

## RESEARCH ARTICLES

# Array Comparative Genomic Hybridization Reveals Frequent Alterations of G1/S Checkpoint Genes in Undifferentiated Pleomorphic Sarcoma of Bone

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Undifferentiated pleomorphic sarcoma of bone (UPSB) is a rare tumor often difficult to differentiate from fibrosarcoma of bone (FSB), diagnostically. We applied array comparative genomic hybridization (array CGH) to screen for genes with potential importance in the tumor and compared the results with alterations seen in FSB. Twenty-two fresh frozen tissue specimens from 20 patients (18 primary tumors and 4 local recurrences) with UPSB were studied. DNA was isolated and hybridized onto Agilent 244K CGH oligoarrays. The hybridization data were analyzed using Agilent DNA Analytics Software. The number of changes ranged from 2 to 168 (average = 66). Losses were most frequently seen at 8p, 9p, 10, 13q, and 18q, and gains at 4q, 5p, 6p, 7p, 8q, 12p, 14q, 17q, 19p, 20q, 22q, and X. Homozygous deletions of *CDKN2A*, *RBI*, *TP53*, and *ING1* were seen in 8/20, 7/20, 3/20, and 2/20 cases, respectively. Hypermethylation of both *p16<sup>INK4a</sup>* and *p14<sup>ARF</sup>* was found in two cases with loss at *CDKN2A*. Inactivation either of *CDKN2A*, *RBI*, or *TP53* was detected in 18/20 cases. One case showed high level gains of *CDK4* and *MDM2*. Frequent gains were seen at *MYC*, *PDGFRA*, *KIT*, and *KDR*. Immunohistochemical positivity of *KIT*, *PDGFRA*, *KDR*, and *PDGFRB* was found in 8/14, 5/14, 4/14, and 4/14 cases, respectively. The regions most significantly discriminating between UPSB and FSB included *RBI* and *MYC*. No homozygous deletions of *RBI* were found in FSB. In conclusion, our analysis showed the disruption of G1/S checkpoint regulation to be crucial for the oncogenesis of UPSB. © 2011 Wiley-Liss, Inc.

## INTRODUCTION

Undifferentiated pleomorphic sarcoma of bone (UPSB), previously known as malignant fibrous histiocytoma (MFH) of bone, is a rare type of sarcoma, which represents less than 2% of all primary malignant bone lesions. The tumor is highly malignant and shows high tendency to metastasis, especially to the lungs. The recommended treatment is usually wide surgical excision. The differential diagnosis is based on histological findings, and in many cases UPSB is difficult to distinguish from osteosarcoma or fibrosarcoma of bone (FSB) (Steiner et al., 2002). New therapeutic strategies, as well as new diagnostic markers, are therefore needed.

Little is known about the genetics of UPSB. In the most extensive study thus far, performed by the authors, conventional comparative genomic hybridization (CGH) was used to screen DNA copy number alterations in 26 UPSB cases (Tarkkanen et al., 2006). In addition, based on the CGH findings, expression of *MYC* was studied using immunohistochemistry, and increased levels of the protein were detected in

33% of the tumors. However, although several other chromosomal regions of frequent gains and losses with potential importance in UPSB were detected, no other candidate genes could be pinpointed because of low resolution of conventional CGH.

Recent genomic profiling studies have yielded promising results for potential exploitation in diagnosis, prediction of outcome, and treatment of soft-tissue sarcomas with complex karyotypes (Barretina et al., 2010; Chibon et al., 2010). Discovery

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of novel specific genetic alterations or alteration patterns is expected to help the improvement of these fields in sarcomas of bone, too.

In this study, we applied array comparative genomic hybridization (array CGH), to find genes and narrow regions with potential role in the pathogenesis of the tumor. Agilent CGH arrays containing about 244,000 oligonucleotide probes covering the whole genome were used to study copy number alterations in 20 UPSb cases. In addition, the results were compared with the copy number alterations in 11 FSb cases, previously published by the authors (Niini et al., 2010), to find potential markers for differential diagnosis between these two tumor types.

## MATERIALS AND METHODS

### **Patients and Material**

Histological diagnosis of UPSb, following the World Health Organization 2002 classification (Fletcher et al., 2002), was performed on morphology, and after exclusion of any other possible more differentiated entity (i.e., leiomyosarcoma of bone, fibroblastic osteosarcoma or metastases from sarcomatoid carcinoma) also using immunohistochemistry. The distinction between UPSb and FSb was based both on neoplastic cell morphology, with pleomorphic component in the former and only spindle cells in the latter, and on the architecture that is storiform in UPSb and with a herring bone structure in FSb.

Twenty-seven fresh frozen tissue specimens from 25 patients with UPSb were originally considered for this study (23 primary tumors and 4 local recurrences). After histological revision, tumor specimens with only a proportion of neoplastic cells over 80% were used for DNA isolation. Enough good quality DNA for array CGH hybridization was available from 22 of these specimens (18 primary tumors and 4 local recurrences) obtained from 20 patients.

All of the UPSb cases included in the study were of Grade 4 according to Broders system (Broders et al., 1939). Other clinical data for the patients are shown in Table 1. Informed consents were collected from all patients in accordance with the standard procedure in each country.

### **Array Comparative Genomic Hybridization and Data Analysis**

DNA isolation, array CGH, and data analysis were performed as in the array CGH study of

FSb previously published by the authors (Niini et al., 2010). Digestion, labeling, and hybridization were performed by following Agilent's protocol version 4.0 for Agilent Human Genome CGH 244A Oligo Microarrays (Agilent Technologies, Santa Clara, California, USA). The array images were analyzed using Agilent Feature Extraction Software (version 9.5.3.1) and the data obtained was analyzed using Agilent DNA Analytics Software (version 4.0).

A loss was regarded as homozygous when  $\log_2$  ratio was lower than -1 and a gain as high level gain when  $\log_2$  ratio was higher than or equal to 1.5. The copy number statuses at the loci of particular interest were visually checked from individual profiles in Agilent DNA Analytics Software, comparing the  $\log_2$  ratio of the region with the  $\log_2$  ratio of the neighboring region and genome-wide to the overall profile of the case. Exceptions from the above-mentioned thresholds could be made on the basis of the profile.

### **Methylation Specific Polymerase Chain Reaction of the *p16<sup>INK4a</sup>* and *p14<sup>ARF</sup>* Genes**

Methylation specific PCR for *p16<sup>INK4a</sup>* and *p14<sup>ARF</sup>* promoter methylation was performed as described previously (Herman et al., 1996; Esteller et al., 2000), in the same way as in our array CGH study of FSb (Niini et al., 2010).

### **Immunohistochemistry of KDR (VEGFR2), KIT, and PDGF-Alpha and PDGF-Beta Receptors**

Immunohistochemistry was performed on tumor specimens obtained from 14 of the 20 UPSb patients, for which enough paraffin-embedded tumor tissue was still available. Immunohistochemistry was carried out by using an avidin-biotin-peroxidase complex method with the following primary antibodies: anti-VEGFR2 (KDR) rabbit polyclonal antibody (1:100 dilution rate; Abcam, Cambridge, United Kingdom), anti-human CD117 (KIT) rabbit polyclonal antibody (1:50 dilution rate; DakoCytomation, Glostrup, Denmark), anti-human PDGF receptor alpha-subunit (1:50 dilution rate), and anti-human PDGF receptor beta-subunit (1:50 dilution rate) mouse monoclonal antibodies (both from Genzyme, Cambridge, Massachusetts, USA). Immunohistochemistry was carried out on decalcified, paraffin-embedded samples. For each specimen, both negative and antigenicity controls for immunostaining were performed. Negative controls were

TABLE I. Clinicopathologic Features of 20 Patients with Undifferentiated Pleomorphic Sarcoma of Bone

Patient code	Sample(s)	Age at diagnosis (years)	Gender	Site	Treatment	Type of surgery	Surgical Margins	Years until 1st relapse	Type of Relapse	Years until last follow-up	Final Outcome
UPSI	Pri, 1st Rec, 2nd Rec	10	Male	Pelvis	Surgery	Resection	Marginal	0.6	Local Rec.	4.0	AWD
UPS4	Pri	77	Male	Humerus	Surgery	Resection	Wide			5.5	NED
UPSS	Rec	78	Female	Femur	Surgery	Amputation	Wide			1.2	DOD
UPS6	Pri	39	Male	Femur	Surgery + Post-OpCH	Resection	Intralesional		Lung Met.	1.9	DOD
UPS7	Pri	58	Male	Femur	Pre-OpCH + Surgery + Post-OpCH	Resection	Wide			5.8	NED
UPS9	Pri	59	Female	Tibia	Surgery	Amputation	Wide	0.6	Lung Met.	2.9	AWD
UPS10	Pri	23	Male	Pelvis	Chemotherapy only	Not done	Not done			0.5	DOD
UPS11	Pri	44	Male	Femur	Surgery + Post-OpCH	Amputation	Wide			9.3	DOD
UPS14	Pri	79	Female	Pelvis	Surgery	Resection	Intralesional			6.3	NED
UPS15	Pri	43	Male	Femur	Pre-OpCH + Surgery + Post-OpCH	Amputation	Intralesional	0.5	Lung Met.	0.6	DOD
UPS16	Pri	59	Female	Femur	Surgery	Amputation	Radical			8.0	NED
UPS17	Pri	35	Male	Clavicula	Surgery + Post-OpCH	Resection	Wide			6.1	NED
UPS19	Pri	52	Male	Femur	Surgery + Post-OpCH	Amputation	Wide			0.9	DOD
UPS20	Pri	54	Female	Pelvis	Pre-OpCH + Surgery	Resection	Wide	2.2	Lung Met.	5.7	DOD
UPS21	Pri	19	Male	Tibia	Surgery	Amputation	Wide			3.2	NED
UPS22	Pri	35	Female	Pelvis	Surgery + Post-OpCH	Resection	Wide			7.8	NED
UPS24 <sup>a</sup>	Pri	32	Male	Femur	Surgery + Post-OpCH	Resection	Wide			5.2	NED
UPS25	Pri	44	Female	Femur	Pre-OpCH + Surgery + Post-OpCH	Resection	Wide			5.8	NED
UPS26	Rec	53	Male	Humerus	Surgery + Post-OpCH	Resection	Wide	1.8	Local Rec.	2.5	DOD
UPS27	Pri	25	Male	Tibia	Pre-OpCH + Surgery + Post-OpCH	Resection	Wide			17.2	NED

<sup>a</sup>Radio-induced disease. Pri, primary tumour; Rec, local recurrence; Met, metastasis; AWD, alive with disease; DOD, dead of disease; Pre-OpCH, pre-operative chemotherapy; Post-OpCH, post-Operative chemotherapy.

carried out by replacing the primary antibody with normal horse or goat serum for mouse monoclonal or rabbit polyclonal primary antibodies, respectively. As positive control for the antigenicity of the specimen, one tumor section was incubated with the V9 anti-vimentin monoclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany). Only vimentin-positive samples were considered for the immunohistochemical analyses. Primary antibodies were incubated overnight at 4°C. Development of immunoreaction was obtained with diaminobenzidine and nuclei were counterstained with Gill's hematoxylin. As positive controls, tissue sections of normal human kidney (KDR-positive), PDGF receptors-positive breast cancer, and KIT-positive gastrointestinal tumor were used. All immunohistochemical stainings were evaluated without knowing the genetic data to avoid any possible bias in data interpretation. Samples were scored as positive or negative by taking into consideration the cellular immunostaining. Only specimens with a clearly evident and diffused immunostaining for each protein were considered as positive.

#### **Association of Inactivation of *RBI*, *CDKN2A*, and *TP53* with Survival**

The associations between patients' survival status and inactivation of *RBI*, *CDKN2A*, and *TP53* were investigated with Kaplan-Meier analysis using the open access software tools in R statistical programming language ([www.r-project.org](http://www.r-project.org)), in particular the "survival" package of BioConductor (Gentleman et al., 2004). No other factors potentially influencing outcome were included in the analysis because of the small number of cases. The significance of the associations was estimated with Cox proportional hazards model (Cox, 1972) with Efron approximation (Efron, 1977); the *P* values were calculated using the log-ratio test. Probes with  $\geq 10\%$  missing values were filtered out as a preprocessing step. The patients without any specimen from the primary tumor and the patients with metastasis at diagnosis were excluded from the analysis.

#### **Comparison of Differences in Copy Number Between UPSb and FSb**

Nearest shrunken centroids classifier (Tibshirani et al., 2002) was constructed based on minimal common regions of copy number change across the

cases to identify genomic aberrations that could be used to predict patient's UPSb/FSb status. Minimal common regions of copy number change across the cases were detected based on the deletion/amplification profiles provided by Agilent DNA Analytics Software. Close-by regions exhibiting nearly identical copy number profiles across the cases were combined (maximum gap of 7 probes; correlation between mean signal  $\log_2$  ratios  $\geq 0.95$ ). The mean signal  $\log_2$  ratio over the probes within each copy number region was used as a measure of deletion/amplification signal. This accounts for the signal strength, which would be missed with binary copy number calls. The classifier produces a list of most significant genomic regions associated with UPSb/FSb status of the cases. Classification performance in leave-one out cross-validation and comparisons to randomly labeled patient data (100 random permutations of the labels) were used to assess generalization capability and statistical significance of the classifier.

## **RESULTS**

#### **Copy Number Alterations—Overview**

The original data of the array CGH hybridizations of all of the samples can be found in the public database Cangem (<http://www.cangem.org/>). The overview of the copy number alterations in all of the cases along the whole genome is shown in Figure 1. For patient UPS1 with three samples of different stages of the disease, only the diagnostic sample is included in the figure, as well as in the overall frequencies presented later, unless otherwise specified. The genomic regions presented in this article are compatible with the UCSC human hg18 assembly. The minimal overlapping regions that were lost or gained in at least eight (40%) of the 20 cases are shown in Supporting Information Tables 1 and 2, respectively. The regions with recurrent homozygous losses are presented in Table 2, and individual gains or amplifications with  $\log_2$  ratio higher than or equal to 1.5, designated as high level gains, are shown in Table 3. The  $\log_2$  ratio 1.5 corresponds to approximately five copies of the region when present in 100% of the cells.

The numbers of copy number alterations per case are shown in Table 4. Alterations were seen in all of the cases, ranging from 2 to 168, with an average of 66. In 8 (40%) of the 20 cases, the number of alterations was below 50, in 7 (35%) cases between 50 and 100 and in 5 (25%) cases



Figure 1. The penetrance overview of array CGH findings in 20 patients with undifferentiated pleomorphic sarcoma of bone. Copy number losses are shown by green color on the left and copy number gains by red color on the right of the vertical line. (Note: Losses and gains consequent upon copy number variants are included in the figure). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

more than 100. Gains were slightly more frequent than losses.

Losses were most frequently seen at the chromosomal arms 8p, 9p, 10p, 10q, 13q, and 18q. The four regions with most frequent losses, seen in 14 (70%) of the cases, were all located at chromosome 13: 13q13.3 (35,171,158–36,491,551), 13q13.3-q14.11 (37,397,428–40,437,353); 13q14.11 (42,260,983–42,944,918), and 13q14.11-q14.2 (43,127,337–48,512,284). Gains were most frequently found at chromosomal arms 4p, 5p, 6p, 7p, 8q, 12p, 14q, 17q, 19p, 20q, 22q, Xp, and Xq. The most commonly gained region of 134 kb with gains in 13 (65%) of the 20 cases was located at 20q11.22 (32,039,324–32,173,642). Gains in 11 (55%) of the cases were seen at regions 8q24.21 (128,108,198–129,081,391); 8q24.3 (142,287,436–146,007,394), 14q11.2 (19,827,054–23,063,177), 20q11.22 (32,952,023–33,010,638).

The array CGH profiles of the local recurrence samples of patient UPS1 were highly similar to the profile of the diagnostic sample with ~156 alterations. The first recurrence had acquired about 10 additional changes compared to the diagnostic tumor. The profiles of the two recurrences were almost identical.

#### Regions Pinpointing Candidate Genes

The 5 Mb minimal overlapping region of frequent losses (14/20, 70%) at 13q14.11-q14.2 (43,127,337–48,512,284) contains the *RBI* tumor suppressor gene (retinoblastoma 1 gene). The closer examination of the array CGH profiles suggested that this gene was homozygously deleted in seven (35%) and heterozygously deleted in eight (40%) of the 20 cases (Table 4). The minimal overlapping region of the homozygous deletions was restricted to the *RBI* locus.

Frequent losses were seen also at 9p21.3 with the minimal overlapping region (21,862,531–22,755,281) that was lost in 12 (60%) of the 20 cases. This, about 900 kb, region contains the *CDKN2A* tumor suppressor gene [cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)]. The array CGH profiles suggested *CDKN2A* to be homozygously deleted in eight (40%) and heterozygously deleted in five (25%) of the cases (Table 4). The minimal overlapping region of the homozygous deletions was restricted to *CDKN2A* and *CDKN2B* [cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)].

Recurrent homozygous deletions were also detected at 17p13.1 with a minimal overlapping

TABLE 2. The Genomic Regions with Recurrent Homozygous Losses in 20 Cases of Undifferentiated Pleomorphic Sarcoma of Bone

Cytoband/s	Start (bp) <sup>a</sup>	Stop (bp) <sup>a</sup>	Size	Genes <sup>b</sup>	Frequency	Patients
3q26.31 9p21.3	176,371,417 2,892,614	176,474,115 22,060,183	103 kb 168 kb	NAALADL2 <b>CDKN2A</b> , CDKN2B	2/20 8/20	UPS7, UPS11 UPS4, UPS6, UPS15, UPS19, UPS21, UPS22, UPS25, UPS26
13q13.1 13q13.3-q14.1	30,649,976 34,848,507 37,397,228	31,448,695 35,798,882 40,669,270	799 kb 950 kb 3.3 Mb	— NBEA, MAB21L1, DCLK1, SOHHLH2, SPG20 UFM1, FREM2, STOML3, C13orf73, NHRC3, LHFPL, COG6, FOXO1, MRPS31, SLC25A15, ELF1, WBP4, KBTBD6	2/20 2/20 2/20	UPS4, UPS15 UPS26, UPS27 UPS1, UPS27
13q14.11	40,974,345	42,514,676	1.5 Mb	KIAA0564, DGKH, AKAP11, TNFSF11, C13orf30, EPST11, DNAC15	2/20	UPS1, UPS27
13q14.2	47,756,288	47,872,285	116 kb	<b>RBI</b> , P2RY5	7/20	UPS1, UPS9, UPS11, UPS14, UPS16, UPS24, UPS27
13q21.1 13q33.3-q34	53,296,268 108,989,914	56,026,263 111,085,616	2.7 Mb 2.1 Mb	— IRS2, COL4A1, COL4A2, RAB20, FLJ10769, CARS2, <b>ING1</b> , ANKRD10, ARHGEF7, C13orf16	2/20 2/20	UPS4, UPS27 UPS1, UPS27
17p13.1 Yp11.31-p11.2 Yq11.221-q11.223	7,522,684 2,710,250 16,139,606	7,538,045 3,318,261 22,930,937	15 kb 608 kb 6.8 Mb	TP53 SRY, RPS4Y1, ZFY numerous	3/20 4/16 5/16	UPS5, UPS6, UPS10 UPS6, UPS15, UPS19, UPS27 UPS6, UPS15, UPS19, UPS21, UPS27

<sup>a</sup>Regions are compatible with the UCSC human hg18 assembly.<sup>b</sup>Strongest candidate genes are shown in bold.

TABLE 3. Genomic Regions with Amplifications or High Level Gains in 20 Patients with Undifferentiated Pleomorphic Sarcoma of Bone

Cytoband/s	Start <sup>a</sup>	Stop <sup>a</sup>	Size	Log <sub>2</sub> ratio	Patient code	Candidate genes
2q11.2	96,054,642	96,936,939	882 kb	1.70	UPS19	
4q12-q13.2	53,081,830	67,479,579	14.4 Mb	1.45	UPS19	PDGFRA, KIT, KDR
4q12	53,680,959	54,133,770	452 kb	1.65	UPS11	
4q12	54,317,343	54,580,599	263 kb	1.62	UPS11	
4q12	57,840,785	58,743,743	902 kb	1.59	UPS27	
4q13.1	59,890,126	64,390,533	4.5 Mb	1.73	UPS19	
<b>4q13.1-q22.1</b>	<b>61,877,043</b>	<b>90,475,825</b>	<b>28.6 Mb</b>	<b>1.73</b>	<b>UPS27</b>	
<b>4q13.1</b>	<b>62,392,987</b>	<b>62,609,320</b>	<b>216 kb</b>	<b>2.46</b>	<b>UPS27</b>	
4q22.1	88,845,279	90,403,230	1.6 Mb	1.96	UPS27	
4q22.1	91,680,016	92,129,752	449 kb	1.87	UPS27	
8p23.1	10,274,715	12,448,574	2.2 Mb	1.47	UPS19	
11q22.1	98,867,085	98,935,475	68 kb	1.81	UPS26	
11q22.1-q22.2	101,481,261	102,007,534	526 kb	1.80	UPS11	
11q23.3	118,181,572	118,281,982	100 kb	2.01	UPS26	
11q23.3	119,599,910	120,116,204	516 kb	2.04	UPS26	
11q24.1	123,099,800	123,181,496	81 kb	1.99	UPS26	
11q32.1	94,533,210	95,549,816	1.0 Mb	1.45	UPS11	
<b>12q13.2-q14.1</b>	<b>54,617,814</b>	<b>58,625,217</b>	<b>4.0 Mb</b>	<b>1.86</b>	<b>UPS26</b>	
<b>12q13.3-q14.1</b>	<b>55,709,104</b>	<b>57,740,187</b>	<b>2.0 Mb</b>	<b>2.43</b>	<b>UPS26</b>	CDK4 MDM2
12q15	67,351,573	68,032,507	680 kb	1.89	UPS26	
14q23.1	58,683,477	60,865,723	2.2 Mb	1.68	UPS26	
15q26.3	97,528,730	100,283,019	2.8 Mb	1.54	UPS19	
20q11.21-q11.23	29,297,070	35,397,310	6.1 Mb	1.61	UPS24	
20q13.11-q13.12	41,558,715	41,854,749	296 kb	1.64	UPS24	
20q13.12	44,725,701	45,001,847	276 kb	1.57	UPS24	
20q13.13-q13.2	46,690,203	49,571,845	2.9 Mb	1.57	UPS24	
22q11.21	19,063,227	19,835,558	772 kb	1.89	UPS11	
22q12.1-q12.2	27,556,536	28,013,310	456 kb	1.71	UPS24	
Xp11.22-p11.21 <sup>b</sup>	52,870,762	55,211,193	2.3 Mb	1.97	UPS10	

Overlapping regions with different levels of gain in the same patient are shown in bold.

<sup>a</sup>Regions are compatible with the UCSC human hg18 assembly.

<sup>b</sup>Region overlaps with copy number variable region.

region at the *TP53* tumor suppressor locus (tumor protein p53) (Table 2). According to the array CGH profiles, the gene was homozygously deleted in at least three (15%) and heterozygously deleted in four to five (20–25%) of the 20 cases (Table 4). In one of the cases (patient UPS27), the visual examination of the array CGH profile suggests a possible homozygous *TP53* deletion, although the log<sub>2</sub> ratio at the *TP53* locus (−0.78) was clearly lower than the log<sub>2</sub> ratio at the *RBI* locus (−1.57) in the same case.

Moreover, a region with recurrent homozygous deletions at 13q33.3-q34 contains the *ING1* tumor suppressor gene (inhibitor of growth family, member 1) which was homozygously deleted in two (10%) of the 20 cases (Table 2). Both homozygous *ING1* deletions occurred in cases with homozygous deletions of *RBI* (Table 4). In addition, *ING1* was heterozygously deleted in seven (35%) of the cases.

Furthermore, two distinct closely located high level gains, one containing *CDK4* (cyclin-

dependent kinase 4) (12q13.2-q14.1) and another containing *MDM2* [Mdm2 p53 binding protein homologue (mouse)] (12q15), were seen in one (5%) of the cases (Table 3). In many cases, the visual examination of the log<sub>2</sub> ratio profile suggested a gain of low log<sub>2</sub> ratio at *CDK4* locus (range: 0.27–0.43; gains with question marks in Table 4). Including these, gains of *CDK4* were seen altogether in 9 (45%) of the 20 of the cases. All of the gains were seen in cases with either homozygous deletion of *RBI* or *CDKN2A*, except for one case, which possessed a heterozygous deletion of *RBI*. *MDM2* was gained altogether in five (25%) of the cases (Table 4).

An about 2 Mb minimal overlapping region at 4q12 (54,317,543–56,092,179), gained in 10 (50%) of the 20 cases, contains *KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue), *PDGFRA* (platelet-derived growth factor receptor, alpha polypeptide), and *KDR* [kinase insert domain receptor (a type III

TABLE 4. Copy Number Changes of a Selected Group of Genes in 20 Patients with Undifferentiated Pleomorphic Sarcoma of Bone

Patient code	No. of changes	Tumour stage	RB1	CDKN2A	TP53	ING1	CDK4	MDM2	MYC	PDGFRA KIT KDR	PDGFA <sup>a</sup>	PDGFBB	VEGFA
UPSI	156	pri	hom. loss	—	het. loss	hom. loss	—	gain	gain	het. loss	—	—	gain
	167	rec	hom. loss	—	het. loss	hom. loss	—	—	gain	gain	gain	gain	gain
UPS4 <sup>b</sup>	167	rec	hom. loss	—	het. loss	hom. loss	—	gain	gain	het. loss	—	—	gain
UPSS	168	pri	het. loss.	hom. loss	—	hom. loss	—	gain	gain	gain	gain	gain	gain
UPS5	79	rec	het. loss.	hom. loss	—	hom. loss	—	gain (?)	gain	het. loss	—	—	gain
UPS6	68	pri	het. loss	hom. loss	het. loss	hom. loss	het. loss	—	gain	gain	gain	gain	—
UPS7 <sup>b</sup>	44	pri	het. loss	hom. loss	het. loss	hom. loss	het. loss	—	gain	gain	gain	—	—
UPS9	18	pri	hom. loss	—	—	—	—	—	—	—	—	—	—
UPS10	116	pri	het. loss	het. loss	hom. loss	hom. loss	hom. loss	—	gain	gain	gain	—	—
UPSI 1	113	pri	hom. loss	het. loss	—	—	—	—	—	—	—	—	—
UPSI 4	19	pri	hom. loss	—	—	het. loss	het. loss	—	gain (?)	—	—	—	—
UPSI 5	51	pri	het. loss	hom. loss	het. loss	het. loss	het. loss	—	gain	gain	gain	gain	—
UPSI 6	26	pri	hom. loss	het. loss	het. loss	het. loss	het. loss	—	gain	gain	gain	gain	—
UPSI 7	23	pri	het. loss	—	hom. loss	het. loss	het. loss	—	—	—	—	—	—
UPSI 9	77	pri	—	—	—	—	—	—	—	—	—	—	—
UPSI 20	2	pri	—	—	het. loss	het. loss	het. loss	—	—	—	—	—	—
UPSI 1	51	pri	gain	hom. loss	hom. loss	hom. loss	hom. loss	—	—	—	—	—	—
UPSI 22	61	pri	—	hom. loss	—	—	het. loss	het. loss	—	—	—	—	—
UPSI 24	37	pri	hom. loss	—	gain	—	—	—	—	—	—	—	—
UPSI 25	22	pri	—	hom. loss	gain	—	—	—	—	—	—	—	—
UPSI 26	86	pri	het. loss	hom. loss	—	het. loss	amp.	amp.	gain	gain	—	gain	—
UPSI 27	109	pri	hom. loss	gain	hom.(?) loss	hom. loss	—	—	gain	gain	—	—	—

pri, primary tumour; rec, local recurrence; hom., homozygous; het., heterozygous; amp., gain or amplification with  $\log_2$  ratio  $\geq 1.5$ . Gains with question marks: the gain was not detected by the algorithm but was seen in the visual examination ( $\log_2$  ratio range: 0.27–0.43).

<sup>a</sup>The copy number status was based on the flanking region (no probes at the locus).

<sup>b</sup>Hypomethylation of CDKN2A.

receptor tyrosine kinase); *VEGFR2*] with only a few other genes. In one of the cases (patient UPS19), the genes were included in a region of high level gain (Table 3). Moreover, the regions containing *PDGFA* and *PDGFB*, the genes encoding the ligands of *PDGFRA*, were gained in 11 (55%) and nine (45%) of the 20 cases, respectively (in patient UPS1 only in the samples from local recurrences) (Table 4). *PDGFA* was also located at a minimal overlapping region of frequent gains at 7p22.3-p22 (140,213–5,836,862). Furthermore, *VEGFA*, encoding the ligand of KDR was located at an overlapping region of frequent gains (9/20, 45%) at 6p21.1 (42,147,971–44,704,049) (Table 4). In patient UPS1, the gain, however, disappeared in the second local recurrence.

Moreover, an about 1 Mb minimal overlapping region at 8q24.21 (128,108,198–129,081,391), with gains in 11 (55%) of the cases, contains the *MYC* oncogene [v-myc myelocytomatosis viral oncogene homologue (avian)] (Table 4). Furthermore, a 4 Mb overlapping region at 12p12.1-p11.23 (23,065,748–27,464,900), gained in nine (45%) of the cases, harbors the *KRAS* oncogene (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue) (Table 4).

The case with only two copy number alterations (patient UPS20) showed a deletion inside the *CREB5* locus at 7p15.1 (28,644,720–28,747,059) and a gain at 4q35.2 (189,103,136–190,330,129). The latter one may also be a copy number variant instead of a true alteration.

#### Methylation of *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>*

Because of the frequent deletion of *CDKN2A* in our cases, methylation status of the two genes located at the *CDKN2A* locus, *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>*, was studied using methylation PCR. As array CGH is fundamentally not a quantitative method, and because of the possibility of small subclones not detected by array CGH, methylation PCR was performed on all of the cases, also on those showing a homozygous deletion of *CDKN2A* in array CGH. Hypermethylation of the promoters of both *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* was found in two (10%) of the 20 cases (Table 4). One of these cases showed a heterozygous deletion, and the other case a homozygous deletion of *CDKN2A* based on array CGH.

#### Immunohistochemistry of KIT, KDR (VEGFR2), PDGFR-Alpha, and PDGFR-Beta

Since the region containing *KDR*, *KIT*, and *PDGFRA* was affected by frequent gains, one

high level gain included, the receptors encoded by the genes (*KDR*, *KIT*, and *PDGF-alpha*) and, additionally the *PDGF-beta* receptor, were selected to be studied by immunohistochemistry. The expression was studied in the 14 primary tumor specimens obtained from the whole group of 20 patients included in this study. Immunohistochemistry results are shown in Table 5. Immunohistochemical positivity was found in eight cases for *KIT* (57%), five cases for *PDGFR-alpha* (36%), and four cases for *KDR* (*VEGFR2*) and *PDGFR-beta* (29%). No significant association was found between the immunohistochemistry results and the copy number status revealed by array CGH.

As previously described in another study (Niini et al., 2010), our system to score the immunostaining was based on a scale of positivity ranging from one plus to three plus, according to the percentage of positive cells. Cases with 5–10% positive cells were classified as +–, cases with 10–50% positive cells as ++–, and cases with more than 50% positive cells as +++. In this study, all positive UPS cases showed a score of ++– or +++, with at least 25–30% of positive cells. Positivity was evident as a membran/intracellular diffuse immunostaining in all cases.

#### Association of Inactivation of *RBI*, *CDKN2A*, and *TP53* with Survival

We studied the association of inactivation of *RBI*, *CDKN2A*, and *TP53* with overall and event-free survival using Kaplan-Meier analysis. Inactivation herein means homozygous deletion for all of the genes, and for *CDKN2A*, also the patient with both hypermethylation and heterozygous deletion of the gene is included. Because of the small number of patients, no reliable conclusions could be drawn from the Kaplan-Meier analysis. In addition, no other factors potentially affecting outcome were taken into account in the analysis. However, the Kaplan-Meier curve suggested a trend toward better overall and event-free survival in the patients with *RBI* deletion compared with the patients without the deletion (averages = 7.6 versus 4.6 years and 6.8 versus 4.1 years, respectively). The *CDKN2A* deletion did not show any association with either overall or event-free survival. Two of the three patients with homozygous *TP53* deletion were excluded from the analysis (one was lacking a primary tumor specimen and the other one showed metastasis at onset), and the patient with a potential

TABLE 5. Immunohistochemistry (IHC) Results and Corresponding Array CGH Results in 14 of the 20 Patients with Undifferentiated Pleomorphic Sarcoma of Bone

Patient code	KIT, PDGFRA, KDR array CGH	KIT IHC	PDGFR-alpha IHC	KDR (VEGF-R2) IHC	PDGFRB array CGH	PDGFR-beta IHC
<b>UPS1</b>						
primary	—	Positive	Positive	Positive	Het. loss	Negative
recurrence	Het. loss	NA	NA	NA	Het. loss	NA
recurrence	—	NA	NA	NA	Het. loss	NA
UPS4	Gain	Negative	Negative	Negative	—	Positive
UPSS5	Gain	Positive	Positive	Negative	Gain	Negative
UPS6	Gain	Positive	Negative	Positive	—	Positive
UPS7	—	NA	NA	NA	—	NA
UPS9	—	NA	NA	NA	—	NA
UPS10	—	NA	NA	NA	—	NA
UPS11	Gain	NA	NA	NA	—	NA
UPS14	—	Positive	Negative	Negative	—	Negative
UPS15	—	Positive	Negative	Negative	Het. loss	Positive
UPS16	Gain	Negative	Negative	Negative	—	Negative
UPS17	Het. loss	Positive	Positive	Positive	Het. loss	Positive
UPS19	Amp.	Positive	Positive	Positive	—	Negative
UPS20	—	Negative	Negative	Negative	—	Negative
UPS21	—	Negative	Negative	Negative	—	Negative
UPS22	Gain	Negative	Positive	Negative	—	Negative
UPS24	—	Negative	Negative	Negative	Gain	Negative
UPS25	Gain	Positive	Negative	Negative	—	Negative
UPS26	Gain	NA	NA	NA	Gain	NA
UPS27	Gain	NA	NA	NA	—	NA

Het., heterozygous; Amp., high level gain; NA, sections not available.

homozygous deletion (patient UPS27) was included in the group of no homozygous *TP53* deletion. The only patient with *TP53* deletion that was included in the analysis, showed very poor overall and event-free survival (both 0.5 years) compared with the patients without the deletion (averages = 6.2 and 5.6 years, respectively) and a trend toward poor survival was seen in the Kaplan-Meier analysis.

#### Differences in Copy Number Between UPSb and FSb

We created a classifier to detect genomic regions with the highest predictive value about patient's UPSb/FSb status (for details, see the Methods section). The selected regions predicted the tumor type correctly with 81% accuracy in cross-validation tests (expectation 65%) with an empirical *P* value of *P* = 0.02. This confirms that the detected copy number regions are significantly associated with the tumor type. Ninety-one regions were found, listed in Supporting Information Table 3. The regions clustered to 44 cytogenetic locations that are presented in Supporting Information Table 4. Note that due to relatively small sample size (*N* = 31), some of the regions identified by the classifier may be

false positives. The testing procedure identifies a set of regions that jointly provide optimal classification performance, taking potential correlations between chromosomal changes into account. Further tests with an independent validation data set are needed to confirm the disease association of each individual region. The 20 statistically most significant regions are presented in Table 6. These 20 regions were located at 1p36.33, 1p36.13, 3q13.31, 4q28.3, 4q34.1, 6p21.32-p21.31, 8q24.21, 8q24.23-q24.3, 11p11.12-q11, 13q14.2, 17q25.3, and 19p13.13-p13.11. The region at 13q14.2 contains the *RB1* tumor suppressor gene and one of the regions at 8q24.21 the *MYC* oncogene.

#### DISCUSSION

The copy number karyotypes in most of our UPSb cases were extremely complex. The average number of aberrations was 66, and 60% of the cases showed more than 50 aberrations, suggesting high chromosomal instability in the tumor. In a recent extensive study on soft tissue sarcomas, the authors identified three types of recurrent array CGH profiles: "simple amplicon profile" with almost exclusively amplifications, "arm profile" with losses and gains mainly

TABLE 6. The 20 Regions Most Significantly Differing in Copy Number Between Undifferentiated Pleomorphic Sarcoma of Bone (UPSB; 20 Cases) and Fibrosarcoma of Bone (FSB; 11 Cases)

Cytoband/s	Genomic location (bp) <sup>a</sup>	Gains				Losses			
		Frequency (%) <sup>b</sup>		Mean log <sub>2</sub> ratio <sup>c</sup>		Frequency (%) <sup>d</sup>		Mean log <sub>2</sub> ratio <sup>e</sup>	
		FSB	UPSB	FSB	UPSB	FSB	UPSB	FSB	UPSB
1p36.13	16,964,267–17,124,610	9	30	0.23	0.37	27	10	-0.32	-0.4
1p36.33	836,543–1,643,898	0	30	—	0.43	18	0	-0.23	—
3q13.31	117,076,648–117,365,745	0	10	—	0.33	45	15	-0.48	-0.26
4q28.3	134,706,213–135,674,944	27	10	0.4	0.26	9	20	-0.22	-0.48
4q34.1	172,551,944–173,666,131	36	10	0.42	0.26	9	35	-0.22	-0.39
4q34.1	173,676,705–173,800,904	27	10	0.4	0.26	18	30	-0.23	-0.45
6p21.32-p21.31	33,514,258–33,649,716	0	40	—	0.39	27	5	-0.23	-0.37
8p11.21-p11.1	41,370,754–43,315,378	0	40	—	0.5	45	15	-0.29	-0.31
8q24.21	128,892,972–130,319,496	27	55	0.24	0.46	27	0	-0.31	—
8q24.21	128,108,198–128,870,640	27	55	0.42	0.48	27	0	-0.31	—
8q24.23-q24.3	139,784,685–142,274,992	27	45	0.24	0.51	36	5	-0.31	-0.32
8q24.3	142,368,577–146,007,394	27	55	0.2	0.54	27	5	-0.31	-0.05
8q24.3	146,013,296–146,056,336	18	50	0.24	0.55	27	0	-0.31	—
8q24.3	146,070,958–146,128,758	18	45	0.24	0.55	27	0	-0.31	—
11p11.12-q11	51,251,929–54,807,338	18	10	0.44	0.37	0	20	—	-0.35
11q11	55,428,743–55,556,617	9	10	0.54	0.37	0	25	—	-0.52
13q14.2	47,756,488–47,954,006	0	5	—	0.5	36	75	-0.29	-0.65
17q25.3	77,442,683–77,564,174	27	50	0.52	0.56	0	0	—	—
19p13.11	19,496,164–19,612,554	18	45	0.43	0.41	0	0	—	—
19p13.13-p13.12	13,566,709–13,951,906	18	45	0.31	0.42	0	0	—	—

<sup>a</sup>Regions are compatible with the UCSC human hg18 assembly.

<sup>b</sup>Frequency of patients with gains overlapping the region.

<sup>c</sup>Average log<sub>2</sub> ratio of the whole region in patients with a gain.

<sup>d</sup>Frequency of patients with losses overlapping the region.

<sup>e</sup>Average log<sub>2</sub> ratio of the whole region in patients with a loss.

involving the full chromosome arm or entire chromosome, and “rearranged profile” characterized by high level of complexity (Chibon et al., 2010). Most of our cases with UPSB would fall into the group of “rearranged profile,” which mainly corresponded to undifferentiated sarcomas and leiomyosarcomas in the study concerned. The authors also established a gene expression signature that predicts metastatic outcome in non-translocation-related sarcomas and that is composed of genes which expression is related to genome complexity (Chibon et al., 2010). This agrees well with our finding of highly complex karyotypes in the UPSB cases and the aggressive character of the tumor.

The present results are concordant with our previous findings seen in UPSB using conventional CGH (Tarkkanen et al., 2006). The regions 9p22-pter, 13q21-q22, and 18q12-q22 with most frequent losses in the conventional CGH study were frequently lost also in array CGH and most of the regions with frequent gains in conventional CGH, especially 1q21-q23, 6p21.1, 7p12-pter, 7q22-q31, 8q21.3-qter, and 9q32-qter, were fre-

quently gained in the present study, too. Some regions showed clearly higher frequency of losses or gains in our array study, most probably reflecting the higher resolution of the array CGH technique leading to higher number of alterations revealed, especially small ones.

Compared to our cases with FSB, the copy number karyotypes were on average more complex in our cases with UPSB (averages, 43 versus 66, respectively). Despite similar copy number patterns, the classification analysis revealed high number of regions which were associated with the tumor type, clustering into 44 cytogenetic locations. To focus on the most reliable findings, we analyzed the 20 regions with the most frequent and significant association with the tumor type in cross-validation tests. These regions were located at 1p36.33, 1p36.13, 3q13.31, 4q28.3, 4q34.1, 6p21.32-p21.31, 8q24.21, 8q24.23-q24.3, 11p11.12-q11, 13q14.2, 17q25.3, 19p13.13-p13.11. Our results support the current opinion that UPSB and FSB are genetically highly similar but, however, constitute two distinct entities.

The copy number patterns seen in osteosarcoma using array CGH and conventional CGH are also very similar to the pattern seen in our UPSb cases (Squire et al., 2003; Lau et al., 2004; Kresse et al., 2009). The most striking differences between the tumors were found at 3p with more frequent losses, and at 1q21-q22 and 17p12-p11 with more frequent gains in osteosarcoma. In addition, at 17p12-p11 amplifications are frequently seen in osteosarcoma (Squire et al., 2003; Lau et al., 2004; Man et al., 2004; Atiye et al., 2005; Kresse et al., 2009), but not detected in our cases with UPSb. Differences were also seen at 12p and 22q11.21, which were more frequently gained in UPSb, as well as at 15q13-21 more frequently lost and at chromosome 21 more frequently gained in osteosarcoma.

Similar copy number patterns were also seen in conventional CGH studies on MFH of soft tissue, previously considered as one large entity but currently classified as different subtypes of undifferentiated pleomorphic sarcoma (Laramendy et al., 1997; Mairal et al., 1999; Simons et al., 2000; Weng et al., 2003). When comparing our results to a recent extensive study on soft-tissue sarcomas (Barretina et al., 2010), the most similar pattern of copy number alterations could be seen in myxofibrosarcoma (previously classified as MFH) and pleomorphic liposarcoma, especially concerning frequent losses at chromosomes 10 and 13 (particularly the *RB1* locus) and frequent gains at 5p and 7p.

Most of the copy number alterations seen in our cases probably just reflect high chromosomal instability and are insignificant for the pathogenesis of the tumor. However, many of the minimal overlapping regions of frequent gains and losses, the regions with high level gains, and, especially, the regions with recurrent homozygous losses are likely to contain genes that play a role in the development or progression of the tumor.

The most striking findings in our study were the high frequencies of homozygous losses with minimal overlapping regions at the *RB1* (13q14.2) and *CDKN2A* (9p21.3) loci (35% and 40%, respectively). In our previous study using conventional CGH, *RB1* was also located in the region with most common losses (42%) (Tarkkanen et al., 2006). We also reported one minimal overlapping region of frequent losses (23%) at 9p22-pter. Because of the low resolution of conventional CGH (one cytoband) we, however, hypothesize that the true overlapping region of the losses in the cases includes the *CDKN2A* locus at 9p21.3.

Using methylation PCR, we also detected hypermethylation of *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>*, the two genes residing at the *CDKN2A* locus, in two of the cases (10%), one with heterozygous deletion and the other with homozygous deletion of *CDKN2A*. The finding of hypermethylation in the case with homozygous deletion of the gene is presumably due to inability of the array CGH technique to distinguish between subclones of a tumor. Probably the tumor contains two subclones, one with homozygous deletion of *CDKN2A*, and another subclone with one allele deleted and another allele hypermethylated.

Previously, the locus causing diaphyseal medullary stenosis with MFH (DMS-MFH), a rare hereditary cancer syndrome, was mapped to a region containing *CDKN2A* using linkage analysis (Martignetti et al., 1999, 2000). Thirty-five percent of the individuals with DMS-MFH develop UPSb. Since the authors found loss of heterozygosity within the same region in sporadic UPSb specimens, they suggested a common genetic etiology underlying both hereditary and sporadic UPSb. They, however, excluded *CDKN2A* and *CDKN2B* as candidate tumor suppressor genes causing UPSb on the basis that no mutations were found in these genes in the individuals with DMS-MFH. Our results, however, suggest the deletion of *CDKN2A* to be one of the first events in the tumorigenesis of sporadic UPSb. The gene may, thus, play a role in the hereditary form of the tumor, too.

In our cases with FSb, homozygous deletions of *CDKN2A* were even more frequent (64%) than in the cases with UPSb (40%) (Niini et al., 2010). In FSb, hypermethylation of both *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* was seen in one case (10%), which suggests approximately the same frequency of *CDKN2A* methylation in FSb and UPSb. Homozygous deletion is, thus, the main mechanism of inactivation of the gene in both tumors. In osteosarcoma, the frequency of homozygous deletions of *CDKN2A* (~10%) is much lower than seen in our cases with UPSb or FSb (Miller et al., 1996; Patino-Garcia and Sierrasumaga, 1997; Nielsen et al., 1998; Wei et al., 1999; Tsuchiya et al., 2000).

Interestingly, the region with *RB1* was among the 20 regions that most significantly differed in copy number between FSb and UPSb. Moreover, no homozygous deletions of *RB1* were detected in our 11 cases with FSb. Only large heterozygous losses including the locus were seen in four cases showing a homozygous deletion of *CDKN2A*.

(Niini et al., 2010). Further studies with different techniques and larger number of cases will show whether *RB1* is inactivated only in UPSb and never in FSb, as our results suggest. If this hypothesis proves to be true, *RB1* might be used as a marker in the differential diagnosis between these two tumors. In osteosarcoma, alterations of *RB1*, including deletions, rearrangements and mutations, have been reported in about 70% of the cases [reviewed in (Ladanyi and Gorlick, 2000)].

In addition to deletions of *RB1* and *CDKN2A*, homozygous deletions with minimal overlapping region at the *TP53* locus (17p13.1) were found in three (15%) of our UPSb cases. Mutations of *TP53* have previously been reported in UPSb, in one study in 11% of the cases (Taubert et al., 1998), and in another, performed on elderly patients, in 22% of the cases (Kawaguchi et al., 2002). Homozygous deletion of *TP53* was also detected in one (9%) of our FSb cases (Niini et al., 2010). In osteosarcoma, alterations of *TP53*, including deletions and mutations, have been reported in up to 50% of the cases [(Tsuchiya et al., 2000; Patino-Garcia et al., 2003; Lopez-Guerrero et al., 2004), reviewed in (Ragland et al., 2002)].

Furthermore, one region with recurrent homozygous deletions (10%) in our UPSb cases contains the *ING1* tumor suppressor gene (13q34). The *ING1* proteins cooperate with *TP53* and are necessary for *TP53* to efficiently regulate cell cycle and apoptosis [(Zhu et al., 2009), reviewed in (Ythier et al., 2008)]. Down-regulation or loss of *ING1* expression has been reported in several carcinomas and hematological malignancies [reviewed in (Ythier et al., 2008)]. To our knowledge, inactivation or reduced expression of *ING1* has not been reported in sarcoma before. Except for cell cycle regulation and apoptosis, both the *TP53*- and the *ING1* proteins have been shown to be involved in DNA repair, which is critical for the maintenance of genomic integrity [reviewed in (Campos et al., 2004)]. Notably, all of the cases with homozygous deletion of either *TP53* or *ING1* showed a high number of copy number alterations (range: 68–156) and the average was much higher than seen in other patients (106 versus 56).

In addition to the homozygous deletions detected in the tumor suppressor genes regulating the G1/S cell cycle checkpoint, distinct high level gains containing the *MDM2* (12q14.3-q15) and *CDK4* genes (12q14) were detected simulta-

neously in one of our UPSb cases also showing a homozygous deletion of *CDKN2A*. Moreover, gains of *CDK4* were frequent (45%), most of them occurring simultaneously with inactivation of either *RB1* or *CDKN2A*. In many of the cases, the gain seemed to be present only in a subclone of the tumor. Based on these findings, we suggest that gain of *CDK4* is a common secondary event in UPSb tumors with either *RB1*- or *CDKN2A* inactivation. In a recent study on osteosarcoma, gains and high level amplifications of the 12q13-15 region were frequently seen especially in the parosteal type of the tumor (67% and 20%, respectively) (Mejia-Guerrero et al., 2010). The region was shown to contain two different amplicons that were frequently coamplified, one centered on *MDM2* and the other on *CDK4*, as was seen in our UPSb case.

Our results show inactivation either of *RB1*, *CDKN2A*, or *TP53* in 18 (90%) of the 20 UPSb cases. Taken into account the high frequency of inactivating mutations of both *RB1* and *TP53* in many tumors, including osteosarcoma, it is highly probable that either the *RB1*- or the *TP53* signaling pathway is disrupted also in the remaining two cases, another one of which showed a heterozygous deletion of *RB1*. On this basis, we suggest that either the *RB1* pathway or the *TP53* pathway, or both, is inactivated not only in the majority, but in all of the cases with UPSb, and that the disruption of the G1/S checkpoint regulation is a necessary event for the oncogenesis of the tumor. Bearing in mind that homozygous deletion of *CDKN2A* inactivates both the *RB1*- and the *TP53* pathway, and assuming that homozygous deletion of *ING1* inactivates the *TP53* pathway, our results suggested the disruption of both two G1/S checkpoint control pathways in 11 (55%) of the 20 cases. Since mutations and rearrangements not changing copy number cannot be detected by array CGH, and since micro RNA coding sequences were not explored in this study, our results do not rule out the possibility that disruption of both the *RB1*- and the *TP53* pathway is needed in the tumorigenesis of UPSb.

One of the regions with most frequent gains in our UPSb cases (55%) contains the *MYC* oncogene (8q24.21). This agrees well with our previous conventional CGH study on UPSb, in which we found frequent gains (35%) in the region, including two high level amplifications (Tarkkanen et al., 2006). We previously also found expression of the *MYC* protein in 33% of the tumors studied. Interestingly, the region

containing *MYC* was one of the 20 regions most significantly discriminating UPSb and FSb. In FSb, the region was gained only in 3 (27%) and lost in 3 (27%) of the 11 cases (in UPSb no losses were detected). It will be interesting to see whether *MYC* is overexpressed only in UPSb and not in FSb. In that case, *MYC* might be used as a marker in the differential diagnosis between these two tumors. Array studies on osteosarcoma show frequent gains at the region containing *MYC* (Squire et al., 2003; Lau et al., 2004; Kresse et al., 2009), and in one of the studies a narrow amplification centered to the *MYC* locus was detected (Squire et al., 2003). In osteosarcoma, gain of *MYC* is believed to be one of the later events occurring during the progression of the tumor, which would suggest this to be the case in UPSb, too (Ladanyi and Gorlick, 2000).

Another minimal overlapping region of frequent gains (50%), including one high level gain, was restricted to the region with the *PDGFRA*, *KIT*, and *KDR* (*VEGFR2*) genes (4q12). The 4q12 region was frequently gained also in our conventional CGH study on UPSb (Tarkkanen et al., 2006). Notably, the genes encoding the ligands of *PDGFRA* (*PDGFA* and *PDGFB*) and *KDR* (*VEGFA*) were also located in regions with frequent gains. Using immunohistochemistry, we detected expression of *KIT* in 57%, *PDGFR-alpha* in 36% and *KDR* and *PDGFR-beta* in 29% of the cases studied. *PDGFR-alpha*, *KIT*, and *KDR* were all found to be expressed in the case with high level gain at 4q12. In many cases with a lower level gain the immunohistochemistry result was negative. One reason could be that a low level gain does not necessarily cause a detectable change in the protein expression in immunohistochemistry. The protein expression seen in some cases with a heterozygous loss of the gene could be caused by activation of the remaining allele.

The 4q12 region containing *PDGFRA*, *KIT*, and *KDR* was frequently gained also in the FSb cases (64%), one high level gain included (Niini et al., 2010). Expression of *PDGFRA* and/or *PDGFRB* was detected in 46% of the primary tumors and in all four local recurrences, and expression of *KIT* in 40% of primary tumors and in one of the four local recurrences (Niini et al., 2010). Frequent gains at the 4q12 region, including high level amplifications, have been reported in osteosarcoma, too (Lau et al., 2004; Man et al., 2004). Involvement of PDGF receptors and their ligands have been suggested in sarcomas, as in

osteosarcoma and Ewing sarcoma [(Sulzbacher et al., 2003; Bozzi et al., 2007), reviewed in (DuBois and Demetri, 2007)], and the expression of *PDGF-AA* homodimer has been reported to associate with tumor progression in osteosarcoma (Sulzbacher et al., 2003). Expression of *VEGFA*, the ligand of *KDR*, has been shown to associate with poor prognosis in sarcomas, including osteosarcoma and Ewing sarcoma [(Lee et al., 1999; Kaya et al., 2000; Fuchs et al., 2004), reviewed in (DuBois and Demetri, 2007)], and in one study on nonmetastatic osteosarcoma, the expression of *KDR* was found in 67% of the patients (Lee et al., 1999). Expression of the normal *KIT* receptor has been reported in many neoplasms, including osteosarcoma and Ewing sarcoma (Sulzbacher et al., 2003; Tamborini et al., 2004; Burger et al., 2005; Entz-Werle et al., 2005; McIntyre et al., 2005; Bozzi et al., 2007).

We studied the association of the *RB1*, *CDKN2A*, and *TP53* deletions with survival using Kaplan-Meier analysis. Although the sample size for the *TP53* deletion was too small for reliable estimate, it is worth to note that two of the three patients with a homozygous *TP53* deletion died after short period of time after diagnosis (0.5 and 1.9 years) and one of them with only a recurrence specimen quite soon after relapse (1.2 years). Moreover, one of the patients had a metastasis already at the time of diagnosis. These facts suggest that the *TP53* deletion may be associated with poor prognosis in UPSb. This agrees with one previous report on the tumor, in which *TP53* mutations were suggested to associate with poor survival (Kawaguchi et al., 2002).

The Kaplan-Meier curve suggested a trend toward favorable clinical outcome in our patients with a homozygous *RB1* deletion. In osteosarcoma, in contrast, *RB1* alterations have been shown to associate with poor survival (Wadayama et al., 1994; Patino-Garcia et al., 2003). No association between inactivation of *CDKN2A* and survival was seen in our UPSb patients. This differs from what was seen in other sarcomas, including osteosarcoma (Benassi et al., 2001; Maitra et al., 2001; Oh et al., 2006) and Ewing sarcoma (Wei et al., 2000; Lopez-Guerrero et al., 2001; Huang et al., 2005; Honoki et al., 2007), in which alterations or loss of expression of *CDKN2A* have been shown to associate with poor prognosis. The trends of survival seen in our analyses need to be confirmed with a larger series of patients to take also other factors potentially influencing outcome, such as treatment or size of tumor, into account.

In conclusion, our analysis showed that the disruption of G1/S checkpoint regulation is crucial for the oncogenesis of UPSb. Homozygous deletions of either *RB1* or *CDKN2A* were detected in 80% of the cases, and recurrent homozygous deletions were also found at the *TP53* locus and at the region containing the *ING1* gene. In addition to the G1/S checkpoint genes, our results suggest, e.g., gains of *MYC*, *PDGFRA*, *KIT*, and *KDR* to be important in the pathogenesis of the tumor. Furthermore, clustering analysis revealed numerous regions differing in copy number between UPSb and FSb, the most significant of which included regions with *RB1* and *MYC*. Our results provide a good foundation for the search of markers for differential diagnosis between these two tumors.

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