Chordoma of the skull base: predictors of tumor recurrence

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Object. Chordomas of the skull base are generally regarded as slow-growing tumors; however, approximately 20% of these lesions have been shown to recur as early as 1 year postsurgery. The classic pathological paradigms are poor predictors of outcome, and additional markers are needed to identify patients at risk for early tumor recurrence. In this study the authors describe such a marker.

Methods. In a series of 26 patients with chordomas of the skull base, the authors investigated the relationship between the biological behavior of the tumor, which was determined according to the interval for its recurrence and volume doubling time; and several pathological and molecular features, which included the histological variant, proliferative activity, mutation of p53 protein, expression of human telomerase reverse transcriptase (hTERT) messenger (m)RNA, loss of heterozygosity (LOH), and microsatellite instability. The major finding in this study was that hTERT mRNA expression in chordoma cells identifies those tumors that exhibit unusually fast rates of growth. The expression of hTERT mRNA was frequently associated with mutation of p53 protein, indicating that telomerase dysfunction combines with abnormal p53 function to initiate the unrestrained clonal expansion of the tumor cells. In cases in which the tumor was partially removed, mutation of p53 protein and expression of hTERT mRNA predicted increased doubling time for residual tumor as well as the probability of tumor recurrence. Cell proliferation, as investigated using the Ki-67 method, was significantly related to the proliferative index that changed by a factor as high as 8 among different regions of the same tumor. The LOH and microsatellite instability do not seem to affect the prognosis of skull base chordomas.

Conclusions. Reactivation of telomerase in chordomas is a reliable predictor of outcome. The ability to predict the biological behavior of chordomas might have immediate implications in the management of this disease in patients who undergo surgery.

KEY WORDS • chordoma • skull base • tumor recurrence • human telomerase reverse transcriptase • p53

Chordomas of the skull base are rare, accounting for only 0.1 to 0.2% of all intracranial tumors. These neoplasms, which originate from remnants of the primitive notochord, represent a challenge for the neurosurgeon because of their proximity to vital neurovascular structures. These lesions are also difficult for the pathologist to interpret because their aggressive behavior frequently contrasts with a differentiated phenotype. The malignant potential of chordomas lies in their tendency to infiltrate the bone, which leads to the high recurrence rate after treatment. Although chordomas are generally regarded as slow-growing tumors, early recurrence after surgical treatment is not uncommon. Analysis of recurrence-free survival has shown that approximately 20% of these tumors are expected to recur as early as 1 year postsurgery. The classic pathological paradigms that have been investigated so far, however, like the histological variant, mitotic index, proliferative activity, extent of necrosis, and so on, are poor predictors of outcome, and additional markers are needed to identify patients at risk of early tumor recurrence.

The ability to predict the biological behavior of chordomas might have immediate implications in the management of the disease in patients who undergo surgery. For example, patients at high risk of early tumor recurrence might be entered into trials featuring aggressive surgical treatment in which multiple and extensive surgical approaches are used, followed by proton beam radiation therapy. Conversely, aggressive surgical approaches, which have been associated with high morbidity rates, might not be indicated in patients with indolent tumors. Such lesions might be treated using more conservative approaches, which yield low operative morbidity.

In a series of 26 chordomas of the skull base, we investigated the relationship between the biological behavior of the tumor and several pathological and molecular features, which included the histological variant, proliferative activity,
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ny, mutation of p53 protein, expression of hTERT mRNA, LOH, and/or microsatellite instability. These paradigms were chosen either because of their established role in cell cycling and proliferation, or because they have reportedly been found to be related to tumor aggressiveness in clival as well as in sacral chordomas. In this report, we demonstrate that the association of hTERT mRNA expression and mutation of p53 protein identifies a tumor phenotype that is prone to recur quickly postsurgery.

Clinical Material and Methods

Patient Population

Twenty-six patients with chordomas of the cranial base who had been surgically treated between January 1988 and the present at the Institute of Neurosurgery, Catholic University School of Medicine were entered in this study. Clinical data for the treated patients are summarized in Table 1. There were 14 men and 12 women who ranged in age from 17 to 80 years (mean 50.3 years).

Locations of Tumors. Tumor locations were as follows: upper clivus five cases; middle clivus, 15; lower clivus, four; and petrous bone, two. The tumor extended to the anterior skull base and orbit in two cases, to the C1–2 in four, and to the infratemporal region in one. The cavernous sinus was involved in six cases. In one case, the tumor was primarily retropharyngeal and infiltrated the clival bone at a later stage; this patient also had pulmonary and hepatic metastases. In one case a vertebral chordoma developed at the thoracic level.

Tumor Types and Surgical Approaches. According to the classification of Al-Mefty and Borba, there were five Type I chordomas (tumor involving one anatomical region and resectable using a single approach), 13 Type II chordomas (tumor involving two anatomical regions but resectable using a single approach), and eight Type III chordomas (tumor involving more than two anatomical regions in which resection required multiple approaches). A total of 48 surgical procedures were performed (1.8 procedures/patient), 38 of them at our institution. These included transsphenoidal approaches in 23, transmaxillary in four, extreme lateral in four, orbitozygomatic in two, and transoral, transbasal, retrosigmoidal, combined transbasal and transmaxillary, and combined subtemporal–infratemporal and extreme lateral in one each.

Extent of Resection. At the initial operation, total removal, that is, absence of tumor on microsurgical inspection and on MR imaging at 3 to 6 months postsurgery, was achieved in that is, absence of tumor on microscopical inspection and on MR study performed 3 months postsurgery, which was used as the baseline, with follow-up MR images. In cases in which the 3-month postoperative MR image demonstrated no residual tumor, the tumor doubling time was represented by the time at which tumor recurrence was noted. Tumor volume was calculated by measuring the enhancing tumor area with planimetry on Gd-enhanced T1-weighted MR images. After the tumor area in square millimeters was calculated on each image, volumes were determined in cubic centimeters by multiplying the area by the distance between adjacent images (2–5 mm).

Histopathological Diagnosis and Immunophenotyping

The tumor specimens were obtained at surgery and were fixed in formalin or cryopreserved in liquid nitrogen. For histological studies, formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and cosin. The tumors were histologically classified as chordoma, chondroid chordoma, low-grade chondrosarcoma, and sarcomatous chordoma according to the scheme of Burger and Scheithauer. All immunostaining techniques were performed in paraffin-embedded tissue sections after an initial step of heat-induced antigen retrieval accomplished by microwave oven processing (two 5-minute cycles at 750 W) in citrate buffer. After incubation with the primary antibody, immunodetection was performed using the ABC-peroxidase method (LSAB-Dako, Glostrup, Denmark), with freshly made diaminobenzidine as a chromogen. Primary sera were directed against EMA, S100 protein, vimentin, and pankeratin cocktail. Chordomas strongly stain with keratin and EMA, unlike chondrosarcomas, which express S100 and vimentin but generally do not stain with keratin and EMA.

A minority of chordomas exhibit cartilaginous differenti-
TABLE 1

Characteristics of 26 patients with chordoma of the skull base

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Tumor Location</th>
<th>Approach or Treatment (mos)</th>
<th>Extent of Resection</th>
<th>Tumor Doubling Time (mos)</th>
<th>Tumor Sample</th>
<th>Histological Diagnosis</th>
<th>MIB-1 (%)</th>
<th>p53</th>
<th>hTERT (mos)</th>
<th>Outcome (mos)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>55, F</td>
<td>upper middle clivus, sphenoid, CS, ethmoid</td>
<td>TS (5)</td>
<td>partial</td>
<td>9</td>
<td>b</td>
<td>chordoma</td>
<td>4.5</td>
<td>+</td>
<td>+</td>
<td>AWD (126)</td>
</tr>
<tr>
<td>2</td>
<td>50, M</td>
<td>middle clivus, sphenoid, sella turcica</td>
<td>TS (20)</td>
<td>subtotal</td>
<td>NA</td>
<td>a</td>
<td>chordoma</td>
<td>1.2</td>
<td>−</td>
<td>−</td>
<td>DD (36)</td>
</tr>
<tr>
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<td>48, M</td>
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<td>TS</td>
<td>total</td>
<td>NA</td>
<td>a</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
<td>DD (64)†</td>
</tr>
<tr>
<td>4</td>
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<td>126</td>
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<td>2.7</td>
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<td>lower clivus, petrous bone, C1–2</td>
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<td>110</td>
<td>c</td>
<td>chordoma</td>
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<td>−</td>
<td>DD (46) †</td>
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<td>80</td>
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<td>2.8</td>
<td>−</td>
<td>−</td>
<td>AWD (108)</td>
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<td>total</td>
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<td>chordoma</td>
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<td>−</td>
<td>−</td>
<td>NED (103)</td>
</tr>
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<td>20</td>
<td>c</td>
<td>chordoma</td>
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<td>−</td>
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<td>partial</td>
<td>58</td>
<td>b</td>
<td>chordoma</td>
<td>3.4</td>
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<td>−</td>
<td>AWD (88)†</td>
</tr>
<tr>
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<td>middle clivus, sphenoid</td>
<td>TS (110)</td>
<td>total</td>
<td>NA</td>
<td>a</td>
<td>chordoma</td>
<td>2.5</td>
<td>−</td>
<td>−</td>
<td>AWD (86)</td>
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<tr>
<td>11</td>
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<td>upper middle clivus, sella turcica, sphenoid, CS</td>
<td>TS (57)</td>
<td>partial</td>
<td>57</td>
<td>b</td>
<td>chordoma</td>
<td>3.2</td>
<td>−</td>
<td>−</td>
<td>AWD (86)</td>
</tr>
<tr>
<td>12</td>
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<td>upper middle clivus, spheno-noid, sella turcica</td>
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<td>partial</td>
<td>57</td>
<td>b</td>
<td>chordoma</td>
<td>3.2</td>
<td>−</td>
<td>−</td>
<td>AWD (86)</td>
</tr>
<tr>
<td>13</td>
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<td>57</td>
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<td>−</td>
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<td>57</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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<tr>
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<td>partial</td>
<td>57</td>
<td>b</td>
<td>chordoma</td>
<td>3.2</td>
<td>−</td>
<td>−</td>
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</tr>
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<td>57</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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<tr>
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<td>TS (57)</td>
<td>partial</td>
<td>57</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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<tr>
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<td>middle clivus, sphenoid</td>
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<td>partial</td>
<td>57</td>
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<td>chordoma</td>
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<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>19</td>
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<td>posterior clinoid, petros apex</td>
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<td>partial</td>
<td>57</td>
<td>b</td>
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<td>−</td>
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<tr>
<td>20</td>
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<td>TS (57)</td>
<td>partial</td>
<td>58</td>
<td>b</td>
<td>chordoma</td>
<td>3.5</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>21</td>
<td>20, M</td>
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<td>TS (57)</td>
<td>partial</td>
<td>20</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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<tr>
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<td>retropharynx, middle clivus</td>
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<td>46</td>
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<td>−</td>
<td>−</td>
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</tr>
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<td>55, M</td>
<td>petrous bone, infratemporal, temporomandibular joint</td>
<td>TS (57)</td>
<td>partial</td>
<td>55</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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</tr>
<tr>
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<td>partial</td>
<td>78</td>
<td>b</td>
<td>chordoma</td>
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<td>76</td>
<td>b</td>
<td>chordoma</td>
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<td>−</td>
<td>−</td>
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<tr>
<td>26</td>
<td>55, F</td>
<td>middle clivus, sphenoid</td>
<td>TS (57)</td>
<td>partial</td>
<td>55</td>
<td>b</td>
<td>chordoma</td>
<td>3.5</td>
<td>−</td>
<td>−</td>
<td>AWD (26)</td>
</tr>
</tbody>
</table>

* AWD = alive with disease; BT = brachytherapy; Cr = craniotomy; CS = cavernous sinus; DD = died of disease or disease-related complications; EL = extreme lateral; FTOZ = frontotemporal–orbitozygomatic; lam = laminectomy; NA = not available; NED = no evidence of disease; PBRT = proton beam radiotherapy; RS = retrosigmoidal; STIT = subtemporal–infratemporal; TB = transbasal; TDT = tumor doubling time; TM = transmaxillary; TO = transoral; TP = transpetrosal; TS = transsphenoidal; + = positive; − = negative.
† Operation performed elsewhere.
‡ Intraoperative death.
§ Died of temporal glioblastoma.
|| Spinal metastasis.
|| Died of acute dienecephalic syndrome.
†† Pulmonary and hepatic metastases.
Recurrence of clival chordomas

...ation with cells situated in small lacunae within a chondroid matrix. In these cases, the distinction between chondroid chordoma and low-grade chondrosarcoma may be difficult, and only convincing EMA and cytokeratin reactivity in a sizable proportion of cartilage-containing tumor allows a diagnosis of chondroid chordoma. Some chordomas may degenerate into malignant neoplasms with various patterns, including an undifferentiated spindle cell tumor or malignant fibrous histiocytoma.

**Proliferative Activity: MIB-1 PI**

The proliferative potential of the tumor cells was assessed using the Ki-67 mouse monoclonal antibody (MIB-1; Ylem, Avezzano, Italy), which is exclusively expressed in the nucleus of proliferating cells. The MIB-1 PI was determined as the percentage of positive nuclei with respect to the total number of nuclei in high-power fields (×400). In each case, approximately 1500 chordoma tumor cells were counted in areas with maximal staining.

**Immunohistochemical Staining for p53**

Anti-p53 antibody (DO-7, Dakopatts, Glostrup, Denmark; 1:300 dilution) determines a determinant of wild-type and mutant p53 protein in formalin-fixed sections. The wild-type protein has a very short half-life and has generally been considered to be undetectable with an immunohistochemical method that uses anti-p53 antibody. Conversely, the mutant p53 protein has a longer half-life of several hours and thus can be visualized with an immunohistochemical method. Sections were examined for p53 immunoreactivity by two pathologists (F.P. and L.M.L.) who were unaware of the outcomes and clinical features. Only cells with dense nuclear staining were interpreted as positive. Tumors were categorized as expressing little or no p53 when staining was observed in less than 10% of cells, or as overexpressing p53 when staining was observed in more than 10% of cells in the high-power field with maximal staining. Therefore, neoplasms were defined p53-positive based on the percentage of p53-expressing cells.

**Use of ISH to Assess hTERT Expression**

Digoxigenin-labeled antisense and sense riboprobes were generated by in vitro transcription of a 2000-bp region of hTERT cDNA cloned in both orientations after addition of T7 RNA polymerase promoter. Alkaline hydrolysis in carbonate buffer was used to reduce the probe length, as described elsewhere. For ISH, the tissue samples were cut through a graded series of ethanol, and air dried. Hybridization procedures were performed as reported elsewhere. In brief, sections were preincubated with hybridization buffer containing 50% vol/vol freshly denioned formamide for 45 minutes at 42°C, and incubated with digoxigenin-labeled riboprobe diluted at a concentration of 50 ng/ml in hybridization buffer overnight at 42°C. After hybridization, the slides were immersed in preheated astringent wash solution (Dako, Glostrup, Denmark) for 20 minutes at 42°C with shaking, and washed for 5 minutes at room temperature in PBS. Sections were then incubated for 90 minutes with antidigoxigenin alkaline phosphatase-conjugated antibody (150 mU/ml; Boehringer Mannheim Corp., Indianapolis, IN) at room temperature in the dark. After two washes with PBS, slides were briefly treated with a solution containing 100 mM Tris HCl, 100 mM NaCl, 50 mM MgCl2 (pH 9.5) and incubated for 15 to 30 minutes with a solution of nitroblue tetrazolium salt (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphates (0.18 mg/ml) containing 0.25 mg/ml levamisole at room temperature in darkness. Control samples hybridized with digoxigenin-labeled sense riboprobe and samples pretreated for 60 minutes with 100 mg/ml RNAsé A (Sigma Chemical Co.) displayed no signal. Sections hybridized with antisense riboprobe for glucose-3-phosphate dehydrogenase were used to check RNA integrity. Preincubation buffer, fluorescein-conjugated peptide nucleic acid probe for glucose-3-phosphate dehydrogenase, astringent wash solution, antifluorescein alkaline phosphatase-conjugated antibody and solution of nitroblue tetrazolium salt (0.34 mg/ml), and 5-bromo-4-chloro-3-indolyl phosphates (0.18 mg/ml) containing 0.25 μl levamisole were used according to the manufacturer’s instructions (PNA ISH Detection Kit; Dako).

**Analysis of LOH and Microsatellite Instability**

Analyses of LOH and microsatellite instability were performed in nine patients (Cases 12, 15, 17, 18, 19, 22, 24, 25, and 26 in Table 1). These patients had no history of hereditary nonpolyposis colorectal cancer syndrome, a condition known to be characterized by high microsatellite instability associated with defects of one or more genes of the DNA mismatch repair system. Cryptopreserved tumor specimens were used for DNA extraction. Peripheral blood lymphocytes were obtained with the patients’ informed consent before surgery, and were used as normal control tissue. Isolation of DNA was performed in blood and tumor specimens by using standard methods. The microsatellite instability/LOH analysis was performed at the same time on tumor and matched normal tissue DNA by using 10 microsatellite loci. These included three mononucleotide (BAT-25, BAT-26, and BAT-40), five dinucleotide (D5S346, D2S123, D9S171, D11S904, and D17S250), one trinucleotide (AR), and one tetraneucleotide (ACTBP2) tandem repeats. The microsatellite loci were chosen to compose the set that is recommended for examining high microsatellite instability in colorectal tumors (BAT-25, BAT-26, D5S346, D2S123, and D17S250), as well as additional markers that are frequently used for microsatellite instability analysis in colorectal cancers and in other neoplasms (BAT-40, D9S171, D11S904, AR, and ACTBP2). This panel of markers has been proven to detect microsatellite instability readily in intracranial tumors. Primer sequences were from the Genome Data Base (http://www.gdb.org/) or as published previously. The PCR was performed as previously described, with an initial denaturation step of 3 minutes at 98°C linked to 35 cycles for 30 seconds at 94°C, 60 seconds at primer-specific annealing temperature, and 30 seconds at 73°C, followed...
by a final extension step of 5 minutes at 73°C. The PCR was performed in a final volume of 25 ml containing 1 X PCR buffer (10 mM Tris HCl, 50 mM KCl), 1.2 to 2 mM MgCl₂, 100 mM deoxynucleotide triphosphate, 0.1 mCi [α³²P]deoxyctydine 5'-triphosphate, 400–600 nM of each primer, and 0.1 U of Taq polymerase). A hot-start reaction was performed by preheating the mixture in the thermocycler at 98˚C for 3 minutes, then cooling to 80˚C before adding the Taq polymerase. The PCR product (2 ml) was diluted two-fold with stop solution (20 mM ethylenediamine tetraacetic acid 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) and denatured at 98˚C for 5 minutes. The mixture was electrophoresed on 6% polyacrylamide/7 M urea gel at 2000 V for 1 hour. The gel was fixed in 10% acetic acid, dried in an oven at 80˚C for 45 minutes, and exposed to film for 18 to 36 hours.

Statistical Methods

Differences in the expression of the various parameters between primary and recurrent chordomas were evaluated using the Student t-test. Correlation between tumor doubling time and the PI was studied using regression analysis and the Pearson correlation coefficient. The log rank test was used to compare recurrence-free survival between p53-positive and p53-negative chordomas, and between chordomas with and without hTERT mRNA expression. Statistical significance was assigned to probability values less than 0.05. The computer softwares used for statistical analysis were Statistica (version 5.5; StatSoft, Tulsa, OK) and Fig. P (version 2.7; Biosoft, Cambridge, UK). Values are expressed as the means ± standard deviation.

Results

Recurrence of Clival Chordomas and Tumor Doubling Time

Tumor recurrence was noted in 14 (53.8%) of 26 patients, who had a total of 22 recurrences (Table 1). Tumor recurrence followed a partial resection in six patients, subtotal resection in four, and total resection in four. Nine patients had a single recurrence, three had two recurrences, one had three, and one had four (Fig. 1). The interval for recurrence was quite variable, ranging from 5 to 147 months (33.9 ± 40.1 months); however, in eight cases (30.7%) early tumor regrowth was found after surgery, with intervals for recurrence of less than 1 year (Fig. 2). Interestingly, early tumor regrowth was noted from the first recurrence in seven of these cases, whereas the tumor regrew faster at its fourth recurrence in only one (Case 22 in Table 1). Early tumor recurrence followed partial resection in six cases and subtotal resection in two. Patients in whom the tumor was totally removed did not have recurrence earlier than 1 year after surgery. The interval for tumor recurrence was 60.8 ± 34.2, 52 ± 53.4, and 6.9 ± 22 months after total, subtotal, and partial tumor resection, respectively. The interval for recurrence was significantly shorter after partial tumor resection compared with total as well as subtotal resection (p < 0.001, t-test); however, there was no significant difference in the interval for recurrence between totally and subtotally resected chordomas (p > 0.05, t-test). This relationship was valid whether we included or excluded Case 23 from the analysis; a chondrosarcoma recurred and was resected 147 months after subtotal removal in this patient.

The tumor doubling time was calculated in 15 patients, which included the recurrent cases and an elderly patient in whom radiotherapy was chosen as the first option (Case 25 in Table 1). It ranged between 6 and 126 months with a mean value of 31.1 ± 34.1 months, which was not significantly different from the interval for tumor recurrence (p > 0.05, t-test). Chordomas that recurred after total, subtotal, and partial resection showed tumor doubling times of 36.1, 48.6 ± 40.3, and 8.4 ± 2.5 months, respectively. Thus, the tumor doubling time was significantly shorter after partial resection than after total or subtotal resection.
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Histological Variant and Tumor Recurrence

Histological examination revealed typical chordoma in 19 cases, chondroid chordoma in three, low-grade chondrosarcoma in three, and sarcomatous chordoma in one (Table 1 and Figs. 2A, 3A, and 4A). In one case, the initial diagnosis of chondroid chordoma was changed to low-grade chondrosarcoma on review of the immunohistochemical profile. In another the diagnosis of sarcomatous chordoma was established at the third recurrence after previous diagnoses of typical chordoma (Case 22 in Table 1 and Fig. 4A). Of the 19 patients with typical chordoma, tumor recurrence was noted in 12 (63.2%), with a total of 19 recurrences; the interval for recurrence was 24.3 ± 23.9 months (range 5–80 months). Seven of these cases showed early tumor regrowth less than 1 year postsurgery. The other patient in whom early tumor recurrence developed had a diagnosis of sarcomatous chordoma. The patients with recurrent chondroid chordoma and chondrosarcoma had intervals for recurrence of 126 and 147 months, respectively (Table 1). For patients with typical chordoma, the survival rates at 5 and 10 years were 61.5 and 53.8%, respectively. The 1-year recurrence-free probability values were 76, 100, and 100% in patients with typical chordoma, chondroid chordoma, and chondrosarcoma, respectively.

Proliferative Activity and Tumor Recurrence

The PI, as estimated using Ki-67 immunostaining, was 4.3 ± 2.5%, 2.5 ± 1.4%, and 1.8 ± 1.8% for chordomas, chondroid chordomas, and chondrosarcoma, respectively (Table 1 and Figs. 2B, 3B, and 4B). The pattern of Ki-67 immunostaining was not homogeneous throughout the chordoma tissue, with a wide variation in the PI among the different regions of any given tumor. In some cases, the PI might change by a factor as high as 8 in the same chordoma specimen. In recurrent cases of typical chordoma, the PI was increased by 39% compared with the primary tumor, because it scored 3.6 ± 2% and 5 ± 2.1% in primary and recurrent chordomas, respectively. In the single case of recurrent chondroid chordoma available for this analysis, however, the PI decreased from 4.3 to 2.7% between the primary and recurrent tumor. Figure 5 shows the relationship between the PI of typical chordomas and tumor doubling time in recurrent cases. The two variables followed an exponential curve, and in a semilogarithmic diagram there appeared to be a significant relationship between them, with a 95% confidence interval (p = 0.032; linear regression Pearson test). Notably, chordomas with tumor doubling times shorter than 1 year exhibited a wide spectrum of PI, which ranged from 2.5 to 11.8% (Fig. 5).

Mutation of p53 Protein

Mutation of p53 protein was found in 17 (44.7%) of 38 tumor specimens, which included 16 chordomas and one chondrosarcoma (Table 1 and Figs. 2C, 3C, and 4C). There was no mutation of p53 in the four specimens of chondroid chordoma, and the single case of sarcomatous chordoma was also negative for the p53 immunoreaction (Fig. 4C). In p53-positive chordomas, the interval for tumor recurrence...
was significantly shorter (7.4 ± 2.2 months) than in p53-negative chordomas (44.8 ± 25.8 months; p < 0.001, log rank test). In cases in which partial or subtotal resection was performed, p53 mutation predicted increased doubling time for residual tumor. In fact, tumor doubling time was 9.4 ± 4.2 and 42 ± 18.2 months, respectively, in chordomas with and without p53 mutation, with a significant difference between the two groups (p < 0.0001, t-test).

Expression of hTERT: ISH Findings

Expression of hTERT mRNA was found in 11 (42.3%) of 26 cases and in 17 (44.7%) of 38 tumor samples, which included typical chordoma in 14 (48.3%) of 29; chordoid chordoma in zero (0%) of four; chondrosarcoma in one (33.3%) of three; and sarcomatous chordoma in two (100%) of two (Table 1 and Figs. 2D, 3D, and 4D). Tumor recurrence was noted in seven (77.8%) of nine chordomas with hTERT mRNA expression and in four (44.4%) of nine chordomas without hTERT mRNA expression. The interval for tumor recurrence was 7.4 ± 2.2 and 44.8 ± 25.8 months for chordomas expressing hTERT mRNA and those without hTERT mRNA expression, respectively (p < 0.0001, Student t-test). Also, the tumor doubling time was significantly shorter in hTERT mRNA-positive compared with hTERT mRNA-negative chordomas (p < 0.0001, Student t-test); it was calculated as 9.4 ± 4.2 and 42 ± 18.2 months, respectively. The recurrence-free probability curves for chordomas with and without hTERT mRNA expression are given in Fig. 6. There was a highly significant difference in recurrence-free probability between the two groups of tumors (p < 0.00002, log rank test). In cases of partial tumor removal, hTERT mRNA expression predicted recurrence because the recurrence-free probability was significantly higher in hTERT mRNA-negative compared with hTERT mRNA-positive chordomas (p < 0.0001, log rank test). The PI was significantly higher in chordomas that expressed hTERT mRNA (5.8 ± 2.4% compared with those that did not express hTERT mRNA (2.7 ± 0.9%) (p < 0.01, Student t-test). The expression of hTERT mRNA was associated with mutation of p53 protein in all but two cases, which included one typical chordoma with mutant p53 but no hTERT expression (Case 14), and the sarcomatous chordoma, which was p53-negative and showed hTERT mRNA expression (Case 22).

Results of LOH and Microsatellite Instability Analysis

Microsatellite analysis was performed using 10 different primers and consisted of a total of 140 analyses, which were repeated twice. Differences between normal and tumor DNA were detected in none of the nine chordomas that were assayed for these tests, including the sarcomatous...
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chordoma (Fig. 7). We did not find microsatellite instability, defined as the presence of an additional band or a difference in length due to insertion or deletion of the amplified allele, in the tumor tissue compared with matched blood samples (Fig. 7). Moreover, the presence of LOH, which was defined as the remarkable attenuation (> 50%) or the loss of one allele in the tumor DNA, was detected in none of the chordoma samples.

Discussion

Recurrence of Chordoma and Extent of Resection

The concept, which has been demonstrated in several previous studies of chordomas,1,3,11,22,32,34,42,45,46,52 that the extent of resection influences the interval for tumor recurrence has been confirmed here. In fact, we did not observe tumor recurrences earlier than 1 year postsurgery in patients who had undergone total tumor resection. It also appears, however, that chordomas that have been resected to the same extent might exhibit different rates of regrowth. Furthermore, we have found no significant difference in recurrence intervals between chordomas in which a total resection was achieved and those in which a subtotal removal was performed. A similar conclusion was also reached by Colli and Al-Mefty.11

From a theoretical point of view, the tumor doubling time should be less related to the extent of resection compared with the interval for tumor recurrence, because to calculate the former value, the amount of residual tumor is used as the baseline. Apart from minor differences, however, analysis of the tumor doubling time replicated the picture that had emerged when considering the recurrence interval. More specifically, in cases in which the tumor recurred soon after surgery, in which there had been partial or subtotal tumor removal, the time to recurrence and the tumor doubling time were not significantly different. This result supports the view that the regrowth rate of chordomas might be dependent on variables other than the amount of resection. It is of interest that all but one of the patients with multiple recurrences had experienced fast tumor regrowth since the first recurrence. We infer from these data that the propensity to recur quickly after surgery might be an original feature of chordomas rather than an acquired characteristic.

It is important to note that, first, there is a subgroup of chordomas that exhibit an unusually fast rate of growth.11,12,23,41 second, these tumors represent approximately 20% of chordomas in series in which extensive surgical approaches have been used;11 and third, this percentage increases to approximately 30% when more conservative surgical approaches are used, as in our series.

Histological and Immunohistochemical Features of Chordomas

Although it is well established that patients with chordosarcoma have a much better prognosis than those with chordoma, the issue of whether the distinction between typical chordoma and chordoid chordoma might be prognostically important is still debated. In the study by Heffelfinger, et al.,24 the chordoid variant of clival chordoma was associated with a significantly better prognosis than in the typical tumor. In later reports from the same institution by Mitchell, et al.,36 and by Forsyth, et al.,21 however, those investigators found no correlation between the histological variant and disease-free survival. A worse outcome in patients with the chordoid variant was noted by Wold and Laws13 in children and young adults, and by Hug, et al.,27 in adult patients. In a recent paper on chordoma of the cranio-cervical junction, Colli and Al-Mefty11 found no significant difference in survival or in the recurrence-free survival curves in patients with typical chordoma and those with chordoid chordoma, who represented approximately 30% of the whole study group. These authors concluded that the distinction between typical chordoma and chordoid chordoma has no practical value. In our study, we characterized the immunophenotype of all chordoma tumors and found chordoid differentiation in approximately 15% only, and these cases appear to have a better prognosis than the typical chordomas. A concluding comment on this issue is that there is still no convincing evidence to indicate that we can formulate reliable predictions on the evolution of chordomas based on the mere histological pattern.

Proliferative Activity

The prognostic value of proliferative activity in chordomas is controversial. In fact, Forsyth, et al.,73 and Matsuno, et al.,83 found that high mitotic activity or an MIB-1 score greater than 10% in the tumor specimens are related to shorter disease-free survival, whereas other authors have found no correlation between proliferative activity and outcome.57,38,44 In chordomas of the sacrum and mobile spine, Ki-67 greater than 5% was found to be an adverse prognos-
tic factor. More recently, Crockard, et al., reported a logarithmic relationship between tumor volume doubling time and the Ki-67 PI in a series of 24 patients with skull base chordoma, and concluded that the cell marker Ki-67 can be used to predict the growth rate of individual chordomas. In another paper by the same group, which included 19 such cases, and in which data were analytically presented, however, there are several patients with a short tumor doubling time (7–21 months) in whom the Ki-67 PI varied by a factor of 8 among different individuals (1.5–12.2%). In that report it also appears that specimens of the same tumor that were obtained in closely repeated procedures exhibited quite different Ki-67 PI values. These results support our finding that, although a somewhat significant relationship exists between the PI and tumor doubling time, the Ki-67 PI is not homogeneously represented among the different regions of chordoma tumors, and that regions of increased cell proliferation are interspersed within areas of indolent tumor growth.

**Mutation of p53 Protein and Expression of hTERT**

One major finding in our study is that mutation of the p53 protein in chordoma tumors is frequently associated with hTERT mRNA expression, and that such an association identifies those chordomas that exhibit unusually fast rates of growth. It has been proposed that at least two different mechanisms should be in place before cell immortalization occurs. The first mechanism generally requires inactivation of the pathways involving tumor suppressor genes, like the p53 protein, and the second involves the reactivation of telomerase. One of the main functions of the p53 protein is to block the progression through G1 into the S phase in response to DNA damage. The loss of normal p53 function leads to genomic instability and other genomic alterations, which supports the model of progressive accumulation of genetic changes with increasing grades of malignancy. Matsumoto, et al., have shown a close relationship among p53 immunoreactivity, proliferative potential, and recurrence of intracranial chordoma. In their study, nine of 10 recurrent chordomas showed positive immunoreaction for p53 protein, and the second involves the reactivation of telomerase. One of the main functions of the p53 protein is to block the progression through G1 into the S phase in response to DNA damage. The loss of normal p53 function leads to genomic instability and other genomic alterations, which supports the model of progressive accumulation of genetic changes with increasing grades of malignancy. Matsumoto et al. have shown a close relationship among p53 immunoreactivity, proliferative potential, and recurrence of intracranial chordoma. In their study, nine of 10 recurrent chordomas showed positive immunoreaction for p53 protein, and the second involves the reactivation of telomerase.

It is generally thought that the tumor cells undergo telomere shortening in the early stages of carcinogenesis, and that telomerase activation is advantageous mainly in the later stages of tumorigenesis. In previous studies of malignant brain gliomas, we showed that telomerase enzyme activity correlates with the histological grade, and that hTERT mRNA is the primary determinant regulating telomerase activity. We have also shown that the expression of hTERT mRNA is an early event in tumor progression from low-grade astrocytoma to malignant astrocytoma and glioblastoma. In a similar investigation, Harada, et al., demonstrated that secondary glioblastomas, which progress from low-grade astrocytoma, exhibit high levels of hTERT mRNA expression. These authors also showed that the increase in hTERT mRNA expression followed a p53 mutation, which was detected in the very early stages of carcinogenesis. In histologically benign neoplasms, the expression of hTERT mRNA might not be directly related to cell proliferation, but it appears to herald the unlimited clonal expansion of the tumor cells.

As stated earlier, cancers are characterized by multiple oncogenic events that collectively contribute to the phenotype of advanced-stage disease. Nevertheless, it is also important to know whether the initial oncogenic event continues to play a functional role at later stages of tumor progression. This question has recently been addressed by using transgenic animal models, and the prevailing opinion is that the primary oncogene is required to maintain the tumor phenotype, despite the presence of numerous additional oncogene and tumor suppressor mutations. In terms of understanding oncogenesis, chordoma is a unique model because in some instances the long survival and repeated surgeries allow a sort of molecular follow-up of the tumor. This was true in the patient suffering from the malignant chordoma (Case 22 in Table 1); he had undergone three previous surgeries for resection of a typical chordoma followed by high-dose radiation therapy, and then presented with a sarcomatous chordoma. At a later stage of the disease, when widespread systemic metastases had occurred, the tumor cells expressed hTERT mRNA but were negative for p53 immunoreaction, indicating that the tumor cells were either p53-defective or contained normal p53. It has recently been shown that the combined loss of the p53 family members p63 and p73, which also seem to be linked to tumor suppression, results in the failure of cells containing normal p53 to undergo apoptosis in response to DNA damage. Overall, our data indicate that in chordoma tumors, telomere dysregulation cooperates with abnormal p53 protein to facilitate initiation of cellular transformation.

**Loss of Heterozygosity and Microsatellite Instability**

Microsatellite instability is an indirect marker of globally defective DNA mismatch repair in neoplastic cells. Frequent microsatellite instability, that is, instability appearing in more than 30 to 40% of the examined loci, has been described in tumor samples obtained in patients with hereditary nonpolyposis colorectal cancer syndrome. Conversely, in frequent microsatellite instability, that is, instability appearing in less than 30 to 40% of the examined loci, has mainly been found in sporadic malignancies, and the molecular mechanisms underlying this phenotype have not been completely elucidated. In a study of 12 patients with sacral chordoma conducted by Klingler, et al., six patients (50%) showed microsatellite instability at least one locus, and two had LOH for at least one locus. Interestingly, one individual with LOH at two different loci presented with lymph node metastases and died of widespread metastatic disease. Their study indicates that LOH might prove to predict a worse prognosis; however, we were unable to confirm these results even in the case of malignant chordoma with systemic metastases in our study. Actually, the percentages of microsatellite instability and LOH that have been reported by Klingler, et al., appear to be exceedingly high compared with intracranial tumors, in which microsatellite instability and LOH range from 1.8 to 37%, with the glioblastoma tumors having the highest values. Thus, analyses of LOH and microsatellite instability do not seem to have much prognostic value in patients with intracranial chordoma.

**Conclusions**

In this study we show that the expression of hTERT
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mRNA by chordoma tumor cells is a reliable predictor of aggressive tumor behavior. The association of hTERT mRNA expression with p53 mutation characterizes the early stages of tumor progression.

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