Epidermal growth factor receptor gene amplification and expression in disseminated pediatric low-grade gliomas

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Object. Pediatric low-grade gliomas (LGGs) are the largest group of central nervous system neoplasms in children. Although these tumors are generally benign, 5 to 10% of patients with pediatric LGGs present with leptomeningeal dissemination. The genetic and biological nature of these tumors is poorly understood. The authors looked for certain molecular abnormalities that may differentiate disseminated gliomas from the other pediatric LGGs.

Methods. Comparative genomic hybridization (CGH) was applied to 18 pediatric LGGs. Six cases featuring disseminated pediatric LGGs were compared with 12 control cases involving nondisseminated pediatric LGGs. Fluorescence in situ hybridization (FISH) analysis and immunohistochemical analysis were used to highlight further specific genetic targets.

The CGH revealed multiple chromosomal abnormalities in five of six cases with disseminated gliomas and in six of 12 control cases. No correlation was found between the number of chromosomal abnormalities and dissemination status. Amplification of chromosome 7 was noted in four of six cases with disseminated gliomas as opposed to one of 12 control cases (p = 0.02). The FISH analysis revealed epidermal growth factor receptor (EGFR) amplification in one case negative to chromosome 7 amplification by CGH, raising the amplification cases to five of six (p = 0.0038). Immunohistochemical analysis for EGFR was positive in six of six cases and in two of 12 control cases (p = 0.0015). At the end of a mean follow-up period of 7.2 years, all patients with disseminated gliomas are alive with variable but slow disease progression.

Conclusions. The high rate of EGFR gene amplification and protein expression in disseminated pediatric LGGs is intriguing and may have implications for our understanding of the role of EGFR in glioma genesis. Targeted therapies may be available for these children. Larger-scale studies are needed to establish further these findings.

Key Words • low-grade glioma • epidermal growth factor receptor • dissemination • pediatric neurosurgery

Pediatric LGGs are a heterogeneous group of neoplasms representing 40 to 50% of pediatric brain tumors. Although most pediatric LGGs tend to have a nonaggressive clinical course, extreme diversity exists regarding factors such as location, clinical course, and progression-free survival.

Three to 10% of patients with pediatric LGGs present with leptomeningeal dissemination either at diagnosis or at the time of progression.9,10,21 The exact incidence of this phenomenon is not known mainly because of the lack of routine spinal MR imaging in all patients at diagnosis or progression. Risk factors for dissemination are not well defined. Tumor location is most commonly reported, such as hypothalamic and optic pathway gliomas and tumors proximate to the ventricular system.2,8,10,21 The clinical course is also extremely variable, but, surprisingly—in contrast to high-grade gliomas—dissemination does not necessarily mean end-stage disease.9 Currently, no pathological subtype is specifically associated with dissemination.21 Biological markers are urgently needed so that tumors at risk for dissemination can be targeted and so that the possibly of better therapy for these children can be explored.

The EGFR is a transmembrane glycoprotein that constitutes one of four members of the erbB family of tyrosine kinase receptors.5 The binding of EGFR to its cognate ligands leads to autophosphorylation of receptor tyrosine kinase and subsequent activation of signal transduction pathways that are involved in regulating cellular proliferation,
invasiveness, and survival. The EGFR was found to be amplified in a significant portion of high-grade gliomas and is associated with tumor aggressiveness and invasion. These characteristics make EGFR an attractive gene to use in examining the behavior of disseminated pediatric LGGs.

The aim of this study was to look for specific genetic and biological characteristics of disseminated pediatric LGGs and to compare them with nonmetastic pediatric LGGs.

**Materials and Methods**

**Tissue Accrual**

Tissue samples were collected from the pediatric neurooncology tissue bank after ethical board approval. For CGH, snap-frozen tissues were used, and FISH was applied to tumors when fresh material was available. Immunohistochemical analysis was performed on representative slides from paraaffin-embedded sections of all tumors. Clinical data were collected from the neurooncology database and clinical charts at the participating institutions. Inclusion criteria included a pathological report consistent with Grade I or II (low-grade) gliomas and cranial plus spinal MRI imaging as a routine metastatic screen at diagnosis.

Specimens from six patients with disseminated disease confirmed by MR imaging at diagnosis were compared with those from 12 control patients with local disease (Table 1).

**Comparative Genomic Hybridization**

High-molecular-weight DNA was isolated from the frozen samples according to standard procedure by using the QIAamp DNA Mini kit (Qiagen, Valencia, CA). Hybridization of differentially labeled tumors and normal DNA to normal metaphase chromosomes was performed essentially as previously described. Briefly, normal lymphocyte metaphase preparations were denatured at 75°C for 2 minutes in a denaturation solution (70% formamide, 2 × SSC, pH 7) and dehydrated in an ethanol series (70, 80, and 100%). Tumor DNA was labeled with Spectrum green deoxyuridine triphosphate and healthy DNA with Spectrum red deoxyuridine triphosphate by using a nick translation labeling kit (Vysis, Downers Grove, IL). After labeling, the DNA fragments ranged in size from 300 to 600 bp. One microgram of labeled tumor and normal DNA was ethanol precipitated with 30 μg of unlabeled Cot-1 DNA, dissolved in 14 μL LSI hybridization buffer (Vysis), denatured at 75°C for 5 minutes, and applied to normal lymphocyte metaphase preparations. The hybridization was performed at 37°C for 3 days in a humid chamber. After hybridization, slides were washed in 0.4 × SSC at 75°C for 2 minutes and in 0.1% NP-40, 2 × SSC for 2 minutes at room temperature. Slides were counterstained with DAPI in an antifade solution.

**Digital Image Analysis and Statistical Methods**

Digital image analysis was used to facilitate the identification of chromosomal regions with abnormal fluorescence ratios. Images of the hybridized metaphases were evaluated with a digital image analysis system modeled on a Zeiss fluorescence microscope and a cooled charge-coupled device camera (Photometrics, Inc., Tucson, AZ) interfaced to a Cytovision Image Analysis System (Applied Imaging, Newcastle upon Tyne, United Kingdom). Tricolor images (green [Spectrum green] for the tumor DNA, red [Spectrum red] for the normal reference DNA, and blue [DAPI] for the DNA counterstain) were acquired from 10 to 15 metaphases per specimen. Calculation of the green/red (tumor/normal tissue) fluorescence ratios was performed using Cytovision software. Briefly, chromosomes were segmented and interchromosomal backgrounds subtracted from the chromosomal fluorescence. The green/red fluorescence ratio (tumor/normal copy number) was displayed as a green/red ratio profile. The ratio profiles were standardized so that the overall green/red ratio for the entire metaphase was set to 1. Copy number changes in the tumor were expressed relative to this baseline ratio. All or partial chromosome gains were defined as having a green/red ratio greater than 1.2 and all or partial chromosome losses were defined as having a green/red ratio less than 0.8. The Student t-test and the Fisher exact test were used to differentiate between the groups or the chromosomes examined.

**Fluorescence in Situ Hybridization**

Touch preparations were generated by gently touching each tumor tissue biopsy specimen to a dry microscope slide. This brief contact allowed an adequate number of single tumor cells to adhere to the slide surface. Specimens were fixed in Carnoy (methanol, acetic acid 3/1, 20 minutes at 37°C). Slides were dehydrated with an ethanol series (70, 80, and 100%) for 2 minutes. Probes (0.5 μL), H2O (1 μL) and hybridization buffer (3.5 μL; Vysis) were added to each slide, and a coverslip was sealed with rubber cement. Code naturation (5 minutes at 75°C) and hybridization (24 hours at 37°C) were performed on the Hybrite system (Vysis). Analysis of the signals was performed with a Zeiss Axioscope 1 fluorescence microscope (Zeiss, Jena, Germany) by using a dual or triple bandpass filter set.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed on formalin-based tissue sections that were 4 μm thick and embedded in paraffin. Tissue sections prepared for antibody application were pretreated with heat-induced epitope retrieval performed with a buffer (pH 6.0 Target retrieval; Zymed, San Francisco, CA) for 12 minutes by a temperature-controlled microwave treatment that used an H2800 model processor (Energy Bean Sciences, Inc., Agawam, MA).

The mouse monoclonal anti–human antibody EGFR (1:75 dilution; Santa Cruz, CA) was applied and incubated for 32 minutes. Slides were developed using an enhanced DAB detection kit (biotinylated Ig/SA-HRPO; Ventam, Tucson, AZ) with endogenous biotin block on an autostainer (ES Automated Stainer; Ventam). Slides were then counterstained with hematoxylin, dehydrated, and covered with a permanent mounting medium for microscopic examination. Staining was interpreted as strong (+3), medium (+2), or weak (+1) and was deemed focal if less than 50% of cells stained positive.

**Statistical Analysis**

Associations among clinical features (patient age at diagnosis, dissemination status, and extent of resection), tumor histopathological analysis, and molecular characteristics (CGH abnormalities, EGFR amplification, and expression) were investigated using the Fisher exact test. Progression-free survival was measured from the date of diagnosis to the date of first progression (or death, whichever came first) or last contact.

**TABLE 1**

**Summary of clinical, pathological, and molecular data in all patients**

<table>
<thead>
<tr>
<th>Type of Disease</th>
<th>Nondisseminated</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of patients</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>mean age at diagnosis (yrs)</td>
<td>4.3</td>
<td>7.1</td>
</tr>
<tr>
<td>range</td>
<td>0.9–9</td>
<td>0.5–17</td>
</tr>
<tr>
<td>M/F</td>
<td>2.4</td>
<td>5.7</td>
</tr>
<tr>
<td>pathological entity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JPA</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>other pediatric LGGs</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>no. of cases expressing chromosomal abnormalities by CGH</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

* Chromosome 7 amplification by CGH, FISH for EGFR locus. Abbreviation: JPA = juvenile pilocytic astrocytoma.
Results

Comparative Genomic Hybridization

The CGH analysis was successfully performed for the 18 pediatric LGGs. Table 1 summarizes the clinical data and copy number changes of the 18 patients (six disseminated tumors and 12 nondisseminated tumors). Numeric chromosomal abnormalities were found in five of six patients with disseminated LLGs. Six of 12 nondisseminated LGGs had chromosomal abnormalities as well (Fig. 1). The number of chromosomal abnormalities did not correlate with the risk of dissemination or disease progression within the group with disseminated glioma. Gain of DNA material of chromosome 7 was encountered in 67% (four of six) of the disseminated tumor cases and in only 8.3% (one of 12) of the nondisseminated cases (p = 0.02). Careful evaluation of the data revealed that the nondisseminated case exhibited a gain of chromosome 7q, whereas all gene amplification in the disseminated tumor group involved the 7p arm (p < 0.001). Other chromosomal abnormalities were neither common nor specific for cases of disseminated gliomas compared with cases of nondisseminated gliomas.

The FISH Analysis

To investigate whether amplification of the EGFR gene located on the short arm of chromosome 7 is involved, we performed FISH analysis with the relevant probe on available samples (Table 2). This analysis revealed that one of the two disseminated samples (Case 5, Fig. 2) that did not show a gain of chromosome 7 by CGH had focal amplification of the EGFR locus, with more than 20 copies of the EGFR locus in 7% of the cells, raising the ratio of disseminated tumor cases with EGFR amplification to five of six patients. Another case (Case 2) that showed amplification of chromosome 7 by CGH exhibited the same pattern of rare cells, with high levels of EGFR locus amplification.

Immunohistochemical Analysis for EGFR

To ascertain whether EGFR is expressed differentially in the two groups, we performed an immunohistochemical analysis to determine the presence of EGFR in all samples. In all six cases with dissemination, EGFR stained positively. Immunohistochemical analysis was positive in only two of 12 nondisseminated tumor control cases (p = 0.0015; Table 1). The pattern of EGFR staining did not correlate with disease progression or magnitude of dissemination.

Follow Up

Long-term follow up was available for all of the patients. All children in the disseminated tumor group are alive at a mean follow up of 7.2 years; however, all six patients had tumor progression requiring at least two different chemotherapy regimens in all cases and radiotherapy in three. Of the control group, seven patients underwent gross-total resection at diagnosis, and one experienced a recurrence. Five of 12 control patients underwent biopsy only. All patients were treated with chemotherapy, and only one died. In the two patients of the control group whose specimens stained positively for EGFR gross-total resections of the tumor were performed and there was no recurrence of disease. There was no correlation between the immunohistochemical findings and tumor progression.

Discussion

The ability of pediatric LGGs to disseminate is still a clinical and biological enigma. We tried to address some of the issues regarding the genetics and biology underlying this phenomenon. The combination of EGFR gene amplification and overexpression in pediatric LGGs found in our study is intriguing.

Disseminated pediatric LGGs are more invasive but are not necessarily more aggressive than are other pediatric LGGs of the same primary locations. High-grade gliomas...
have an extremely aggressive clinical course that correlates with more complex karyotypes and CGH abnormalities. The CGH findings in this study did not reveal a significant difference between cases involving disseminated gliomas and control cases with local tumors, which correlated with the rather benign clinical behavior of the disseminated group. These data imply that one should look for specific genetic pathways regarding the invasive nature of disseminated LGGs rather than for global genomic instability.

A gain of chromosome 7 is the most common chromosomal abnormality of LGGs, implying that our findings may not be specific. Critical review of the literature reveals that the most common finding in pediatric LGGs is normal karyotype (80–90% of cases). In adults with LGGs, a gain of chromosome 7 occurs in 10 to 15% of cases but usually involves the long arm of the chromosome. In published pediatric series, this frequency is less than 10%, compatible with the findings in our control group. The combination of FISH and CGH revealed amplification of the short arm of chromosome 7 (the EGFR locus) in 83% of our patients with disseminated tumors. Although the numbers are small, these findings are statistically significant and require further investigation.

Amplification of EGFR is associated with adult primary glioblastomas. As opposed to the secondary type, they do not have p53 and other mutations, and EGFR amplification has been shown to endow these tumors with a highly invasive nature. Recent reports have shown that EGFR signaling promotes activation and expression of certain metalloproteinases, as well as adhesion molecules on the extracellular matrix. The combination of clinical and biological data makes the EGFR pathway an attractive candidate for the dissemination ability of certain pediatric LGGs.

The expression of EGFR has not been studied extensively in pediatric LGGs. In adults, gene expression profiling has shown that although EGFR is highly expressed in high-grade glial tumors, it is expressed in variable degrees in low-grade tumors (compared with normal brain tissue). In our study, EGFR expression as determined by immunohistochemical analysis was variable between tumors ranging from low to high intensity and from local to diffuse. The significance of these findings in adult glioblastomas is controversial. In children with disseminated pediatric LGGs, a biopsy sample is not regularly taken from the disseminated region and so may not represent the metastatic cell subpopulation. Furthermore, Okada, et al., applied FISH to secondary glioblastomas and found EGFR-amplified cells in the periphery of a tumor that was rendered EGFR-nonamplified by other techniques. Analysis of this intriguing finding indicates that in pediatric LGGs, subpopulations of cells that express EGFR can disseminate locally or leptomeningeally according to the tumor location.

The main caveats of this study pertain to the small number of patients and the lack of sufficient material with which to perform FISH for all of the patients. We did not

**TABLE 2**

Clinical and molecular characteristics of cases with disseminated tumors*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pathological Entity</th>
<th>Primary Location</th>
<th>Dissemination</th>
<th>CGH +7p</th>
<th>FISH for EGFR</th>
<th>Immunohistochemical Analysis for EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>protoplasmic astrocytoma</td>
<td>spinal cord</td>
<td>holocord</td>
<td>+</td>
<td>ND</td>
<td>+2</td>
</tr>
<tr>
<td>2</td>
<td>JPA</td>
<td>optic pathway</td>
<td>brain</td>
<td>+</td>
<td>28 total, 8.5 HL</td>
<td>+2 focal</td>
</tr>
<tr>
<td>3</td>
<td>astroblastoma</td>
<td>lat ventricle</td>
<td>brain &amp; spinal cord</td>
<td>+</td>
<td>ND</td>
<td>+2</td>
</tr>
<tr>
<td>4</td>
<td>JPA</td>
<td>spinal cord</td>
<td>holocord</td>
<td>−</td>
<td>ND</td>
<td>+1 focal</td>
</tr>
<tr>
<td>5</td>
<td>PXA</td>
<td>supracellular</td>
<td>brain</td>
<td>−</td>
<td>31.5 total, 7 HL</td>
<td>+3</td>
</tr>
<tr>
<td>6</td>
<td>JPA</td>
<td>none</td>
<td>brain &amp; spinal cord</td>
<td>+</td>
<td>ND</td>
<td>+1–2</td>
</tr>
</tbody>
</table>

* HL = high level of EGFR signal; ND = not determined; PXA= pleomorphic xanthoastrocytoma.

**FIG. 2.** Case 5. An amplification and expression profile of chromosome 7. **Left:** The CGH profile of chromosome 7, revealing a balanced profile. **Center:** A FISH capture revealing a variety of cells, some highly amplified for EGFR (shown in red; chromosome 7 centromere appears in green). **Right:** Photomicrograph showing that immunohistochemical analysis was positive for EGFR. H & E, original magnification × 40.
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look for EGFR gene mutations, which turns out to be an important issue, in these tumors. More tumor banking and cooperation between centers will enable us to undertake further comprehensive studies, including RNA extraction for expression of the gene in these rare cases.

Epidermal growth factor receptor is an important target for further research because of the small molecules that inhibit this receptor.20,21 In the context of disseminated pediatric LGGs, it will probably be better to add these agents as adjuncts to the standard chemotherapy protocols, because current protocols are successful in disease control19 and because only subsets of the cells express EGFR.

Conclusions

To our knowledge, this is the first study to address the biological and genetic background of disseminated pediatric LGGs. The high frequency of EGFR amplification correlated well with protein expression. Biologically, this study broadens our understanding of the role of EGFR in glioma genesis and invasion. The diagnostic, prognostic, and possible therapeutic implications of these findings will need validation in a larger comprehensive study.

References


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