**Objective:** To examine the expression of c-Met, c-Erb-b2 (HER2/neu), and epidermal growth factor (EGFR) in a cohort of 12 chordomas, based on the current and future availability of targeted molecular inhibitors.

**Design:** Protein expression levels were analyzed by immunohistochemical analysis from archival tissue using multitumored tissue microarray and by Spearman rank correlation test.

**Setting:** Tertiary care facility.

**Subjects:** We assembled the cohort of 12 chordomas from patients treated at the Yale–New Haven Hospital from 1986 to 2003 and compared them with 51 other tumors from 17 assorted solid tissue tumor types along with 1 sample of healthy tissue from each site.

**Main Outcome Measure:** The expression of c-Met, HER2/neu, and EGFR.

**Results:** Most chordomas displayed strong expression of EGFR and c-Met, whereas a variable level of expression of HER2/neu was seen. In addition, we noted a strong correlation between EGFR and c-Met expression, especially for primary chordomas ($P = .006$).

**Conclusions:** Chordomas, like many other solid-tissue tumors, express HER2/neu, EGFR, and the hepatocyte growth factor/scatter factor receptor c-Met. Most chordomas had strong expression of both the hepatocyte growth factor/scatter factor receptor and EGFR. Inhibitors to EGFR are already in clinical use for other solid-tissue tumors and represent a potentially viable experimental treatment option for refractory chordoma. Further studies are required to investigate these findings.


C HORDOMAS ARE RARE NEOPLASMS ARISING FROM NOTOCHORDAL REMNANTS IN THE MIDLINE SKELETAL AXIS. THE MOST COMMON SITES ARE THE SKULL BASE AND THE SACROCOCCYGEAL REGION. BECAUSE THERE IS AN ANNUAL INCIDENCE OF JUST 1 IN 2 MILLION FOR SKULL BASE CHORDOMAS, RELATIVELY LITTLE IS KNOWN ABOUT THE MOLECULAR BIOLOGY OF THESE TUMORS. THEY ARE USUALLY SLOW-GROWING TUMORS, AND INITIAL SYMPTOMS ARE USUALLY RELATED TO LOCAL PROGRESSION OF THE DISEASE WITH SUBSEQUENT COMPRESSION OF ADJACENT STRUCTURES. VAGUE HEADACHE, VISUAL CHANGES, AND CRANIAL NERVE PALSY ARE COMMON MANIFESTATIONS OF SKULL BASE CHORDOMAS. BY THE TIME MOST PATIENTS REPORT THESE SYMPTOMS, MOST CHORDOMAS HAVE ALREADY GROWN QUITE LARGE.

The natural course of chordoma is quite grim; most patients do not survive 10 years because of high local recurrence rates. The current treatment of choice is aggressive surgical excision followed by local irradiation, but even with the best treatments available, overall survival time remains roughly 5 years.

Given the rare nature of these tumors, current understanding of molecular marker expression in chordomas is limited. With the recent introduction of targeted molecular therapeutics into clinical practice, an enhanced understanding of the molecular biology of chordomas is needed. One important cell-signaling pathway in cancer progression involves a class of molecules known as receptor tyrosine kinases (RTKs). These molecules serve as membrane-bound cytokine receptors and initiate an intracellular signaling cascade on ligand binding. There are multiple examples of RTK proto-oncogenes, including c-sis (the platelet-derived growth factor receptor), the erb-b/HER epidermal growth factor receptor family (EGFR), and c-met (the hepatocyte growth factor/
and lung cancer. In addition, use of inhibitors of c-erb-b/HER family EGFR and HER2/neu are already in clinical use and have demonstrated efficacy in other more common solid-tissue tumors, including breast and lung cancer. In addition, use of inhibitors of c-met is currently planned for clinical trials and in the future may be approved for widespread clinical use. Because of the rare nature of chordomas, large-scale clinical trials of these agents in chordomas seem unlikely. If a molecular basis for predicted efficacy of a targeted agent could be discovered, however, such agents may represent a viable experimental treatment option for individual patients with refractory disease.

Basing our study on the current or future availability of targeted molecular inhibitors, we examined the expression of c-Met, c-Erb-b2 (HER2/neu), and epidermal growth factor receptor (EGFR) in a cohort of 12 chordomas and compared them with 51 tumors from 17 assorted tumor types. Given the limited number of chordomas available for analysis, these other tumor types provided an internal comparison for the results.

METHODS

CHORDOMA COHORT SELECTION

Following institutional review board approval, we assembled the cohort of chordoma tumors from all patients with a pathological diagnosis of chordoma treated at Yale–New Haven Hospital, New Haven, Conn, from 1986 to 2003. All patients were included; we had no exclusion criteria other than lack of available material for immunohistochemical analysis.

MULTITUMOR TISSUE MICROARRAY CONSTRUCTION

A multitumor tissue microarray had been previously assembled and was used for comparison of chordoma molecular marker expression with various other malignancies. We assembled the multitumor cohort from patients treated at Yale–New Haven Hospital between 1980 and 1999 with the appropriate pathological diagnosis. We included 3 tumors of each type plus 1 normal tissue for each site. Following institutional review board approval, we constructed the tissue microarray as previously described and included 51 tumor cases from varying sites. We obtained tissue cores from paraffin-embedded formalin-fixed tissue blocks from the archives at the Department of Pathology, Yale–New Haven Hospital.

A pathologist (D.K.) reviewed slides from all blocks to select representative areas of invasive tumor or normal tissue to be cored. The cores were placed on the recipient microarray block using a tissue microarrayer (Beecher Instruments, Silver Spring, Md). The tissue microarray was then cut to yield 3-µm sections, which were placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc, Hackensack, NJ) with UV cross-linking.

IMMUNOHISTOCHEMICAL ANALYSIS

All slides were processed simultaneously in identical conditions using standard methods. Chordoma slides were processed as standard 5-µm tissue sections mounted on sialinized glass slides (Dako, Carpenteria, Calif). Multitumor tissue microarray slides were prepared as described in the previous subsection. Slides were deparaffinized in xylene followed by 2 rinses in absolute ethanol. Following a 1-minute rinse in deionized water, heat-induced epitope retrieval was performed by pressure cooking in citrate buffer, pH 6.0, for 7 minutes. Nonspecific primary antibody interactions were blocked by incubation with 0.3% bovine serum albumin in 0.1M tris-buffered saline (BSA/TBS), pH 8.0, for 30 minutes at room temperature. Slides were then incubated with primary antibodies diluted in BSA/TBS overnight at 4°C (Table 1). Slides were then washed 3 times (for 5 minutes each time) with BSA/TBS containing 0.05% Tween-20 to remove unbound primary antibody. Goat anti-rabbit secondary antibody conjugated to a horseradish peroxidase decorated dextran-polymer backbone (Envision; Dako) was then applied for 1 hour at room temperature. Diaminobenzidine chromagen was used to visualize antibody binding. All slides were run simultaneously under identical conditions and included negative control (no primary antibody) slides.

IMMUNOHISTOCHEMICAL SCORING FOR EGFR, c-Erb-b2, AND c-Met

Consensus scoring by 2 observers (P.M.W. and D.K.) determined molecular marker expression. Membranous staining intensity was evaluated and scored on an ordinal 0 to 3+ scale for c-Met, EGFR, and HER2/neu. Occasional cytoplasmic staining was seen with c-Met but was not included in scoring. We scored each paraffin section of tumor and each tumor core using a 4-tiered grading system: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. Samples with scores of 2+ or 3+ were considered overexpression of that protein.

STATISTICAL ANALYSIS

We evaluated correlation of marker expression by Spearman rank correlation test. All calculations and analyses were performed with SPSS 11.5 for Windows (SPSS Inc, Chicago, Ill) and, where appropriate, were 2-tailed. Significance was considered to be P<.05.

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Table 1. Summary of Antibodies Used

<table>
<thead>
<tr>
<th>Target</th>
<th>Identifier</th>
<th>Type</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>c-Met</td>
<td>c28</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology, Santa Cruz, Calif</td>
<td>1:1000</td>
<td>Pacucci et al.11 1998</td>
</tr>
<tr>
<td>c-Erb-b2 (HER2/neu)</td>
<td>a0485</td>
<td>Rabbit polyclonal</td>
<td>Dako Cytomation, Carpenteria, Calif</td>
<td>1:200</td>
<td>Pai-Scherf et al.12 1999</td>
</tr>
<tr>
<td>EGFR</td>
<td>2232</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technologies, Beverly, Mass</td>
<td>1:100</td>
<td>Endo et al.13 2002</td>
</tr>
</tbody>
</table>

Abbreviation: EGFR, epidermal growth factor.
RESULTS

COHORT DEMOGRAPHICS

After review of the Department of Pathology records at Yale–New Haven Hospital, we identified 13 surgical specimens from 11 patients diagnosed as having chordomas. One of these blocks was unavailable, leaving 12 chordoma specimens (from 10 patients). Two patients experienced recurrence and subsequent resection. These recurrent specimens were included in the cohort but separated out for statistical analysis, leaving 10 unique specimens for comparison. The patients (7 women and 3 men) ranged in age from 16 to 86 years (median age, 58 years). Locations of the chordomas were skull base (3), sacrum (5), and other vertebra (2). Demographic information is summarized in Table 2.

The multitumor tissue microarray consisted of 51 tumors of 17 malignancies (3 tumors from each type) along with 1 healthy tissue from each site. Tumor types included head and neck squamous cell, renal cell, and gastric carcinomas; melanoma; prostate, brain, ovarian, germ cell, colon, bladder, endometrial, thyroid, hepatic, pancreatic, and lung cancers; lymphoma; and sarcoma.

IMMUNOHISTOCHEMICAL ANALYSIS FOR HER2/neu, c-Met, AND EGFR EXPRESSION

Twelve (100%) of 12 chordoma tumors and 37 (73%) of 51 comparison tumor specimens had sufficient representative tissue for analysis of HER2/neu expression. Chordomas displayed a spectrum of intensities ranging from no (0) expression to very strong (3+) expression of HER2/neu. Among primary chordomas there were 3 tumors with no expression (0), 1 with mild (1+), 2 with moderate (2+), and 4 with strong (3+) expression. Both recurrent chordomas lacked expression of HER2/neu. Among comparison tumors, prostate and bladder carcinoma displayed predominately strong (3+) expression, whereas lymphomas, hepatic carcinomas, and sarcomas displayed predominately weak to no staining (0 to 1+).

A representative micrograph of moderate (2+) HER2/neu expression in chordoma is shown in Figure 1A.

For c-Met, 12 (100%) of 12 chordomas and 39 (76%) of 51 comparison tumors had sufficient representative tissue for analysis. Chordomas displayed predominately strong expression of c-Met, with no tumors displaying absent expression, 1 with mild (1+), 2 with moderate (2+), and 7 with strong (3+) expression. The recurrent chordoma tumors both displayed strong (3+) expression of c-Met. Among comparison tumors, gastric, prostate, ovarian, and colon carcinomas displayed predominately strong (3+) expression, whereas thyroid carcinoma and lymphomas displayed predomi-
Numerous studies\textsuperscript{14-16} have yielded a consensus on characteristic immunohistochemical staining parameters that define chordomas. These include positive expression of cytokeratin and epithelial membrane antigen. While this aids pathological diagnosis, few studies have been conducted to determine expression patterns of other potentially useful molecular markers. A recent study by Deniz et al\textsuperscript{22} found that expression levels of basic fibroblast growth factor, transforming growth factor $\alpha$, and fibronectin were all correlated with local recurrence and aggressive biological behavior. Kilgore and Prayson\textsuperscript{18} found no association between cyclin D1, MIB-1, p53, and BCL-2 and prognosis. In addition, the receptor tyrosine kinase c-Met was found to be highly expressed in chordomas in one study\textsuperscript{19} of benign and malignant bone tumors.

In the present study, we decided to focus our investigations on the differential expressions of c-Met, EGFR/HER1, and HER2/neu RTKs between chordomas and a wide variety of tumors. As inhibitors of these RTKs become more widely used in the clinical setting, a better understanding of the signaling pathways in vivo, their pattern of expression and activation, and their modulation by these therapies is of critical importance because it may be helpful in predicting the target tumor population that will benefit from these therapies.

We found that all 3 of these RTKs are expressed to varying degrees in chordomas. For EGFR and c-Met, most chordomas displayed strong expression, whereas for HER2/neu we saw a variety of expression levels. In addition, we noted a strong correlation between EGFR and c-Met expression, especially for primary chordoma tumors.

The ErbB/HER receptor family is composed of the following 4 related receptors: EGFR (Erb-b1/EGFR/HER1), Erb-b2 (HER2/neu), Erb-b3 (HER3), and Erb-b4 (HER4). Ligand binding promotes receptor dimerization, which results in high-affinity ligand binding, activation of the intrinsic protein kinase activity, and tyrosine autophosphorylation. These events result in the activation of a signal transduction cascade that is mitogenic and possibly transforming.\textsuperscript{20} The c-Met/hepatocyte growth factor (HGF) pathway is also very important in carcinogenesis. The HGF is the ligand of c-Met receptor and is also known as the scatter factor. Signaling pathways activated by c-Met–HGF interaction mediate several cellular processes essential for life. These cellular processes lead to a broad range of biological events, including embryological development, wound healing, tissue regeneration, angiogenesis, growth, invasion, and morphogenic differentiation.\textsuperscript{6} Because several of these physiological processes are known to be crucial in tumorigenesis and metastasis, the c-Met/HGF pathway plays a significant role in carcinogenesis.

Our findings of increased c-Met expression in most chordomas is important. The chromosomal site of the c-met proto-oncogene has been located at 7q31.\textsuperscript{21} Scheil et al\textsuperscript{22} found that gains of 7q were among the most common chromosomal alterations noted; 69% of chordomas studied demonstrated 7q gain. Thus, gain of c-Met expression via 7q amplification may represent an early event in chordoma progression.

We also demonstrated a significant and strong correlation between c-Met and EGFR expression, especially in primary chordomas. Peghini et al\textsuperscript{19} noted a similar relationship in gastrinomas and found this to correlate with aggressive behavior. This is not surprising given the mounting body of evidence that EGFR and c-Met inter-

**Figure 2.** Receptor tyrosine kinase expression patterns in primary chordoma (n=10). A, c-Met expression in primary chordoma was noted to be primarily strong (3+). B, HER2/neu expression was variable from no expression to strong expression. C, Epidermal growth factor (EGFR) expression was primarily strong (3+).
act in a variety of ways to modulate downstream mitogenic signaling following HGF stimulation. Schieving et al demonstrated that specific pharmacologic inhibition of EGFR kinase did not affect c-Met kinase activity but blocked the proliferative effects of HGF binding. This is clinically important because it raises the possibility that inhibition of EGFR alone may mediate antitumor effects on multiple RTK signaling pathways.

Our study's main limitation was the small sample size, necessitating a hypothesis-generating experimental design. Despite obvious limitations, such studies are essential in stimulating new research into rare neoplasms. Efforts are currently under way to create a larger follow-up experiment through multi-institution collaboration. Additional in vitro studies that assess the impact of RTK inhibitors on chordoma cell culture would also be extremely useful. In 2001, Scheil et al described the creation of the only known chordoma cell line, U-CH1. We are currently investigating this possibility.

Our study demonstrated that EGFR, HER2/neu, and c-Met may play a significant role in the growth of chordomas. Given the natural course of chordomas, any possible new treatment options deserve exploration. Our results may suggest the potential clinical utility of the use of RTK inhibitors gefitinib (or erlotinib), trastuzumab, and/or c-Met inhibitors in the treatment of patients with refractory chordomas. These molecule-targeted therapies will certainly revolutionize cancer therapy. The use of these inhibitors alone or in combination with chemotherapy or radiation shows promise for therapy in a wide variety of cancers whose growth depends on aberrant signaling via these pathways. Our findings should be viewed with caution, but for patients with few other treatment options, experimental use of one of these agents on an "of 1" trial basis may be a viable option. However, while certainly less toxic than traditional chemotherapy, these inhibitors are not devoid of adverse effects. A decision to use them experimentally, even for a patient with no alternative treatment options, should again be made with due caution.

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