Recurrent 12q13-15 chromosomal aberrations, high frequency of isocitrate dehydrogenase 1 mutations, and absence of high mobility group AT-hook 2 expression in periosteal chondromas

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Abstract. Periosteal chondroma is a benign cartilage tumor that accounts for <2% of chondromas. In the present study, four cases of periosteal chondromas were cytogenetically investigated and studied for the expression of high-mobility group AT-hook 2 (*HMGA2*), mutations in codons 132 of isocitrate dehydrogenase *(IDH)*1 and 172 of *IDH2*; mutations -C228T and -C250T in the promoter region of telomerase reverse transcriptase (*TERT*); and for methylation in the promoter regions of *O*-6-methylguanine-DNA methyltransferase (*MGMT*) and cellular retinol binding protein 1 (*CRBP1*). Chromosome aberrations of 12q13-15 were found in two out of the four tumors, while two had a normal karyotype. Two periosteal chondromas carried the mutation IDH1R132C (CGT>TGT), and two carried the mutation IDH1R132L (CGT>CTT). However, none of the four tumors had methylated *MGMT* and *CRBP1* promoters or mutations at codon 172 of *IDH2*. In addition, -C228T and -C250T mutations were not present in the promoter region of *TERT*, nor was *HMGA2* demonstrated to be expressed. The present study indicated that in periosteal chondromas, the involvement of 12q13-15 in structural rearrangements may be recurrent but that *HMGA2* is not expressed. Additionally, the periosteal chondromas investigated in the study carried a heterozygous IDH1R132 mutation, the *MGMT* and *CRBP1* promoters were not methylated, and -C228T and -C250T mutations in the promoter region of *TERT* were absent.

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Introduction

Periosteal chondroma is a benign cartilage tumor that accounts for <2% of chondromas. This type of tumor grows in the periosteal region and frequently erodes the underlying bone cortex. Periosteal chondroma can lead to sclerosis of underlying bone, and the formation of a 'dished' area under the tumor with a 'buttress' or peripheral wall of reactive bone at its edge. The bone cortex may be abnormal or thinned adjacent to the tumor. Periosteal chondromas typically present in the second and third decades of life with a male predilection (2:1), are usually asymptomatic, and are commonly identified incidentally on radiographs obtained for other reasons (1). Common sites of occurrence include the proximal humerus, proximal and distal femur and the phalanges of the hands and feet (1). A tender swelling or the feeling of a mass may bring the lesion to clinical attention. Periosteal chondroma may be confused with chondrosarcoma or periosteal and parosteal osteosarcoma. Therefore, diagnosis requires a thorough investigation that includes radiological examination and biopsy. Simple excision of the tumor is often curative.

Previous studies have described clonal cytogenetic abnormalities in 7 periosteal chondromas, but no specific aberration pattern has been detected (2-4). In other studies, *IDH1* mutations were observed in 6 out of the 8 periosteal chondromas examined (5,6). The present study investigated cytogenetic and molecular genetic data in 4 cases of periosteal chondroma.

Materials and methods

Ethics statement. The study was approved by the regional ethics committee (Regional komité for medisinsk forskningsetikk Sør-Øst, Norge, http://helseforskning.etikkom.no).

Patients. A total of 3 females and 1 male, between 8 and 47 years of age, were included in the study. Clinical data are presented in Table I.

Chromosome banding analysis. Samples from the surgically resected tumors were mechanically and enzymatically disaggregated and short-term cultured as described previously (6). The cultures were harvested and the chromosomes were G-banded using Wright's stain (Sigma-Aldrich; St Louis, MO, USA) (7). The subsequent cytogenetic analysis and karyotype description followed the recommendations of the International System for Human Cytogenetic Nomenclature (8).

Polymerase chain reaction (PCR) for IDH1 and IDH2 mutations. Genomic DNA was extracted using a Maxwell 16 Research Instrument system and a Maxwell 16 Tissue DNA Purification Kit (Promega Corporation, Madison, WI, USA). For the detection of possible *IDH1* and *IDH2* mutations, quantitative (q)PCR with high resolution melt curve analysis (HRM) was performed followed by Sanger sequencing to confirm positive HRM screens (9). As positive and negative controls, plasmids containing the wild-type IDH1R132 and IDH2R172 and the mutated IDH1R132H and IDH2R172M were used. The 20-μl PCR mixture contained 10 μl Precision Melt Supermix (Bio-Rad Laboratories AB, Oslo, Norway), 0.2 μM of each of the forward and reverse primers (custom-made; Invitrogen Life Technologies, Carlsbad, CA, USA), and 20 ng of genomic DNA. To identify possible IDH1R132 mutations, the following primers were used: The forward primer was IDH1-F1, 5'-TCA GAG AAG CCA TTA TCT GCA-3' and the reverse was IDH1-R1, 5'-AAT CAC ATT ATT GCC AAC ATG A-3'. To identify possible IDH1R172 mutations the following primers were used: The forward was IDH2-F1New, 5'-TAG TCC CTG GCT GGA CCA-3' and the reverse was IDH2-R1New, 5'-TGC CCA GGT CAG TGG ATC-3'. The PCRs were conducted on a CFX96 Touch Real-Time PCR Detection System using the Bio-Rad CFX Manager 2.1 software (Bio-Rad Laboratories AB). The PCR cycling and melt curve conditions were as follows: An initial denaturation at 95˚C for 2 min followed by 40 cycles of 10 sec at 95˚C, 30 sec at 55˚C (plus plate read). Next, the melt curve started with denaturation at 95˚C for 30 sec and annealing at 60˚C for 1 min. The melt curve program then continued from 65˚C to 95˚C with increments of 0.2˚C for 10 sec, plus a final plate read. The HRM of the data was made using the Precision Melt Analysis software, version 1.2 (Bio-Rad Laboratories AB). To confirm positive HRM screens, Sanger sequencing was performed as follows: The PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel GmbH, Düren, Germany) and direct Sanger sequencing was performed using the LIGHTrun Sequencing service (GATC Biotech AG, Konstanz, Germany; www.gatc-biotech.com/en/products/sanger-services/lightrun-sequencing.html). BLAST software (blast.ncbi.nlm.nih.gov/Blast.cgi) was used for computer analysis of the sequence data.

PCR for -C228T and -C250T mutations. In order to detect the possible -C228T and -C250T mutations in the promoter region of telomerase reverse transcriptase (*TERT*), which correspond to positions 124 and 146 bp, respectively, upstream of the *TERT* ATG start site (10), PCR was conducted as follows: The 25 μl PCR volume contained 1X PrimeSTAR GXL Buffer (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), 200 μM of each dNTP, 0.4 μM of each of the primers (custom-made; Invitrogen Life Technologies), 1.25 units of PrimeSTAR GXL DNA polymerase and 20 ng of genomic DNA. The primer sequences were as follows: TRETpromF2, 5'-GCC GGG CTC CCA GTG GAT TCG-3' and the reverse primer TRETpromR2, 5'-GGC TTC CCA CGT GCG CAG CAG-3'. The PCR was conducted on a C-1000 Thermal Cycler (Bio-Rad Laboratories AB) with an initial denaturation at 94˚C for 30 sec, followed by 35 cycles of 7 sec at 98˚C, 90 sec at 68˚C, and a final extension for 5 min at 68˚C. A total of 4 μl PCR products were stained with GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA), electrophoresed through a 1.0% agarose gel (certified molecular biology agarose (Bio-Rad Laboratories AB) and images for analysis were captured using a camera. The remaining amplified products were purified using the NucleoSpin Gel and PCR Clean-up kit followed by direct Sanger sequencing as above.

Methylation analysis. Methylation analysis of the *O*-6-methylguanine-DNA methyltransferase (*MGMT*) promoter was performed using the primers and PCR conditions described by Esteller *et al* (11). Methylation analysis of the cellular retinol binding protein 1 (*CRBP1*) promoter was performed using the primers and PCR conditions described by Jerónimo *et al* (11).

Total RNA and cDNA synthesis. Total RNA was extracted using an miRNeasy mini kit and QIAcube (Qiagen AB, Sollentuna, Sweden) according to the manufacturer's recommendations. Human Universal Reference Total RNA was used as a control (Clontech Laboratories, Inc., Mountainview, CA, USA); it is a mixture of total RNAs from male and female adult human tissues selected to represent a broad range of expressed genes. Reverse transcription (RT) was conducted as follows: 400-500 ng total RNA was reverse transcribed in a 20 μl reaction volume using iScript Advanced cDNA Synthesis Kit for RT-qPCR according to the manufacturer's instructions (Bio-Rad Laboratories AB). Next, the cDNA was diluted to the equivalent of 10 ng/μl of RNA and 2 μl was used as a template in subsequent PCR assays.

High-mobility group AT-hook 2 (HGMA2) expression analysis. To assess *HMGA2* expression in the periosteal chondroma tissue samples by PCR, the 25 μl PCR volumes contained 12.5 μl Premix Taq polymerase (Takara Bio Europe SAS) 2 μl diluted cDNA and 0.4 μM of each of the forward and reverse primers (custom-made; Invitrogen Life Technologies). The PCRs were conducted on a C-1000 Thermal Cycler. The PCR conditions were as follows: An initial denaturation at 94˚C for 30 sec followed by 35 cycles of 7 sec at 98˚C, 120 sec at 68˚C, and a final extension for 5 min at 68˚C. The primer sequences used for the amplification of transcripts of *HMGA2* exons 1-3 were as follows: HMGA2-846F1, 5'-CCA CTT CAG CCC AGG GAC AAC CT-3' and HMGA2-1021R, 5'-CCT CTT GGC CGT TTT TCT CCA GTG-3'. The quality of the cDNA synthesis was assessed by amplification of a cDNA fragment of the *S100A10* gene, which is expressed in chondrocytes (12). The following primer sequences were used:

Table I. Cytogenetic and molecular analysis of four periosteal chondromas.

Case	Gender	Age	Location	Tumor size (cm)	Karyotype	*IDH1* mutation	*IDH2* R172 mutation	*TERT* promoter -C228T/-C250T	*MGMT* promoter methylation	*CRBP1* promoter methylation	*S100A10* expression (mean Cq)	*HMGA2* expression (mean Cq)
1	F	8	Humerus	6.0	46,XX	R132C (CGT>TGT)	No	No/No	No	No	Unknown	Unknown
2	F	21	Humerus	2.5	46,XX	R132C (CGT>TGT)	No	No/No	No	No	28	-/-
3	F	22	Femur	4.3	46,XX,t(12;13)(q13;p11) [10]/46,XX[3]	R132L (CGT>CTT)	No	No/No	No	No	34	-/-
4	M	47	Humerus	2.8	47,XY,+8[11]/45~87<2n>, t(5;8)(q31;q12~13),t(9;10)(p13;q11),del(12)(q15q23), -13,add(22)(p11)[cp3]/46,XY[3]	R132L(CGT>CTT)	No	No/No	No	No	24	-/-

IDH, isocitrate dehydrogenase; *TERT*, telomerase reverse transcriptase; *MGMT*, *O*-6-methylguanine-DNA methyltransferase; *CRBP1*, cellular retinol binding protein 1; *HMGA2*, high-mobility group AT-hook 2.

S100A10-555F, 5'-TTC ACA AAT TCG CTG GGG ATA AAG G-3' and S100A10-840R, 5'-GAT TCC TTA AGC GAC CCT TTG GGA C-3'. The PCR for the amplification of transcripts of *HMGA2* were repeated three times. A total of 3 μl PCR products were stained with the GelRed Nucleic Acid Gel Stain, electrophoresed through a 1.0% agarose gel and images for analysis were captured using a camera.

qPCR was also conducted to determine the expression level of *HMGA2*. The TaqMan Gene Expression Assays Hs00171569_m1 (which probes HMGA2 exons 1-2), Hs00971725_m1 (which probes HMGA2 exons 4-5) and the *S100A10* gene (control) assay Hs00237010_ml (Applied Biosystems Life Technologies, Foster City, CA, USA) were used. Four replicates of each sample and endogenous control were used to ensure reliability. The 20 μl PCR reaction volume contained 1X TaqMan Universal Master Mix II with uracil n-glycosylase, 1X TaqMan gene expression mix, and cDNA (equivalent to 10 ng RNA). The PCR was run on the CFX96 Touch Real-Time PCR Detection System. The thermal cycling included an initial step at 50˚C for 2 min, followed by 10 min at 95˚C, 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The Bio-Rad CFX Manager software, version 2.1, was used to analyze the data and to calculate the mean quantification cycle (mean Cq).

Results

Clonal chromosomal aberrations. The clinical, cytogenetic and molecular analysis data are presented in Table I. The histologic examination of case 1 showing a nodular tumor of benign chondrocytes covered by periosteum is presented in Fig 1A. Clonal chromosomal aberrations were identified in two out of the four examined periosteal chondromas, while the other two tumors presented with a normal karyotype. Rearrangements of chromosome 12 q13-15 were observed in the two tumors with chromosome abnormalities (Table I). Case 3 has a simple balanced translocation between chromosomes 12 and 13, t(12;13)(q13;p11), as the sole aberration. The tumor in case 4 had two cytogenetically unrelated clones. The first clone displayed trisomy of chromosome 8, while the second clone had numerical and structural aberrations, including a deletion in the long arm of chromosome 12, del(12)(q15q23) (Table I, Fig. 1B).

Molecular genetic analyses. All four periosteal chondromas had the R132 mutation in *IDH1*, whereas none had mutations in codon 172 of *IDH2* (Table I, Fig. 1C). Two tumors carried an IDH1R132C (CGT>TGT) mutation while the other two carried an IDH1R132L (CGT>CTT) mutation. None of the periosteal chondromas had methylated *MGMT* or *CRBP1* promoters or mutations at positions -C228T and -C250T in the promoter region of *TERT* (Table I).

The total RNA was extracted from the three tumors (cases 2-4) and a 310-bp *S100A10* cDNA fragment was amplified in all of them, indicating that the synthesized cDNA was of good quality (Fig. 1D). RT-PCR with the primer set HMGA2-846F1/HMGA2-1021R did not amplify any cDNA fragments from the periosteal chondromas, whereas an amplified *HMGA2* cDNA fragment was observed in the positive control (Fig. 1D). This result indicated that the *HMGA2*

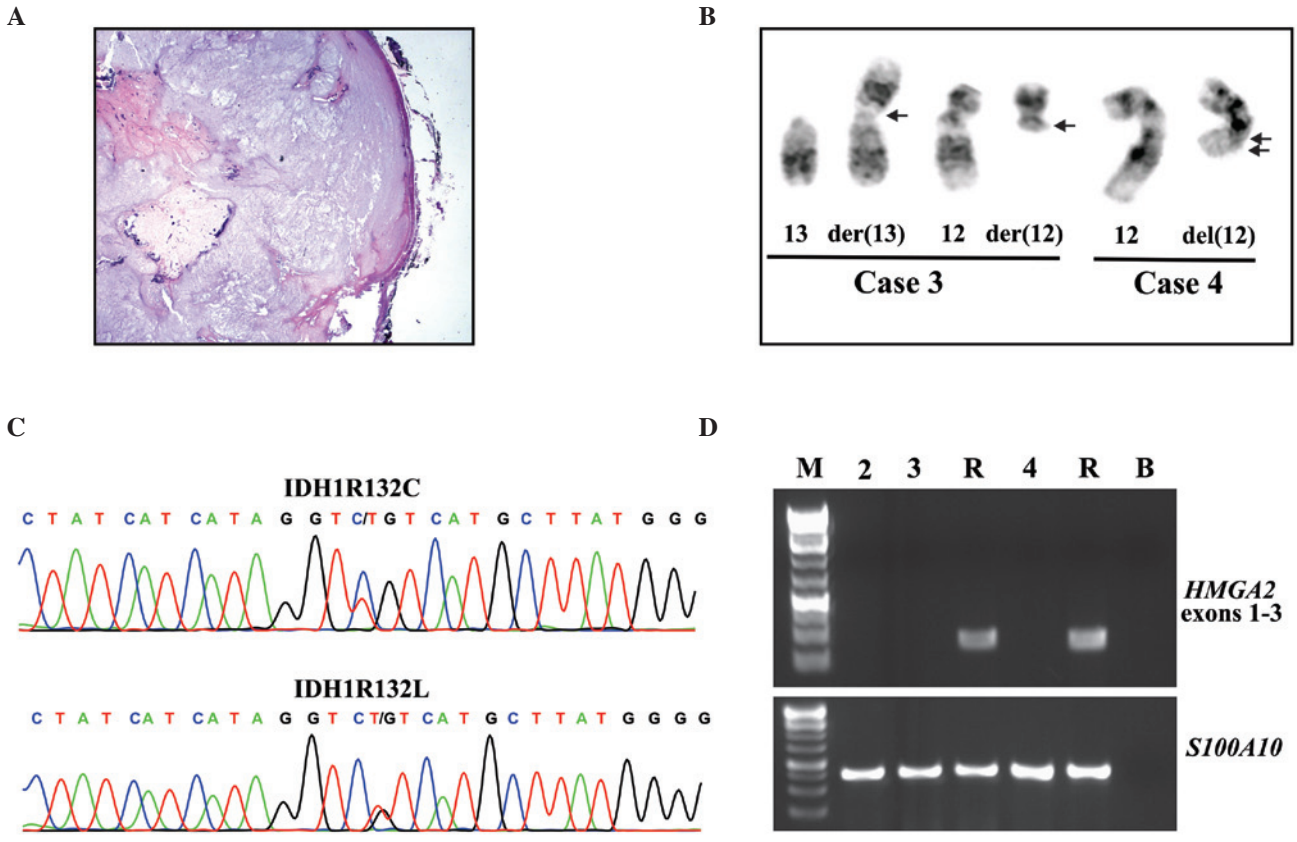

Figure 1. Histology, cytogenetics, *HMGA2* expression, and *IDH1* mutations in periosteal chondromas. (A) Histologic examination of case 1 exhibiting a nodular tumor of benign chondrocytes covered by periosteum. (B) Partial karyotypes presenting chromosome aberrations der(13)t(12;13)(q13;p11) and der(12) t(12;13)(q13;p11) in case 3, and del(12)(q15q23) in case 4, with the corresponding normal chromosome homologs; breakpoint positions are indicated by arrows. (C) Partial sequence chromatogram displaying the CGT>TGT (IDH1R132C; cases 1 and 2) and CGT>CTT in (IDH1R132L; cases 3 and 4) *IDH1*. (D) Polymerase chain reaction results demonstrating the amplification of *HMGA2* exons 1-3 and *S100A10*. M, GeneRuler 1 Kb Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA); R, cDNA synthesized from Human Universal Reference Total RNA; B, no RNA in cDNA synthesis. *HMGA2*, high-mobility group AT-hook 2; IDH, isocitrate dehydrogenase.

transcript was not expressed in the three examined periosteal chondromas. Similar results were also obtained with qPCR: For the control Human Universal Reference Total RNA, the mean Cq was 25, 30 and 31 for *S100A10*, HMGA2 exons 1-2, and *HMGA2* exons 4-5, respectively (data not shown). The mean Cq for *S100A10* was 28, 34 and 24 for cases 2, 3 and 4, respectively, indicating that the gene expression assay was successful. However, there were no mean Cq values for *HMGA2* exons 1-2/*HMGA2* exons 4-5 since the TaqMan gene expression assays did not amplify any product (Table I). These results further indicate that *HGMA2* was not expressed in the periosteal chondromas examined in the present study.

Discussion

To the best of our knowledge, prior to the present study, karyotypic information on periosteal chondromas was restricted to seven cases and no consistent abnormality was recognized (4). Changes observed in periosteal chondromas in the previous study included loss of chromosome 6 and rearrangements of 2q37, 4q21-25, 11q13-15 and 12q13 (4). The present study demonstrated that the q arm of chromosome 12 was involved in two out of the two periosteal chondromas with an informative karyotype. Taken together, the data from the present study and those previously reported (3) demonstrate that rearrangements of 12q13-15 may be recurrent in this type of tumor. The involvement of the chromosome bands 12q13-15 in periosteal chondromas may not be random, particularly as they are frequently aberrant in benign connective tumors, such as lipoma and leiomyoma (14). In addition, 12-q rearrangements that result in the transcriptional activation of the *HMGA2* gene, were reported in mesenchymal chondromas by Dahlén *et al* (15). They also demonstrated that *HMGA2* was expressed in four of six soft tissue chondromas, of which three tumors possessed a truncated (exons 1-3) transcript and one possessed the full-length (exons 1-5) transcript of *HMGA2*. In addition, Dahlén *et al* (15) observed that *HMGA2* was expressed in two skeletal chondromas: One of the tumors, which possessed a pericentric inversion of chromosome 12, expressed a truncated transcript of *HMGA2*; whereas the other case, which had no visible involvement of 12q by cytogenetic analysis, expressed the full-length *HMGA2* transcript (15). The description of these two tumors was inconclusive as to whether they were enchondromas or periosteal chondromas.

In the present study, neither conventional RT-PCR nor qPCR demonstrated expression of *HMGA2* in the examined periosteal chondromas.

The mutation analysis of *IDH1* and *IDH2* revealed that the tumors carried heterozygous *IDH1* mutations at R132, which are in accordance with previous observations that a majority of periosteal chondromas carry heterozygous mutations at R132 of *IDH1* (5,6). From the results of the present study it can be hypothesized that the rearrangements and expression of *HMGA2* are mutually exclusive with *IDH1* and *IDH2* mutations in periosteal chondromas.

The R132L mutation in *IDH1* that was observed in two of the periosteal chondromas in the current study has also been identified previously by Amary *et al* (6), who observed this mutation in central low-grade cartilaginous tumors (1/23), chondrosarcomas GII and GIII (3/23) and dedifferentiated chondrosarcomas (2/13). Of the eight periosteal chondromas previously analyzed for the presence of mutations in *IDH1*, mutations were observed in six: Four possessed IDH1R132C (CGT>TGT) and two possessed R132S (CGT>AGT) (4,5). Viewing the present and previously published data in concert (5,6), mutations in codon 12 of *IDH1* have been present in the majority (83%, 10/12) of examined periosteal chondromas. Of those mutations in the *IDH1,* R132C comprises 60% (6/10), whereas R132L and R132S are observed in 20% (2 cases each). However, R132H, the most common mutation in gliomas, has not been reported thus far in periosteal chondromas. The exact role that *IDH1* mutations serve in neoplasia is not fully understood, however it has been demonstrated that presence of a heterozygous R132H mutation induces genome-wide alterations in DNA methylation, leading to hypermethylation and hypomethylation (16,17). *IDH1* mutations are associated with *MGMT* and *CRBP1* promoter methylation in certain brain tumors (18-21). However, this association was not observed in the periosteal chondroma tissue specimens in the present study, since none of them possessed methylated MGMT or CRBP1 promoters, as determined using the MSP methodology (11,12).

Mutations at positions -C228T and -C250T in the promoter region of *TERT*, which correspond to 124 and 146 bp upstream of the *TERT* ATG start site, have been reported in gliomas and other tumors (10) but have yet to be studied in periosteal chondromas. *TERT* promoter mutations have been demonstrated to be inversely associated with *IDH1*/*IDH2* mutations in tumors of the nervous system (22,23). In the present study, none of the four periosteal chondromas had -C228T or -C250T mutations. In conclusion, the present study revealed that the involvement of 12q13-15 in structural chromosomal aberrations is a relatively recurrent and common event in periosteal chondromas, that *HMGA2* is not frequently expressed, that the majority of periosteal chondromas carry heterozygous IDH1R132 mutation, that *MGMT* and *CRBP1* promoters are not methylated, and that neither -C228T nor -C250T is present in the promoter region of *TERT*. Rearrangements of *HMGA2* resulting in fusion genes and expression of *HMGA2*-fusion transcripts appear to be mutually exclusive with *IDH1* and *2* mutations.

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