Array-Comparative Genomic Hybridization of Central Chondrosarcoma

Identification of Ribosomal Protein S6 and Cyclin-Dependent Kinase 4 as Candidate Target Genes for Genomic Aberrations

Leida B. Rozeman, PhD[1]
Karoly Szuhai, MD, PhD[2]
Yvonne M. Schrage, MSc[1]
Carla Rosenberg, PhD[2,3]
Hans J. Tanke, PhD[2]
Antonie H. M. Taminiau, MD, PhD[4]
Anne Marie Cleton-Jansen, PhD[1]
Judith V. M. G. Bovée, MD, PhD[1]
Pancras C. W. Hogendoorn, MD, PhD[1]

[1] Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands.

[2] Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.

[3] Department of Biology, Bioscience Institute, University of Sao Paulo, Sao Paulo, Brazil.

[4] Department of Orthopedic Surgery, Leiden University Medical Center, Leiden, the Netherlands.

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Address for reprints: Pancras C. W. Hogendoorn, MD, PhD, Department of Pathology, L-1-Q, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, the Netherlands; Fax: (011) 31 715248158; E-mail: p.c.w.hogendoorn@ lumc.nl

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BACKGROUND. Enchondromas are benign lesions that can occur as solitary tumors or multiple tumors (Ollier disease) and may be precursors of central chondrosarcomas. Recurrent chondrosarcomas can be of a higher grade compared with primary tumors, suggesting possible progression.

METHODS. Genome-wide array-comparative genomic hybridization (CGH) was used to investigate copy number changes in enchondromas and central chondrosarcomas to elucidate both primary genetic events and the events related to tumor progression. Analyses of variance, Student *t* tests, and hierarchical clustering were used for the current analyses. Array-CGH data were compared with complementary DNA (cDNA) and quantitative reverse-transcriptase polymerase chain reaction expression array data.

RESULTS. Genomic imbalances were rare in enchondromas and in grade I chondrosarcomas, whereas they were frequent in high-grade tumors. No genomic imbalances that were specific for Ollier disease were found. The authors identified 22 chromosome regions that were imbalanced in ≥25% of tumors, and 3 of those regions were located on chromosome 12 (12p13, 12p11.21-p11.23, and 12q13, containing among others the PTPRF-interacting protein-binding protein 1 (*PPFIBP1*) gene. Loss of chromosome 6 and gain of 12q12 were associated with higher grade. Comparison of array-CGH with cDNA expression showed correlations for the ribosomal protein S6 (*RPS6*) and cyclin-dependent kinase 4 (*CDK4*) genes.

CONCLUSIONS. In the current study the authors identified genomic regions and new candidate genes (*RPS6*, *CDK4*, and *PPFIBP1*) that were associated with tumor progression and prognosis in patients with high-grade chondrosarcomas. ***Cancer* 2006;107:380–8.** © *2006 American Cancer Society.*

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Chondrosarcoma of bone is a slowly growing, malignant tumor characterized by the formation of cartilage. These tumors have an equal gender incidence and principally occur in adults ages 30 to 60 years. The majority of chondrosarcomas (≈83%) arise centrally within the medullary cavity of bone and are called primary conventional central chondrosarcomas or secondary central chondrosarcomas if they develop from a preexisting enchondroma.[1] The latter can be solitary or multiple tumors in the context of Ollier disease (enchondromatosis). A minority of chondrosarcomas (≈17%) are subclassified as secondary peripheral tumors.[2] Conventional central

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and secondary peripheral chondrosarcoma share similar cytonuclear features, and 3 grades of malignancy are discerned[3] that are correlated with prognosis.[1] However, there is clear evidence for genetic differences between central and peripheral chondrosarcomas.[4,5]

Only a few publications have made a distinction between central and secondary peripheral chondrosarcomas. Those reports indicated that central chondrosarcomas are predominantly near-diploid,[5] whereas peripheral chondrosarcomas are aneuploid.[4–6] A broad range of presumably mostly random genomic alterations is seen in high-grade central chondrosarcomas with some indications that chromosome 9 is affected more often.[6,7]

Cytogenetic studies in which no distinction was made between central and peripheral chondrosarcomas revealed several recurrent aberrations (for review, see Sandberg and Bridge[7]), some of which (e.g., the loss of 13q) reportedly had prognostic impact.[8] Enchondromas show mainly a normal karyotype.[7,9]

Several genes have been tested for the presence of mutations in central chondrosarcomas. For instance, it was observed that *TP53* on chromosome 17p13 was deleted or mutated in some chondrosarcomas, mainly in high-grade tumors.[10] Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) at 9p21 has been studied extensively in central chondrosarcomas. Although cytogenetics, comparative genomic hybridization (CGH), and loss of heterozygosity (LOH) point to the 9p21 region as an important candidate locus for central chondrosarcoma development,[10] mutations and methylation of the *CDKN2A* gene combined with absent p16 protein expression is found only in a subset of mainly high-grade central chondrosarcomas.[11,12]

For enchondromas in the context of Ollier disease, the overall percentage of malignant transformation is much higher, from approximately 25% to 30% per patient with Ollier disease compared with <1% for patients with solitary enchondromas.[1,13] Genetic data on patients with Ollier disease are sparse. LOH was identified at 13q14 and 9p21,[14] whereas cytogenetic studies revealed no[6] or only 1 alteration per tumor (deletion of 1p[15]; inversion on chromosome 9[6]). A mutation in the parathyroid hormone receptor 1 (*PTHR1*) gene was reported in 2 of 6 patients with Ollier disease,[16] although another study in 31 patients with Ollier disease could not confirm this finding.[17]

To identify genomic alterations further, we performed a genome-wide screen by high-resolution array-CGH on solitary and Ollier disease-related enchondromas and conventional central chondrosarcomas. We looked for genomic alterations that were specific for central chondrosarcoma and Ollier disease and those related to tumor progression and prognosis. The results were compared with expression array data and were verified by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis. Tumors from patients with Ollier disease were included to identify putative genetic changes specific for this syndrome.

MATERIALS AND METHODS

Samples

Fresh-frozen samples were collected from the archives of the Department of Pathology of Leiden University Medical Center. In total, 21 tumor samples (3 enchondromas, 7 grade I chondrosarcomas, 7 grade II chondrosarcomas, and 4 grade III chondrosarcomas) were used for the BAC array (Table 1). Chondrosarcomas were either primary conventional or secondary to a radiologic, longstanding, documented enchondroma. No samples that originated from recurrent tumors were used. One of the patients was diagnosed previously with a breast carcinoma.[18] Patient data were obtained by review of clinical charts and radiographs. Grading was performed according to Evans et al.[3] Additional samples were used for verification experiments. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, "*Code for Proper Secondary Use of Human Tissue in the Netherlands*" (Dutch Federation of Medical Scientific Societies).

DNA Isolation

DNA was isolated from samples that contained ≥70% tumor cells estimated in hematoxylin and eosin-stained sections. Four samples were microdissected to enrich the tumor cell percentage.[4] DNA was isolated using a Wizard genomic DNA purification kit (Promega), according to the manufacturer's instructions. The samples were hybridized against a gender-matched control DNA pool that was created from >10 normal blood DNA samples (Promega).

Array-CGH

A BAC/PAC clone set provided by the Welcome Trust Sanger Institute (United Kingdom) was used to construct 1-Mb resolution arrays. Information regarding the full set is available at the Sanger Center mapping data base site, Ensembl (http://www.ensembl.org/). Array production, hybridization, and image-acquisition procedures were performed as described pre-

TABLE 1
Clinicopathologic Data on the Samples Used in Genomic Array Analysis

Sample no.	Gender	Diagnosis (Grade)	Ollier disease	Location	Size (cm)	Follow-up in months (outcome)
L206*	Female	EC	Yes	Phalanx	1 × 0.3 × 0.3	42 (Remission)
L1251*	Male	EC	Yes	Phalanx	Greatest dimension, 1.3	8 (Recurrence)
L892	Male	EC	No	Phalanx	?	2 (Remission, lost to follow-up)
L185	Female	C-CS (I)	No	Femur	Greatest dimension, 1	101 (Remission)
L321	Male	C-CS (I)	No	Femur	7 × 2.6 × 3.2	54 (Remission)
L738*	Female	C-CS (I)	No	Humerus	5.9 × 2.5 × 3.4	59 (Remission)
L761*	Male	C-CS (I)	No	Femur	Greatest dimension, 2.5	16 (Remission)
L803*	Female	C-CS (I)	No†	Femur	4.5 × 2.5 × 2	28 (Remission, DOC)
L853	Female	C-CS (I)	No	Humerus	3 × 3 × 3	31 (Remission)
L1212	Female	C-CS (I)	No	Humerus	6.5 × 5 × 6	16 (Remission)
L172*	Male	C-CS (II)	Yes	Scapula	Greatest dimension, 4	7 (Remission, lost to follow-up)
L130	Male	C-CS (II)	No	Rib	12 × 83.5	23 (Recurrence)
L646	Female	C-CS (II)	No	Femur	11 × 2.5	72 (Remission)
L654*	Male	C-CS (II)	No	Fibula	2.5 × 3.5 × 4.5	17 (Recurrence)
L813*	Male	C-CS (II)	Yes	Humerus	?	2 (Remission, lost to follow-up)
L861*	Male	C-CS (II)	No	SI joint	Greatest dimension, 1	1 (Metastasis, DOD)
L908*	Male	C-CS (II)	No	Humerus	Greatest dimension, 4.8	30 (Remission)
L171*	Male	C-CS (III)	No	Humerus	13 × 10 × 9	6 (Metastasis, DOD)
L795*	Male	C-CS (III)	No	Scapula	11 × 9 × 6.5	8 (Recurrence)
L903	Female	C-CS (III)	No	Femur	21 × 5.5 × 4.2‡	32 (Recurrence)
L1066*	Male	C-CS (III)	No	Humerus	20.3 × 14	16 (Metastasis)

EC indicates enchondroma; C-CS, conventional central chondrosarcoma; ?, unknown size; DOC, died of other causes; SI, sacroiliac; DOD, died of disease.

* Combinational DNA expression data were available for these samples (see Rozeman et al., 2005[22]).

† This patient also had a breast carcinoma (see Odink et al., 2001[18]).

‡ Contaminated margin.

viously.[19] In brief, all samples were labeled with indocarbocyanine-deoxycytidine triphosphates (dCTPs) and hybridized on the slides together with indodicarbocyanine-dCTP-labeled reference DNA. Hybridized slides were scanned with an Agilent DNA microarray scanner.

Data Analysis

Spot intensities were measured by GenePix Pro 4.1 software. Spots with low intensity (<5 times the average of the background) or with nonsimilar values among replicates were excluded.[19] Further analysis and averaging of the triplicate spotted clones was performed by using Excel algorithms[19] and CGH Analyzer MeV (University of Pennsylvania, Abramson Cancer Research Institute[20]). The determination of significant copy number changes detected in array-CGH for tumor samples, in which a substantial part of the genome is altered, is not straightforward. Identification of breakpoints and determination of the true copy number values are problematic because of aneuploidy and admixture of nontumor cells. To facilitate and standardize the data analysis, aCGH-Smooth was used.[21] Clones that showed imbalances in normal controls were considered polymorphic and were excluded from further analysis.

Correlation of Genomic Alterations and Expression Levels

Expression array data were available for 13 of 21 tumors studied.[22] RNA from these tumors was hybridized to a complementary DNA (cDNA) array that contained 8696 cDNA clones, representing common genes and a selection of genes (≈500) with known expression in cartilage and growth plate or involvement in carcinogenesis in general. Of the smallest regions of overlap (SROs), array GCH and cDNA-array data were compared to relate gene expression effects to DNA copy number alterations.

qRT-PCR

Verification of cDNA expression levels was performed as described previously.[22] For ribosomal protein S6 (RPS6), the primers ATTCAGCGTCTTGTTACT CCAC (forward) and CCTTAGCCTCCTTCATTCTCTTG (reverse) were used. In total, 40 samples were used, consisting of phalangeal enchondromas ($n = 7$ samples) and central chondrosarcomas (grade I, $n = 11$ samples; grade II, $n = 7$ samples; and grade III, $n = 9$ samples). Two normal cartilage samples and 4 growth plate samples were used for comparison. Normalization was performed by using G-NORM.

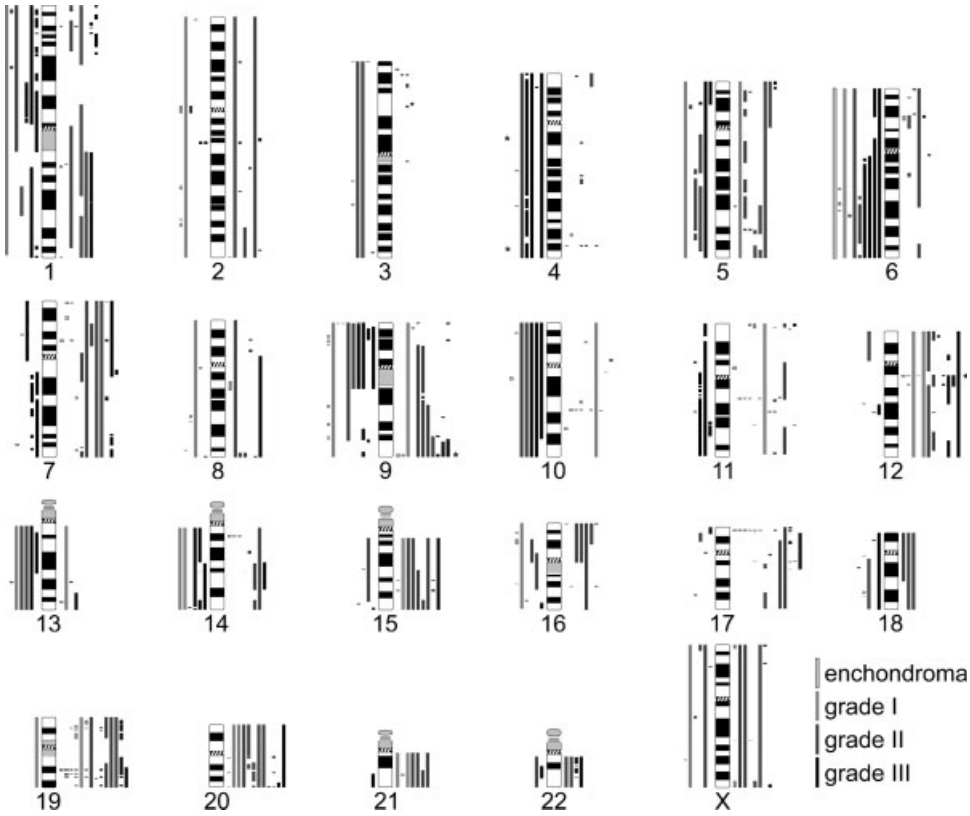

FIGURE 1. Genomic alterations were identified in enchondromas and in conventional central chondrosarcomas. These ideograms show the distribution of numerical aberrations subdivided into enchondroma and the 3 different grades of chondrosarcoma. Gains are shown on the right, and losses are shown on the left. Gain of chromosomal region 12q12 is associated with grade III chondrosarcomas. Polymorphisms have been left out of this figure, except if they were seen as only loss or gain in the tumors (for instance, 17p) Loss of chromosomes 10, 4q13, and 4q34.3 and gain of 9q34 are associated with adverse prognosis. Asterisks mark the locations of 4q13, 4q34.3, 9q34, and 12q12. (Chromosome ideograms were obtained from http://www.pathology.washington.edu/research/cytopages/)

RESULTS

Array-CGH

After hybridization, from 95% to 99% of the spotted clones fulfilled the quality criteria[19] and were used in the analysis. In 10 tumors with statistically significant copy number alterations, the samples showed high normal cell contamination despite efforts to enrich the tumor percentage by microdissection in some instances. In these samples, extra adjustments (aCGH-Smooth) were applied to enable data input with the MeV software package (Samples L761, L1212, L172, L654, L813, L861, L908, L795, L903, and L1066).

Genomic alterations were found mainly in high-grade samples. No DNA copy number alterations were found in Samples 2 through 6 or Sample 8 (2 enchondromas and 4 grade I chondrosarcomas). Overall, grade III chondrosarcomas showed the largest number of DNA imbalances: Enchondromas had an average of 8 imbalances (range, 4–11 imbalances), grade I chondrosarcomas had an average of 18 imbalances (range, 3–26 imbalances), grade II chondrosarcomas had an average of 28 imbalances (range, 9–39 imbalances), and grade III chondrosarcomas had an average of 43 imbalances (range, 26–74 imbalances) (Fig. 1). The SROs (comprising $\geq$3 adjacent clone DNA copy number alterations) present in $\geq$5 different samples are listed in Table 2.

Analysis of Genomic Alterations in Ollier Disease-Related Tumors

Specific Ollier disease alterations were searched for in the 4 samples with Ollier disease (2 phalangeal enchondromas and 2 grade II chondrosarcomas). Of the 2 phalangeal enchondromas, 1 revealed no alterations, whereas the other showed loss of chromosome 6. The 2 grade II chondrosarcomas showed more alterations: gain of almost the entire chromosomes 2, 5, 8, 15, 19, 20, 21, and 22 and gain of parts of chromosomes 1, 5, 7, 9, 16, 17, and 18 in 1 sample

TABLE 2
Smallest Regions of Overlap Amplified or Deleted in at Least 5 Samples*

Chromosome	Type	Region	SRO start clone	SRO stop clone	SRO size (Mb)	No of tumors affected	Genes of interest
1	Del	p36.22-p36.31	RP3-438L4	RP3-438L4	3.2	5	
1	Del	p13.2-p22.1	RP5-1033H22	RP4-770C6	21	5	EXTL2 (=)
5	Amp	q23.3	RP1-241C15	RP1-241C15	0.55	5	P4HA2 (–)
6	Del	p21.32-p25.3 pter	PAC62L11	RP5-1077I5	33	5	Histones
6	Del	q22-q25	RP1-94G16	RP11-13P5	43	8	ESR1 (=), PERP (–)
7	Amp	p12.3-p15.3	RP11-99O17	RP11-21H20	24	5	RALA (–)
7	Amp	p11.2-q11.23	RP5-905H7	RP11-107L23	17	5	GUSB (=)
7	Amp	q36.1-q36.3 qter	RP11-422E4	CTB-3K23	6.4	5	SHH (–), C7orf2 (–)
8	Amp	q24.3 qter	RP5-1056B24	CTC-489D14	5.5	7	MAFA (–)
9	Del	p21.3-p24.1	RP11-527D15	RP11-149I2	15	7	CDKN2A (=), RPS6 (↓)
9	Amp	q33.3-q34.3	RP11-373J8	GS1-135I17	14	7	ABL1 (–), VAV2 (–)
10	Del	pter-q25.2		RP11-426E5	113	5	RSU1 (–), PTEN (–), NDUFB8 (=)
12	Amp	p13	RP11-277E18	RP11-277E18	2.9	6	
12	Amp	p11.21-p11.23	RP11-425D17	RP11-50I19	5.9	5	PTHLH (=), PPFIBP1 (–)
12	Amp	q13	RP5-1057I20	RP11-571M6	11	6	ERBB3 (–), SAS (–), CDK4 (↑), GLI (=)
15	Amp	q25.3	RP11-133L19	CTB-154P1	21	7	FES (–)
19	Amp	p13.11-p13.3	RP11-500M22	CTD-3149D2	17	7	VAV1 (=), JUNB (=), JUND (=)
19	Amp	q13.11-q13.31	CTD-2527I21	RP11-569M1	9.2	7	AKT2 (=)
20	Amp	q11.21	RP11-410N8	RP11-410N8	0.66	9	
20	Amp	q12	RP11-122O1	RP5-892M9	3.2	8	MAFB (–)
20	Amp	q13.33 qter	RP4-563E14	RP13-152O15	4.0	9	BIRC7 (–)
21	Amp	q22.11-q22.3	RP11-410P24	CTB-63H24	19	6	ETS2 (–)

SRO indicates smallest regions of overlap; Del, deletion; Amp, amplification; RP, ribosomal protein; (=), similar expression levels between tumors with the aberration and tumors with normal content; (↑), higher expression levels in tumors with genomic gain versus tumors with normal content; (↓), lower expression levels in tumors with genomic loss versus tumors with normal content; (–) not informative/no data (see Rozeman et al., 2005[22]).

* The size of SRO presented in the table represents the distance between the first and last BAC clone in a recurrent DNA copy number alteration.

and both losses (on chromosomes 1, 3, 4, 6, 9, 10, 13, 15, 16, and 22) and amplifications (on chromosomes 6, 7, 12, 14, 15, 16, 17, 18, and 19) in the other sample. None of the alterations recurred in all 4 samples or even in 3 of 4 samples, nor were any of the changes specific for Ollier disease (i.e., absent in solitary tumors).

Correlation with Grade

The number of aberrations between low-grade and high-grade tumors clearly differed. However, no specific alterations for the grades were seen when all grades were taken in account. Comparison of only grade II with grade III chondrosarcomas identified gain in grade III chondrosarcomas of 4 clones on chromosome 12 ($P < .001$) (Fig. 2), 3 of which were adjacent on chromosomal region 12q12 (≈1.3 Mb).

Previously, it was reported that loss of 6q was associated with impaired metastasis-free survival.[8] Hierarchical clustering solely with clones from chromosome 6 partly separated the grade III chondrosarcomas from the other tumor grades ($P < .05$) (Fig. 3A). Hierarchical clustering on chromosome 10 showed separate clusters for 6 tumors from patients with recurrent disease, metastasis, and/or death from disease ($P < .05$; not shown). Other previously reported chromosomal areas[8] revealed no separate cluster formation for grade and/or outcome.

Correlation with Prognosis

To identify possible regions associated with adverse prognosis, we compared patients who had no evidence of disease with patients who had recurrent disease (excluding patients who had tumors with positive surgical margins), metastasis, and/or who died of disease (minimum follow-up, 1 year, unless an event occurred within the 1st year). Student *t* tests identified 165 BAC clones ($P < .001$) that showed differences between the 2 groups, and subsequent hierarchical clustering identified a near perfect distinction (Fig. 3B) that was correlated with histologic grade. These BAC clones formed consecutive regions on chromosome 10 (from 10pter to 10q25.3), chromosome 4 (≈4.2 Mb on 4q13 and ≈4.4 Mb on 4q34.3), and chromosome 9 (≈6.2 Mb on 9q34).

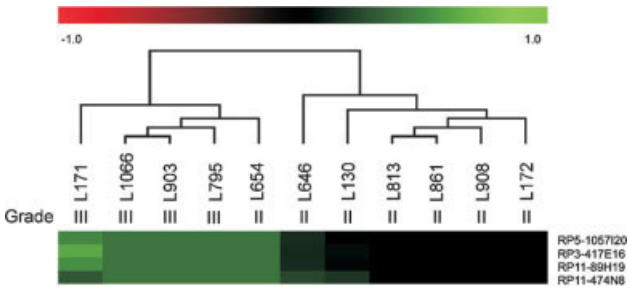

FIGURE 2. Hierarchical clustering of the 4 clones of chromosome 12 is illustrated with differentiation between grade II and grade III chondrosarcomas. Two clusters can be discerned: 1 that contains mainly grade III (amplified) samples and 1 that contains grade II chondrosarcomas.

Correlation of Genomic Alterations and Expression Levels

Subsequent analysis of gene expression profiles (available for 13 tumors) (see Table 1)[22] in the SROs was performed. Gene expression levels of tumors with genomic loss/gain of the SRO were compared with tumors that did not have genomic loss/gain. Overall, the genomic alterations did not result in significant alterations of gene expression levels (Table 1). However, the expression in 2 of ≈300 genes tested in this experiment, *CDK4* (12q13) and *RPS6* (9p21-24), appeared to correlate (Pearson *r*: $P = .000$ and $P = .004$, respectively) with the genomic alteration (Fig. 4A,B). These correlations were verified by qRT-PCR. Tumors with gain of 12q13 ($n = 4$ tumors) showed higher expression levels than tumors without deletion ($n = 8$ tumors; Student *t* test; $P = .01$) (Fig. 4C). Tumors with loss of 9p21-24 ($n = 7$ tumors) showed lower expression levels than tumors without deletion ($n = 5$ tumors), although the *P* value did not reach significance (Student *t* test; $P = .128$) (Fig. 4D). In an extended group of 27 chondrosarcomas, *RPS6* expression was correlated negatively with grade ($P = .006$) (Fig. 4E), with lower expression in high-grade tumors.

DISCUSSION

We used array-GCH to examine DNA imbalances in solitary and Ollier disease-related enchondromas and conventional central chondrosarcomas. It was demonstrated previously in osteosarcoma and other tumor types that array-CGH may uncover previously undetected DNA copy number changes. The current study represents the results of genome-wide copy number screening by array-GCH in chondrosarcomas.

Our array-GCH results are in agreement with genomic changes previously reported at the cytogenetic level (4–10 Mb resolution) by G-banding and chromosome CGH[7,23] with absent or limited numbers of alterations in enchondromas and the presence of several genomic alterations in chondrosarcomas (such as deletion of 9p, gain of parts of chromosome 12, and gain of chromosomes 20 and 21) (see Fig. 1). The number of changes increased in higher grade tumors. Although we did observe recurrent changes on chromosome 8 (8q24.3), we did not observe a recurrence of the previously reported gain at 8q24.12-q24.13.[24]

The genetic changes that underlie the origin of Ollier disease and that distinguish its behavior from solitary tumors remain unclear.[1,13] Comparison of tumors from patients with Ollier disease with one another and with solitary tumors revealed no specific genomic alteration that was present either in all samples or exclusively in Ollier disease samples. Our previous cDNA array analysis also did not reveal differences between Ollier disease-related and solitary enchondromas and chondrosarcomas.[22] Nevertheless, we cannot exclude the possibility that more subtle changes, which cannot be identified with array-CGH, may underlie this syndrome, such as amplifications/deletions $\lessapprox 1$ Mb or balanced rearrangements. However, balanced rearrangements have not been reported in Ollier disease-related tumors or conventional central chondrosarcomas. Another possibility is that Ollier disease may be caused by a genetic point mutation. Previously, we excluded the suggested role of *PTHR1*.[17]

The current study revealed that gains and losses of large DNA segments (≥1 Mb) are not present in all tumors, and 2 of 3 enchondromas and 4 of 7 grade I chondrosarcomas exhibited no detectable alterations in DNA copy numbers. High numbers of alterations, mainly including large DNA segments (arms/whole chromosomes), were present predominantly in high-grade tumors. These alterations can be both recurrent and random, with the latter presumably representing chromosomal instability. However, which alterations are primary and secondary cannot be determined.

By applying a cut-off minimum of SRO presence in 5 samples, we identified 22 SROs (Table 2), ranging from 0.55 Mb to 113 Mb. One SRO involved a deletion of 9p21.3-p24.1. It was reported previously that this region was deleted in chondrosarcomas[7] and contained the locus INK4A/INK4A-ARF, coding for the tumor suppressor genes *CDKN2A*/*CDKN2C*. Previously we[12] and others[11] investigated this locus and found that loss of this locus or the protein was associated with high histologic grade. In the current study, we have identified another gene involved in

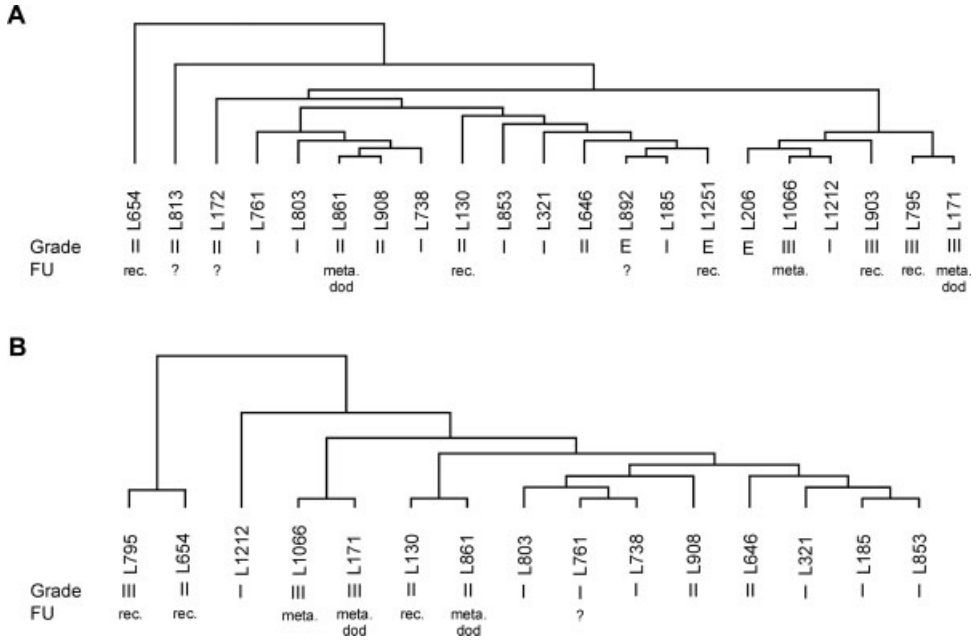

FIGURE 3. Hierarchical clustering of tumors. (A) Hierarchical clustering on clones of chromosome 6. A distinct cluster that contains all grade III chondrosarcomas, 1 grade I chondrosarcoma, and 1 enchondroma is evident and separates the grade III chondrosarcomas from almost all other grade tumors. (B) Hierarchical clustering on the 131 BAC clones, as determined with Student *t* tests for prognosis, shows the distribution of patients who had no evidence of disease compared with patients who had recurrence, metastasis, or death from disease. III indicates grade III chondrosarcoma; II, grade II chondrosarcoma; I, grade I chondrosarcoma; E, enchondroma; FU, follow-up; rec., recurrence; ?, lost to follow-up; meta., metastasis; dod, dead of disease.

the 9p deletion, *RPS6*, in which low expression levels were correlated significantly with the deletion. In addition, significantly lower expression of *RPS6* was found in high-grade tumors. The RPS6 protein belongs to the S6E family of ribosomal proteins, which are regulated by phosphorylation. The protein contributes to the control of cell growth and proliferation through the selective translation of particular classes of mRNA[2], and, thus, is a candidate tumor suppressor gene in chondrosarcomas.

Chromosome 12 contained 3 SROs. Gain of chromosomal region 12q13 was found in 6 tumors (2 grade I, 1 grade II, and 3 grade III chondrosarcomas). This region has been reported previously in chondrosarcomas and other sarcomas.[7,26] Several genes in this region have been identified as important for tumorigenesis. For instance, sarcoma-amplified sequence (*SAS*), *CDK4*, and glioma-associated oncogene homologue (*GLI*) are located in the amplified zone. Two other often-implicated genes in sarcomas, high-mobility group AT-hook 2 (*HMGA2*) and *MDM2*, are located just outside the region we identified. RNA expression analysis revealed higher expression levels of *CDK4* in tumors in which this region was amplified (Fig. 4), as also reported for other tumors.[27] Expression profiles of other genes in this region were not correlated with genomic gain. However, additional studies are required to confirm these findings in other tumor samples.

Along with the correlation between the genomic aberration and expression of *RPS6* and *CDK4*, no other genes that were present in the cDNA array and that were located in the identified SROs showed a significant correlation. This may be explained in part by the regulation of expression, which also can be regulated at the RNA or protein level, and not only by haploinsuficiency. Another reason that may obscure results from expression analysis is the tumor content. Although we only used samples with a tumor content $>70\%$ on hematoxylin and eosin-stained sections, the results from aCGH Smooth revealed that, in some sections, the actual percentage most likely was lower. In those samples, we observed significant alteration of the genomic content; however, prior to smoothing of the data, the threshold for amplification or deletion was not reached. Whereas, in the genomic array, the alterations still could be observed, the expression array data could be obscured more by the normal content, resulting in an underestimate of the actual differences in expression.

Chromosome 1 contained 2 SROs that were deleted (1p36.22-p36.31 and 1p13.2-p22.1), as reported previously.[7] The region 1p13.2-p22.1 contains, among others, *EXTL2*. In patients with multiple osteochon-

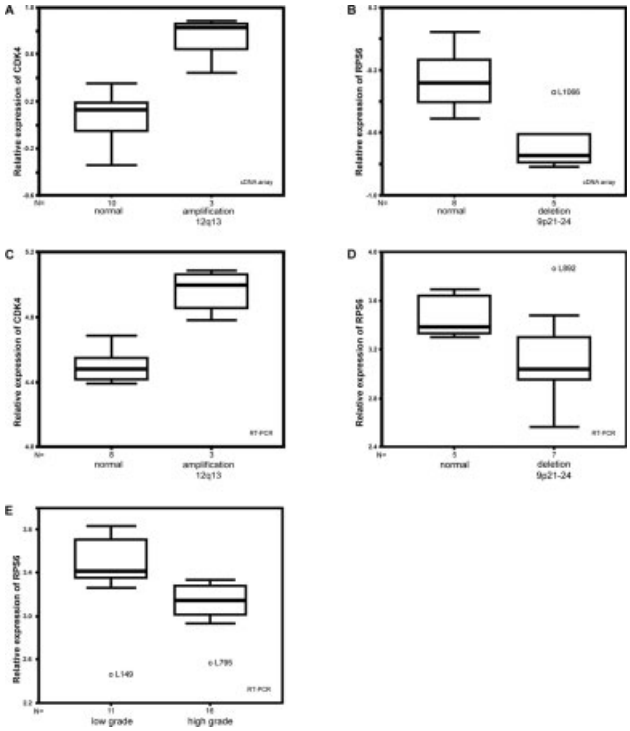

FIGURE 4. Correlation RNA expression with amplifications/deletions identified by array comparative genomic hybridization: cyclin-dependent kinase 4 (CDK4) from a complementary DNA (cDNA) array (A); ribosomal protein S6 (RPS6) from a cDNA array (B); CDK4 from quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis (C); RPS6 from qRT-PCR (D); and correlation of RPS6 expression with histologic grade from qRT-PCR analysis (E).

dromas, which is a hereditary syndrome with multiple exostoses that may transform into secondary peripheral chondrosarcomas, 2 other members of this gene family are involved. These patients have mutations in the *EXT1* or *EXT2* genes that encode for the proteins involved in the heparan-sulphate side-chain elongation. *EXTL2* is homologous to *EXT1* and *EXT2*, and it initiates heparan-sulphate synthesis.[2] Because conventional central chondrosarcomas resemble the secondary peripheral chondrosarcomas histologically, *EXTL2* may be a target for deletion. However, cDNA microarray analysis revealed no difference in RNA expression of *EXTL2* between the tumors that contained a deletion of this SRO and those without this deletion.

Gain of chromosomal region 12p11.21-p11.23 was observed in 5 tumors (2 grade I, 1 grade II, and 2 grade III chondrosarcomas). One of the genes in this region is parathyroid hormone-like hormone (*PTHLH*), which is an important gene for chondrocyte growth and differentiation. Previously, we reported that this protein is expressed in almost all enchondromas and chondrosarcomas.[29,30] The region also contains PTPRF-interacting protein-binding protein 1 (*PPFIBP1*), which reportedly interacts with S100A4, a calcium-binding protein related to tumor invasiveness and metastasis.[31] However, for these genes, no cDNA microarray data were available.

In general, most genomic alterations were found only in high-grade tumors. Analysis of the different grades identified 2 regions that were capable of partly separating the different grades. Copy number gain of region 12q12 separated grade I from grade II chondrosarcomas in hierarchical clustering (Fig. 2). This region (≈1.3 Mb) contains, among others, histone deacetylase 7A (*HDAC71*) and SUMO1/sentrin specific protease 1 (*SENP1*). SENP1 is capable of reducing the deacetylase activity of HDAC1.[32] Chromosome 6 contains several histone genes (deleted SRO, 6p22-p21.3), and hierarchical clustering of the clones on this chromosome also partly separated the grade III chondrosarcomas from the other chondrosarcomas (Fig. 3A). Alterations in 6p21 and 12q12, therefore, may affect genome stability through histones, resulting in damage of DNA. Two other regions that contained a cluster of histone genes, 1q21 and 1q42, were not affected.

Investigating a potential correlation with prognosis revealed that patients who have tumors with loss on chromosome 4 (4q13 and 4q34) and chromosome 10 and gain on chromosome 9 (9q34) may have a poor prognosis. These aberrations also correlate with increase in histologic grade and tumor size. However, the results reported here should be tested further in a separate group of tumors to confirm their validity. In our cluster analysis, 1 tumor performed somewhat unexpectedly, clustering together with tumors that carried an adverse prognosis, whereas no recurrence or metastasis was reported in that patient. However, follow-up for this patient was available only for 16 months, which is relatively short for chondrosarcomas, because recurrences still may occur within 5 years after surgery, and metastases may occur after 10 years.

In conclusion, recurrent alterations (SROs) were found in chondrosarcomas along with nonspecific genomic instability, predominantly in high-grade chondrosarcomas. These alterations involve chromosome 12, multiple regions of which are amplified (3 SROs: 12p13, 12p11.21-p11.23, and 12q13), and chromosome 6. Therefore, we hypothesize that these parts of the chromosome play an important role in the tumor progression of chondrosarcoma. The expression of *CDK4* was correlated with the genomic alteration on 12q13. For the well known loss of chromosomal region 9p21, we propose *RPS6* as a possible other gene of interest in addition to *CDKN2A*.

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