699–709.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57.

Schulz A, Battmann A, Heinrichs CM, Kern A, Tiedemann A, Bürger H, Fohr B, Hani N, Pietruck C, Busk H, Jundt G. 1993. Properties and reactivity of a new human osteosarcoma cell line (HOS 58). Calcif Tissue Int 52:30.

Skubitz KM, D'Adamo DR. 2007. Sarcoma. Mayo Clin Proc 82:1409–1432.

Ternovoi VV, Curiel DT, Smith BF, Siegal GP. 2006. Adenovirus-mediated p53 tumor suppressor gene therapy of osteosarcoma. Lab Invest 86:748–766.

Urano F, Umezawa A, Yabe H, Hong W, Yoshida K, Fujinaga K, Hata J. 1998. Molecular analysis of Ewing's sarcoma: Another fusion gene, EWS-E1AF, available for diagnosis. Jpn J Cancer Res 7:703–711.

vanden Berg H, Kroon HM, Slaar A, Hogendoorn PCW. 2008. Incidence of biopsy-proven bone tumors in children: A report based on the Dutch pathology registration "PALGA." J Pediatr Orthop 28:29–35.

van Valen F. 1998. Ewing's sarcoma family of tumors. In: Masters JR, Palsson B, editors. Human Cell Culture, Vol. 1. London: Kluwer Academic Publisher, pp. 55–85.

Vogelstein B, Lane D, Levine AJ. 2000. Surfing the p53 network. Nature 408:307–310.

Wang H, Huang S, Shou J, Su EW, Onyia JE, Liao B, Li S. 2006. Comparative analysis and integrative classification of NCI60 cell lines and primary tumors using gene expression profiling data. BMC Genomics 7:166.

Wunder JS, Gokgoz N, Parkes R, Bull SB, Eskandarian S, Davis AM, Beauchamp CP, Conrad EU, Grimer RJ, Healey JH, Malkin D, Mangham DC, Rock MJ, Bell RS, Andrulis IL. 2005. TP53 mutations and outcome in osteosarcoma: A prospective, multicenter study. J Clin Oncol 23:1483–1490.