Oncogene (2010) 29, 3827–3834
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www.nature.com/onc

ORIGINAL ARTICLE

Genetic models of osteochondroma onset and neoplastic progression: evidence for mechanisms alternative to *EXT* genes inactivation

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Osteochondroma, the most common benign bone tumor, may occur as a sporadic lesion or as multiple neoplasms in the context of multiple osteochondromas syndrome. The most severe complication is malignant transformation into peripheral secondary chondrosarcoma. Although both benign conditions have been linked to defects in *EXT1* or *EXT2* genes, contradictory reports are present in the literature regarding the requirement of their biallelic inactivation for osteochondroma development. A major limitation of these studies is represented by the small number of samples available for the screening. Taking advantage of a large series of tissues, our aim was to contribute to the definition of a genetic model for osteochondromas onset and transformation. *EXT* genes point mutations and big deletions were analyzed in 64 tissue samples. A double hit was found in 5 out of 35 hereditary cases, 6 out of 16 chondrosarcomas and 2 recurrences; none of the 11 sporadic osteochondromas showed two somatic mutations. Our results clearly indicate that, in most cases, biallelic inactivation of *EXT* genes does not account for osteochondromas formation; this mechanism should be regarded as a common feature for hereditary osteochondromas transformation and as an event that occurs later in tumor progression of solitary cases. These findings suggest that mechanisms alternative to *EXT* genetic alteration likely have a role in osteochondromas pathogenesis.
Oncogene (2010) **29,** 3827–3834; doi:10.1038/onc.2010.135; published online 26 April 2010

Keywords: solitary osteochondroma; multiple osteochondroma; chondrosarcoma; *EXT* genes; tumor suppressor; mutation screening

Introduction

Osteochondroma or osteocartilaginous exostosis is a cartilage-capped benign bony neoplasm, which mainly arises in the juxta-epiphyseal regions of long bones of affected individuals (Solomon, 1964). Osteochondromas may occur as non-familial, sporadic lesions (solitary osteochondroma, SO) or as multiple neoplasms in the context of multiple osteochondroma hereditary syndrome (MO (MIM 133700, 133701)).

SO are estimated to affect approximately 1–2% of the general population; they typically develop from early childhood to the end of adolescence and represent approximately 85% of all osteochondromas. Factors, such as radiation, iterated trauma, retinoid therapy and genetic defects have been suggested to contribute to the development of SO (Essadki *et al*., 2000).

MO is the most common genetic skeletal dysplasia, with an estimated prevalence of 1/50 000 and an autosomal dominant inheritance (Schmale *et al*., 1994). Patients with MO show multiple benign osteochondromas that usually develop gradually until puberty and are characterized by the same anatomical and histological appearance of SO (Porter and Simpson, 1999). MO is a clinically heterogeneous disease with a great variability in size and number of osteochondromas; complications include different grade of pain, skeletal deformities, range of motion limitations, fractures and nerve compression (Hennekam, 1991). The most severe complication of osteochondroma is the malignant transformation into secondary peripheral chondrosarcoma (PCS), which develops within the cartilage cap of a pre-existing osteochondroma (see Springfield *et al*., 1996 for a review). Malignant transformation of SO is rare (<1%), whereas it is estimated to occur in 1–5% of cases of MO (Hennekam, 1991; Schmale *et al*., 1994; Wicklund *et al*., 1995).

Both conditions have been linked to genetic alterations in two main loci, *EXT1* on chromosome 8q24.11–q24.13 (MIM 608177) and *EXT2* on chromosome 11p12-p11 (MIM 608210) (Wu *et al*., 1994; Wuyts *et al*., 1995; Ludecke *et al*., 1997). Exostosin-1 (EXT1) and exostosin-2 (EXT2) belong to the larger *EXT* family of homologous genes; their products, EXT1 and EXT2, are ubiquitously expressed glycoproteins with glycosyltransferase activity involved in the GAG(HS) chain elongation of heparan sulfate proteoglycans (HSPGs) (McCormick *et al*., 1998). Extracellular matrix heparan sulfate proteoglycans have a fundamental role during cartilage development and skeletal growth by regulating

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Received 18 December 2009; revised 9 March 2010; accepted 28 March 2010; published online 26 April 2010

gene expression, presentation of growth factors, establishment of morphogen gradients and modulation of blood homeostasis (Farach-Carson *et al*., 2005).

The most common class of mutations in *EXT1* and *EXT2* consist of inactivating mutations (frame shift, nonsense and splice-site) that represent 77–80% of the MO causing mutations (Wuyts and Van Hul, 2000; Jennes *et al*., 2009). These alterations determine a premature termination of encoded EXT proteins, inducing a rapid inactivation and degradation with a final result of a nearly complete loss of their function (Wuyts *et al*., 1998; Cheung *et al*., 2001). Missense mutations are less frequent and cluster mainly in residues that are crucial for proper functioning of the EXT proteins (McCormick *et al*., 2000; Cheung *et al*., 2001).

In the past, osteochondroma has been regarded as a perversion in the direction of normal bone growth resulting from aberrant epiphyseal development (Huvos, 1991). However, later studies detected cytogenetic aberrations suggesting that loss or mutation of *EXT1* and *EXT2* are important in the pathogenesis of sporadic as well as hereditary osteochondromas, thus indicating the neoplastic origin of these lesions (Mertens *et al*., 1994; Bridge *et al*., 1998). In 1995, two separated studies found loss of heterozygosity for markers linked to *EXT1* and *EXT2*, and suggested that osteochondromas development follows the Knudson's two-hit model for tumor suppressor gene inactivation (Hecht *et al*., 1995; Raskind *et al*., 1995). In this model, a germ line mutation (first hit) causes the predisposition for a disease and the somatic inactivation of the remaining wild-type copy of the gene (second hit) leads to the abnormal growth (Knudson, 1971).

Currently, contradictory reports are present in the literature regarding the requirement of a complete inactivation of *EXT* genes for osteochondroma development. In 1999, Bovée *et al*. found loss of heterozygosity at the *EXT1* locus in three of eight SO and two of six MO cases (Bovée *et al*., 1999a, b); more recently, the same group reported of *EXT1* homozygous deletions in seven of eight SO by array-CGH and multiplex ligation-dependent probe amplification (MLPA) analysis (Hameetman *et al*., 2007). In a study performed on 12 MO and 4 SO, Hall *et al*. found only one solitary case in which two somatic mutations were present, concluding that available data indeed provided limited support for the two-hit hypothesis (Hall *et al*., 2002).

To address this, we analyzed the mutational status of *EXT1*/*EXT2* in a series of MO, SO and PCS samples larger than all case studies reported thus far; we report germline and somatic mutational analysis of 11 sporadic and 35 hereditary osteochondromas, along with 18 PCS including 2 tumor recurrences. Our findings indicate that, in most cases, *EXT* genes biallelic inactivation does not account for osteochondromas onset, paving the way to future studies aimed at identifying alternative pathogenetic mechanisms and their contribution.

Results

Selection of tissue samples

Patients were selected as described in the Subjects and methods section. A total of 64 samples from 49 patients were analyzed; the type and location of each tumor sample along with patients clinical data are summarized in Table 1.

Mutational and copy number analysis of EXT1/EXT2 *genes in sporadic osteochondroma patients*

As described in the 'Subjects and methods' section, we used a denaturing high-performance liquid chromatography (DHPLC)/direct sequencing and MLPA protocol that allows detection of mutations located in *EXT1*/*EXT2* coding regions and exon-intron junctions.

Our mutational screening did not reveal any mutations in the osteochondroma samples from five SO patients (24, 26, 28, 32 and 33), while in the other six cases we found big deletions (patients 27, 29, 30, 31), one small deletion (patients 25) and one small insertion (patient 34) on *EXT1* (Table 2). No samples with two mutational hits were observed. Similarly, the DHPLC analysis did not detect point mutations in the five chondrosarcoma resections obtained from SO patients.

Two tumor samples (FEXST238 and CST3) showed the loss of one copy of *EXT1* and *EXT2* respectively by MLPA screening; in the recurrence of this latter sample (FEXST249), loss of both *EXT2* alleles was found (Table 2). All hemizygous and homozygous deletions were confirmed by quantitative real-time PCR (Q–PCR).

The same MLPA and Q–PCR normalized values were used both for tissue and germline DNA samples to define hemizygous or homozygous losses, similarly to previous studies (Hameetman *et al*., 2007). In fact, we reckon that sensitivity of these techniques, along with high tissue specificity offered by microdissection procedure, may allow us to detect similar values for germline or somatic genetic losses (Traeger-Synodinos, 2006; Hameetman *et al*., 2007).

Mutational and copy number analysis of EXT1/EXT2 *genes in MO patients*

Table 3 describes the results of our mutational screening of 35 MO tissue samples, obtained from 23 patients, and 11 PCSs along with 1 tumor recurrence from 11 subjects; germline mutations detected in related constitutional DNA samples are also listed.

In all MO and chondrosarcoma samples, the germline mutation was confirmed; all these variants have been included in the online MOs Mutation Database (Jennes *et al*., 2009). 30 out of 35 MO cases showed only this heterozygous mutation, while in five tissues a double hit was found. In particular, in two MO samples (FEXST53 and FEXST218B) the germline frameshift mutation was found in homozygosity, while two resections (FEXST137 and FEXST218A) showed an additional somatic mutation (frameshift or nonsense mutation respectively) on the same gene affected by the germline alteration. MLPA analysis of these MO

Table 1 Patients selected for the study and related tumor samples analyzed

Patient	*Sample ID*	*Diagnosis (MO/SO/PCS)*	*Age at surgery (years)*	*Sex*	*Tumor location (right/left)*
1	FEXST160	MO	40	M	Iliac crest
2	FEXST32	MO	23	M	Femur
3	FEXST196	MO	10	F	Tibia
4	FEXST52	MO	25	M	Proximal femur
5	FEXST53	MO	6	M	Scapula
5	FEXST284A	MO	10	M	Femur (right)
5	FEXST284B	MO	10	M	Distal femur (right)
5	FEXST284C	MO	10	M	Distal tibia (left)
5	FEXST284D	MO	10	M	Proximal tibia (left)
6	FEXST54	MO	43	F	Femur
7	FEXST55	MO	8	M	Radius
8	FEXST80	MO	6	M	Forearm
9	FEXST79	MO	10	M	Tibia
10	FEXST137	MO	9	M	Distal femur
11	FEXST119t	MO	35	M	Tibia
11	FEXST119c	PCS gr.I (MO)[a]	35	M	Tibia
12	FEXST100	MO	15	M	Proximal humerus (left)
12	FEXST166	MO	16	M	Distal ulna (left)
13	FEXST234	MO	12	F	Fibula
14	FEXST130	MO	13	M	–
15	FEXST136	MO	17	F	–
16	FEXST208	MO	9	F	Radius
17	FEXST123	MO	9	M	Tibia
17	FEXST218A	MO	9	M	Proximal humerus (right)
17	FEXST218B	MO	9	M	Distal ulna (right)
17	FEXST304A	MO	12	M	Femur (right)
17	FEXST304B	MO	12	M	Tibia (right)
17	FEXST304C	MO	12	M	Tibia (left)
18	FEXST341	MO	11	F	Tibia
19	FEXST275A	MO	10	F	Scapula (left)
19	FEXST275B	MO	10	F	Radial head (left)
20	FEXST286A	MO	14	M	Tibia (right)
20	FEXST286B	MO	14	M	Tibia (left)
21	FEXST109b	MO	20	M	Fibula (right)
22	FEXST112	MO	17	M	Femur
23	FEXST122	MO	14	M	Femur
24	FEXST69	SO	10	M	Tibia
25	FEXST70	SO	19	M	Humerus
26	FEXST75	SO	15	F	Tibia
27	FEXST90	SO	11	M	Humerus
28	FEXST181	SO	13	M	Femur
29	FEXST150	SO	17	M	Proximal humerus
30	FEXST289	SO	21	M	Femur
31	FEXST290	SO	38	M	Femur
32	FEXST302	SO	32	F	Scapula
33	FEXST342	SO	27	F	Iliac crest
34	FEXST350	SO	9	M	Humerus
35	CST13	PCS gr.II (MO)	26	M	Pelvis
36	CST2	PCS gr.I (MO)	36	F	Pelvis
37	CST7	PCS gr.I (MO)	50	M	Femur
38	FEXST11	PCS gr.I (MO)	36	M	Proximal femur
38	CST12	PCS gr. II (REC)[b]	37	M	Femur
39	CST8	PCS gr.I (MO)	31	F	Scapula
40	CST1	PCS gr.I (MO)	37	M	Proximal tibia
41	FEXST182	PCS (MO)	46	M	Pelvis
42	CST9	PCS gr.II (MO)	35	M	Iliac crest
43	CST11	PCS gr.II (MO)	35	M	Pelvis
44	CST10	PCS gr.I (MO)	41	M	Femur
45	CST3	PCS gr.II (SO)	31	M	Pelvis
45	FEXST249	PCS (REC)	34	M	Pelvis
46	CST16	PCS gr.I (SO)	48	M	Proximal humerus
47	CST14	PCS gr.I (SO)	32	M	Proximal femur
48	CST5	PCS gr.I (SO)	65	M	Femur
49	FEXST238	PCS (SO)	31	F	Pubis

Abbreviations: MO, multiple osteochondroma; PCS, peripheral chondrosarcoma; SO, solitary osteochondroma; –, information not available.
[a]Tumor histological grading (grade I—II—III).
[b]REC = tumor recurrence.

Table 2 Somatic mutations detected by combined DHPLC/MLPA molecular screening in patients with SO or derived chondrosarcomas

Patient	*Sample ID*	*SO/PCS*	*Gene*	*Somatic mutation*	*HE/HO*
24	FEXST69	SO			
25	FEXST70	SO	EXT1	c.1286-2delAGAT	HE
26	FEXST75	SO			
27	FEXST90	SO	*EXT1*	ex1_11del	HE
28	FEXST181	SO			
29	FEXST150	SO	*EXT1*	ex2_11del	HE
30	FEXST289	SO	*EXT1*	ex1_11del	HE
31	FEXST290	SO	EXT1	ex1_8del	HE
32	FEXST302	SO			
33	FEXST342	SO			
34	FEXST350	SO	*EXT1*	c.1955_1956insG	HE
45	CST3	PCS	*EXT2*	ex1_14del	HE
45	FEXST249	PCS REC[a]	*EXT2*	ex1_14del	HO
46	CST16	PCS			
47	CST14	PCS			
48	CST5	PCS			
49	FEXST238	PCS	*EXT1*	ex1_11del	HE

Abbreviations: DHPLC, denaturing high-performance liquid chromatography; HE, heterozygous; HO, homozygous somatic mutation; MLPA, multiplex ligation-dependent probe amplification; PCS, peripheral chondrosarcoma from SO; SO, solitary osteochondroma.
[a]REC = tumor recurrence.

samples did not show the presence of big deletions, thus indicating that the genetic status of tissues with homozygous mutations is not due to the loss of wild-type allele and likely results from mitotic recombination; only in one MO tumor sample (FEXST341), the loss of one copy of *EXT1* was detected by MLPA and Q–PCR analyses.

Moreover, for patients 5, 12, 17, 19 and 20 multiple tissue samples from different affected sites were available for analysis; among these, only one out of five (for patient 5) and two out of six (for patient 17) osteochondromas derived from a same subject were shown to carry multiple mutations (Table 4).

We then analyzed the mutational status of 11 MO-derived PCS resections (Table 3); our DHPLC/MLPA screening showed the presence of a somatic *EXT1/2* mutational hit in four cases. In samples from patients 11, 36 and 44, the mutations detected in constitutional DNA were found at homozygous status in the tumor resections; only for sample CST2 this was due to the deletion of the wild-type allele. One PCS sample (CST1) with the *EXT2* germline mutation c.124delA showed also the deletion of the first two exons of *EXT1*. It is important to note that the benign lesion from patient 11 (FEXST119t), unlike the malignant resection obtained from the same subject (FEXST119c), presented only the germline genetic alteration.

The screening of sample CST12, representing a tumor recurrence from the same patient of FEXST11, showed homozygosity for the germline mutation on *EXT2* gene because of deletion of the wild-type allele.

Discussion

The aim of this study was to evaluate whether the occurrence of a second mutational hit in *EXT* genes is actually required for the development of sporadic and hereditary osteochondromas and/or for their neoplastic transformation into chondrosarcoma. In fact, the debate on the possible role of *EXT1/2* as classical tumor suppressor genes, and thus the necessity of their complete inactivation for osteochondroma onset, is still open in the literature.

To address this issue, we analyzed the genetic status of *EXT* genes in a wide series of sporadic and hereditary osteochondromas and PCS, analyzing both point mutations and big rearrangements by DHPLC/direct sequencing and MLPA, respectively. The appropriate resolution of this latter technique to detect small homozygous deletions in osteochondroma tissue samples has recently been reported (Hameetman *et al.*, 2007). In this study, all MLPA results were further validated by Q–PCR.

The analysis of 11 SO tissue samples identified in five of them the deletion of one copy of *EXT1* gene or part of it; this observation is in agreement with previous studies reporting that small or big deletions of *EXT1* are the most common alteration in SO (Bovée *et al.*, 1999a, b; Bernard *et al.*, 2001; Hameetman *et al.*, 2007), while single-base pair mutations are indeed rare (1 out of 11 SO samples in the present analysis). No mutations were found for 5 of 11 SOs (45%), with a percentage of negative SO similar to those reported from other authors (Hall *et al.*, 2002; Trebicz-Geffen *et al.*, 2008). In spite of the size of SO series here analyzed and the methods used are comparable to those of previous reports (Bovée *et al.*, 1999a, b; Hameetman *et al.*, 2007), our screening did not evidence the presence of second mutational events that would substantiate the need of a complete *EXT* inactivation for nonhereditary osteochondromas development. Actually, considering the percentage of SO without *EXT* mutations described in this and previous studies (Hall *et al.*, 2002; Trebicz-Geffen *et al.*, 2008), also the presence of a first alteration on *EXT1/2* does not seem to be a rule, suggesting possible alternative mechanisms for SO pathogenesis.

Table 3 Germline and somatic mutations detected by combined DHPLC/MLPA molecular screening in patients with MO or MO-related chondrosarcomas

Patient	*Sample ID*	*MO/PCS*	*Gene*	*Germline mutation*	*Somatic mutation*[a]	*HE/HO*
1	FEXST160	MO	EXT1	c.1649_1650delTT	–	–
2	FEXST32	MO	*EXT2*	c. 659G>A	–	–
3	FEXST196	MO	*EXT1*	c.1298_1299delGA	–	–
4	FEXST52	MO	*EXT1*	c.599G>A	–	–
5	FEXST53	MO	*EXT2*	c.459delT	c.459delT	HO
5	FEXST284A	MO	*EXT2*	c.459delT	–	–
5	FEXST284B	MO	*EXT2*	c.459delT	–	–
5	FEXST284C	MO	*EXT2*	c.459delT	–	–
5	FEXST284D	MO	*EXT2*	c.459delT	–	–
6	FEXST54	MO	*EXT1*	c.1469delT	–	–
7	FEXST55	MO	*EXT1*	c.1468_1469insC	–	–
8	FEXST80	MO	*EXT1*	c.1633-2A>G	–	–
9	FEXST79	MO	*EXT2*	c.151G>T	–	–
10	FEXST137	MO	*EXT2*	c.627_628delGG	c.363_364insCT	HE
11	FEXST119t	MO	*EXT1*	c.218delA	–	–
12	FEXST100	MO	*EXT2*	Ex8del	–	–
12	FEXST166	MO	*EXT2*	Ex8del	–	–
13	FEXST234	MO	*EXT1*	c.461_462delTT	–	–
14	FEXST130	MO	*EXT2*	c.536+1G>A	–	–
15	FEXST136	MO	*EXT2*	Ex1_14del	–	–
16	FEXST208	MO	*EXT1*	c.1018C>T	–	–
17	FEXST123	MO	*EXT1*	c.1468_1469insC	–	–
17	FEXST218A	MO	*EXT1*	c.1468_1469insC	c.1902C>G	HE
17	FEXST218B	MO	*EXT1*	c.1468_1469insC	c.1468_1469insC	HO
17	FEXST304A	MO	*EXT1*	c.1468_1469insC	–	–
17	FEXST304B	MO	*EXT1*	c.1468_1469insC	–	–
17	FEXST304C	MO	*EXT1*	c.1468_1469insC	–	–
18	FEXST341	MO	*EXT1*	c.1644delC	ex1_11del	HE
19	FEXST275A	MO	*EXT1*	c.1624G>T	–	–
19	FEXST275B	MO	EXT1	c.1624G>T	–	–
20	FEXST286A	MO	*EXT1*	c.847_855 del	–	–
20	FEXST286B	MO	*EXT1*	c.847_855 del	–	–
21	FEXST109b	MO	*EXT1*	c.1536+1G>T	–	–
22	FEXST112	MO	*EXT1*	c.1720G>T	–	–
23	FEXST122	MO	*EXT1*	c.1720G>T	–	–
35	CST13	PCS	*EXT2*	c.900C>A	–	–
36	CST2	PCS	*EXT1*	c.2038G>T	ex1_11del	HE
37	CST7	PCS	*EXT2*	c.940-1G>A	–	–
38	FEXST11	PCS	*EXT2*	c.772C>T	–	–
38	CST12	PCS REC[b]	*EXT2*	c.772C>T	ex1_14del	HE
39	CST8	PCS	*EXT1*	c.934delT	–	–
11	FEXST119c	PCS	*EXT1*	c.218delA	c.218delA	HO
40	CST1	PCS	*EXT2*	c.124delA	EXT1, ex1_2del	HE
41	FEXST182	PCS	*EXT1*	c.45_46delTT	–	–
42	CST9	PCS	*EXT1*	c.1948_1955del	–	–
43	CST11	PCS	*EXT2*	c.1132C>T	–	–
44	CST10	PCS	*EXT2*	c.270T>A	c.270T>A	HO

Abbreviations: DHPLC, denaturing high-performance liquid chromatography; HE, heterozygous; HO, homozygous somatic mutation; MLPA, multiplex ligation-dependent probe amplification; MO,multiple osteochondroma; PCS, peripheral chondrosarcoma from MO.
[a]If not specified, the somatic mutation affects the same *EXT* gene carrying the germline mutation.
[b]REC = tumor recurrence.

Mutational screening of 35 MO samples from unrelated probands identified all germline mutations in both osteochondromas and constitutional DNA samples; in addition, 5 MO tissues were found to have a somatic alteration on the *EXT* genes. Similarly to what observed for solitary cases, the absence of a second mutational hit in 30 of 35 MO samples (86%) clearly indicate that hereditary osteochondromas onset does not necessarily require multiple genetic alterations in *EXT1*/*EXT2*; this result is even more relevant considering the extensive number of samples here analyzed compared with previously reported case studies. Taken together, the results of our mutational screening on solitary and hereditary osteochondromas indicate that only the minority of cases carries multiple *EXT* mutations.

Moreover, according to the double-hit model, each osteochondroma from a same patient requires a somatic mutation to develop; this was not confirmed from the results here obtained analyzing multiple samples from five subjects, because a somatic mutation was found only in the minority of tissues. In our opinion, this finding deserves further investigations because it could be hypothesized that these osteochondromas actually represent tumors progressing toward PCS.

Loss of heterozygosity at the *EXT* loci has been described in chondrosarcomas arising within the

Table 4 Somatic mutations detected in five MO patients with osteochondromas from different affected sites available for analysis

Patient	*Sample ID*	*Osteochondroma location*	*Germline mutation*	*Somatic mutation (HE/HO)*
	T53	Scapula		*EXT2*, c.459delT (HO)
	T284A	Proximal femur (r)		–
5	T284B	Distal femur (r)	*EXT2*, c.459delT	–
	T284C	Distal tibia (l)		–
	T284D	Proximal tibia (l)		–
12	T100	Proximal humerus (l)	*EXT2*, ex8del	–
	T166	Distal ulna (l)		–
	T123	Tibia		–
	T218A	Humerus (r)		*EXT1*, c.1902C>G (HE)
17	T218B	Distal ulna (r)	*EXT1*, c.1468_1469insC	*EXT1*, c.1468_1469insC (HO)
	T304A	Femur (r)		–
	T304B	Tibia (r)		–
	T304C	Tibia (l)		–
19	T275A	Scapula (l)	*EXT1*, c.1624G>T	–
	T275B	Radial head (l)		–
20	T286A	Tibia (r)	*EXT1*, c.847_855del	–
	T286B	Tibia (l)		–

Abbreviations: HE, heterozygous; HO, homozygous; l, left; r, right.

cartilage cap of both sporadic and hereditary osteochondromas (Hecht *et al*., 1995; Raskind *et al*., 1995). Bovée *et al*. confirmed this observation and extended their study to other 10 putative tumor suppressor genes, observing a high percentage of loss of heterozygosity along with ploidy ranging from half to twice the normal DNA content (Bovée *et al*., 1999a, b). They therefore suggested that PCSs gain genetic alterations during malignant transformation. In this study, we analyzed a total of 18 PCS, 5 arising within SOs and 11 MO-related, along with 2 tumor recurrences. In only two PCS from sporadic exostosis, the somatic loss of one copy of *EXT1* or *EXT2* gene was detected by MLPA analysis; interestingly, the corresponding recurrence of this latter sample showed the loss of both *EXT2* alleles. These results indicate that in PCS from sporadic osteochondromas a double hit in *EXT* genes is not a common event; instead, new genetic alterations appear in the tumor recurrence, suggesting that there is a progressive accumulation of mutations involving these genes during neoplastic progression. The mutational status of these samples is remarkable also considering the rarity of mutations affecting *EXT2* described so far in SO samples.

EXT genes mutation analysis performed on 11 MO-related chondrosarcomas and one tumor recurrence confirmed the presence, in all tissues, of the germline mutations detected in correlated constitutional DNA samples. In addition, in approximately 40% of PCS samples (4 out of 11) a second somatic mutational hit was detected, substantiating the observation that multiple genetic alterations of *EXT* genes accumulate during malignant transformation of hereditary osteochondromas (Porter and Simpson, 1999; Bovée *et al*., 1999a, b).

To gain a critical and complete interpretation of these results, it should be considered that our genetic screening focused on coding regions and exon–intron junctions, while regulatory elements and untranslated regions were not analyzed. Therefore, we can not rule out the possibility that rare mutations occurring in these sequences were missed, although they may be infrequent (Lonie *et al*., 2006; Jennes *et al*., 2009).

A possible limitation of our study is actually represented by the lack of information regarding the percentage of non-mutated cells that may be present in the analyzed cartilage tissues. However, although the assessed sensitivity for point mutation detection in mosaics of cells may vary according to different mutation types, the techniques used in this study can detect very low levels of mosaic mutations, such as 5% of mutated cells for DHPLC and 15% for direct sequencing (Rohlin *et al*., 2009).

In conclusion, relevance of this study is represented by the analysis of a comprehensive case study that allowed us to trace back the *EXT* mutations from chondrosarcomas and recurrences samples to osteochondromas and constitutional DNA. Our results clearly indicate that biallelic inactivation of *EXT1* or *EXT2* genes does not account for both SO and MO formation in the majority of cases. In fact, in approximately 85% of MO samples here analyzed only germline mutations were found, without additional somatic events, and 45% of SO resulted negative to our molecular screening. The presence of double hits in the *EXT* genes should instead be regarded as a common feature of MO malignant transformation and as an event that occurs later in tumor progression of SO-related PCS; however, this latter observation needs to be confirmed in a wider series of chondrosarcomas and recurrences samples.

All these evidences strongly suggest that, in most osteochondromas, mechanisms alternative to *EXT* genetic alteration likely have a role in tumors development. These may include mutations/polymorphisms in *EXT* regulatory sequences as well as post-transcriptional regulation pathways; otherwise, genes involved in endochondral bone formation not belonging to *EXT* family may be the target of secondary genetic events or functional polymorphisms.

As we performed an accurate screening of a large number of cases, we believe that our results provide a

significant contribute to the eventual definition of a genetic model for sporadic and hereditary osteochondromas onset and malignancy, paving the way to future studies aimed at investigating new pathogenetic mechanisms.

Materials and methods

Patients and tissue samples
Fresh-frozen tissue samples were obtained with informed consent from 49 patients undergoing surgery at Rizzoli Orthopaedic Institute (Bologna, Italy) between 1990 and 2008. Patients were selected based on clinical and radiographic diagnosis of osteochondroma (SO or MO) and/or PCS (derived from SO or from MO).

For each osteochondroma, microdissection of cartilage cap was performed at the Pathology Department at the time of pathological evaluation and all histologic slides were reviewed by a pathologist for diagnosis confirmation (MA).

We analyzed a total of 46 osteochondromas (11 SO and 35 MO) and 18 PCS (6 derived from SO and 12 from MO); 16 of 18 PCS analyzed were primary tumors and 2 were recurrences. For five MO patients (patients 5, 12, 17, 19 and 20), we obtained samples from multiple affected sites; moreover, for all probands with hereditary conditions blood samples were analyzed as the source of germline DNA. All patients are unrelated probands, except for patient 22 and 23 that are brothers.

This project was approved by the ethics committee of Rizzoli Orthopaedic Institute; all samples were handled in a coded manner.

DNA isolation
Genomic DNA was extracted from frozen tumor tissues and lymphocytes using GENTRA Puregene Tissue kit and QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany), respectively. DNA quality was checked with a NanoQuant Infinite M200 instrument (Tecan Group Ltd, Männedorf, Switzerland) before analyses.

Mutation analysis
Complete mutational screening of *EXT1*/*EXT2* coding regions and exon–intron junctions was performed on DNA obtained from tissues by analyzing samples with DHPLC followed by direct sequencing of samples with abnormal elution profile. The 11 coding exons of *EXT1* and the 13 of *EXT2*, along with exon–intron junctions, were PCR-amplified using primer pairs and PCR condition previously described (Pedrini *et al*., 2005). The results of amplification and the presence of right sized PCR reaction products were confirmed by agarose gel electrophoresis. DHPLC analysis was carried out using the WAVE DNA Fragment Analysis System 3500HT (Transgenomic, Crewe, UK) equipped with a DNASep column (Transgenomic as already described (Pedrini *et al*., 2005).

Amplification products showing abnormal elution profiles were re-amplified, purified with QIAquick PCR Purification Kit (QIAGEN GmbH) and sequenced in both forward and reverse direction using BigDye Terminator chemistry version 3.1 and ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The reference *EXT1*/*2* sequences were obtained from GenBank accession numbers NM_000127.2 and NM_000401.2, respectively. Mutation numbering is based on complementary DNA, such that +1 is the A of the ATG initiation codon.

When regions with pathogenic mutations or polymorphisms were detected in a tissue sample, direct sequencing of the same regions was performed also on DNA from related peripheral lymphocytes. All identified mutations were compared with those reported in the literature as being disease-causing for MOs (Jennes *et al*., 2009; MOs Mutation Database: http://medgen.ua.ac.be/LOVD). The pathogenic effect of novel mutations was confirmed by testing other family members, when available, and by screening at least 200 control chromosomes from 100 unrelated healthy individuals as previously described (Pedrini *et al*., 2005).

Gene copy number evaluation by MLPA and Q–PCR
MLPA analysis has been performed to detect copy number variations in all tissue and blood samples. The analyses were performed using SALSA MLPA kit for *EXT1*/*EXT2* genes, Probemix P215, according to supplier instructions (MRC-Holland, Amsterdam, The Netherlands). Each sample was analyzed in at least two independent experiments. FAM-labelled ligation products were capillary electophoresed using ABI Prism 3100 automated DNA sequencer instrument with the GeneMapper 4.0 suite (Applied Biosystems) and statistically analyzed using an Excel Spreadsheet for normalization of peaks' height and area results. Both for tumor and germline DNA, normalized values over 1.30 were considered as duplications, whereas values beyond 0.80 or 0.30 were considered as hemizygous or homozygous deletions, respectively.

MLPA results were all validated by Q–PCR performed on a Corbett RotorGene 6000 instrument using SYBR Green Dye I chemistry (RT^2 Real Time SYBR Green PCR Mix, SABiosciences, Frederick, MD, USA). The ΔΔCt method for relative quantitation was optimized using carefully designed specific primers to estimate copy numbers of all *EXT1*/*EXT2* exons (primers sequences are available on request); the method was validated by the accurate measure of previously characterized DNA samples from blood of MO patients harboring exon copy number variations. Human β-actin (NC_000007.12) was used as endogenous control; DNA from healthy individuals was used as calibrator (ΔΔCt = 1; ΔΔCt for deleted exons = 0.5; ΔΔCt for duplicated exons = 2).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by the European Project EuroBoNeT.

References

Bernard MA, Hall CE, Hogue DA, Cole WG, Scott A, Snuggs MB *et al.* (2001). Diminished levels of the putative tumor suppressor proteins EXT1 and EXT2 in exostosis chondrocytes. *Cell Motil Cytoskeleton* **48**: 149–162.

Bovée JV, Cleton-Jansen AM, Kuipers-Dijkshoorn NJ, van den Broek LJ, Taminiau AH, Cornelisse CJ *et al.* (1999a). Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in

the origin of peripheral and central chondrosarcoma. *Genes Chrom Cancer* **26**: 237–246.

Bovée JV, Cleton-Jansen AM, Wuyts W, Caethoven G, Taminiau AH, Bakker E *et al*. (1999b). EXT-mutation analysis and loss of heterozygosity in sporadic and hereditary osteochondromas and secondary chondrosarcomas. *Am J Hum Genet* **65**: 689–698.

Bridge JA, Nelson M, Orndal C, Bhatia P, Neff JR. (1998). Clonal karyotypic abnormalities of the hereditary multiple exostoses chromosomal loci 8q24.1 (EXT1) and 11p11–12 (EXT2) in patients with sporadic and hereditary osteochondromas. *Cancer* **82**: 1657–1663.

Cheung PK, McCormick C, Crawford BE, Esko JD, Tufaro F, Duncan G. (2001). Etiological point mutations in the hereditary multiple exostoses gene EXT1: a functional analysis of heparan sulfate polymerase activity. *Am J Hum Genet* **69**: 55–66.

Essadki B, Moujtahid M, Lamine A, Fikry T, Essadki O, Zryouil B. (2000). Solitary osteochondroma of the limbs. Clinical review of 76 cases and pathogenic hypothesis. *Acta Orthop Belg* **66**: 146–153.

Farach-Carson MC, Hecht JT, Carson DD. (2005). Heparan sulfate proteoglycans: key players in cartilage biology. *Crit Rev Eukaryot Gene Expr* **15**: 29–48.

Hall CR, Cole WG, Haynes R, Hecht JT. (2002). Reevaluation of a genetic model for the development of exostosis in hereditary multiple exostosis. *Am J Med Genet* **112**: 1–5.

Hameetman L, Szuhai K, Yavas A, Knijnenburg J, van Duin M, van Dekken H *et al*. (2007). The role of EXT1 in nonhereditary osteochondroma: identification of homozygous deletions. *J Natl Cancer Inst* **99**: 396–406.

Hecht JT, Hogue D, Strong LC, Hansen MF, Blanton SH, Wagner M. (1995). Hereditary multiple exostosis and chondrosarcoma: linkage to chromosome II and loss of heterozygosity for EXT-linked markers on chromosomes II and 8. *Am J Hum Genet* **56**: 1125–1131.

Hennekam RC. (1991). Hereditary multiple exostoses. *J Med Genet* **28**: 262–266.

Huvos AG. (1991). *Bone Tumors: Diagnosis, Treatment, and Prognosis*, 2nd edn. WB Saunders: Philadelphia.

Jennes I, Pedrini E, Zuntini M, Mordenti M, Balkassmi S, Asteggiano CG *et al*. (2009). Multiple osteochondromas: mutation update and description of the multiple osteochondromas mutation database (MOdb). *Hum Mutat* **30**: 1620–1627.

Knudson Jr AG. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* **68**: 820–823.

Lonie L, Porter DE, Fraser M, Cole T, Wise C, Yates L *et al*. (2006). Determination of the mutation spectrum of the EXT1/EXT2 genes in British Caucasian patients with multiple osteochondromas, and exclusion of six candidate genes in EXT negative cases. *Hum Mutat* **27**: 1160.

Ludecke H-J, Ahn J, Lin X, Hill A, Wagner M J, Schomburg L *et al*. (1997). Genomic organization and promoter structure of the human EXT1 gene. *Genomics* **40**: 351–354.

McCormick C, Leduc Y, Martindale D, Mattison K, Esford LE, Dyer AP *et al*. (1998). The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nature Genet* **19**: 158–161.

McCormick C, Duncan G, Goutsos KT, Tufaro F. (2000). The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the Golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proc Natl Acad Sci USA* **97**: 668–673.

Mertens F, Rydholm A, Kreicbergs A, Willen H, Jonsson K, Heim S *et al*. (1994). Loss of chromosome band 8q24 in sporadic osteocartilaginous exostoses. *Genes Chromosom Cancer* **9**: 8–12.

Pedrini E, De Luca A, Valente EM, Maini V, Capponcelli S, Mordenti M *et al*. (2005). Novel EXT1 and EXT2 mutations identified by DHPLC in Italian patients with multiple osteochondromas. *Hum Mutat* **26**: 280.

Porter DE, Simpson AH. (1999). The neoplastic pathogenesis of solitary and multiple osteochondromas. *J Pathol* **188**: 119–125.

Raskind WH, Conrad EU, Chansky H, Matsushita M. (1995). Loss of heterozygosity in chondrosarcomas for markers linked to hereditary multiple exostoses loci on chromosomes 8 and 11. *Am J Hum Genet* **56**: 1132–1139.

Rohlin A, Wernersson J, Engwall Y, Wiklund L, Björk J, Nordling M. (2009). Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. *Hum Mutat* **30**: 1012–1020.

Schmale GA, Conrad III EU, Raskind WH. (1994). The natural history of hereditary multiple exostoses. *J Bone Joint Surg Am* **76**: 986–992.

Solomon L. (1964). Hereditary multiple exostosis. *Am J Hum Genet* **16**: 351–363.

Springfield DS, Gebhardt MC, McGuire MH. (1996). Chondrosarcoma: a review. *Instr Course Lect* **45**: 417–424.

Traeger-Synodinos J. (2006). Real-time PCR for prenatal and preimplantation genetic diagnosis of monogenic diseases. *Mol Aspects Med* **27**: 176–191.

Trebicz-Geffen M, Robinson D, Evron Z, Glaser T, Fridkin M, Kollander Y *et al*. (2008). The molecular and cellular basis of exostosis formation in hereditary multiple exostoses. *Int J Exp Pathol* **89**: 321–331.

Wicklund CL, Pauli RM, Johnston D, Hecht JT. (1995). Natural history study of hereditary multiple exostoses. *Am J Med Genet* **55**: 43–46.

Wu Y-Q, Heutink P, de Vries BBA, Sandkuijl LA, van den Ouweland AM, Niermeijer MF *et al*. (1994). Assignment of a second locus for multiple exostoses to the pericentromeric region of chromosome 11. *Hum Mol Genet* **3**: 167–171.

Wuyts W, Ramlakhan S, Van Hul W, Hecht JT, van den Ouweland AM, Raskind WH *et al*. (1995). Refinement of the multiple exostoses locus (EXT2) to a 3-cM interval on chromosome 11. *Am J Hum Genet* **57**: 382–387.

Wuyts W, Van Hul W, De Boulle K, Hendrickx J, Bakker E, Vanhoenacker F *et al*. (1998). Mutations in the EXT1 and EXT2 genes in hereditary multiple exostoses. *Am J Hum Genet* **62**: 346–354.

Wuyts W, Van Hul W. (2000). Molecular basis of multiple exostoses: mutations in the EXT1 and EXT2 genes. *Hum Mutat* **15**: 220–227.