Biological and Molecular Analysis of a Low-Grade Recurrence of a Glioblastoma Multiforme

Adrienne C. Scheck,2 Joan Rankin Shapiro, Stephen W. Coons, Sylvia A. Norman, and Peter C. Johnson

Neuro-Oncology Research [A. C. S., J. R. S., S. A. N.] and Division of Neuropathology [S. W. C., P. C. J.], Barrow Neurological Institute of St. Joseph’s Hospital and Medical Center, Phoenix, Arizona 85013

ABSTRACT
We and others have reported that human malignant gliomas demonstrate intratumor heterogeneity in which many regions may be benign; however, the presence of regions of increased malignancy in these same tumors is generally indicative of poor patient prognosis. These data suggested that tumor progression may be a local phenomenon, resulting in regions that progress to a more malignant type prior to the progression of the entire tumor. Implicit in this premise is the idea that molecular markers of tumor progression may be detectable prior to histological evidence of progression. This report details analyses performed on a primary and recurrent tumor obtained from the same patient in which the primary tumor was of a higher histological grade than the recurrent tumor. Results of molecular, cytogenetic, flow cytometric, and histological analyses of the primary tumor were indicative of a grade 4 glioblastoma multiforme. Standard cytogenetic and flow cytometric analyses demonstrated that the cells were near-diploid with a stem line population of 46,XX normal G-banded karyotypes. In contrast, tissue resected from the recurrent tumor 5 months later was histologically less malignant; however, the molecular, cytogenetic, and flow cytometric analyses of this sample demonstrated the presence of specific genetic abnormalities typically found in more malignant tumors. These data demonstrate that specific molecular and/or genetic changes leading to tumor progression may become detectable in a glioma prior to the appearance of histological features of a higher grade tumor.

INTRODUCTION
Genomic instability is inherent in cancer cells (1, 2) including gliomas (3). As tumor cells divide, the resulting daughter cells contain the genetic alterations present in the parent cell, but they may also continue to evolve additional karyotypic and/or molecular changes. Local expansion of these divergent clones results in a regionally heterogeneous tumor. Thus, specific genetic abnormalities present in some tumor regions will not occur in others. This can result in local areas of altered phenotypic characteristics such as therapy resistance, increased malignancy, growth rate, microvascular proliferation, and invasiveness (4–13). We recently analyzed 38 separate regions of a low-grade oligoastrocytoma and demonstrated the presence of regional heterogeneity using both cytogenetic and flow cytometric analyses (14). These data demonstrated the presence of regions with enhanced genetic instability that generated several distinct clonal abnormalities occupying different regions of the tumor. Although one clone appeared to be more widely disseminated than the other three clonal populations, each was adjacent to an area in which >50% of the cells consisted of nonclonal aberrations.

Difficulties in the diagnosis of glial tumors due to their regional heterogeneity is recognized, and has spawned the search for molecular markers of progression that may be detected in tumor specimens which erroneously reveal low histopathological malignancy. A number of potential markers have been identified such as various karyotypic abnormalities (15–20), aberrant expression of the EGFR3 (10, 21–30), loss of expression of the tumor suppressor gene DCC (31), increased expression of proliferation markers (32–35), aberrant expression of p53 and mouse double minute 2 (36–39), and aberrant expression of growth factors (40–45). In addition to impacting tumor diagnosis, the use of prognostic markers to study regional heterogeneity has further implications for clinical tumor management. Surgical removal of regions with local malignant transformation may be more effective in promoting regression and improving survival than a less directed resection. Reports of patients whose recurrent tumor is of a lower grade than the primary tumor are largely anecdotal, and an in-depth analysis of such tumor pairs has not been reported. We now report the molecular, cytogenetic, flow cytometric, and histological analysis of tumor samples from a primary and recurrent tumor from the same patient in which the recurrent tumor was of a lower histological grade than the primary tumor.

MATERIALS AND METHODS

Patient Data and Tumor Histopathology. The patient was a 30-year-old female undergoing craniotomy for resection of a primary malignant brain tumor in the right frontal lobe. A subtotal gross resection was performed, and the tumor was identified as a GBM (grade 4 astrocytoma) using the Ringertz-

1 This work was supported by NIH Grants CA50931 and CA25956.

2 To whom requests for reprints should be addressed, at Neuro-Oncology Research, Barrow Neurological Institute of St. Joseph’s Hospital and Medical Center, 350 West Thomas Road, Phoenix, AZ 85013. Phone: (602) 406-3647; Fax: (602) 406-7172; E-mail: ascheck@mha.chw.edu.

Received 7/17/95; revised 9/8/95; accepted 9/15/95.

The abbreviations used are: EGFR, epidermal growth factor receptor; DCC, deleted in colon cancer; GBM, glioblastoma multiforme; FISH, fluorescence in situ hybridization; FCM, flow cytometry; DI, DNA index; RPTPβ, receptor protein tyrosine phosphatase β; RT, reverse transcription.
Total cellular RNA was isolated from frozen tumor tissue using LHR-A, LHR-B, LHR-C, LHR-D, and LHR-E in compliance with conditions specified by the manufacturer. Six-txl aliquots of this reaction were used for PCR with primers described in Table 1. Since these primers span introns, they will amplify the appropriate size fragments from cDNA only. The use of one RT reaction for multiple PCR reactions allowed us to use the "housekeeping" gene histone 3.3 as a "loading" control (31, 48). Samples were analyzed by electrophoresis through a 1.8% agarose gel, visualized using ethidium bromide, and a Polaroid photograph was taken of the resulting gel. Quantitation was done by densitometric analysis of the photographic negative (49). Negative controls without RNA were performed for each experiment.

**Table 1 Primers used for PCR Analyses**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Primer location</th>
<th>Fragment size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td>5'-TTCCGCCATGGTATTATTTAATCA-3'</td>
<td>Exon O</td>
<td>233</td>
<td>84</td>
</tr>
<tr>
<td>EGFR</td>
<td>5'-GGCTCTCTCATGTTGTAAGAGG-3'</td>
<td>Exon P</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>(TM)</td>
<td>5'-TCCTCCTCATGTTGTAAGAGG-3'</td>
<td>1467-1484</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Cyt.)</td>
<td>5'-TATCTACCCTTTGTTGTTCTAT-3'</td>
<td>1645-1666</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ext)</td>
<td>5'-AGGCTCTCTCATGTTGTAAGAGG-3'</td>
<td>3001-3020</td>
<td>832f</td>
<td>25</td>
</tr>
<tr>
<td>RT(PP)</td>
<td>5'-ATGTTGCAAGTGCTCTTCTT-3'</td>
<td>3813-3832</td>
<td>577/507</td>
<td></td>
</tr>
<tr>
<td>(intact)</td>
<td>5'-TGCAGAATAGTACACTGCTG-3'</td>
<td>5176-5178</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPTPβ</td>
<td>5'-GCGTGACTGAGAAGGAAAGAAA-3'</td>
<td>254-273</td>
<td>1151d</td>
<td>A. C. Scheck</td>
</tr>
<tr>
<td>(secreted)</td>
<td>5'-AGGCTCTCTCATGTTGTAAGAGG-3'</td>
<td>1385-1404</td>
<td>331</td>
<td></td>
</tr>
<tr>
<td>(truncated)</td>
<td>5'-AGGCTCTCTCATGTTGTAAGAGG-3'</td>
<td>5716-5736</td>
<td>534</td>
<td></td>
</tr>
<tr>
<td>Histone 3.3</td>
<td>5'-TGCAGAATAGTACACTGCTG-3'</td>
<td>6250-6270</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGTGTGAAGTGCTCTTCTT-3'</td>
<td>2295-2313</td>
<td>509</td>
<td>S. A. Norman</td>
</tr>
<tr>
<td></td>
<td>5'-TCATCCTCCGTGGTCATGCT-3'</td>
<td>2787-2804</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-TGCAGAATAGTACACTGCTG-3'</td>
<td>2295-2313</td>
<td>2945d</td>
<td>S. A. Norman</td>
</tr>
<tr>
<td></td>
<td>5'-ATGTTGCAAGTGCTCTTCTT-3'</td>
<td>5222-5240</td>
<td>375</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GCGTGACTGAGAAGGAAAGAAA-3'</td>
<td>5716-5736</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGTGTGAAGTGCTCTTCTT-3'</td>
<td>172-191</td>
<td>215</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>5'-TTCCGCCATGGTATTATTTAATCA-3'</td>
<td>1385-1404</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-AGGCTCTCTCATGTTGTAAGAGG-3'</td>
<td>3001-3020</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-ATGTTGCAAGTGCTCTTCTT-3'</td>
<td>5716-5736</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Nucleotide number or exon location.
b Size expected from amplification of cDNA.
c TM, transmembrane domain; Ext., extracellular domain; Cyt., cytoplasmic domain.
d Lower number is the size of the truncated message. RT-PCR will not demonstrate the 2945-bp fragment due to its length. The cytoplasmic domain of the gene-encoding EGFR has two truncated forms that can be demonstrated by this primer pair; however, only the 507-bp fragment was detected in tumors LH and LHR.
e Primers were designed to different exons using the Oligo 4.1 primer design software.
f Primers were designed using the sequence given in Kreuger and Saito (85).

Burger (46) and revised WHO (47) systems for astrocytoma. The patient was treated with 6060 cGy to the tumor and a chemotherapeutic regimen that included 10-ethyl-10-deazaaminopterin (edatrexate, 10-EDAM) and carboplatin. A second treatment regimen with focused ultrasound was also administered; however, the tumor recurred 5 months after the original surgery. At this time, a second large resection was performed and only diffusely infiltrative low-grade astrocytoma (grade 2) was found. The large tissue samples were dissected into 1-cm regions, and each region was further divided, with one sample going into formalin for routine paraffin-embedding, a second sample being frozen and stored in liquid nitrogen for molecular analysis, and the third sample used for cytogenetics. For cytogenetic and molecular analyses, the primary tumor sample was obtained as a single sample and coded LH. The recurrent tissue was received as five separate samples and coded LHR-A, LHR-B, LHR-C, LHR-D, and LHR-E in compliance with our Internal Review Board.

**Isolation of Total Cellular RNA and RT-PCR Analysis.**

Total cellular RNA was isolated from frozen tumor tissue using RNAzol B (Cinna/Biotex Laboratories, Inc. Houston, TX) and conditions specified by the manufacturer. RT followed by the PCR (RT-PCR) was used to determine gene expression in a semiquantitative manner. Total RNA (1 μg/20 μl RT reaction) was reverse transcribed using the GeneAmp PCR kit and random hexamer primers (Perkin-Elmer Corp., Norwalk, CT) with conditions specified by the manufacturer. Six-μl aliquots of this reaction were used for PCR with primers described in Table 1. Since these primers span introns, they will amplify the appropriate size fragments from cDNA only. The use of one RT reaction for multiple PCR reactions allowed us to use the "housekeeping" gene histone 3.3 as a "loading" control (31, 48). Samples were analyzed by electrophoresis through a 1.8% agarose gel, visualized using ethidium bromide, and a Polaroid photograph was taken of the resulting gel. Quantitation was done by densitometric analysis of the photographic negative (49). Negative controls without RNA were performed for each experiment.

**Tumor Dissociation for Cytogenetics and Cell Culture.**

The tumor samples were dissociated mechanically into single-cell suspensions by mincing the tissue to a fine pulp with scalpels, then aspirating several times through an 18-gauge needle as previously reported (15, 50). The single-cell suspension was plated in 25-cm² flasks at a concentration of 3–5 × 10⁴ cells/flask to obtain short-term chromosome preparations for the analysis of first division cells (15). The remaining tissue fragments and single cells were plated to establish monoclonal cultures. Conditioned medium was applied to all monolayer cultures. Conditioned medium was a 1:1 ratio (v/v) of standard growth medium (Waymouth 87/3 medium; Grand Island Biological Company, Grand Island, NY) containing 25% FCS (HyClone, Logan, UT), and “spent” medium removed from a vigorously growing glioma cell line A02V4 (51). When cell cultures became semiconfluent or demonstrated active growth, the medium was changed completely. No antibiotics were used. All primary and passage 1 cell cultures not used in cytogenetic studies or drug resistance assays were frozen and maintained in liquid nitrogen.

The primary untreated freshly resected tumor sample coded LH was a large mass of tissue. For the purpose of cytogenetic analysis, multiple small pieces were removed from different parts of this sample; these were combined and handled as a single specimen. The recurrent tumor consisted of five individual samples removed from different regions of the brain which were dissociated and cultured separately. Only the LHR-E sample yielded viable cells for cytogenetic analysis and monolayer
cultures. Each of the other LHR regions had limited cellular outgrowth from one or more of the tissue explants. The cells migrating from these explants were very abnormal, containing multiple or gigantic nuclei. After several weeks in culture, large vacuoles formed and degeneration ensued (52). This is not an unusual feature in recurrent tumors. One explanation for the frequent death of recurrent tumor cells is that the bulk of such samples consists of necrotic tissue resulting from radiation and/or chemotherapy treatment.

Cytogenetic analyses of first-division cells (primary cells) were performed on short-term cultures (15) within 72 h after plating. A minimum of two flasks were prepared for each sample, and the karyotypic deviation had to occur in both flasks before being scored. The cytogenetic analysis of the tumors in this report follows the International System for Human Cytogenetic Nomenclature (53, 54). Rearranged chromosomes were considered clonal markers if present in two or more karyotypes, and the gain or loss of a whole chromosome(s) had to occur in five or more karyotypes to be defined as a clonal population. Nonclonal cells were cells in which: (a) a rearranged chromosome occurred only once; (b) a specific loss and/or gain of chromosome(s) occurred in less than five metaphases; and/or (c) a metaphase contained a chromosome exchange, telomeric fusions, and single-stranded chromatin strands as previously reported (14, 50).

Fig. 1  A, primary tumor LH: GBM with marked nuclear pleomorphism, vascular proliferation, and focal necrosis; B, recurrent tumor LHR: low-grade astrocytoma composed of well-differentiated fibrillary astrocytes with low cellularity, little pleomorphism, and no necrosis or vascular proliferation.
A biopsy of galea was obtained from the patient at the time of primary resection prior to therapy. The constitutional karyotype of the patient was determined by counting the chromosome number of 25 metaphases and karyotyping 5 cells. All metaphases were analyzed by standard G- or Q-banding techniques.

**FISH.** Slides containing 4-5-μm-thick paraffin-embedded sections were air dried and baked at 65°C overnight. The slides were incubated for 1 h in a 1:1 alcohol:chloroform mixture at room temperature, then treated with tRNAse for 1 h at 37°C, rinsed four times with 2× SSC (150 mM NaCl, 15 mM Na2HPO4) at room temperature, and dehydrated using cold, graded alcohols. The slides were air dried, denatured in 1% formamide in PBS at 70°C, dehydrated in cold, graded alcohols, and air dried. The slides were hybridized with chromosome 7-specific α satellite DNA probe in hybridization mix (Oncor, Inc., Gaithersburg, MD), sealed with a glass coverslip, and incubated at 37°C for 4 to 16 h. The sealant was removed, and the slides were incubated in 65% formamide at 43°C for high stringency (single probe) or in 50% formamide at 37°C for 2 min for less stringent conditions. The slides were washed twice with 2× SSC (pH 7.0) at 37°C for 4 min and once with 1× phosphate-buffered detergent (Oncor, Inc., Miami, FL). Following centrifugation at 400 × g for 5 min the pellet was resuspended in a staining solution consisting of 100 g/liter propidium iodide (Sigma Chemical), 1 g/liter ribonuclease (Sigma Chemical), and 1 ml/liter NP40 detergent (Sigma Chemical) in PBS.

**FCM.** FCM was performed on paraffin-embedded material. After 3-μm sections were removed for routine diagnostic purposes, three to five 50-μm sections were cut for FCM. The tissue was processed using our modifications of the method of Hedley (34, 55). The 50-μm sections were dewaxed in xylene and rehydrated through graded alcohols overnight in an Auto-technicon (Technicon Corp., Tarrytown, NY). After mincing, the specimens were incubated at 37°C for 2 h in a solution of 5 g/liter pepsin (Sigma Chemical, St. Louis, MO) in saline, with the pH adjusted to 1.8. After mechanical dissociation by forceful passage through a 22-gauge needle, the cell suspensions were filtered through 150- and 30-μm mesh (Small Parts, Inc., Miami, FL). Following centrifugation at 400 × g for 5 min at room temperature, the supernatant was discarded, and the pellet was resuspended in a staining solution consisting of 100 g/liter propidium iodide (Sigma Chemical), 1 g/liter ribonuclease (Sigma Chemical), and 1 ml/liter NP40 detergent (Sigma Chemical) in PBS.

The propidium iodide-stained cell suspensions were analyzed on an Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL) using 15 mW 488 nm light from a 25-mW argon laser. The DNA histograms were analyzed using Multicycle (Phoenix Flow Systems, San Diego, CA). DI (ratio of the DNA content of aneuploid and diploid populations) between 1.90 and 2.10 were considered near-tetraploid. Controls were not used; the first peak was defined as diploid by convention.

**Ki-67 Immunohistochemistry.** Ki-67 immunostaining was performed using modifications of the original methods of Gerdes et al. (56). Five-μm sections were cut from the frozen tissue block, fixed for 10 min in cold acetone, and air dried for at least 2 h. The slides were incubated for 20 min at room temperature with a 1:50 dilution of Ki-67 antibody (DAKO, Inc., Santa Barbara, CA) and developed using an alkaline phosphatase-antialkaline phosphatase kit with fast red chromogen. Intrinsic alkaline phosphatase activity was blocked with levamisole (Vector Laboratories, Burlingame, CA). The labeled sections were lightly counterstained with hematoxylin.

The percentage of Ki-67-positive cells (labeling index) was determined from the area with the highest number of labeled cells. One thousand nuclei in contiguous microscopic fields were counted at ×400. Cells pertaining to blood vessels (endothelial cells and pericytes) were not counted. Reactive astrocytes and residual non-neuronal parenchymal cells could not be distinguished from tumor cells on the frozen section slides. A nucleus was considered positive if it demonstrated either a diffuse or punctate (nucleolar) distribution of reaction product.

**RESULTS**

**Histopathology**

The primary tumor was a highly pleomorphic malignant astrocytic neoplasm with often bizarre tumor giant cells. Mitoses were easily found. There was scattered individual cell necrosis as well as confluent areas of necrosis accompanied by marked microvascular proliferation (Fig. 1A). By way of contrast, the recurrent tumor was only modestly cellular and composed of well-differentiated fibrillary cytology with little pleomorphism, no tumor necrosis nor vascular proliferation being identified. Mitoses were not found (Fig. 1B).

**Gene Expression**

**DCC.** The primary tumor did not express DCC-specific mRNA; however, recurrent tumor expressed this mRNA to a degree that was approximately half that of normal brain tissue (Fig. 2 and Table 2). Extensive histopathological analysis detected <50% contaminating normal cells in the recurrent tumor tissue sample, suggesting that this expression is not solely due to contaminating normal cells.

**EGFR.** Numerous studies have demonstrated overexpression of the mRNA encoding the EGFR as well as the presence of truncated forms of the message in high-grade gliomas. Our analysis of the primary and recurrent tumor samples from this patient show truncation in both the cytoplasmic and extracellular domains of the message in the primary and recurrent tumor samples. Expression of the transmembrane portion of the gene can be used as a measure of total expression, since there have been no reports of mRNA truncation in this region of the message. Neither tumor sample had overexpression of this message as determined by analysis of the expression of the transmembrane domain of the EGFR gene (Fig. 2 and Table 2).

**RPTPβ.** RPTPβ mRNA is alternatively spliced into three transcripts encoding two transmembrane forms (one “intact” and one truncated) and one secreted form that consists of only the extracellular domain and is the human homologue of rat 3F8PG (57). In our studies to date (58), all three variants appear...
to be expressed in the low-grade tumors assayed, including the recurrent tumor LHR (58). However, 50% of the GBMs assayed, including LH, express only the variant encoding the secreted form of RPTPβ. Expression of RPTPβ transcripts appears to correlate with the histological classification of LH as a GBM and LHR as a low-grade tumor.

**Cytogenetics and in Vitro Characteristics**

The cytogenetic analysis of first-division cells was performed 72 h after plating and at serial passages 3 and 33 for the primary LH sample. A total of 86 metaphases was obtained from the primary cytogenetic analysis of LH. The majority of cells were clustered in the near-diploid 2n± range. A smaller near-tetraploid 4n± population was observed in addition to giant cells consisting of several hundred chromosomes per metaphase (Fig. 3A). The modal chromosome number of the 2n± cells was 46 with a stem line karyotype, 46,XX, with normal G-bands using standard banding techniques. The 4n± population was endoreduplicated cells (92,XXXX), and random chromosome loss occurred in all metaphases karyotyped in this chromosome range. The monolayer culture established from the dissociated single cells from primary tumor LH grew rapidly, and at serial passage 3 cytogenetic analysis was repeated. The cells were

---

4 S. A. Norman, unpublished results.
predominately 46,XX[15] with normal G-bands (Fig. 4A) with a
side population whose karyotypic deviation was 45,X,-X[6].
The few 4n± cells observed were endoreduplicated, 92.XXX[6]/
90.XX,-X,-X[5]. The karyotypes prepared on the remaining 2n±
and 4n± cells illustrated only random chromosome loss from these
cells. The LH monolayer culture continued to grow vigorously, and
a third chromosome harvest was prepared at serial passage 33. The
majority of LH cells retained a 2n± chromosome constitution with
a smaller peak clustered in the 4n± chromosome range. The
cytogenetic analysis of first-division
karyotypes prepared on the near-diploid population were:
46,XX[23]/45.X,-X[9] and for the near-tetraploid population,
92.XXX[8]/90.XX,-X,-X[6]. No giant cells were observed in
either serial passage 3 or 33.

In the primary analysis of the recurrent tumor sample
LHR-E (Fig. 3B), the modal chromosome number was 46; the
der(1) inv(1)(p22q25), del(1)(q42), ins(2)(q23p24q33), del(2)
(q24p33), der(3)inv(3)(p21q37), add(5)(q35), del(6)(q13), del(10)
(q23), der(11)(t;6;11)(q13;p15.3), der(21)(t;12)(q42;q22.3)
(Fig. 4B). The LHR sample at serial passages 3, 7, and 18
retained this modal chromosome number. At serial passage 3,
the cells were more heterogeneous than the serial passage 7
cells. Karyotyping demonstrated random loss of chromosomes; the clonal markers were the same nine clonal markers for both serial passages 3 and 7, as was identified in the primary cytogenetic analysis. In contrast to the primary untreated LH monolayer culture, this cell line grew very slowly, a characteristic that was retained until the cell line was discontinued at passage 18. The modal chromosome number at passage 18 was 46. No informative karyotypes were prepared from this preparation.

An analysis of paraffin-embedded tissue utilizing chromosome 7-specific α centromeric probes confirmed the diploid nature of both the primary (LH) and recurrent (LHR-E) tumor tissue. The tissue from primary tumor LH (Fig. 5A) has large areas in which only two hybridization signals per nucleus can be seen. The presumed endoreduplicated cells contain four signals per nucleus and are approximately twice the size of those cells with two signals/nucleus. Not seen in Fig. 5A were the giant cells scattered throughout the tissue that contained three to seven signals. The recurrent LHR-E sample also has predominately two hybridization signals/nucleus (Fig. 5B). Giant cells observed in the primary analysis were not detected in this paraffin-embedded section, although an occasional cell with increased nuclear size had three to four signals per nucleus, suggesting that endoreduplication was also occurring in the recurrent tumor sample.

**Table 2 Gene expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primary tumor</th>
<th>Recurrent tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DCC</strong></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>EGFR TM</strong></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>EGFR Ext. (normal)</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>EGFR Ext. (truncated)</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>EGFR Cyt. (normal)</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>EGFR Cyt. (truncated)</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>RPTPβ (intact)</strong></td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>RPTPβ (truncated)</strong></td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>RPTPβ (secreted)</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Expression was determined using RT-PCR. Histone 3.3 was used as a “loading” control for RNA concentration. Glial tissue from trauma patients was used as a normal control for quantitation of DCC and EGFR TM expression. + +, expression equal to that observed in normal brain; +, approximately half of normal; —, no detectable expression; Yes, presence of the truncated or normal size mRNA fragments.

a TM, transmembrane domain of the EGFR gene; Ext., extracellular domain of the EGFR gene; Cyt., cytoplasmic domain of the EGFR gene; normal, normal size mRNA; truncated, truncated form of the message that results from alternative splicing of the mRNA.

b Intact, full-length receptor; truncated, truncated extracellular domain; secreted, extracellular domain only.

**FCM**

**FCM Ploidy.** Both tumors contain two aneuploid populations, one of which is near-diploid and the other with a DI between 1.8 and 1.9. The resolution limits of FC are such that the DI of the populations in the tumors must be considered indistinguishable. There is intratumor regional variability in the percentage of the two aneuploid populations. As a result, the
Fig. 4  Cytogenetic analysis of the primary LH first-division cells and recurrent LHR-E cells. In A, the stem line karyotype observed in the primary LH cells was 46,XX. Giemsa-trypsin banding. ×1100. In B, the stem line karyotype observed in the recurrent LHR-E cells was 46,XX, der(1)inv(1)(p22q25), del(1)(q42), ins(2)(q23q24q33), del(2)(q24q33), der(3)inv(3)(p21q27), add(5)(q35), del(6)(13), del(10)(q23→qter), der(11)t(6;11)(q13;p15.3), der(21)t(1;21)(q42;q22.3).
relative percentages of the populations between tumors cannot be meaningfully compared.

**FCM-SPF.** The primary tumor had an average SPF of 7.8 (range of the six regions, 4.6–13.2). The recurrent tumor had an SPF of 3.8 (range, 0.5–8.8). The SPF of the recurrent tumor is significantly lower than that of the primary tumor, consistent with a lower grade tumor.

**Proliferation**

**Ki-67 Labeling Index.** The average labeling index of the primary tumor was 18.8 and was determined by averaging the results from six regions (range, 10.0–29.0). This result is quite high and is indicative of a high-grade tumor. The labeling index of the recurrent tumor was 3.8 (only one region of this tumor was available for analysis). Although this is quite a bit lower than the results found for the primary tumor, it is slightly higher than that usually found in low-grade astrocytomas.

**DISCUSSION**

Most diagnostic specimens from patients with a diagnosis of malignant glioma are subtotal resections or biopsies, and pathologists base their diagnoses on the most malignant regions of these samples. Although we assume that the regions that remain are of a similar histological grade, it is possible that the tumor cells left in the brain can be of a more or less benign grade. Whatever the grade and adjuvant therapy received by the patient, tumor cells generally evolve into a more malignant neoplasm with the result that the recurrent tissue is equally or more malignant than was the originally resected tissue. The primary tumor LH was received as a single mass with histopathology that was clearly identified as a glioblastoma multiforme. Necrosis, the hallmark of a GBM, was observed as were microvascular proliferation, numerous mitotic cells, and giant cells (Fig. 1A). The entire large recurrent tumor was submitted for histological examination and demonstrated only low-grade as-
tretoma. In addition, although median survival of a patient with a GBM is approximately 1 year, this patient survived for 4 years, again suggesting that the tumor remaining following surgical resection of the primary tumor and adjuvant therapy was more benign. The LH/LHR tumor pair provided an opportunity to compare the molecular, genetic, and/or phenotypic markers of the two samples.

The expression of the DCC, EGFR, and RPTPβ genes were determined using RT-PCR. These genes were chosen because of their potential usefulness as prognostic markers of glial tumors. Cytogenetic, molecular cytogenetic, and flow cytometric analyses were done to determine the extent of aneuploidy and genetic rearrangement in the primary tumor as compared to the recurrent tumor. Finally, flow cytometric analysis of the S-phase fraction and histological examination of Ki-67 expression were done to analyze the proliferative nature of these tumors.

**Molecular Studies.** The DCC gene is a tumor suppressor gene that was originally identified in colon cancer using loss of heterozygosity analysis of chromosome 18. The gene encodes a protein that is highly homologous to the neural cell adhesion molecule family, and its aberrant expression has subsequently been demonstrated in numerous tumor types (59–63). Work in our laboratory demonstrated that expression of the DCC gene is frequently reduced in human malignant gliomas, particularly in higher grade tumors such as GBMs (31). This work has recently been confirmed by Ekstrand et al. (64) using human glioblastoma xenografts. The expression of DCC in the LH and LHR tumors was somewhat consistent with the histological grade: there was no expression in the primary high-grade tumor; and the recurrent low-grade tumor expressed the DCC gene to an extent less than that found in normal brain. Histopathological analysis of the tumor tissue samples demonstrated that the expression found in tumor LHR was not due solely to contaminating normal brain parenchyma since the contribution of normal brain to the tumor sample was clearly <50%. However, the presence of even a small amount of normal brain in our tumor sample would bias the result toward apparent increased expression of DCC. This suggests that the LHR tumor cells actually expressed less than half the normal amount of DCC mRNA. Thus, reduced expression of the DCC gene may be indicative of a higher grade tumor prior to histologically recognizable morphological changes. The fact that expression of this gene was altered in this tumor in the absence of obvious alteration of chromosome 18 is not surprising, and our work as well as the work of others has demonstrated that reduced expression of this gene can occur in the absence of reduced gene copy number (31).

The EGFR gene is mapped to chromosome 7 (65), and its altered expression has also been correlated with tumor grade. Gene rearrangement, overexpression, and/or mRNA truncation has been identified in 40–60% of high-grade gliomas, less frequently in anaplastic astrocytomas, and only rarely in low-grade tumors (24–26, 30, 43, 66–68). In this study, we have found that both the primary (LH) and the recurrent (LHR) tumor express normal and truncated forms of the extracellular and cytoplasmic regions of EGFR mRNA. Expression of the transmembrane region was approximately equal to that observed in normal brain tissue, suggesting that this gene was not highly overexpressed in either of these tumors. Although truncated forms of the EGFR message are frequently associated with gene amplification and/or overexpression, aberrant forms of the mRNA can sometimes be identified without concomitant overexpression. The detection of truncated forms of mRNA in the low-grade recurrent tumor suggests that although this tumor was histologically categorized as a low-grade tumor, it retained some molecular markers typically present in higher grade tumors.

RPTPβ is predominantly expressed in the central nervous system, although its role, if any, in glioma growth and/or progression has not been identified. The extracellular portion of the phosphatase has a domain with high sequence similarity to a rat brain chondroitin sulfate proteoglycan (3F8PG) and a region with fibronectin type 3 repeats. Chondroitin sulfate proteoglycans have been shown to inhibit cell adhesion, cell migration, and neurite outgrowth. The cytoplasmic region contains tandemly repeated phosphatase domains. Alternate splicing of the message results in three variant transcripts: a truncated and an intact transmembrane form that both contain the cytoplasmic phosphatase domains and a secreted form that consists of the extracellular domain alone. We have found that although low-grade tumors (including LHR) and some GBMs express all three variant forms, approximately 50% of the GBMs studied to date (including LH) express only the secreted form. Thus, as is the case for the gene encoding DCC, the absence of the normal expression of RPTPβ may be indicative of a higher grade tumor. The association of loss of expression of two variant forms of RPTPβ with high-grade gliomas is particularly interesting in light of its localization to chromosome 7q31.3 (69), a chromosome frequently overrepresented in gliomas.

**Cytogenetic and Molecular Cytogenetic Studies.** Although we have viewed the trisomy of chromosome 7 as one of the early numerical changes occurring in the progression of human gliomas (70, 71), the cytogenetic analysis of first division cells (15) and serial passages 3 and 33 of the primary LH cells demonstrated karyotypically normal cells (46,XX) with the first appearance of a sideline (45,X,-X) at serial passage 3. However, both of these cells appeared to undergo endoreduplication as karyotypes with 92,XXXX and 90,XX,-X,-X were also observed in later serial passes (passages 3 and 33).

Numerous reports cite a 46,XX chromosome constitution for a certain proportion of freshly resected adult glioblastomas multiforme either as the sole karyotypic finding (19, 72, 73), or in combination with an abnormal stem line and/or sidelines (15, 20, 49, 50, 52, 74–77). It has never been clear whether this finding represents sampling error, reactive astrocytes, or transformed cells with molecular lesions. We attempted to reduce the sampling error by performing all analyses on the same block of tissue used in the histopathological diagnosis. We also prepared a hematoxylin and cosin-stained slide on serially sectioned tissue to determine the characteristics of tissue under investigation, which can then be compared back to the original histopathology. The primary cytogenetic analysis of first division cells from primary tumor LH reflects the histopathology of the LH tumor. The size of the nuclei within the fields demonstrating frequent mitotic figures are consistent with a 2n= chromosome number. Scattered among these cells are cells with approximately twice the nuclear size, representative of the 4n= cells. Also consistent with the histopathology was the identification of giant cells. The molecular cytogenetics of the paraffin-embed-
ded sections of LH tissue also reflects the histopathology. Utilizing a chromosome 7-specific DNA probe, we demonstrated that the majority of cells contained one to two hybridization signals in large regions, suggesting that the majority of cells were diploid. Occasional cells containing three to four signals per nucleus and approximately twice the nuclear size, as well as giant cells (containing three to seven signals per nucleus), were identified in the FISH analysis. This further supported the histological and karyotypic data which illustrated the presence of endoreduplicated cells that could then progress to sidelines if aberrant segregation occurred during subsequent cell division. This could also explain the DI of 1.8–1.9 demonstrated by flow cytometric analysis.

The freshly dissociated LH cells used for in vitro cultivation and serial passaging grew rapidly. This in vitro vigor was retained at serial passage 33. Normal glia cells established from trauma patients and maintained by the same protocol as our tumor cells do not grow this vigorously and rarely can be passaged beyond 25 serial passages. This length of in vitro cultivation also applies to normal tissue obtained from the pediatric population; however, fetal tissue is capable of 40–45 serial passages in this culture system. Despite the vigorous growth of the LH cells, the predominant cell at serial passages 3 and 33 still retained a 46,XX karyotype with normal G-bands, although a sideline, 45,X,-X was detected at serial passages 3 and 33. This would further suggest that the LH cells, while cytogenetically normal, are not reactive astrocytes, but are in fact transformed cells with molecular genetic lesions.

The recurrent LHR tumor was regionally sampled; however, of the five regions received in the laboratory, only one sample (LHR-E) yielded viable cells that could be cytogenetically analyzed within 72 h. We have frequently found that samples from recurrent tumor may contain apparently viable cells within the necrotic regions. Many of these cells are capable of cell division, although most are in the process of terminalizing because of damage inflicted by therapy (52). Thus, it is possible to obtain occasional metaphases from such cells. However, the cytogenetic abnormalities are extensive and most cells contain multiple chromosome exchanges and fragmented chromosomes. Other than illustrating the effects of radiation and/or drug damage, they do not contribute to our understanding of recurrent tumor growth. However, within these same tumor samples foci of viable cells may be present that can contribute to the growth of recurrent tumor. Tumor sample LHR-E was one such sample that was obtained from an infiltrating region of the tumor. The metaphases collected from such cells will contain clonal markers and/or the gain and loss of chromosomes that are similar to each other, if not identical. The abnormalities identified in this population of cells may be more indicative of chromosomal change associated with survival and growth. Although the chromosome number remained 46,XX, 9 chromosome aberrations were observed in the primary analysis that were retained during in vitro cultivation. Despite the rearrangements that gave rise to the nine markers, no major band of genetic material appears to have been lost and such rearrangement is consistent with other reports on recurrent tumor that has been treated with chemotherapy and/or irradiation (78). It should also be noted that while the LH and LHR samples were resected as a single mass (LH) and in five separate pieces (LHR-A, LHR-B, LHR-C, LHR-D, and LHR-E), respectively, the manner in which they were sampled for cytogenetics should have provided the best opportunity to detect multiple unrelated clonal abnormalities. The single resected LH sample was cut into several pieces, and multiple samples were removed from each of those pieces. Approximately 650 mg tissue were dissociated from this pooled sample. At least 100 metaphases were counted from each of the two flasks harvested from the 72-h time point. In addition to the metaphase counts, 86 karyotypes were prepared from these two preparations (Fig. 3A). If unrelated stem line populations were present in the sample, it should have been detected with the number of cells counted and karyotyped, unless such a population represented <1–2% of the sample (14). This could explain the appearance of the 45,X-X stem line observed at serial passages 3 and 33 but not in the primary analysis. The LHR tissue was received as five individual pieces (approximately 2.1 g tissue) that were dissociated and retained separately. Only LHR-E contained a sufficient number of cells to perform a cytogenetic analysis within 72 h after plating. The results were described above. The other four samples had only a few viable cells evident in the cultures. Chromosome preparations obtained from these cultures between days 6 and 12, yielded less than five metaphases per sample. The metaphase from each of these samples was very abnormal because they each contained multiple rearrangements, chromosome exchanges, and chromosome fragments. These same four samples also failed to establish viable cell lines with a 12-week period and were discarded. As indicated above, the LHR-E culture, while growing slowly, was maintained in vitro until serial passage 18. FISH analysis of paraffin-embedded sections supported the diploid nature of chromosome 7 in the LHR-E region. FISH analysis also demonstrated an occasionally endoreduplicated cell and the absence of giant cells again supporting the cytogenetic and histopathological analyses.

**FCM and Proliferation Studies.** Proliferative activity of astrocytomas has been correlated with the histological grade and outcome by us and others (11, 34, 35, 79–83). In flow cytometry, we found that an S-phase fraction ≥6% was indicative of a high-grade astrocytoma with short patient survival. Similarly, Montine et al. (35) reported that Ki-67-labeling indices <3% are indicative of a low-grade astrocytoma with longer patient survival, while labeling indices >3% suggest a high-grade tumor and an aggressive course. Our preliminary data support this report. Both the Ki-67-labeling index (18.8%) and the FCM S-phase fraction (7.8%) of the primary tumor predicted a high-grade tumor which would follow a rapidly progressive course and be associated with short patient survival. In contrast, the SPF (3.8%) and Ki-67-labeling index (3.8%) of the recurrent tumor were indicative of a low-grade or borderline malignant glioma, respectively.

FCM ploidy studies did not reveal significant differences between the primary and recurrent tumors. Both have two major populations, one of which was near-diploid and one of which was aneuploid with DNA indices of 1.8 and 1.9. Because of the limited resolution of flow cytometry, the small difference in

---

5 S. W. Coons, unpublished data.
DNA index between the two tumors does not allow a conclusion that these are different populations. Similarly, the near-diploid populations may also have chromosomal or genetic differences which are too small to be assessed by flow cytometry. This limitation of flow cytometry in regard to the analysis of astrocytomas has been noted (35), and we use cytogenetic data to provide proof of relatively small ploidy differences in cell populations. Both cytogenetic and molecular cytogenetic analysis of primary tumor LH demonstrated a majority of near-diploid cells with some giant cells that probably arose through endoreduplication. The recurrent tumor was also primarily near-diploid, although there were nine markers that may account, in part, for the flow cytometry results.

The molecular, cytogenetic, flow cytometric, and histological data presented here have demonstrated that some markers of progression may be detectable in a tumor prior to the appearance of the histological features that define such progression. The identification of markers such as these may prove to be useful tools to augment current histopathological methods of prognostication. The data reported here, taken together with our documentation of the low-grade recurrence of a high-grade tumor, also demonstrate the clinical relevance of continued studies of regional heterogeneity and tumor cell evolution in human malignant gliomas.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Susan N. Rhodes for the molecular studies, Sarah Demarest and Humaira Anne Sinnott for the FISH studies, Dorothy Haskett for the FCM studies, and Brian Hamilton for photography. We also wish to thank student assistant Kamal Chollera for his assistance in G-banding and karyotype preparation. We thank the Palo Verde Laboratory (Chandler, AZ) technologists, Linda Sheppard, David Sheppard, Louise Adam, Gordon Hart, Kim Kobojeck, and Tanya Respass, for confirmations of the karyotypic abnormalities of these tumors. We appreciate the assistance of Dr. William R. Shapiro in his helpful discussions, and Linda Gower for patient information.

REFERENCES

Biological and molecular analysis of a low-grade recurrence of a glioblastoma multiforme.

A C Scheck, J R Shapiro, S W Coons, et al.


**Updated version**

Access the most recent version of this article at:

http://clincancerres.aacrjournals.org/content/2/1/187

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.