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ORIGINAL ARTICLE

Secondary peripheral chondrosarcoma evolving from osteochondroma as a result of outgrowth of cells with functional *EXT*

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Secondary peripheral chondrosarcoma is the result of malignant transformation of a pre-existing osteochondroma, the most common benign bone tumor. Osteochondromas are caused by genetic abnormalities in *EXT1* or *EXT2*: homozygous deletion of *EXT1* characterizes sporadic osteochondromas (non-familial/solitary), and germline mutations in *EXT1* or *EXT2* combined with loss of heterozygosity define hereditary multiple osteochondromas. While cells with homozygous inactivation of *EXT* and wild-type cells shape osteochondromas, the cellular composition of secondary peripheral chondrosarcomas and the role of *EXT* in their formation have remained unclear. We report using a targeted-tiling-resolution oligo-array-CGH (array comparative genomic hybridization) that homozygous deletions of *EXT1* or *EXT2* are much less frequently detected (2/17, 12%) in sporadic secondary peripheral chondrosarcomas than expected based on the assumption that they originate in sporadic osteochondromas, in which homozygous inactivation of *EXT1* is found in ~80% of our cases. FISH with an *EXT1* probe confirmed that, unlike sporadic osteochondromas, cells from sporadic secondary peripheral chondrosarcomas predominantly retained one (hemizygous deleted loci) or both copies (wild-type) of the *EXT1* locus. By immunohistochemistry, we confirm the presence of cells with dysfunctional *EXT1* in sporadic osteochondromas and show cells with functional *EXT1* in sporadic secondary peripheral chondrosarcomas. These immuno results were verified in osteochondromas and secondary peripheral chondrosarcomas in the setting of hereditary multiple osteochondromas. Our data therefore point to a model of oncogenesis in which the osteochondroma creates a niche in which wild-type cells with functional *EXT* are predisposed to acquire other mutations giving rise to secondary peripheral chondrosarcoma, indicating that *EXT*-independent mechanisms are involved in the pathogenesis of secondary peripheral chondrosarcoma.
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Introduction

Secondary peripheral chondrosarcomas arise in the cartilaginous cap of a pre-existing osteochondroma (Bertoni *et al*., 2002). Osteochondromas are the most common benign bone tumors of adolescence (Khurana *et al*., 2002; van den Berg *et al*., 2008). They occur as sporadic (non-familial/solitary) or multiple (hereditary) cartilage-capped bony projections from the metaphyses of endochondral bones adjacent to the growth plate (Khurana *et al*., 2002; Bovee *et al*., 2010). Osteochondromas develop during skeletal growth (13–15 years) and cease to grow when the growth plate fuses at puberty (van den Berg *et al*., 2008).

Sporadic and multiple osteochondromas have been linked to genetic alterations in two main loci, *EXT1* at 8q24 (65%) or *EXT2* at 11p11-13 (35%) (Bovee *et al*., 2010). The majority of the multiple osteochondromas (70–75%) are caused by point mutations. Deletions involving single or multiple exons are found in about 10% of all hereditary cases and no genomic alterations are detected in about 10–15%. In some of these negative cases, somatic mosaicism with large genomic deletions of *EXT1* and *EXT2* have been described as the underlying mechanism of multiple osteochondroma formation (Szuhai *et al*., 2011). In sporadic osteochondromas, homozygous deletions of *EXT1* are identified (Hameetman *et al*., 2007b; Reijnders *et al*., 2010; Zuntini *et al*., 2010; Szuhai *et al*., 2011). Recent experimental studies using mice and zebrafish knockdown models show that homozygous inactivation of *Ext1* or *Ext2* is required for osteochondromagenesis (Clément *et al*., 2008; Jones *et al*., 2010; Matsumoto *et al*., 2010). In humans, loss of the remaining wild-type allele of *EXT* has been detected in approximately 40% of sporadic and multiple osteochondromas (Hameetman *et al*., 2007b; Reijnders *et al*., 2010; Zuntini *et al*., 2010). In sporadic osteochondromas, we have described homozygous deletions of *EXT1* in ~80% of the cases (Hameetman *et al*., 2007b; Reijnders *et al*., 2010; Zuntini *et al*., 2010). It was

shown recently that during the formation of osteochondromas, chondrocytes with functional *EXT* from the growth plate or the neighboring tissue are being integrated into the cartilaginous cap, generating a mosaic mixture of wild-type cells and cells with homozygous inactivation of *EXT1* or *EXT2* (de Andrea *et al*., 2010, 2011; Jones *et al*., 2010; Matsumoto *et al*., 2010).

Less than 1% of patients with sporadic osteochondromas and 1–3% of patients with multiple osteochondromas at the age of 30–60 years will eventually develop a secondary peripheral chondrosarcoma (Dorfman *et al*., 2002). Secondary peripheral chondrosarcomas are malignant cartilage-producing tumors. Contrary to what is observed in osteochondromas, homozygous inactivations of the *EXT* genes are detected in a much smaller subset (~15%) of secondary peripheral chondrosarcomas (Hecht *et al*., 1995; Raskind *et al*., 1995; Bovée *et al*., 1999; Hallor *et al*., 2009; Zuntini *et al*., 2010).

Although osteochondromas and secondary peripheral chondrosarcomas are associated, many aspects on the pathogenesis of this malignant bone tumor remain unestablished. The homozygous inactivation of the *EXT* genes required for osteochondromagenesis, the mixture of cells with distinct genetic background within the osteochondroma cap and the low frequency of homozygous inactivation of the *EXT* genes in secondary peripheral chondrosarcomas lead to two central questions: (i) Are the cells with homozygous inactivation of *EXT1/2* of osteochondromas the cellular origin of secondary peripheral chondrosarcomas? (ii) Do the *EXT* genes act as tumor-suppressor genes in the formation of secondary peripheral chondrosarcomas?

To address these unsolved questions, we studied 17 clinically and radiologically well-documented secondary peripheral chondrosarcomas derived from sporadic osteochondromas. The copy-number alterations of *EXT1* or *EXT2* were assessed by targeted-tiling-resolution oligo-array-CGH and the genetic changes involving the *EXT1* locus were investigated by fluorescence *in situ* hybridization (FISH). Additionally, the potential function of *EXT1* was explored by immunohistochemistry. Sporadic osteochondromas were used as a reference to understand the formation of a secondary peripheral chondrosarcoma. Multiple osteochondromas and secondary peripheral chondrosarcomas derived from patients with multiple osteochondromas were used to validate the generated model.

Results

Clinical findings and imaging studies

The anatomical distribution and the cartilaginous cap thickness of the 17 sporadic peripheral chondrosarcomas are shown in Table 1. Sixteen patients showed no adverse outcome (metastases or death of disease). However, one patient with a pelvic lesion developed a large local recurrence approximately 3 years after the primary surgery and died a few months later of the disease. No germline mutations in *EXT1* or *EXT2* were detected.

In the imaging studies, a pre-existing osteochondroma and/or its stalk were identified in all 17 cases (Supplementary Figure 1). All cases diagnosed as grade-I secondary peripheral chondrosarcoma fulfilled the criteria for recommending resection for malignant concerns. These criteria included histological features in accordance with the WHO classification (Khurana *et al*., 2002; van den Berg *et al*., 2008) or a cartilage cap thickness of 1.5 cm or greater, or a clinical history and/or radiographic signs of tumor growth (Supplementary Figure 1B–B4). Case L-868 was radiologically classified as a borderline lesion. The criteria used for this case included age of the patient (18-year-old man) with a growing lesion on an almost mature skeleton, in which the growth plates were nearly closed (Supplementary Figure 2).

Homozygous deletions of EXT1/2 *are uncommon in sporadic secondary peripheral chondrosarcomas*

A custom-made, oligonucleotide-based microarray with a tiling coverage of *EXT1/2* genes was used to detect deletions involving any part of *EXT1* or *EXT2* genes. At least 90% of tumor tissue was isolated and analyzed in each case. Table 1 summarizes the results of the array comparative genomic hybridization (array-CGH) analysis. In the 17 sporadic secondary peripheral chondrosarcomas, homozygous deletion of *EXT1* was found in two cases (L-1245, grade-I; and L-224, grade-II) (Figure 1). L-1245 showed deletions involving exon-2 to exon-11. Based on the array profile of L-1245, the proportion of the cell fraction containing homozygous deletion of *EXT1* was ~25% (Figure 1). L-224 showed deletions in exon-10, which is smaller than the bacterial artificial chromosome probe used for FISH analysis. Hemizygous deletion of *EXT1* was found in 12 cases (not shown). A significant gain of chromosome arm 8q was identified in one case (L-123, not shown). No alterations involving the *EXT1* gene were seen in two cases (Figure 1). Additionally, no alterations involving the *EXT2* gene were identified (not shown).

Retention of one or both copies of EXT1 *in the cells of sporadic secondary peripheral chondrosarcomas*

To further investigate the genetic changes in *EXT1*, FISH was performed in the setting of sporadic lesions and was successful in 10 out of 17 secondary peripheral chondrosarcomas and in 4 out of 5 sporadic osteochondromas. Failures were due to DNA damage as a result of decalcification. In sporadic osteochondromas, the majority of the cells showed loss of both copies (homozygous loss) of the *EXT1* locus (mean of all cases: 91%; Figure 2 and Table 1), as expected. Surprisingly, within the tumor, cells retaining both copies of the *EXT1* locus (wild-type alleles) were also observed (mean of all cases: 7%; Figure 2 and Table 1), which were commonly located in the hypertrophic layer of the osteochondroma cap (Supplementary Figure 3).

Table 1 Clinical and radiological information. FISH and EXT-tiling array results

Case number	Sample	Age	Location	Cartilaginous cap thickness	Fluorescent in situ hybridization with an EXT1 probe				EXT tiling array
					No loss	Hemizygous loss	Homozygous loss	Gain[a]	
L-741	GP	8	-	-	86%	10%	4%	0	
L-1142	GP	12	-	-	89%	9%	2%	0	
L-2390	AC	54	-	-	93%	5%	2%	0	
L-2776	AC	60	-	-	93%	6%	1%	0	
		Cut-off values:			-	15%	6%	-	
L-0657	OC	12	Distal Femur	1.5 cm	NA	NA	NA	NA	Homozygous loss *EXT1*[b]
L-0673	OC	12	Proximal Humerus	0.2 cm	7%	2%	91%	0	Homozygous loss *EXT1*[b]
L-1455	OC	17	Proximal Fibula	1 cm	7%	3%	90%	0	Homozygous loss *EXT1*[b]
L-1520	OC	16	Proximal Humerus	0.5 cm	9%	2%	89%	0	Homozygous loss *EXT1*[b]
L-1649	OC	21	Proximal Humerus	1.5 cm	5%	0	95%	0	Homozygous loss *EXT1*[b]
L-1480	grade I PCS	16	Distal Femur	1.5 cm	NA	NA	NA	NA	Normal CGH Profile
L-2644	grade I PCS	46	Proximal Fibula	1 cm	NA	NA	NA	NA	Normal CGH Profile
L-868	Borderline lesion	18	Proximal Humerus	1 cm	24%	34%	42%	0	Hemizygous loss of *EXT1*
L-163	grade I PCS	49	Proximal Tibia	2 cm	NA	NA	NA	NA	Hemizygous loss of *EXT1*
L-626	grade I PCS	21	Proximal Femur	4 cm	60%	28%	12%	0	Hemizygous loss of *EXT1*
L-627	grade I PCS	32	Scapula	2 cm	NA	NA	NA	NA	Hemizygous loss of *EXT1*
L-724	grade I PCS	83	Pelvis	15 cm	NA	NA	NA	NA	Hemizygous loss of *EXT1*
L-739	grade I PCS	37	Pelvis	10 cm	48%	47%	5%	0	Hemizygous loss of *EXT1*
L-1655	grade I PCS	40	Proximal Femur	2 cm	NA	NA	NA	NA	Hemizygous loss of *EXT1*
L-2361	grade I PCS	25	Femur	2 cm	8%	77%	15%	0	Hemizygous loss of *EXT1*
L-11	grade II PCS	49	Pelvis	8 cm	9%	74%	11%	6%	Hemizygous loss of *EXT1*
L-76	grade II PCS	49	Pelvis	20 cm	44%	54%	2%	0	Hemizygous loss of *EXT1*
L-1165	grade II PCS	38	Pelvis	14 cm	NA	NA	NA	NA	Hemizygous loss of *EXT1*
L-1618	grade II PCS	44	Pelvis	30 cm	36%	43%	21%	0	Hemizygous loss of *EXT1*
L-224	grade II PCS	26	Pelvis	11 cm	40%	42%	16%	2%	Homozygous loss *EXT1* exon 10[c]
L-1245	grade I PCS	25	Proximal Fibula	0.8 cm	4%	84%	12%	0	Homozygous loss *EXT1* exon 2-11
L-123	grade II PCS	60	Distal Femur	13 cm	73%	7%	0	20%	Gain of the *EXT1* region

Abbreviations: AC, articular cartilage; FISH, fluorescence *in situ* hybridization; GP, epiphyseal growth plate; NA, not available owing to bad FISH; OC, sporadic osteochondroma; PCS, sporadic secondary peripheral chondrosarcoma.
Blue areas, significant retention of both copies of *EXT1*; green areas, hemizygous loss of EXT1; red areas, homozygous loss of EXT1 and gray areas, gain of the *EXT1* genomic region.
[a]Increase of both centromere-8 and EXT1 FISH signals.
[b]Results reported previously (Hameetman *et al*., 2007b).
[c]The size of the deletion is smaller than the used BAC probe.

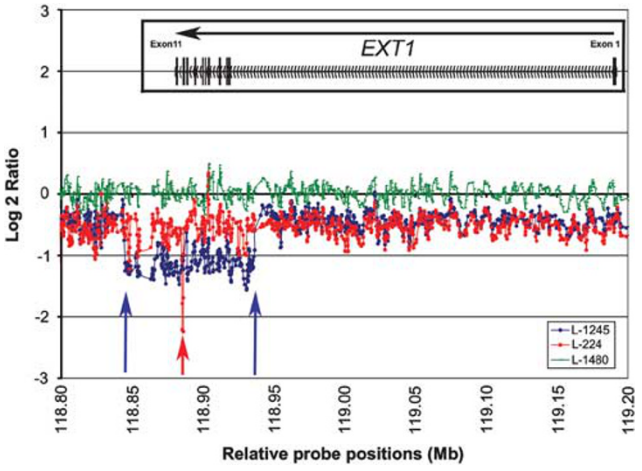

Figure 1 Array-CGH results overview of the genomic region on chromosome-8, including the *EXT1* gene area, shows the profile of samples with homozygous deletions (L-224, a grade-II secondary peripheral chondrosarcoma; and L-1245, a grade-I secondary peripheral chondrosarcoma). In both cases, loss of an area that exceeded the *EXT1* region (indicated by the values lower than 0) containing a region with additional deletion ranging from ~100 kb (blue arrows) to ~10 kb (red arrow) reflecting the homozygous deletion were detected. As reference, a sample (L-1480, a grade-I secondary peripheral chondrosarcoma) with no alteration involving *EXT1* was plotted in green.

Although hemizygous losses were detected, the number of cells with one copy of the gene was below the cut-off value based on the correction factors obtained from FISH data from growth plate and articular cartilage, indicating that these losses reflected truncation artifacts. In each analyzed sporadic peripheral chondrosarcoma, the patterns of copy-number alterations involving the *EXT1* locus compared with the chromosome-8 centromere signals varied widely (Figure 2 and Table 1). Five cases showed cells with no losses (wild-type alleles), as well as hemizygous and homozygous deletions. Two cases showed cells with wild-type and hemizygous deleted loci. One case showed cells with no loss, hemizygous deletion and gain of the *EXT1* locus. Two cases showed cells with no losses, hemizygous and homozygous deletion, as well as gain of the *EXT1* locus. Additionally, all except three cases (L-739, grade-I; L-868, borderline cases; L-2361, grade-I) showed aneuploid cells (>2 signals with a centromere-8 probe) ranging from 4 to 50% of the total amount of cells. In three remarkably large lesions (L-76, L-123 and L-739) no significant homozygous losses were identified; however, hemizygous loss was detected (Table 1). There was no correlation between the size of the lesion and the percentage of cells carrying a homozygous deletion (Spearman's coefficient = 0.31).

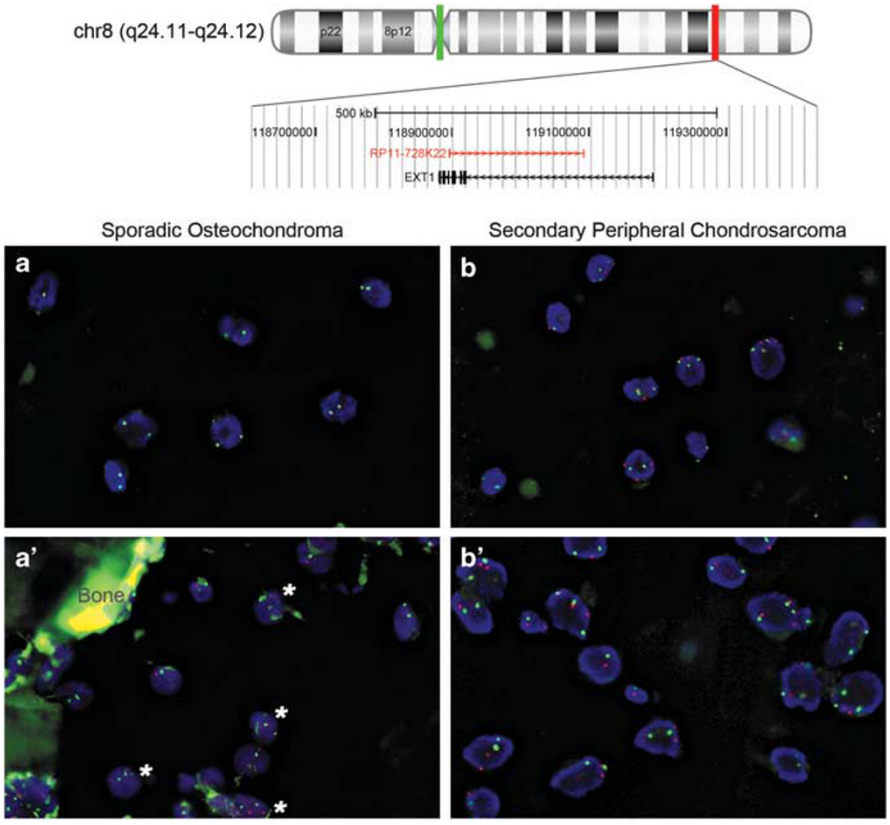

Figure 2 Genomic deletions detected by FISH using an EXT1 probe. The illustration (upper panel) is showing the exact locations and the assigned colors of the used *EXT1* locus (red) and centromere-8 (green) probes. Samples were counterstained using 4′,6-diamidino-2-phenylindole (DAPI), indicating the nucleus of individual cells in blue. (**a**) The lack of red signals with retained green signals indicates a homozygous loss of the *EXT1* loci, which characterize the superficial layer of a sporadic osteochondroma (L-673). (**a**′) Cells with retained red signals (wild-type, asterisks) and loss of both copies were observed in the hypertrophic layer of a sporadic osteochondroma (L-673), indicating the presence of two cell populations. (**b**, **b**′) *EXT1* wild-type cells and gain of the *EXT1* genomic region were detected in secondary peripheral chondrosarcomas (L-123, grade-II).

Cells with functional EXT in sporadic and hereditary secondary peripheral chondrosarcomas

EXT1 and *EXT2* genes encode ubiquitously expressed type-II transmembrane glycosyltransferases (EXT1 and EXT2) (McCormick *et al*., 1998), which localize to the endoplasmic reticulum and Golgi complex, and are required for the synthesis of heparan sulfate chains on proteoglycans (Zak *et al*., 2002; Bulow and Hobert, 2006). To assess the potential function of *EXT1*, we evaluated the expression of the EXT1 protein, using an EXT1 antibody, and the production of heparan sulfate, using the 10E4 antibody, in the setting of sporadic and multiple lesions. The results are shown in Figures 3 and 4, and Supplementary Table 1. In both sporadic and multiple lesions, there were more EXT1- and heparan sulfate-positive chondrocytes in the hypertrophic layer than in the superficial layer. The control growth plate is shown in Supplementary Figure 4.

In the setting of sporadic lesions (Figure 3), no significant changes in the number of cells staining with EXT1 and 10E4 were seen in the hypertrophic layer when comparing osteochondromas to secondary peripheral chondrosarcomas ($P=0.70$). Significant changes were observed in the superficial layer of these tumors; an increasing number of cells staining with EXT1 and 10E4 were observed with increasing histological grade (Figure 3). The number of cells staining with EXT1 and 10E4 were significantly higher in the superficial layer of grade-II secondary peripheral chondrosarcomas when compared with osteochondromas and grade I secondary peripheral chondrosarcomas ($P=0.035$ and $P=0.032$, respectively). No correlation was found between age of the patient and the percentage of EXT1/10E4-positive cells (Spearman's coefficient = 0.02 each), nor between size of the cartilage cap and the percentage of EXT1/10E4-positive cells (Spearman's coefficient = 0.27 and 0.20, respectively).

In the setting of multiple lesions (Figure 4), the number of cells staining with EXT1 and 10E4 were significantly higher in secondary peripheral chondrosarcomas when compared to osteochondromas (for EXT1 at hypertrophic layer: $P=0.006$; and at superficial layer: $P<0.001$; and for heparan sulfate at hypertrophic layer: $P=0.007$; and at hypertrophic layer: $P=0.008$).

Discussion

Osteochondromas originate either from the chondrocytes of the growth plate or the cells of the

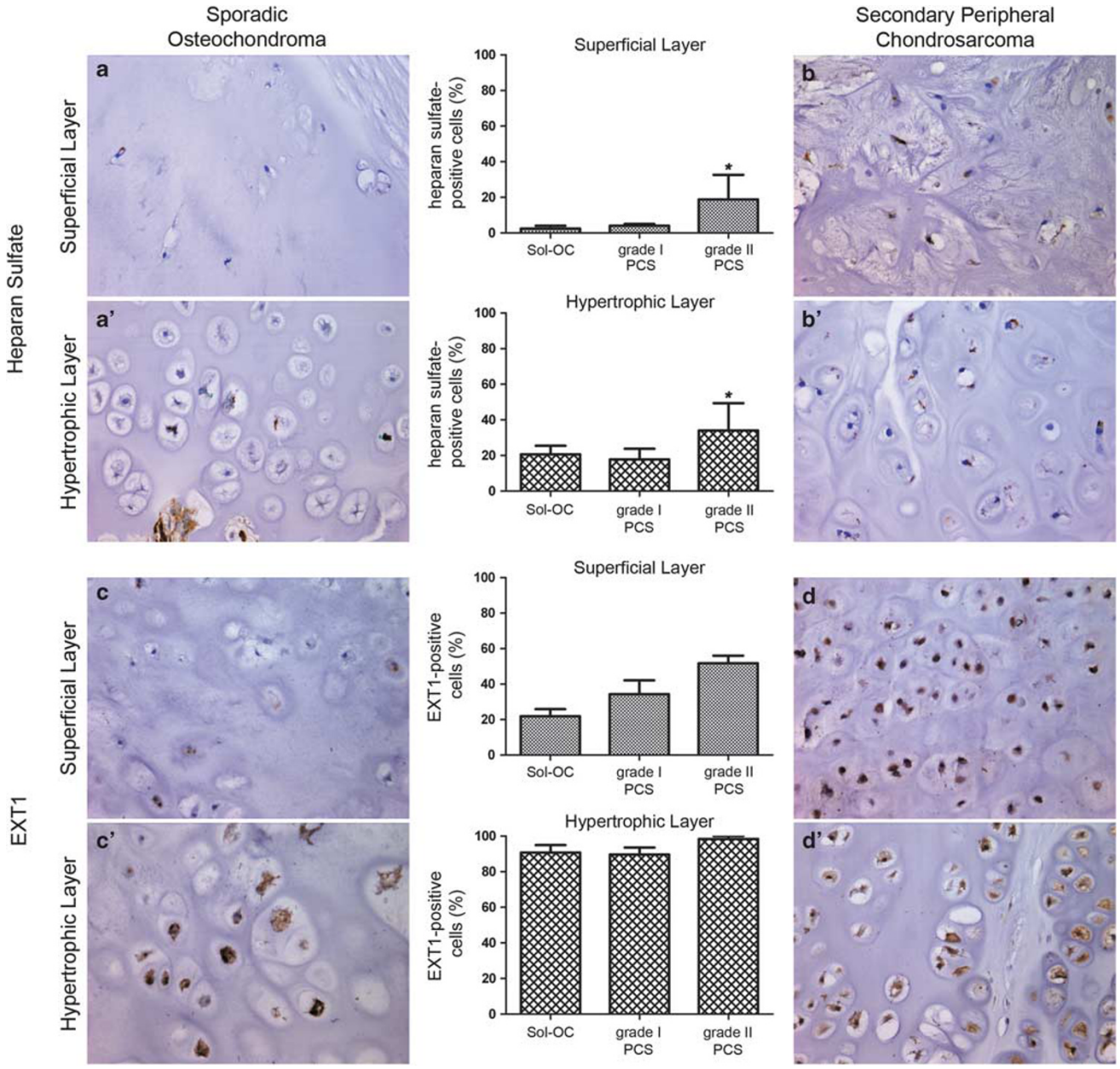

Figure 3 Immunohistochemical screening of cells with functional *EXT1* in sporadic osteochondromas and peripheral chondrosarcomas. In the superficial layer of the cartilage cap, there was an increase of cells with functional *EXT1* (10E4- and EXT1-positive cells) upon neoplastic transformation from osteochondromas (**a**, **c**, respectively) to peripheral chondrosarcomas (**b**, **d**, respectively). In the hypertrophic layer, no significant changes were observed in the percentage of cells stained with EXT1 and 10E4 when comparing osteochondromas (**a**′, **c**′, respectively) to peripheral chondrosarcomas (**b**′, **d**′, respectively). The asterisks indicate $P<0.05$. Sol-OC, sporadic osteochondromas; PCS, sporadic secondary peripheral chondrosarcoma.

perichondrium due to homozygous inactivation of *EXT1* or *EXT2* (Clément *et al*., 2008; Jones *et al*., 2010; Matsumoto *et al*., 2010). During osteochondromagenesis, wild-type chondrocytes and cells with homozygous inactivation of *EXT* are shown to intermingle in the cartilaginous cap (de Andrea *et al*., 2010, 2011; Jones *et al*., 2010; Matsumoto *et al*., 2010). We here show that, in human osteochondromas, the wild-type chondrocytes showing both *EXT1* alleles are predominantly located in the hypertrophic layer of the osteochondroma cap. As the chance of detecting the second hit in the *EXT1* gene depends on the ratio of wild-type versus mutated cells, our data indicate that the probability to detect a homozygous deletion in osteochondromas increases when tumor sampling is more superficial (near the perichondrium), which may explain the conflicting data that have been published by independent institutions (Hameetman *et al*., 2007b; Reijnders *et al*., 2010; Zuntini *et al*., 2010).

The current multistep genetic model for secondary peripheral chondrosarcoma formation assumes that osteochondroma cells with homozygous inactivation of

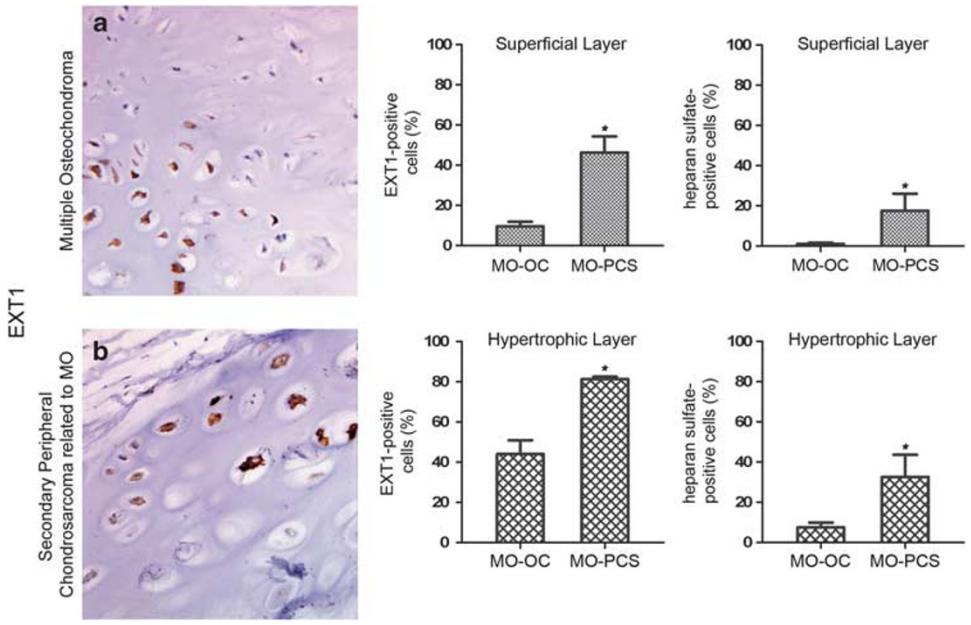

Figure 4 Immunohistochemical screening of cells with functional *EXT1* in multiple osteochondromas (**a**) and peripheral chondrosarcomas (**b**) related to patients with multiple osteochondromas. In the superficial layer and in the hypertrophic layer of the cartilage cap, there was a significant increase of cells with functional *EXT1* (10E4- and EXT1-positive cells) upon neoplastic transformation from osteochondromas to peripheral chondrosarcoma. The asterisks indicate $P<0.05$. MO-OC, multiple osteochondromas; MO-PCS, peripheral chondrosarcoma related to patients with multiple osteochondroma.

EXT1 or *EXT2* acquire one or more additional genetic alterations to progress into malignancy (Bovée *et al*., 2000). By analyzing clinically and radiologically well-documented peripheral cartilaginous tumors, we demonstrate that homozygous inactivation of the *EXT1* locus in sporadic secondary peripheral chondrosarcoma (2/17, 12%) is much less frequently detected than expected based on the assumption that it originates in a sporadic osteochondroma, in which homozygous inactivation of *EXT1* is found in ~80% of the cases reported by our institution (Hameetman *et al*., 2007b; Reijnders *et al*., 2010). EXT tiling array demonstrated that the most frequent event in sporadic secondary peripheral chondrosarcomas is hemizygous loss of *EXT1* (12/17, 70%), followed by no alterations in *EXT1* (2/17, 12%) and gain of the *EXT1* region (1/17, 6%). No deletions involving the *EXT2* gene were identified. FISH using an EXT1 probe confirmed the presence of cells with hemizygous loss and, in addition, revealed a wide intra-tumoral heterogeneity for the *EXT1* gene including homozygous losses, retentions and gains.

Immunohistochemistry against EXT1 and heparan sulfate (10E4) was used to evaluate the spatial distribution of cells with functional or dysfunctional *EXT1* in sporadic osteochondromas and sporadic secondary peripheral chondrosarcomas. The findings were further validated in multiple osteochondromas and secondary peripheral chondrosarcomas derived from patients with multiple osteochondromas. Cells with dysfunctional *EXT1* (that is, non-staining with EXT1 and heparan sulfate) are mainly populating the superficial layer of sporadic and multiple osteochondromas, which is in line with our previous results showing homozygous loss of *EXT1* in sporadic and multiple osteochondromas (Hameetman *et al*., 2007b; Reijnders *et al*., 2010). By contrast, cells with functional *EXT1* are present in the superficial layer (region near the perichondrium) of secondary peripheral chondrosarcomas related to sporadic or multiple osteochondromas, which is in line with the absence of homozygous deletions in the majority of sporadic peripheral chondrosarcomas. In addition, the amount of cells with functional *EXT1* increases with increasing tumor grade. We also observed that the percentage of EXT1-positive cells is slightly higher than the percentage of heparan sulfate-positive cells, which suggests that the cell's environment may enhance or diminish the production of heparan sulfate by modulating the activity of EXT1 in a cell.

Taken together, whereas cells with homozygous inactivation of *EXT1* constitute an osteochondroma, cells with wild-type, functional *EXT1* compose a fully developed secondary peripheral chondrosarcoma (Figure 5). Therefore, it is possible to envision a model where secondary peripheral chondrosarcoma cells with functional *EXT1* outgrow osteochondroma cells with dysfunctional *EXT1*. This model can be demonstrated in two distinct sets of tumors: (i) In lesions with a thin cartilaginous cap thickness, cells that lost both copies of *EXT1* and cells that retain one or both copies of *EXT1* are found (that is, L-868 and L-1245, both ~1 cm). In this situation, osteochondroma cells and secondary peripheral chondrosarcoma cells may be coexisting. (ii) In remarkably large lesions, cells that lost

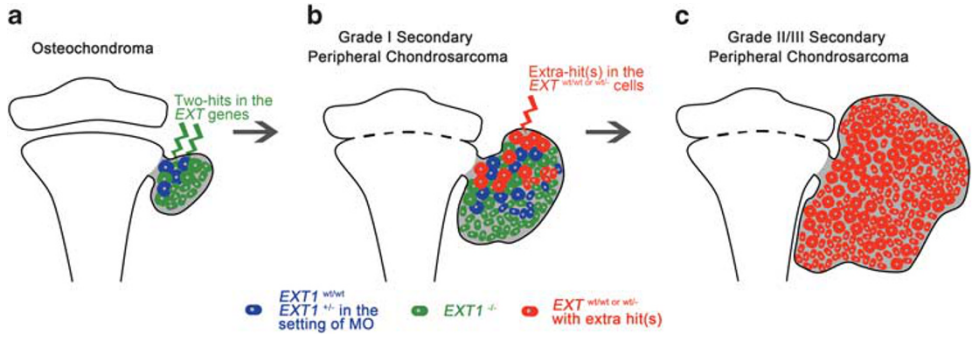

Figure 5 Secondary peripheral chondrosarcomagenesis. (**a**) *EXT*-null cells (green) and *EXT* wild-type cells (blue) compose the osteochondroma cap. (**b**) The *EXT*-null cells form a niche, a permissive microenvironment, which predisposes the *EXT* wild-type cell within the osteochondroma or from the perichondrium to acquire mutation(s) not related to *EXT*s to give rise to a secondary peripheral chondrosarcoma. The chondrosarcoma cells (red) get a proliferative advantage over the osteochondroma *EXT*-null cells. During neoplastic progression, the chondrosarcoma cells may or may not lose one copy of the *EXT1* gene. (**c**) Only chondrosarcoma cells, which retained one or both copies of the *EXT* genes, form the fully developed secondary peripheral chondrosarcoma. MO, multiple osteochondromas.

both copies of *EXT1* are absent (that is, L-76, 30 cm; L-123, 15 cm and L-739, 11 cm). In this instance, only secondary peripheral chondrosarcoma cells that retain one or both copies of *EXT1* may be growing. The proposed model is reinforced by the fact that cells carrying a homozygous deletion (having no copies of the involved genomic region left at all) are not able to regain the lost alleles during neoplastic progression.

In myelodysplasia and secondary leukemia, a 'niche-based' model of oncogenesis has been described, in which a change in a specific niche/microenvironmental cell can serve as the primary moment in a multi-step process toward the malignancy of a supported, but distinct, cell type (Raaijmakers *et al*., 2010; Raaijmakers, 2011). This model of oncogenesis seems to explain the formation of a secondary peripheral chondrosarcoma. The osteochondroma cells with homozygous inactivation of *EXT* (loss of both copies of *EXT*) create a niche (a permissive microenvironment), which facilitates cells with functional *EXT* (retention of one or both copies of *EXTs*) located either in the osteochondroma cap or in the perichondrium that closely surrounds the lesion to acquire secondary genetic changes. Although the secondary peripheral chondrosarcoma-driving mutations are so far unknown, they differ from *EXT* mutations, indicating that *EXT*-independent mechanisms have a role in the pathogenesis of secondary peripheral chondrosarcoma. The need of functional *EXT* and heparan sulfate for cell proliferation is supported by the fact that inactivation of *EXT1* in multiple myeloma leads to decreased tumor growth (Reijmers *et al*., 2010) and that *EXT*-null chondrocytes do not grow *in vitro* (Reijnders *et al*., 2010). Additionally, cells with functional *EXT* in the mosaic osteochondroma cap are suggested to provide a certain threshold level and distribution of heparan sulfate, creating an environment conducive to cells with homozygous inactivation of *EXT* to proliferate and form a tumor (de Andrea *et al*., 2010, 2011; Jones *et al*., 2010; Matsumoto *et al*., 2010).

Osteochondromas and secondary peripheral chondrosarcomas composed by distinct cell types is strengthened by some of our previous results. First, the mRNA expression level of *EXT1* in osteochondromas is not completely absent, which suggests the presence of cells with functional *EXT1* in the tumor. Second, although not significant, the mRNA level of *EXT1* seems to increase from low- to high-grade in secondary peripheral chondrosarcomas (Hameetman *et al*., 2007a). We have also shown that signaling pathways dependent on heparan sulfate, such as fibroblast growth factor and parathyroid hormone-related protein, are inactive in osteochondromas and active in secondary peripheral chondrosarcomas. The heparan sulfate with normal chain length synthesized by the overgrown *EXT1* wild-type cells might be related to the activation of fibroblast growth factor and parathyroid hormone-related protein in secondary peripheral chondrosarcoma.

Although we show that the vast majority of sporadic secondary peripheral chondrosarcomas do not have homozygous deletions involving *EXT1* or *EXT2*, the few reported cases with homozygous deletions of *EXT1* or *EXT2* (2/17 in the present series) suggest that malignant transformation of osteochondroma cells with homozygous inactivation of *EXT1* or *EXT2* is a rare event. Usually, these are small deletions of *EXT1* (that is, case L-224) unable to be detected by FISH.

While our data clarify the pathogenesis of secondary peripheral chondrosarcoma, a number of questions remain. For instance, which is the causative gene for peripheral chondrosarcoma formation? What factors make chondrocytes with functional *EXT1* and *EXT2* within/surrounding an osteochondroma more prone to acquire mutations that lead to malignancy?

In summary, our study shows that (i) an osteochondroma is a niche that facilitates the *EXT1* wild-type cells to acquire other mutations, distinct from mutations involving the *EXT* genes, for secondary peripheral chondrosarcoma formation; (ii) secondary peripheral chondrosarcoma is not *per se* originating from cells

of osteochondroma with homozygous inactivation of *EXT1*, but arises predominantly and maybe even exclusively from cells with functional *EXT1* (Figure 5).

Materials and methods

Patient data and tumor samples

Based on clinical information and radiological studies, 17 sporadic secondary peripheral chondrosarcomas, in which paraffin-embedded and frozen material were available, were retrieved from the Department of Pathology at Leiden University Medical Center collected between 1991 and 2008. In addition, paraffin-embedded material from sporadic ($n=10$) and multiple ($n=10$) osteochondromas, and secondary peripheral chondrosarcomas related to patients with multiple osteochondromas ($n=5$), were selected for comparison in the different used techniques. For controls, epiphyseal growth plate ($n=2$) and articular cartilage ($n=2$) were obtained from orthopedic resections for pathological conditions not related to osteochondroma or chondrosarcoma. Chondrosarcoma histological grading was evaluated according to the Evans classification (Evans *et al*., 1977). Imaging studies (radiography, computed tomographic scans and magnetic resonance imaging scans) were reviewed in all 17 sporadic secondary peripheral chondrosarcomas and in five sporadic osteochondromas. The cartilaginous cap thickness was measured in accordance with Bernard *et al*. (2010). All samples were handled in a coded manner and all procedures were performed according to the ethical guidelines in Code for Proper Secondary Use of Human Tissue in The Netherlands (Dutch Federation of Medical Scientific Societies).

DNA isolation

Per sample about 60 frozen tumor sections (16-μm thick) were cut. DNA was isolated by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's manual. DNA concentration and purity were measured for each sample by using a NanoDrop spectrophotometer (Isogen, De Meern, the Netherlands) and a Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively, according to the manufacturer's protocol. The percentage of tumor cells within each sample was determined on hematoxylin and eosin-stained sections and occasionally normal/reactive tissue was removed by macrodissection to achieve at least 90% of tumor tissue.

Mutation screening

The genomic DNA of five sporadic peripheral chondrosarcomas was tested for mutations in *EXT1* and *EXT2*. The mutation status from 12 cases has been reported previously (Hameetman *et al*., 2007a). Mutation screening of *EXT1* or *EXT2* genes was performed and analyzed as described earlier (Hameetman *et al*., 2007a; Reijnders *et al*., 2010).

EXT tiling array

Seventeen sporadic peripheral chondrosarcomas, well-known for not showing point mutations in the *EXT* genes (Bovée *et al*., 1999; Hallor *et al*., 2009; Zuntini *et al*., 2010), were collected to study DNA copy-number alterations by using a custom-made Agilent oligonucleotide-based microarray containing 44 000 probes with a tiling coverage of *EXT1/2* genes and additional 68 genes involved in heparan and chondroitin sulfate biosynthesis as described by Szuhai *et al*. (2011). Only alterations of *EXT* genes were investigated. EXT tiling array in sporadic osteochondromas has been described previously by our group in a series of 11 tumors (Hameetman *et al*., 2007b; Reijnders *et al*., 2010). Labeling and hybridization of genomic DNA from freshly frozen tumor, as well as data processing, were performed as described earlier (Reijnders *et al*., 2010; Szuhai *et al*., 2011).

Fluorescent in situ *hybridization*

Interphase FISH on 16-μm-thick paraffin sections was performed in all 17 sporadic secondary peripheral chondrosarcomas, as described previously (Szuhai *et al*., 2009). The five sporadic osteochondromas used for comparison were reported previously to show homozygous deletion of *EXT1* (Hameetman *et al*., 2007b). Two sets of FISH probes were applied: Set 1 with the RP11-728K22 bacterial artificial chromosome clones covering the *EXT1* genomic region and Set-2 with the probe for the centromere of chromosome-8. RP11-728K22 was selected by using the UCSC Genome Browser (NCBI 36.1/hg18) (http://genome.ucsc.edu) (Figure 2). Copy-number and image analysis were performed as described by Mohseny *et al*. (2010) and Pansuriya *et al*. (2011). At least 100 cells were scored per case (more than 200 for most of the cases) counting the centromere-8 probe and the *EXT1* probe. Cells with more than two centromere-8 probes were considered aneuploid; loss of one copy of *EXT1* probe was considered as a hemizygous gene loss and loss of all copies of the *EXT1* probe was considered as a homozygous deletion of the gene. Gain was defined as the presence of at least three copies of the *EXT1* probe in diploid cells. To determine the cut-off values indicating true loss of the genomic region, the percentage of gene loss was determined in two epiphyseal growth plates and two articular cartilages. From the four cases the mean (7% for hemizygous loss and 2% for homozygous loss) and the s.d. of false positive losses (2% for hemizygous loss and 1% for homozygous loss) were calculated. The mean plus three times the s.d. (15% for hemizygous loss and 6% for homozygous loss) was used as cut-off for evident loss of the gene (Table 1) (Ventura *et al*., 2006; Mohseny *et al*., 2010).

Immunohistochemistry

Immunohistochemistry was performed in all 17 sporadic secondary peripheral chondrosarcomas, as described previously (Pansuriya *et al*., 2011). For comparison, 10 sporadic osteochondromas were used and, for validation, 10 osteochondromas and five secondary peripheral chondrosarcomas both related to multiple osteochondromas were investigated. For EXT1, antigens were retrieved by using microwave heating with EDTA buffer for 10 min and blocked in 5% non-fat milk in phosphate-buffered saline for 30 min and incubated overnight at 4 °C with anti-EXT1 (1:400; Aviva System Biology, San Diego, CA, USA). For 10E4 (1:400; US Biological, Marblehead, MA, USA), the protocol was described earlier (Reijnders *et al*., 2010). Positive controls were growth plate and placenta for the EXT1 antibody, and growth plate and skin for the 10E4 antibody. Each tumor section was divided into two regions: hypertrophic layer—next to the bone of origin, morphologically constituted by medium/large and round cells; and superficial layer—near the perichondrium, constituted by spherical/flattened cells either irregularly arranged or forming clusters. Two pathologists estimated the percentage of positive cells independently (CEA and JVMGB). Results are expressed as the means with a measure of variability (s.d.). Statistical significance was calculated by one-way analysis of variance with Bonferroni's multiple comparison tests by using the SPSS 16.0 software package (IBM, Somers, NY, USA). P values <0.05 were considered significant.

Conflict of interest

The authors declare no conflict of interest.

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