Original Paper

Molecular analysis of the INK4A/INK4A-ARF gene locus in conventional (central) chondrosarcomas and enchondromas: indication of an important gene for tumour progression

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Abstract

Loss of heterozygosity (LOH) at chromosomal band 9p21 is one of the few consistent genetic aberrations found in conventional chondrosarcoma. This locus harbours two cell-cycle regulators, CDKN2A/p16/INK4A and INK4A-p14ARF, which are inactivated in various human malignancies. It was therefore hypothesized that this locus also plays a role in the development of chondrosarcoma and this locus was investigated at protein, genetic, and epigenetic levels. Loss of p16 protein expression was detected by immunohistochemistry in 12 of 73 central chondrosarcomas and it correlated with increasing histological grade (p = 0.001). Loss of p16 protein expression was not found in 51 enchondromas, which are presumed to be potential precursors of conventional central chondrosarcoma. LOH at 9p21 was found in 15 of 39 chondrosarcomas (38%) but it did not correlate with loss of p16 protein expression. SSCP analysis of p16 did not reveal any mutations in 47 cases. Also, p14 was not the target of LOH, since it gave no aberrant bands on SSCP. To investigate whether an epigenetic mechanism was operating, methylation-specific PCR was used to look at p16 promoter methylation, which was identified in 5 of 30 tumours. However, this did not correlate with protein expression, or with LOH at 9p21. Cytogenetic data were available in a subset of cases. All tumours that showed chromosome 9 alterations also showed LOH and loss of INK4A/p16 protein expression. It is concluded that although some alterations were found at the DNA level and at the promoter expression level, the lack of correlation between LOH, promotor methylation, and protein expression indicates that a locus other than CDKN2A/p16 must be the target of LOH at 9p21. The correlation between INK4A/p16 protein expression and tumour grade, and the retention of expression in enchondromas, indicates that loss of INK4A/p16 protein expression may be an important event during tumour progression from enchondroma to conventional central chondrosarcoma, and in the progression in grade after recurrence of chondrosarcoma.

Keywords: bone neoplasm; cartilaginous tumours; CDKN2A/p16; chondrosarcoma; enchondroma

Introduction

Chondrosarcomas are malignant cartilage-forming bone tumours, with an occurrence of about 1 in 100 000 in the general population [1,2]. There are three grades of malignancy that correlate with prognosis [3]. Based on their location in the bone, there are two major subtypes of conventional chondrosarcomas. The majority of the tumours are localized in the medullary cavity of long bones, so-called conventional central chondrosarcomas. Enchondroma may be its benign precursor in rare cases. Malignant transformation of enchondroma is, however, a rare event, especially in enchondroma of the phalanx, a site where chondrosarcomas are extremely rare, despite the fact that hands and feet harbour 35% of enchondromas [4]. A hereditary form of conventional central chondrosarcomas is not known. Enchondromas arising in the context of Ollier’s disease and Maffucci syndrome, two non-hereditary conditions displaying multiple enchondromas, carry a much higher risk of malignant transformation, ie 30–35% (OMIM #166 000) [5]. A minority of chondrosarcomas arise secondary to a pre-existing osteochondroma and are termed secondary peripheral chondrosarcomas [1,2,6]. We have shown that
secondary peripheral chondrosarcomas are characterized by gross chromosomal instability reflected by a high percentage of LOH with almost all chromosomes involved, and a broad ploidy range [7–9]. In contrast, genetic aberrations in conventional central chondrosarcomas are sparse and the tumours are often (peri)diploid. This suggests that only limited genetic alterations are sufficient for tumourigenesis in conventional central chondrosarcomas. Previously, we demonstrated LOH at 9p21 in 3 of 12 (25%) conventional central chondrosarcomas. Moreover, a cytogenetic study of 7 out of 16 conventional central chondrosarcomas showed cytogenetic aberrations, of which five involved chromosome 9p12–22 [7]. These data suggest that the 9p21 region may be important in its tumourigenesis. At chromosome 9p, an important region is the INK4A/ARF locus (9p21), encoding the proteins INK4A/p16 and INK4A/p14ARF. The activation of INK4A/p16 and INK4A/p14ARF results in blockage of cell-cycle progression and inhibition of cellular proliferation. Mutational or transcriptional inactivation of the CDKN2A/p16 and p14 genes can lead to uncontrolled growth. CDKN2A/p16 is inactivated in several tumour types, including bone sarcomas [10–13], either by homozygous deletion, mutation or extensive de novo methylation-inhibiting gene transcription [14]. To investigate whether this locus is the target of the LOH at chromosome 9p in conventional central chondrosarcomas, we present the results of immunohistochemical, LOH analysis, SSCP analysis (INK4A/p16 and INK4A/ARF), and promoter methylation status of the INK4A locus on a large well-characterized patient series.

Materials and methods

Patient material

All tissue samples were handled in a coded fashion. The specimen codes indicating patient data were only available via physicians involved in the diagnosis and treatment of these patients. All procedures were performed according to local ethical guidelines. Table 1 shows the clinicopathological data of the patients in this study. In total, 73 tumour specimens from patients treated between 1986 and 2001 with a histological diagnosis of conventional central chondrosarcoma were retrieved from our files. Peripheral, dedifferentiated, mesenchymal, juxtacortical, and clear-cell chondrosarcomas were excluded. Conventional central and secondary peripheral chondrosarcomas were distinguished on the basis of accepted clinicopathological and radiological criteria [1]. Histological grading was performed according to Evans et al [3].

In addition, tumour material from 51 enchondromas was included: 14 enchondromas of the phalanx and 37 enchondromas from other locations in the long bones. According to the 2002 WHO definition, all enchondromas were located centrally in the bone, as documented by X-ray. Phalangeal and non-phalangeal localizations were analysed separately. An expert bone-tumour pathologist reviewed all cases to confirm the diagnosis. Formalin-fixed, paraffin wax-embedded tissue was available from all patients; for a subset of chondrosarcomas (n = 47), fresh frozen tissue was also available. Follow-up data were retrieved from clinical charts and from the files of The Netherlands Committee on Bone Tumours.

Immunohistochemistry

Immunohistochemical staining was performed with the monoclonal INK4A/MTS1 antibody (Neomarkers, Fremont, CA, USA) according to standard laboratory methods [15]. In short, antigen retrieval was performed using 0.01 M citrate solution. Sections of tonsil tissue were used as a positive control. The staining intensity (0 = negative, 1 = weak, 2 = moderate, and 3 = strong intensity) and the percentage of positive cells (0% 0%, 1 = 1–24%, 2 = 25–49%, 3 = 50–74%, and 4 = 75–100%) were evaluated by two observers independently [8]. In discrepant cases, the sections were re-analysed to obtain a consensus. Haematopoietic cells in bone marrow or endothelial cells served as an internal positive control to evaluate whether negative tumour cells were truly negative or whether prolonged decalcification might have altered the conformation of the antigen, resulting in a false-negative result. INK4A/p16-negative tumours without a positive internal control were excluded.

Loss of heterozygosity and CDKN2A/p16 and p14ARF SSCP analysis

DNA isolation

Paraffin wax-embedded cartilaginous tissue is not suitable for DNA analysis because the decalcification process with formic acid results in DNA degradation. From 40 patients with chondrosarcomas, DNA from fresh-frozen tissue and matched normal DNA were available. Only tumour tissue was available from seven patients. Normal DNA was prepared from

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Table 1. Clinicopathological features

<table>
<thead>
<tr>
<th></th>
<th>Enchondroma (n = 51)</th>
<th>Conventional central chondrosarcoma (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male vs female</td>
<td>28 vs 23</td>
<td>40 vs 33</td>
</tr>
<tr>
<td>Median age at diagnosis, years (range)</td>
<td>36.1 (7–74)</td>
<td>50.8 (18–85)</td>
</tr>
<tr>
<td>Histology grade I</td>
<td>na</td>
<td>28</td>
</tr>
<tr>
<td>grade II</td>
<td>na</td>
<td>33</td>
</tr>
<tr>
<td>grade III</td>
<td>na</td>
<td>12</td>
</tr>
<tr>
<td>Ollier’s disease</td>
<td>4 of 51</td>
<td>4 of 73</td>
</tr>
<tr>
<td>Phalanx vs non-phalanx</td>
<td>14 vs 37</td>
<td>0 vs 73</td>
</tr>
<tr>
<td>Median follow-up, months (range)</td>
<td>75 (2–178)</td>
<td>56.5 (1–187)</td>
</tr>
<tr>
<td>Mean disease-free survival, months (range)</td>
<td>68.8 (2–178)</td>
<td>49.8 (1–169)</td>
</tr>
</tbody>
</table>
peripheral blood drawn from patients after informed consent, or from normal muscle or skin obtained from resected specimens. Tumours with tumour cell percentages over 60% were included for genetic analysis [8]. No frozen material was available from enchondromas. DNA isolation from frozen sections was performed using proteinase K treatment and subsequent purification with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. DNA from blood samples was isolated using a salting-out procedure [16].

LOH analysis

No matching normal DNA was available from seven samples and therefore those seven samples were not suitable for LOH analysis, leaving 40 tumours. For the study of LOH on chromosome 9p, the following polymorphic microsatellite markers were used: D9S269, D9S274, D9S319, and D9S304 (http://gdbwww.gdb.org).

The location of the markers in relation to CDKN2A/p16/INK4A is shown in Figure 1.

LOH analysis and scoring have been described previously [17]. LOH results had to be reproducible in a consecutive analysis.

CDKN2A SSCP analysis

Genomic DNA from 47 tumours was used for SSCP analysis. Both genes encoded by the CDKN2A locus, p16 and p14/ARF, were investigated. The CDKN2A/p16 gene was screened as described previously and covers the entire open reading frame [18]. Additional primers were designed for the p14/ARF exon 1β. Exon 1β was divided into two overlapping fragments. Primers for the two fragments of exon 1β were F1: 5′-CACCTCTGGTGCCAAAGG-3′ and R1: 5′-GCCTCTCGTAGGATCGACG-3′ (A fragment 219 bp), and F2: 5′-GCCGCAGTGGAGGGTTTT-3′ and R2: 5′-CACCACGTTATCTCCTC-3′ (B fragment 257 bp).

Methylation-specific PCR (MSP) analysis

Promoter methylation was determined according to Herman et al [19], based on bisulphite modification and subsequent PCR specific for the methylated versus the unmethylated CDKN2A/p16 promoter sequence.

One microgram of genomic DNA from 30 fresh-frozen tumours was used for MSP analysis and modified using the CpGenome DNA Modification Kit (Intergen, New York, USA). Subsequent CDKN2A/p16-promoter methylation status was analysed using the CpG WIZ Amplification Kit (Intergen, New York, USA). After amplification, samples, methylated, unmethylated, and wild-type controls were analysed on a 2% agarose gel.

Statistical analysis

The SPSS package was used for all statistical analyses. Histological parameters were analysed using the chi-square test for trend. Kaplan–Meier curves and the log-rank test were used to study the effect of INK4A/p16 protein expression and LOH on disease-free survival.

Results

Immunohistochemistry

In total, 14 of 69 (20%) chondrosarcomas demonstrated complete loss of INK4A/p16 protein expression (Figure 2C), which was restricted to higher-grade lesions: 0 (0%) of 24 grade I chondrosarcomas demonstrated loss of expression, compared with 9 (27%) of 33 grade II and 5 (42%) of 12 grade III chondrosarcomas (p = 0.001, chi-square test for trend). In contrast, none of the enchondromas showed loss of INK4A/p16 expression. One of eight patients with Ollier’s disease demonstrated loss of INK4A/p16 protein expression in a grade II conventional central chondrosarcoma. The results of the immunohistochemical analysis of INK4A/p16 staining are shown in Table 2. Immunohistochemical INK4A/p16 staining can result in positive nuclear or cytoplasmic staining. In the tumours tested, we found both nuclear and cytoplasmic staining (Figure 2A). Five of 73 samples showed only cytoplasmic staining. There were also some cases showing focal positive INK4A/p16 staining as shown

![Figure 1. Schematic representation of the CDKN2A/p16 and p14ARF genes, flanked by the markers on chromosome 9p21 that were used for LOH analysis](image-url)
Figure 2. Light micrograph showing the immunohistochemical nuclear and cytoplasmic localization of INK4A/p16. (A) Positive staining for INK4A/p16 in a grade II conventional central chondrosarcoma. (B) Some conventional central chondrosarcomas showed focal positive INK4A/p16 staining (arrows). (C) Fifteen per cent of conventional central chondrosarcomas were entirely negative for INK4A/p16; the inset shows the internal positive control (endothelial cells).

Table 2. Results of p16 immunohistochemistry stratified for diagnosis and tumour grade

<table>
<thead>
<tr>
<th></th>
<th>p16-negative</th>
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</thead>
<tbody>
<tr>
<td>Enchondroma</td>
<td></td>
</tr>
<tr>
<td>non-phalanx</td>
<td>0/25 (0%)</td>
</tr>
<tr>
<td>phalanx</td>
<td>0/14 (0%)</td>
</tr>
<tr>
<td>Total enchondroma</td>
<td>0/39 (0%)</td>
</tr>
<tr>
<td>Conventional central chondrosarcomas</td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>0/24 (0%)</td>
</tr>
<tr>
<td>Grade II</td>
<td>9/33 (27%)*</td>
</tr>
<tr>
<td>Grade III</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>Total conventional central chondrosarcomas</td>
<td>14/69 (20%)</td>
</tr>
</tbody>
</table>

* One INK4A/p16-negative grade II tumour originated from an Ollier’s disease patient.

in Figure 2B. Four specimens could not be evaluated due to repeated loss of tissue attachment during microwave procedures.

LOH and CDKN2A SSCP analysis

SSCP

To investigate if CDKN2A/p16 and p14/ARF are inactivated by a genetic mutation, we carried out SSCP analysis on the four exons encoding these genes and performed LOH analysis with polymorphic microsatellite markers adjacent to the CDKN2A locus. SSCP was chosen as the technique to determine mutations because the method has proven useful for the identification of INK4A mutations previously in melanoma [18] and because it is able to detect up to 90% of mutations in a background of non-tumour tissue, which is not possible by sequencing. In two samples from melanoma patients with previously confirmed CDKN2A/p16 mutation in exon 2 from CDKN2A/p16, an aberrant SSCP pattern could be detected, validating the SSCP analysis. None of the 47 chondrosarcomas revealed aberrant bands at the CDKN2A/p16 and p14/ARF loci. Figure 3 shows an example of SSCP analysis on five chondrosarcomas and a melanoma with a known mutation in p16.

LOH

As shown in Table 3, LOH at 9p21 for one or more markers was found in 15 of 40 (37.5%) cases. LOH was mainly found in grade II and III tumours. LOH for one or more markers in the 9p21 region was found
Remarkably, for all three patients with Ollier’s disease, LOH for one or more markers in the 9p21 region was found.

As shown in Table 3, there was no clear correlation between LOH at 9p and the loss of protein expression.

**Promoter methylation**

Table 4 shows the results of promoter methylation analysis by MSP. An example of MSP is shown in Figure 4. DNA was available to study promoter methylation from six of ten patients with absent INK4A/p16 protein expression. Twenty-four additional patients were selected with varying INK4A/p16 staining intensities with INK4A/MTS1 antibody.

Methylation of the CDKN2A/p16 promoter was detected in 5 of 30 (13%) cases. Detectable promoter methylation was not clearly associated with loss of INK4A/p16 protein expression (Table 4).

**Statistical analysis**

There was a significant relationship between loss of INK4A/p16 protein expression and increasing tumour grade (chi-square test for trend, $p = 0.004$). Both LOH for one or more markers in the 9p21 region and complete loss of INK4A/p16 expression also seemed to correlate with poor prognosis; this was, however, not statistically significant ($p = 0.0828$ and $p = 0.1271$, respectively, log-rank test) and was not independent of histological grade, which was a strong prognostic parameter ($p = 0.000$, log-rank test).

**Table 3.** Correlation between loss of p16 protein expression and LOH at 9p21

<table>
<thead>
<tr>
<th>Tumours with LOH</th>
<th>Tumours with no LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumours with LOH at 9p21</strong></td>
<td><strong>Negative p16 immunohistochemistry</strong></td>
</tr>
<tr>
<td>Grade I</td>
<td>2</td>
</tr>
<tr>
<td>Grade II</td>
<td>9</td>
</tr>
<tr>
<td>Grade III</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
</tr>
</tbody>
</table>

* No corresponding p16 protein expression data were available from eight tumours with LOH data.

**Table 4.** Correlation between CDKN2A/p16 methylation status, tumour grade, and INK4A/p16 immunohistochemistry

<table>
<thead>
<tr>
<th>Conventional central chondrosarcoma</th>
<th>p16 Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>0/7 (0%)</td>
</tr>
<tr>
<td>Grade II</td>
<td>4/17 (24%)</td>
</tr>
<tr>
<td>Grade III</td>
<td>1/6 (17%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conventional central chondrosarcoma</th>
<th>p16 intensity</th>
<th>p16 % positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 expression 0</td>
<td>1/6 (17%)</td>
<td>0</td>
</tr>
<tr>
<td>p16 expression 2</td>
<td>2/6 (33%)</td>
<td>1</td>
</tr>
<tr>
<td>p16 expression 3–4</td>
<td>1/10 (10%)</td>
<td>1</td>
</tr>
<tr>
<td>p16 expression 5–6</td>
<td>1/5 (20%)</td>
<td>2</td>
</tr>
<tr>
<td>p16 expression 7</td>
<td>0/3 (0%)</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 4. Example of MSP analysis. All DNAs were modified with bisulphite. The samples in lanes 1–4 were amplified with primers for the methylated state and those in lanes 5–8 were amplified with primers for the unmethylated state. Lanes 1 and 5 are of a conventional central chondrosarcoma; lanes 2 and 6 are unmethylated control DNAs; lanes 3 and 7 are methylated control DNAs; and lanes 4 and 8 are samples amplified without DNA to serve as a negative control. Lane M is a 100 bp ladder.

Ten of 27 chondrosarcomas that showed INK4A/p16 expression also showed LOH. The staining intensity and the percentage of cells demonstrating INK4A/p16 expression seemed slightly lower in INK4A/p16-positive tumours with LOH than in INK4A/p16-positive tumours without LOH, although the difference was not statistically significant (mean score for intensity 1.38 versus 1.74, mean score for percentage of positive cells 1.54 versus 2.05, mean total score 2.92 versus 3.79).

Discussion

Few genetic aberrations have been identified in conventional central chondrosarcomas, suggesting that limited genetic alterations are required for tumourigenesis. LOH and other cytogenetic alterations at 9p21 are, however, repeatedly found, suggesting an important role in tumourigenesis for genes located in this region. The CDKN2A/p16 locus at 9p21 is an excellent candidate, since it has been shown to be inactivated in multiple tumour types including bone sarcomas [14]. The CDKN2A/p16 gene has been investigated previously in chondrosarcoma, but a heterogeneous group of chondrosarcomas was used and only one genetic alteration was found [20]. These authors identified promoter methylation in 5 of 22 cases, but loss of expression was not confirmed by immunohistochemistry.

Here we present a carefully selected group of conventional central chondrosarcomas only, stratified according to differentiation, grade and aetiology, and their potential precursors, namely enchondromas. A study for CDKN2A/p16 was performed at the level of protein, genetic alterations, promoter methylation, and mutational analysis of p14/ARF. Furthermore, we investigated whether INK4A/p16 protein expression level can be used as a predictive marker for tumour progression in conventional central chondrosarcomas.

Two separate subgroups were discerned within our tumours:

1. Tumours located in the phalanx versus those located elsewhere, since phalangeal chondrosarcomas are extremely rare and have a far better prognosis than chondrosarcomas located elsewhere [4]. Both enchondromas of the phalanx and enchondromas located elsewhere failed to demonstrate loss of INK4A/p16 protein expression.

2. Conventional central chondrosarcomas that occur in the context of Ollier’s disease [5,21] versus sporadic cases. Chondrosarcomas in the context of Ollier’s disease may have a worse prognosis than normal chondrosarcomas [22,23]. Four chondrosarcomas from patients with Ollier’s disease were available for this study and one showed loss of INK4A/p16 protein expression. Remarkably, however, all three patients with Ollier’s disease used for the LOH analysis demonstrated LOH for one or more markers in the 9p21 region. This is consistent with previous data [24] and suggests that INK4A/p16 is not more often involved in Ollier’s disease than in non-Ollier’s disease-related chondrosarcomas. Rather, another gene located in the 9p21 region may be important in chondrosarcoma in Ollier’s disease.

This study shows that conventional central chondrosarcomas demonstrate loss of INK4A/p16 expression by immunohistochemistry in 20% of cases tested and that this loss increases with histological grade. Enchondromas show no loss of INK4A/p16 expression. This is concordant with the low proliferative activity in enchondromas and the role of INK4A/p16 in inhibition of cell-cycle progression. In dysplastic nevi, LOH, mutations, and loss of protein expression of INK4A/p16 have been reported but only in a small fraction, whereas melanomas often show INK4A/p16 alterations, suggesting a role for this gene in tumour progression in this situation too [25–28]. Five tumours showed only cytoplasmic and no nuclear staining. It has previously been described that breast tumours with only cytoplasmic INK4A/p16 staining behave more aggressively [29], but this cannot be concluded for chondrosarcomas because the tumours that showed only cytoplasmic staining were low grade.

In many other tumours, especially melanoma, the CDKN2A/p16 locus is the target for LOH at 9p21 [18,30,31]. Our LOH analysis shows that CDKN2A/p16 is included in the region in all cases with LOH of at least one marker at 9p21. LOH at

9p21 was relatively frequent (37%) and comparable to previous results [8,32,33].

LOH at 9p increased with histological grade, which is most probably a reflection of an overall increase in genetic instability in higher-grade tumours [34]. Whether CDKN2A/p16 is the target of LOH at chromosome 9p21 could not be assessed because SSCP analysis did not identify mutational inactivation of the retained copy of CDKN2A/p16 and p14ARF in any of the tumours tested. Furthermore, there was no association between LOH at 9p21 and immunohistochemical loss of INK4A/p16 protein expression. The 9p12–22 region was involved in five of seven conventional central chondrosarcomas with an aberrant karyotype [35]. Two of the five cases demonstrating −9, t(9:10)(p22;q22) and add(9)(p21) could be included in the present analysis and both showed loss of INK4A/p16 protein expression and LOH at 9p21.

Homozgous deletions of the CDKN2A region have been described occasionally in cell lines and primary tumours of different origins [14,36,37]. We cannot exclude the possibility that such deletions have been missed in our study. However, because there is no obvious relationship between LOH and INK4A/p16 protein expression in our study, homozgous deletions of CDKN2A/p16 in conventional central chondrosarcomas are unlikely.

Another mechanism for tumour suppressor gene inactivation is epigenetic promoter methylation, which has been described for the CDKN2A/p16 gene [19,38]. Indeed, we found methylation in 5 of 30 patients. However, there was no association between LOH at 9p21 and methylation, since only two of these cases showed LOH. There was no significant relationship between protein expression and CDKN2A/p16 promoter methylation, since we identified four tumours that showed promoter methylation without loss of INK4A/p16 protein expression. With increasing age, methylation of several target genes can be detected in normal tissue [39–41]. The average age of the patients that showed CDKN2A/p16 promoter methylation was 41.4 years. This is even less than the average age of all patients with conventional central chondrosarcomas, namely 50.8 years, thereby indicating that age-dependent promoter methylation is not likely. An alternative explanation for the discordance between CDKN2A/p16 promoter methylation and protein expression is the presence of focal INK4A/p16-positive areas next to areas with no INK4A/p16 expression (Figure 2B), indicating tumour heterogeneity with regard to INK4A/p16 promoter methylation. Interestingly, we did not find promoter methylation in low-grade chondrosarcomas.

CDKN2A/p16 alterations have also been identified in other bone sarcomas, such as osteosarcomas [10,11]. Ewing sarcomas [10,12], and malignant fibrous histiocytomas of bone (MFH-b) [13]. About 30% of Ewing sarcomas and osteosarcomas show abnormalities (mutations, deletions, and promoter methylation) of the INK4A locus. For MFH-b, only 1 of 19 tumours showed a CDKN2A/p16 mutation. Ewing sarcomas show a negative correlation between mutations and deletions of the INK4A locus and prognosis.

In conclusion, loss of INK4A/p16 protein expression occurs in one-third of conventional central chondrosarcomas by an as yet unknown mechanism and is associated with higher tumour grade. Although we found LOH at 9p21 in 38% of cases, there was no association between LOH and loss of INK4A/p16 protein expression. Thus, neither the CDKN2A/p16 nor the p14ARF locus appears to be the target and therefore another gene is most likely involved by this recurrent genetic aberration. Also, CDKN2A/p16 promoter methylation does not explain this phenomenon, because we did not find an association with loss of protein expression. It is of importance that CDKN2A/p16 loss is more frequent in high-grade tumours and is absent in enchondroma. It has been reported that recurrences of chondrosarcoma sometimes demonstrate an increase in the degree of malignancy [1,2]. This suggests that loss of INK4A/p16 protein expression is an important event during tumour progression in enchondroma, as well as for the progression in grade in recurrent chondrosarcoma.

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