

# Involvement of *MET/TWIST/APC* Combination or the Potential Role of Ossification Factors in Pediatric High-Grade Osteosarcoma Oncogenesis<sup>1</sup>

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## Abstract

Dysregulated cell growth or differentiation due to mis-expression of developmental critical factors seems to be a decisive event in oncogenesis. As osteosarcomas are histologically defined by malignant osteoblasts producing an osteoid component, we prospectively in pediatric osteosarcomas treated with OS94 protocol the genomic status of several genes implied in ossification processes. In 91 osteosarcoma cases, we focused on the analysis of the fibroblast growth factor receptors (*FGFRs*) *TWIST*, *APC*, and *MET* by allelotyping, real-time quantitative polymerase chain reaction, gene sequencing, and protein polymorphism study. Our study supports the frequent role of *TWIST*, *APC*, and *MET* as osteosarcoma markers (50%, 62%, and 50%, respectively). *TWIST* and *MET* were mainly found to be deleted, and no additional *APC* mutation was identified. Surprisingly, *FGFRs* are abnormal in only < 30%. Most of these factors and their abnormalities seem to be linked more or less to one clinical subgroup, but the most significant correlation is the link of *MET*, *TWIST*, and *APC* abnormalities to a worse outcome and their combination within abnormal tumors. A wider cohort is mandatory to define more robust molecular conclusions, but these results are to be considered as the beginning of a more accurate basis for diagnosis, in search of targeted therapies, and to further characterize prognostic markers.

*Neoplasia* (2007) 9, 678–688

**Keywords:** Pediatric osteosarcoma, osteogenesis, *TWIST*, *APC*, *MET*.

## Introduction

Osteogenesis is a complex and coordinated process involving several intricate signaling pathways, as well as many transcription factors. In the 10 past years, the understanding of skeletogenesis has made tremendous advances due to progress in genetic abnormality diagnoses, mice skeletogenesis studies, and cultured osteoblast or chondroblast models. Bone differentiation occurs both by intramembranous ossification, which concerns flat bones of the skull and the lateral part of clavicles, and by endochondral ossification, which concerns the entire appendicular and axial skeleton [1,2]. Intramembranous ossification is characterized initially by the local proliferation of mesenchymal cells, followed by osteoblast differentiation and local osteogenesis [3,4]. As a more complex process, endochondral ossification needs initially calcified cartilage cores as rigid scaffold, followed by a vascularization step of this cartilage structure and ended by a process of cartilage resorption concomitantly to bone production [5,6]. Skeletal elements derive mainly in the head from neural crests; in limbs, they originate from lateral plate mesoderm, whereas axial skeleton is formed from somites [2].

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<sup>†</sup>This work was supported specifically by funds from Projet Hospitalier de Recherche Clinique, Association pour la Recherche contre le Cancer, and the GIS-Maladies Rares Foundation, in addition to Institut National de la Sant e et de la Recherche M edicale, Centre National de la Recherche Scientifique, and the Medicine Faculty of Strasbourg.

Received 30 April 2007; Revised 29 June 2007; Accepted 3 July 2007.

Disorders caused by mutations in organogenesis are expected to affect skeletal elements in a generalized fashion, whereas mutations in early patterning genes might target only specific elements. Thus, mutations in *TWIST*, *MSX2*, and *FGFR1–FGFR3* genes alter more specifically the dynamics of the ossification of cranial sutures [3,4,7], whereas *SOX9*, bone morphogenetic proteins (BMPs), fibroblast growth factors (especially *FGF2*), *FGFR3*, *PTHrP* and its receptor (*PPR*), Indian HedgeHog (*IHH*), and vascular endothelial growth factor (*VEGF*) are some of the genes acting in multistep long bone development [8–12]. Factors such as *Cbfa1* or *Fgf2* are known to have a role in both processes [13]. *FGF2* has been described to upregulate *Twist* expression during the mesenchymal proliferation of intramembranous ossification [3,4], but it also interacts with *Hgf* and its receptor *Met*, promoting chondrocyte differentiation and seemingly increasing osteoblast proliferation rate through this pathway, during endochondral osteogenesis [14]. One of the other common markers is *Fgfr3*, acting at the osteogenic front of intramembranous process [4] and also playing a role in preformed cartilage [9]. The other example is the canonical *Wnt– $\beta$ -catenin–APC* pathway, which has been recently implicated in osteoblast lineage differentiation in both types of ossifications [15] and which contributes to repressing chondrocyte gene expression during skeletogenesis through *Twist* signaling [16]. Most of these factors are upregulated or downregulated at different check points of normal development to act through a signaling network of dorsal–ventral specification and mesoderm determination. For example, upregulation of *Twist* is involved in mesoderm cell proliferation, whereas downregulation of this protein implies osteoblastic differentiation [1–3].

These osteogenesis signaling pathways are especially active during embryogenesis and postnatal bone development, but their aberrant activation or suppression can lead also to development of cancers in differentiated tissues. For example, fibroblast growth factor receptors (FGFRs) [17–21], as well as *TWIST* [22–24] or canonical *Wnt* signaling that has been well studied in colon carcinoma multistep oncogenesis [25], had been frequently described as progression markers in several cancers. Some of these biomarkers, such as the *Twist* or the *Wnt– $\beta$ -catenin–APC* pathway, are also known to have a high rate of proliferative effect on cancer cells with inhibition of apoptotic signals [23,25]. In high-grade osteosarcoma characterized by the presence of malignant osteoblasts and the production of osteoid component, it could then be hypothesized that such markers could play a role in multistep tumorigenesis. As concerns specifically this malignant bone tumor, only a few of these markers, such as BMPs, *FGFR4*, *PTHrP*, *MET*, or *TWIST*, had been proven to be involved [20,21,26–30]. In addition, the recent development of biphosphonate, particularly targeting *PTHrP* in osteosarcomas and based on mice model and *in vitro* results [31–33], confirmed experimentally the interest of using new treatments targeting ossification functions in this cancer.

Therefore, understanding fundamental and developmental ossification processes will probably help to unravel

bone cancer oncogenesis as it will pinpoint pathways whose alterations contribute to malignant bone tumor onset and progression. Furthermore, identifying new marker genes with disordered expression in cancer might be a putative target for new therapeutic agents.

Then, to gain insight into ossification pathways in bone cancers, our work focused previously on three markers, namely, *APC*, *MET*, and *TWIST* genes and loci in a clinically well-known pediatric population of 91 high-grade osteosarcomas [29,30]. These previous studies showed striking results correlating most of these markers to a worse outcome or to poor response to chemotherapy. They also showed a significant correlation between deletion at *TWIST* and *APC* region rearrangements [30]. Based on these promising results and because of close interactions in both ossification processes, the status of *FGFR1–FGFR4* genes was also analyzed at DNA level. Then, the first step of this screening was performed by using microsatellites surrounding *MET*, *APC*, and *TWIST* genes to confirm the high percentage of these loci abnormalities found in our previous work. Complementary real-time quantitative polymerase chain reaction (QPCR) targeting *MET*, *TWIST*, and *FGFR* (*FGFR1–FGFR3*) genes was performed concomitantly to the sequencing of an *APC* mutation cluster region to understand the role and alterations of these targeted genes. As one specific single nucleotide polymorphism (SNP) of *FGFR4* gene (G→A), resulting in Gly388Arg substitution [19,20,27,34], was described as a marker of progression and invasion in several cancers, we investigated both the genotype status of our cohort on codon 388 and the copy number of *FGFR4* by QPCR. All these molecular results were finally correlated with clinical and histologic features characterizing this homogeneous pediatric population.

## Methods

### *Population, Tumor Banking, and DNA Isolation*

Ninety-one pediatric primary high-grade osteosarcomas, treated homogeneously with OS94 protocol and followed from November 1994 to June 2006, were included in this molecular study. The entire study was performed in accordance with the Declaration of Helsinki. The clinical and histologic characteristics of this population are detailed in Table 1, and most of them were used for statistical correlations. Most of these patient data had been already described in our previous works and recently updated [29,30].

Frozen tumor biopsies were collected from each child at diagnosis, before any treatment. They were stored at  $-80^{\circ}\text{C}$ . Because they were dedicated mainly for histologic diagnosis, only small tumor samples were obtained, allowing only assessable DNA extraction for these 91 patients. Control tissue was also obtained from peripheral blood and conserved on Whatman paper at room temperature. Tumor and blood paired DNA were purified as already described in previous publications [29,30,35]. Tumor and blood DNA concentrations, quantified by fluorometry, ranged from 50 to 400 ng/ $\mu\text{l}$  and from 1 to 10 ng/ $\mu\text{l}$ , respectively.

**Table 1.** Data for Patients (N = 91) Included in This Molecular Study.

Characteristics	Values
Age in years	
Mean (range)	12.9 (4–20)
Median	13
Sex	
Female	36
Male	55
Localization	
Femur	51
Tibia	17
Fibula	2
Talus	1
Humerus	12
Cubitus	2
Pelvis	3
Mandible	1
Metastasis at diagnosis	14
Histologic response	
GR	46
PR	41
Unknown	4
Histologic subtypes	
Osteoblastic	53
Chondroblastic	10
Fibroblastic	8
Others	4
Unknown	16
Survival	
Alive	69
Dead	22 (9 metastatic patients)
OS in months [median (range)]	77 (6–134)
EFS in months (median)	60

#### Allelotyping of 5q21, 7p21, and 7q31 Loci

These 91 paired normal and tumor DNA were investigated by allelotyping with two microsatellites surrounding the *APC* gene in 5q21 (*D5S346* and *D5S492*), four microsatellites surrounding the *TWIST* gene in 7p21 (centromeric *D7S2495* and *D7S2559*; telomeric *D7S1683* and *D7S2532*), and two microsatellites surrounding the *MET* gene in 7q31 (*D7S486* and *D7S677*) (see primer description at <http://www.ncbi.nlm.nih.gov/genemap99> and <http://www.gdb.org> Web sites). As previously described [29,30], DNA from both paired samples were amplified by fluorescent PCR, which was performed in an Omnigen Hybaid Thermocycler (Hybaid Ltd., Ashford, UK) using the following protocols: 5 minutes at 95°C, 35 cycles

of 1 minute at 95°C, 1 minute at 55°C (*D5S346*, *D5S492*, *D7S2495*, *D7S2559*, *D7S1683*, *D7S2532*, and *D7S677*) or 53°C (*D7S486*), and 1 minute at 72°C, followed by 5 minutes at 72°C. PCR products, after separation with denaturing urea gel on a sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden), were directly detected and quantified using an Alfin Fragment Analyzer software package (Amersham Pharmacia Biotech, Uppsala, Sweden). In the last samples, allelotyping was performed using the automated 3100 Applied Biosystems (Foster City, CA) combining microsatellites in multiplex PCRs. This highly reproducible and sensitive technique is devoted to detecting two genomic abnormalities: microsatellite instability (MSI) in case of the presence of additional peaks in tumor samples compared to blood DNA, and allelic imbalance (AI) characterized by the modification of the allele ratio in tumor DNA linked to chromosome instability. MSI is the witness of a mismatch repair defect or RER phenotype, whereas AI detects both locus deletion and locus amplification without precision on the real state of the locus. The intensity of AI is calculated as follows: AI percentage = absolute value[( $B_b/B_a$ ) - ( $T_b/T_a$ ) × 100/( $B_b/B_a$ )], where  $B_a$  and  $B_b$  represent the height of two alleles in control blood, and  $T_a$  and  $T_b$  represent the height of two alleles in the tumor. The significant cutoff of allelic variation was set above 20% [35]. Each result was confirmed by a duplicate PCR.

#### Real-Time QPCR of *MET*, *TWIST*, and *FGFR1*–*FGFR4*

Six genes (*MET*, *TWIST*, *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*) were quantified by QPCR using SYBR Green I dye (Roche Molecular Biochemicals, Mannheim, Germany) with Light Cycler technology (Roche Molecular Biochemicals). Primer sequences were chosen to target introns of each specific gene and are summarized in Table 2. The two internal control genes *DCK* and *APP*, localized at loci 4q13.3–21.1 and 21q21, respectively, were those used on *TWIST* in the same tumor collection in our previous work [30]. The thermal cycle conditions for the six target and two reference genes were exactly the same as previously described [30]. For each experiment, in addition to tumor DNA, a calibrator sample, a normal control DNA, and a negative control were amplified. QPCR was performed in duplicate for tumor samples. Result

**Table 2.** Real-Time QPCR Primers and Cutoffs Used for the Whole Population.

Genes	Forward Primers	Reverse Primers	Normal Cutoffs	Between
<i>MET</i>	5'-CATGCCGACAAGTGCAGTA-3'	5'-CCTAATCTGCAAAGGCCAAAG-3'	<i>MET/APP</i> <i>MET/DCK</i>	0.70 and 1.30 0.60 and 1.40
<i>TWIST</i>	5'-CCTCGGACAAGCTGAGCAAGAT-3'	5'-CCCCACGTCGCCGCGCCAGGAATG-3'	<i>TWIST/APP</i> <i>TWIST/DCK</i>	0.5 and 1.5 0.5 and 1.5
<i>FGFR1</i>	5'-CTTGCCATGGTTCTTCTCCCT-3'	5'-AGGGGCGAGGTCATCACTGC-3'	<i>FGFR1/APP</i> <i>FGFR1/DCK</i>	0.5 and 1.5 0.5 and 1.5
<i>FGFR2</i>	5'-CTTGCCCTTGAGAATGGTCGTC-3'	5'-AGTCTGGGGAAGCTGTAATCT-3'	<i>FGFR2/APP</i> <i>FGFR2/DCK</i>	0.6 and 1.4 0.5 and 1.5
<i>FGFR3</i>	5'-ACTCACTGGCGTTACTGACT-3'	5'-GAGGATGCCTGCATACACAC-3'	<i>FGFR3/APP</i> <i>FGFR3/DCK</i>	0.5 and 1.5 0.4 and 1.6
<i>FGFR4</i>	5'-TACAGCTTCTCCGTGTGTG-3'	5'-GCGTACAGGATGATGTCCGT-3'	<i>FGFR4/APP</i> <i>FGFR4/DCK</i>	0.5 and 1.5 0.6 and 1.4
<i>APP</i>	5'-TCAGGTTGACGCCGCTGT-3'	5'-ACCCAGAGGAGCGCCACCTG-3'		
<i>DCK</i>	5'-GCCGCCACAAGACTAAGGAAT-3'	5'-AGCTGCCCGTCTTCTCAGCCAGC-3'		

analyses, using the Light Cycler software version 3.5 (Roche Molecular Biochemicals), were based on the following relative calculation:

$$2^{-[(CP \text{ target} - CP \text{ reference})_{\text{sample}} - (CP \text{ target} - CP \text{ reference})_{\text{calibrator}}]}$$

CP is the number of cycles for which a fluorescent product could be measured just above a fluorescent noisy background. For each couple of target/reference genes, a cohort of 20 normal samples was investigated to determine the confidence interval and standard deviations of calculated ratios. Established normal cutoffs are detailed in Table 2.

#### PCR Restriction Fragment Length Polymorphism Analysis of FGFR4

This analysis is based on the detection of SNP variant in codon 388 of the *FGFR4* gene (located in exon 9). Exon 9 of *FGFR4* was amplified by PCR using the following primer set: forward primer 5'-GACCGCAGCAGCGCCGAGGCC-AG-3' and reverse primer 5'-AGAGGGAAGCGGGAGC-TTCTGC-3' [20]. The PCR protocol was as follows: a denaturation step of 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute, and a final extension step of 72°C for 10 minutes. Then, these PCR products were prepared for digestion with the restriction enzyme *Bst*NI (New England Biolaboratories, Beverly, MA) and separated with a 4% agarose gel containing ethidium bromide. Electrophoresis allowed us to distinguish between fragment lengths corresponding to Gly allele at codon 388 (109 bp) and fragment lengths corresponding to Arg allele (fragments of 80 and 29 bp). Direct sequencing, using a 3100 automated ABI sequencer (Applied Biosystems), was also performed to confirm the genotype on PCR products in a majority of cases.

#### APC Mutation Analysis

PCR amplification of the *APC* mutation cluster region (codons 1286–1513) was performed on tumor samples using five couples of sense and antisense primers, localized from codons 1200 to 1700 and summarized in Table 3. Purified PCR fragments were then directly sequenced in both directions using the BigDye sequencing kit and a 3100 automated ABI sequencer following the manufacturer's instructions (Applied Biosystems, Foster City, CA). In each mutated sample, a duplicate experiment will be required to confirm the mutation in two independent amplification products of the same sample.

**Table 3.** Primers for *APC* Gene Sequencing.

Forward Primers	Reverse Primers	Codons	Nucleotides
5'-GTAAGCCAGTCTTTGTGTC-3'	5'-CAGCTGATGACAAAGATGAT-3'	1125–1284	3378–3852
5'-AGACTTATTGTGTAGAAGATAC-3'	5'-ATGGTTCACCTCTGAACGGA-3'	1260–1410	3780–4230
5'-TCTGTCAGTTCACCTTGATAG-3'	5'-CAT TTGATTCTTTAGGCTGC-3'	1389–1547	4167–4641
5'-ACAGAAAGATGTGGAATTAAG-3'	5'-TTCTCCAGCAGCTAACTCAT-3'	1516–1673	4548–5019
5'-GCTACATCTCTAAGTGATCT-3'	5'-CTTATCATTGAAGTCCTTGG-3'	1654–1826	4962–5478

#### Statistical Analysis

The association with clinical and histologic parameters described in Table 1 was examined using Pearson's chi-square test. Survival curves and correlations with survival outcome for each molecular marker were performed according to Kaplan-Meier and used log-rank statistics. Statistical significance was determined in case of  $P \leq .05$ . All statistical tests were performed using SPSS 11.5 (SPSS, Inc., Chicago, IL).

#### Results

These 91 children and teenagers are representative of the whole population treated with OS94 protocol in terms of clinical characteristics and therapeutic results (Table 1) [36], allowing us to consider this tumor collection as one of the larger homogeneous pediatric osteosarcoma banks. With data updates, the overall survival (OS) in our population is 77%, and event-free survival (EFS) is 60.2% at 5 years. Furthermore, response to chemotherapy was statistically significant for OS ( $P = .004$ ) and EFS ( $P = .06$ ). Thirty-six of the 91 patients included in this molecular study relapsed, and their median time to relapse was 29 months after diagnosis (range, 2–102 months).

#### Ossification Markers Are Significantly Involved in Pediatric High-Grade Osteosarcomas

As previously published [29,30], loci 7q31, 7p21, and 5q21 are frequently rearranged by allelotyping. As there were only few discordant results between microsatellites of the same locus (1 of 91 for *MET* region; 3 of 76 for *TWIST* region; and 2 of 91 for *APC* locus), we could process the following analysis, and patients considered as informative for these loci either were heterozygous for both markers (*D7S486/D7S677*, *D7S2495/D7S2532*, and *D5S346/D5S492*) or presented with one altered or normal zygosity and with homozygosity at the second marker. In case of homozygous results for *D7S2495/D7S2532* surrounding the *TWIST* locus, we did consider, in addition, the results of two more distant microsatellites *D7S2559* and *D7S2532*. No MSI was observed, and AI was found in 52% (37 of 71) for locus 7q31 containing *MET*, in 46.9% (38 of 81) for locus 7p21 containing *TWIST*, and in 62.3% (48 of 77) for locus 5q21 containing *APC*. Looking closely at the gene status itself by QPCR, *MET* was normal in 50% (44 of 88), deleted in 41% (36 of 88), and amplified in 9% (8 of 88) (Table 4). Three patients were not included in our analysis because two tumor DNA could not be amplified with *MET* primers, and the other one was homozygous by allelotyping and had discordant results with the two control genes (normal with *MET/APP* and deleted with *MET/DCK*).

**Table 4.** Summary of *TWIST*, *MET*, and *FGFR1 – FGFR4* Real-Time QPCR Results; Fgfr4 Protein Polymorphism; and *APC* Locus Allelotyping (Using Microsatellites Targeting 5q21 and in Close Proximity to Each Extremity of the *APC* Gene).

	QPCR												Fgfr4 Protein Polymorphism				Microsatellite		QPCR	
	<i>TWIST</i>		<i>FGFR1</i>		<i>FGFR2</i>		<i>FGFR3</i>		<i>FGFR4</i>		Gly/Gly		Gly/Arg		Arg/Arg		<i>APC</i>		<i>MET</i>	
																	N	A	N	A
Total patients	50	36.5	13.5	80	11	9	2	84	9	7	70	6	24	25.3	20	38	62	50	41	9
Nonmetastatic	52	38	10	80	13	7	3	85	11	6	67	6	27	26	25	40	60	48	43	9
Metastatic at diagnosis	33	33.5	33.5	79	0	21	0	92	0	8	86	7	7	21	0	29	71	61	31	8
		<i>P</i> = .05		<i>P</i> = .38		<i>P</i> = .07		<i>P</i> = .73			<i>P</i> = .12			<i>P</i> = .02			<i>P</i> = .32		<i>P</i> = .44	
Metastatic, deceased	25	37.5	37.5	78	0	22	0	100	0	0	89	0	11	33	0	11	89	43	28.5	28.5
		<i>P</i> = .04		<i>P</i> = .46		<i>P</i> = .07		<i>P</i> = .22			<i>P</i> = .40			<i>P</i> = .2779			<i>P</i> = .07		<i>P</i> = .20	
No relapse	61	28	11	78	10	12	2	78	13	9	63	8	29	28	17	43	57	62	33	5
Relapse	32	52	17	82	12	6	3	94	4	2	83	11	4	21	25	29	71	30	55	15
		<i>P</i> = .03		<i>P</i> = .52		<i>P</i> = .7		<i>P</i> = .08			<i>P</i> = .05			<i>P</i> = .6			<i>P</i> = .15		<i>P</i> = .004	
GR	52	37	11	76	12	12	0	79	13	8	69	8	23	31	19	42	58	57	35	8
PR	44	39	17	85	11	4	5	89	6	5	71	5	24	19	23	29	71	50	38	12
		<i>P</i> = .67		<i>P</i> = .43		<i>P</i> = .29		<i>P</i> = .50			<i>P</i> = .91			<i>P</i> = .5			<i>P</i> = .26		<i>P</i> = .81	
Alive	59	30	11	79	11	10	2	81	12	7	67	7	26	23	20	44	56	56	38	6
Deceased	21	58	21	81	9.5	9.5	5	100	0	0	80	5	15	32	21	17	83	30	52	18
		<i>P</i> = .01		<i>P</i> = .92		<i>P</i> = .84		<i>P</i> = .07			<i>P</i> = .54			<i>P</i> = .72			<i>P</i> = .03		<i>P</i> = .03	
Osteoblastic	49	40	11	78	10	12	4	85	9	6	66	8	26	35	22	37	63	52	42	6
Fibroblastic	50	37	13	63	25	12	0	72	14	14	75	0	25	0	33	20	80	0	75	25
Chondroblastic	37	38	25	100	0	0	0	100	0	0	75	0	25	0	33	50	50	62	13	25
		<i>P</i> = .37		<i>P</i> = .23		<i>P</i> = .49		<i>P</i> = .40			<i>P</i> = .73			<i>P</i> = .66			<i>P</i> = .68		<i>P</i> = .39	

Results are presented as percentages. For each QPCR, normal (N), deleted (D), and amplified (A) subpopulations are listed in the overall cohort and in different clinical subgroups [considering metastatic disease, relapses, response to chemotherapy (GR = good responder; PR = poor responder), survival, and histologic subgroups]. The same statistical analysis was performed for *APC* gene results in normal and rearranged subpopulations and for Fgfr4 protein polymorphism, detecting the following SNP variants in codon 388: Gly/Gly, Gly/Arg, and Arg/Arg.

*TWIST*, as already published [30], was normal in 50% (37 of 74), deleted in 36.5% (27 of 74), and amplified in 13.5% (10 of 74) (Table 4). Results by real-time QPCR are matched with allelotyping in 58 of 71 informative patients for *MET*, and in 64 of 68 initially informative patients for *TWIST*. Deletion or amplification was detected in 4 of 4 discordant results with *TWIST* QPCR, and in 6 of 13 discordant results with *MET* QPCR, allowing us to conclude for these genes and primers that the QPCR technique, as previously described, is less sensitive than allelotyping.

No additional *APC* mutation in the mutation cluster region was detected in the normal population by allelotyping, and neither was there in the rearranged subgroup.

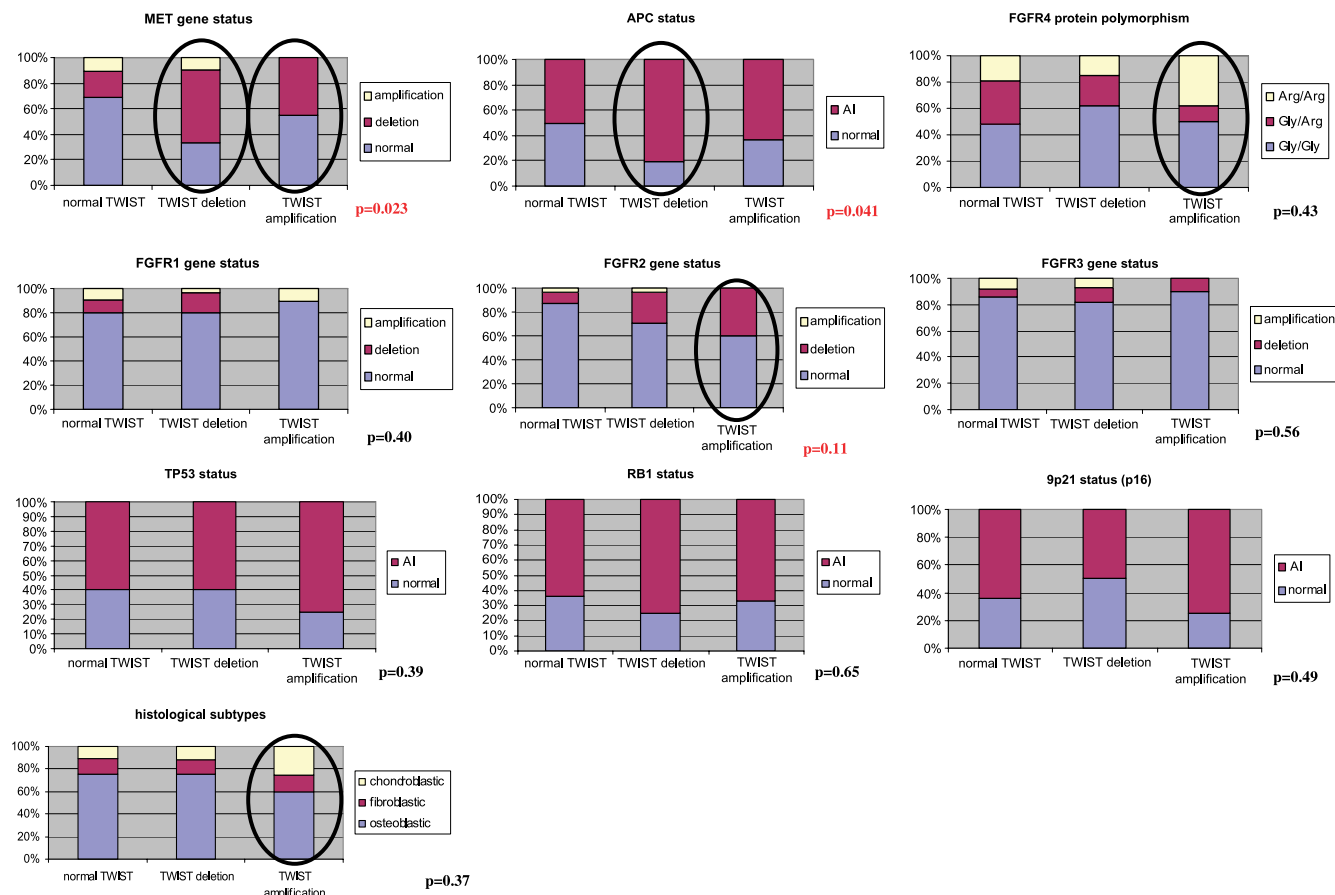
QPCR targeting *FGFR1–FGFR4* revealed surprisingly low rates of abnormalities (20%, 23%, 16%, and 30%, respectively). When rearranged, *FGFR1–FGFR3* are more often deleted (11%, 21%, and 9%, respectively), whereas *FGFR4* is slightly more often rearranged (30%) and mainly amplified (24%; 19 of 81 assessable tumors). Complementary analysis of SNP in *FGFR4* at codon 388 revealed Gly/Gly homozygosity in 54.7% tumors (41 of 75), Gly/Arg heterozygosity in 25.3% (19 of 75), and Arg/Arg homozygosity in 20% (15 of 75).

Statistical analyses combining the results of the different markers (Figures 1 and 2) showed significant links between *TWIST* deletion and loss of heterozygosity (LOH) at the

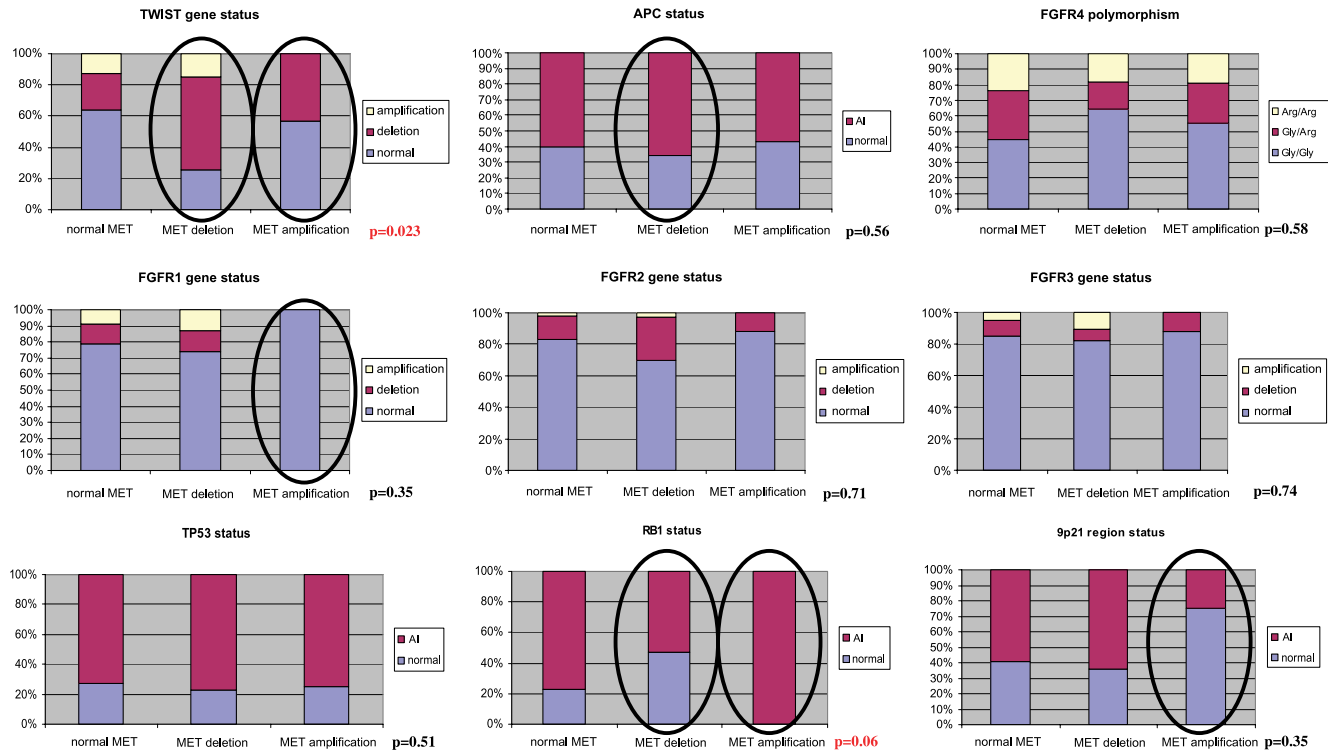
*APC* locus ( $P = .04$ ), as already published [30], between *TWIST* and *MET* deleted subsets ( $P = .023$ ), and a tendency between *TWIST* amplification and *FGFR2* deletion ( $P = .11$ ). A higher frequency of Arg allele in *Fgfr4* protein polymorphism was also shown with *TWIST* amplification. Surprisingly, when cross-table analyses were performed, no *MET* amplification was found to be associated with *FGFR1*, *FGFR2*, *FGFR3*, or *FGFR4* amplification, and the highest rate of normal *FGFR* populations, especially for *FGFR1*, was found in this specific *MET* subgroup. Furthermore, a *MET*-amplified subset was not correlated to *TWIST*, neither to the *PDGFRA* amplification previously described in our population [37]. *MET* amplification was found in only one case associated with *KIT* amplification (data previously published in Entz-Werle et al. [37]). *MET* deletion was associated with the highest percentage of *APC* (5q21) alterations. If we consider our previously published data on *P53*, *RB*, and *P16* loci [29], no real differences were observed for *TWIST* alterations, but in *MET* alterations, a link between a rearranged *RB1* and *MET* amplification ( $P = .06$ ), and between a normal 9p21 locus containing *P16* and this amplified subset was obvious.

*Do These Markers Characterize Clinical Subgroups?*

Our analysis also aimed to prospect the potential predictive value of each marker in different subgroups of pa-



**Figure 1.** Gene status within *TWIST* subgroups and statistical combinations. For each gene, we considered the proportion of normal and abnormal subpopulations in each *TWIST* subgroup (i.e., normal *TWIST*, *TWIST* deletion, and *TWIST* amplification patients). Black circles underline the main associations.



**Figure 2.** Gene status within MET subgroups. A combination of MET subpopulations (normal MET gene, MET deletion, and MET amplification) was made, and the percentages of normal and altered populations for each gene are given in each graph. Black circles underline the main associations.

tients, namely, localized tumor *versus* metastatic disease at diagnosis, no relapse *versus* relapse(s), the number of relapses, good responders (GR subgroup) *versus* poor responders (PR subgroup) to chemotherapy, alive *versus* deceased, histopathological subsets (osteoblastic, fibroblastic, and chondroblastic subtypes), and locations of primary tumor. The main cross-table statistical analyses are summarized in Table 4.

Metastatic osteosarcomas (14 children), especially in the smaller subgroup of deceased metastatic patients (9 children), are significantly characterized by a higher rate of *TWIST* abnormalities (67% and 75%,  $P = .05$  and  $.04$ , respectively) and a higher range of *TWIST* amplification. This subgroup also tends to be linked to *APC* rearrangement with 89% of 5q21 rearrangements. Neither *FGFR2* nor *FGFR3* was rearranged in deceased metastatic patients, and no *FGFR* deletions were observed. As concerns *FGFR4* polymorphism, the Arg/Arg 388 genotype is significantly absent in the metastatic subgroup ( $P = .02$ ).

Interestingly, significant statistical links were observed in relapsed tumors with *TWIST* abnormalities (69%, predominantly deleted;  $P = .03$ ) and *MET* abnormalities (70%, also predominantly deleted;  $P = .004$ ), but in a higher percentage of normal *FGFR3* (94%;  $P = .08$ ) and *FGFR4* (83%;  $P = .05$ ) genes. In case of abnormalities in these genes, they are mainly deleted, and no case of amplified *FGFR3* was found in the relapse population. The number of relapses (among 36 relapses, 4 patients relapsed twice) did not matter to molecular profiles. Considering the response to chemotherapy, no significant statistical data distinguishing the GR

subgroup from the PR subgroup were found. The only noticeable result is the increase in *TWIST* (56% vs 46%) and *APC* (71% vs 58%) abnormalities, normal *FGFR3* gene percentage (89% vs 79%), and absence of amplification cases at *FGFR2* in the GR subgroup. Comparing molecular profiles between still-alive patients and deceased children, statistically significant trends link a high frequency of *TWIST* and *MET* deletions ( $P = .01$  and  $P = .03$ ), a more frequent *APC* rearrangement ( $P = .03$ ), and a normal *FGFR3* gene ( $P = .07$ ) to the deceased population. Due to the small size of the fibroblastic and chondroblastic histologic subgroups, the following observations might not be statistically significant. However, we observed in chondroblastic osteosarcomas, known to be more aggressive tumors [36], that the *FGFR1* and *FGFR3* genes are perfectly normal, that there is no *FGFR2* amplification or *FGFR4* deletion, and that *MET* is mainly amplified. The fibroblastic subset had also noticeable higher frequencies of *FGFR1* and *FGFR3* gene abnormalities and *MET* gene abnormality. Finally, considering tumor locations, 12 humerus osteosarcomas are significantly characterized by *TWIST* deletion (seven deleted tumors of eight rearranged tumors) ( $P = .01$ ), whereas among 58 lower-limb osteosarcomas, 34 of 58 were normal, 16 of 58 had *TWIST* deletion, and 8 of 58 presented *TWIST* amplification.

#### The Prognostic Significance of Differentiating Factors

*APC*, *TWIST*, and *MET* remain as prognostic markers in pediatric osteosarcoma biopsies, as already published [29,30]. Indeed, *TWIST* deletion and amplification are significantly linked to a worse outcome ( $P = .026$  for EFS;  $P = .019$

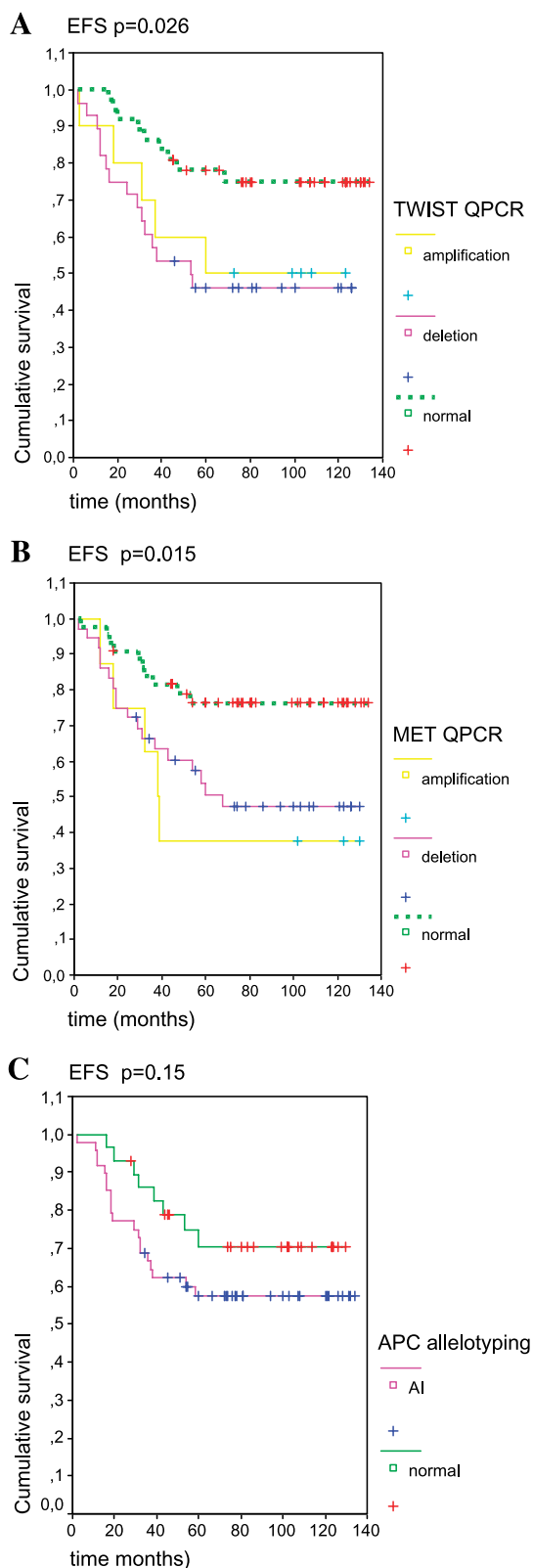
for OS) (Figures 3A and 4A). *MET* abnormalities are also significant witnesses of poor EFS, especially *MET* amplification ( $P = .015$ ; Figure 3B), and tend to be linked to a worse OS ( $P = .10$ ; Figure 4B), whereas *APC* rearrangement tends only to be correlated to a worse EFS ( $P = .15$ ; Figure 3C) and is correlated to a worse OS ( $P = .035$ ; Figure 4C). No significant correlation was established for *FGFR* genes.

## Discussion

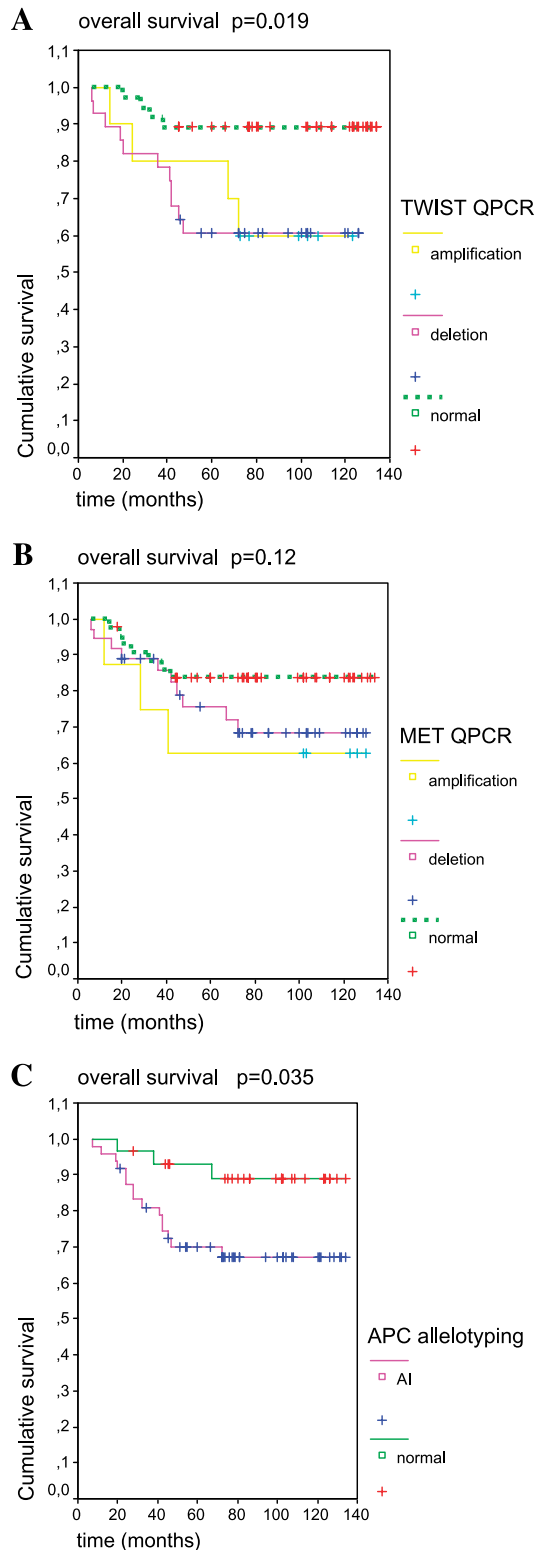
### Ossification Factors Are Involved in Pediatric High-Grade Osteosarcomas

That rearrangements in *APC*, *TWIST*, and *MET* genes are detected in around half of the pediatric osteosarcoma population strongly point toward a role for these ossification factors in pediatric osteosarcoma oncogenesis. Unlike other cancers that are mainly described as overexpressing c-Met or Twist proteins [22–24,38–41], these genes are mostly deleted in pediatric osteosarcomas (three quarters of abnormal tumors for *TWIST* and *MET* presented deletions). Sixty-two percent of *D5S346/D5S492* AI could be also considered as LOH because we used *D5S346* to target the *APC* locus, which is relevant to diagnosing *APC* LOH in colon cancers [42]. As described above, the decrease in *TWIST*, *APC*, and *MET* expressions is involved in normal bone production [1,3,4,15,16,43]. Such observation could explain why gene deletions in osteosarcoma are detected because undifferentiated and malignant osteoblasts usually produce an osteoid or a bone component. A significant combination of these deletions, linking *TWIST* and *MET* deletions and *APC* alterations, was observed. This subgroup of *TWIST* or *MET* deletion seems to need additional deleted genes to deregulate differentiation to obtain a malignant osteoblast status. However, *TWIST* or *MET* amplification, described as a proliferative cancer factor [22–24], does not need the upregulation of another biomarker to allow osteoblast proliferation (Figures 1 and 2). The links between this combination of *TWIST*, *MET*, and *APC* genes seems to be widely explained through interactions during normal ossification [15,16,43]. In addition, the decrease in *APC* and *MET* expression is specifically dedicated to endochondral ossification to decrease chondrocyte component and explains the slight increase in *MET*-amplified tumors in our chondroblastic subpopulation [4,43]. Furthermore, the higher number of *TWIST*-amplified chondroblastic tumors might be also explained by the role of *TWIST*-increased expression during the intramembranous ossification process [3,4]. All these rearrangements suggest a greater role in “osteogenic” profile rather than a role in progression phenotype, as described by Patane et al. [44] for c-Met.

In contrast, *FGFR1–FGFR4*, although documented as implied in bone growth and/or differentiation, appear only to be rearranged in about 16% to 30% of osteosarcomas. Both deletion and amplification are found in these pediatric osteosarcomas (*FGFR1*, *FGFR2*, and *FGFR3* are mainly deleted, and *FGFR4* is amplified) and have also been described in several adult cancers. For example, Fgfr3 protein is



**Figure 3.** EFS correlations considering *TWIST* (A), *MET* (B), and *APC* (C) molecular abnormalities. A statistical correlation for *TWIST* and *MET* analyses is found between a worse outcome and rearranged populations, and only a tendency for *APC* study.



**Figure 4.** OS correlations for TWIST (A), MET (B), and APC (C) molecular abnormalities. A significant correlation is validated between TWIST and APC alterations and a worse OS, and only a tendency is obtained for both MET deletion and amplification.

overexpressed in hepatocarcinoma [45], whereas the expression is lost in higher grades and stages of bladder cancers [46]. Loss of *FGFR2* has already been described in osteosarcomas [21] and brain tumors [47], whereas Fgfr4 protein has

been mainly overexpressed in adult cancers [48]. As less rearranged genes, *FGFRs* do not appear as major specific markers in this pathology. However, *FGFRs*, which are involved in late bone differentiation [1,2,4], seem to modulate osteosarcoma oncogenesis, in addition to the main combination of *MET*, *TWIST*, and *APC* alterations. Indeed, *FGFR2* deletion is significantly associated with a *TWIST*-amplified subgroup, whereas the absence of alterations in *FGFR1–FGFR3* is frequently observed in the *MET*-amplified subset (Figures 1 and 2). As described in calvarial formation, Twist upregulation and Fgfr2 downregulation are concomitantly observed [1,4–6]. Considering the *FGFR* results in Table 4, no alterations could be associated with an aggressive subtype of pediatric osteosarcoma. From the point of view of the osteogenesis process, no statistically significant correlations for *FGFR* genes and histologic subgroups could be found. However, hyperactivation of these receptors was usually involved during normal bone production of endochondral ossification and fusion of cranial sutures [1,2], and such observation could explain the absence of any *FGFR1–FGFR3* amplifications in chondroblastic histologic subtypes (Table 4).

#### Ossification Markers, Progression Factors, and/or Survival Biomarkers

Multiple progression factors, which have been previously described in other cancers, were analyzed in this study [19, 20,22,23] as *TWIST*, *MET*, and *APC* genes or Fgfr4 protein polymorphism. Most of them are frequently altered in our homogeneously treated population of pediatric osteosarcomas. Concerning *FGFR4* genotyping, different percentages (compared to the study of Morimoto et al. [27] on bone tumors and soft-tissue sarcomas) were shown. In our population (Table 4), most of the tumors (54.7%) are characterized by a Gly/Gly *FGFR4* genotype, whereas in the bone tumor subgroup of Morimoto et al., which combined pediatric and adult patients and multiple types of bone malignancies, most of them showed an Arg/Gly *FGFR4* genotype (49%). If we compare our results to the published control series by Bange et al. [20], Morimoto et al. [27], or Wang et al. [48], our cohort seems to be quite different, with a higher frequency of Arg/Arg *FGFR4* genotype (19.7%), usually linked to an increased mobility in mammary tumor cell lines [20], and a lower subgroup of Arg/Gly *FGFR4* genotype (25.8%), usually linked to early metastasis in colon cancers [24].

Looking closely at our statistical analyses, significant gene correlations seem to characterize patients who were diagnosed initially with a more aggressive disease (metastatic patients) and those who progressed after diagnosis (tumor relapses). First of all, one marker is common to both patient subgroups: *TWIST* alteration. We observed predominantly deletions in relapses and deletion or amplification in metastatic populations. Considering our results, which are summarized in Table 4, the molecular profile of an initial metastatic osteosarcoma could be an abnormal *TWIST* gene with a normal *FGFR2* gene ( $P = .07$ ) and no Arg/Arg *FGFR4* genotype ( $P = .02$ ), modulated toward a worse outcome by an *APC*

alteration ( $P = .07$ ). During relapse, another molecular profile combining predominant *TWIST* ( $P = .03$ ) and *MET* deletions ( $P = .004$ ) and normal *FGFR3* and *FGFR4* genes ( $P = .08$  and  $P = .05$ , respectively) seems to be highlighted. Among these markers, abnormal *APC* and *MET* genes are probably the differential factor witnesses of initial invasiveness versus later progression. Surprisingly, but concordant with previous conclusions [27], *FGFR4* genotype at codon 388 had no impact on late progression but seemed to have a role in initial metastatic disease.

The major altered genes *TWIST*, *MET*, and *APC* and their combination are also found to be significantly linked to a worse outcome, as exemplified by statistics in alive and deceased subpopulations and survival analyses. Indeed, some of these factors are involved in patient outcome and could be considered as prognostic markers. *TWIST* deletions and amplifications seem to have the same worst outcome consequence, whereas *MET*-amplified tumors are doing worse than those that are deleted. In fact, patients characterized by a *MET*-amplified tumor would only have a 5-year EFS of 28% and a 5-year OS of 53% (Figures 3B and 4B). This last statement lets us hypothesize that detection of *MET* abnormalities could also subgrade the whole worse outcome group and select the worst subset of patients. The major difficulty in both amplified populations is the low number of tumors, which does not allow us to complete significant analyses. However, these altered subpopulations for *TWIST* and *MET* genes are characterized by differential molecular profiles, as shown in Figures 1 and 2. *TWIST* deletion seems to be associated with *MET* deletion and *APC* alteration, whereas *TWIST* amplification is linked to *FGFR2* deletion and Arg/Arg *Fgfr4* protein polymorphism. Conversely, *MET* amplification seems to be associated with normal *FGFRs* and *RB1* and with an increase in the number of 9p21 locus alteration. No clinical factors seem to be associated with *MET* amplification, except for increase in chondroblastic histology, which is usually linked to a worse outcome [36]. For *FGFR* genes, to date, no evident links between outcome and those genes have been described because of small abnormal populations. A study on a larger population would probably be able to define their precise role in osteosarcoma survival.

#### Could These Factors Be Potential Therapeutic Targets and/or Used as Routine Biomarkers?

Furthermore, *MET* and *FGFR* code for tyrosine kinase receptors, which are therapeutic targets of recently developed drugs. Most of these recent treatments inhibit multiple receptors such as SU06668 or PTK787, targeting, namely, VEGFR, PDGFR, FGFR, or *MET* [49,50]. Our findings of multiple DNA amplifications, leading probably to hyperexpression of wild-type receptors, could promote the use of these new therapeutic strategies. Even though *FGFR* rearrangements are less frequent and amplification is rarer than deletion, all these findings could help us to refine accurately the subgroup of patients who are able to respond to these new drugs, targeting the same signaling pathways at multiple levels. Furthermore, the rapid, sensitive, and useful

techniques described in this study could be used routinely to analyze the status of these genes as surrogate biomarkers at diagnosis on biopsies in pediatric osteosarcomas.

Another therapeutic involvement for these analyses could be an ability to predict a worse clinical outcome at diagnosis, allowing us to stratify the high-risk group of pediatric osteosarcoma cases that should be treated by new strategies to intensify present treatment. Thus, defining molecular profiles of patients would be a helpful tool to determine therapeutic strategies that fit each critical patient and to grade osteosarcomas.

To summarize, our findings further expand knowledge on the role of ossification factor in homogeneous pediatric osteosarcomas. Much more than being involved in the oncogenesis of pediatric osteosarcomas, these factors are also new witnesses to tumor progression and invasiveness and to a worse outcome group. Their combination seems to be the key to further understanding osteosarcoma oncogenesis. To confirm these interesting data, this molecular analysis should be performed on a larger prospective group to obtain significantly statistical analyses in *FGFR* gene studies.

#### Acknowledgements

We are thankful to the French working group of clinicians and pathologists involved in pediatric bone tumors who allowed us to collect whole blood and tumor samples in each French center.

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