Glioblastoma multiforme is the most malignant astrocytic tumor. Two major types of GBM can be distinguished by their differing courses of disease and by different genetic changes. Primary (also called de novo) GBMs develop rapidly without evidence of a lower-grade precursor lesion and occur frequently in older patients. Genetic alterations associated with primary GBMs are EGFR amplification and overexpression, PTEN mutation, and CDKN2A deletion. Secondary GBMs gradually progress from lower-grade astrocytomas and are genetically characterized by frequent TP53 mutations, LOH at chromosome arm 19q, and overexpression of platelet-derived growth factor and its receptor. These lesions are most commonly found in younger patients.

Glioblastoma multiforme has a poor prognosis. The invasive and migratory behavior of this tumor, the risk of neurological damage to the patient, and problems determining the exact extent of residual tumor during surgery make complete resection difficult and often impossible. Therefore, surgery alone achieves a median patient survival of only 3 to 5 months. Adjuvant radiotherapy (60 Gy in 6 weeks) improves the median survival of patients with GBMs to 9 to 12 months.

Recently, temozolomide has become part of the standard therapy; a Phase III trial produced data demonstrating significant improvement in patients who received radiotherapy plus concomitant and adjuvant temozolomide treatment compared with those who received only radiotherapy.

Despite intensive radiotherapy, patients face a high risk of tumor recurrence. Reappearance of high-grade astrocytomas occurs in approximately 90% of cases within a margin of 2 to 3 cm from the original tumor site. In this paper we describe two patients in whom a second glioblastoma multiforme after radiotherapy: recurrence or second primary tumor? Genetic profiling of a distant second glioblastoma multiforme after radiotherapy: recurrence or second primary tumor?

KRISTA A. VAN NIETERIK, M.SC., PAULA H. M. ELKHUZEN, M.D., PH.D., ROB J. VAN ANDELI, LUKAS J. A. STALPERS, M.D., PH.D., SIEGER LEENSTRA, M.D., PH.D., M. VINCENT M. LAFLEUR, PH.D., W. PETER VANDERTOP, M.D., PH.D., BEN J. SLOTMAN, M.D., PH.D., THEO J. M. HULSEBOS, PH.D., AND PETER SMINIA, PH.D.

Departments of Radiation Oncology and Neurosurgery, Vrije Universiteit Medical Center; and Departments of Neurogenetics and Radiotherapy and Neurosurgery, Academic Medical Center, Amsterdam, The Netherlands

Object. In nearly all patients with glioblastoma multiforme (GBM) a local recurrence develops within a short period of time. In this paper the authors describe two patients in whom a second GBM developed after a relatively long time interval at a site remote from the primary tumor. The genetic profiles of the tumors were compared to discriminate between distant recurrence and a second primary tumor.

Methods. Both patients harboring a supratentorial GBM were treated with surgery and local high-dose radiotherapy. Local control of the disease at the primary tumor site was achieved. Within 2 years, a second GBM developed in both patients, not only outside the previously irradiated target areas but infratentorially in one patient and in the opposite hemisphere in the other. The tumors were examined for the presence of several genetic alterations that are frequently found in GBMs—a loss of heterozygosity at chromosome regions 1p36, 10p15, 19q13, and 22q13, and at the CDKN2A, PTEN, DMBT1, and TP53 gene regions; a TP53 mutation; and EGFR amplification.

In the first patient, genetic profiling revealed that the primary tumor had an allelic imbalance for markers in several chromosome regions for which the second tumor displayed a complete loss. In the second patient, genetic profiling demonstrated the presence of genetic changes in the second tumor that were identical with and additional to those found in the primary tumor.

Conclusions. Based on the similarities between the genetic profiles of the primary and the second tumors in these patients, the authors decided that in each case the second distant GBM was a distant recurrence rather than a second independent primary tumor.

KEY WORDS • second glioblastoma multiforme • de novo tumor • recurrent tumor • genetic profiling
ond GBM developed within 2 years after treatment and in whom the lesion was located outside the previously irradiated target area, far from the primary tumor site. To differentiate between a distant recurrence of the original lesion and a true second primary GBM, we compared the genetic profiles of the tumor pairs in each case. The genetic profiles were determined by screening the tumors for several genetic changes that frequently occur in GBM. This genetic differentiation may improve our insight into the origin and biological behavior of GBMs, and may eventually have implications for the future treatment of these tumors.

Materials and Methods

Case Backgrounds

Both patients gave written informed consent to the surgical, radiotherapeutic, and experimental procedures that were performed.

Case 1. This 31-year-old man presented with focal right-sided epileptic seizures. Four weeks later, the cause of the seizures was determined to be a 7-cm left frontal tumor, which displayed a contrast-enhancing rim on MR images. The patient underwent craniotomy and subtotal resection of a typical GBM (World Health Organization Grade IV astrocytoma; Sample VU-11). Postoperatively, he received hypofractionated conformal external-beam radiotherapy (42 Gy in 3-Gy daily fractions) directed at the preoperative tumor volume plus a 2-cm margin, followed by a single-fraction stereotactic boost (15 Gy) to the residual mass (Fig. 1A).

Twenty months later the patient presented with a progressive disturbance in his coordination. The cause of these symptoms was identified on MR images to be a 3.5-cm contrast-enhancing mass located in the center of the cerebellum (Fig. 1B). The second tumor was found in the 35 to 95% isodose area targeted by radiation treatment of the primary lesion. There was no sign of progression at the primary tumor site. The patient underwent a second craniotomy and resection revealing a second GBM (Sample VU-91). Postoperatively, he received hypofractionated conformational external-beam radiotherapy (42 Gy given in 3-Gy daily fractions) directed at the preoperative tumor volume plus a 2-cm margin. Radiotherapy was stopped before the last two fractions could be given because of the patient’s worsening condition. He died 2 months later due to local progression of the second tumor.

Case 2. This 61-year-old man presented with headaches and focal epileptic seizures of his right hand. Four months afterward, MR images revealed a 42-mm left parietal cystic tumor with an irregular contrast-enhancing rim. The patient underwent a craniotomy and macroscopic subtotal resection of a typical GBM (Sample VU-28). Following surgery, he received hypofractionated conformal external beam radiotherapy (42 Gy given in 3-Gy daily fractions) directed at the preoperative tumor volume plus a 2-cm margin (Fig. 1C), followed by a single-fraction stereotactic boost (15 Gy) to the residual mass.

Sixteen months later, the patient presented with a return of headaches due to a 3.6-cm right frontal mass that displayed contrast enhancement on neuroimages (Fig. 1D). The second tumor was found in the 3 to 70% isodose area that had been targeted by radiation treatment of the primary lesion. An inspection of the primary tumor site demonstrated no sign of recurrence. The patient underwent a second craniotomy and subtotal resection of a GBM (Sample VU-90). Postoperatively, he received hypofractionated conformal radiotherapy directed to the preoperative tumor volume plus a 2-cm margin as well as a stereotactic boost (18.75 Gy) to the residual mass. He died 3 months later due to progression of the right frontal tumor.

Tumor and Blood Samples

Tumor samples were obtained during surgery, frozen in liquid nitrogen, and stored at −80°C until needed. Hematoxylin and eosin-stained sections of the frozen tumor samples were examined to estimate the percentage of tumor cells in three of the four samples (Samples VU-11, VU-90, and VU-91). The percentage of tumor cells in these samples was at least 80%. Leukocytes were collected from blood samples obtained in both patients to serve as a source of normal reference DNA. The cells were stored at 4°C until further analysis could be conducted. The DNA was extracted from the frozen tumor samples and corresponding leukocytes according to standard procedures.

Analysis of LOH

The LOH analysis is based on the presence of microsatellite markers in the genome. Primer sequences for amplification of these markers and conditions for PCR were obtained from the Genome Database (http://www.gdb.org). The LOH statuses of chromosome regions 1p36, 10p15, 19q13, and 22q13, and of the CDKN2A, PTEN, DMBT1, and TP53 gene regions were determined by analyzing markers that have been used in previous studies conducted by Hulsbos and colleagues. The PCR and subsequent analysis of the products on denaturing acrylamide gels were performed in a manner described previously. The LOH status of each chromosomal region was inferred from an analysis of at least two informative markers.

The TP53 Mutation and EGFR Amplification

The TP53 status was determined by screening tumor DNA for the presence of mutations in exons 5, 6, 7, and 8, in which more than 95% of TP53 mutations are normally found. Exons 5 and 6 of TP53 were directly sequenced. Exons 7 and 8 were screened for the presence of mutations by performing denaturing gradient gel electrophoresis.

The amplification level of EGFR was determined by performing a differential PCR, according to a protocol described previously. An amplification level of more than 4.2 was taken as evidence of true EGFR amplification.

Results

The primary and second tumors in both patients were analyzed for the presence of genetic changes that frequently occur in GBM, that is, LOH at chromosomes regions 1p36, 9p21 (with CDKN2A), 10p15, 10q23 (with PTEN), 10q25 (with DMBT1), 19q13, and 22q13; LOH and mutation of TP53; and EGFR amplification. The results are summarized in Table 1.

In Case 1 the patient’s primary and second tumor had almost complete LOH at TP53 (located at 17p13.1), which affected the same allele (Fig. 2). Screening of exons 5 through 8 of the remaining TP53 allele did not reveal a second mutation in this gene. Furthermore, the primary tumor exhibited a considerable reduction in intensity for one allele (allelic imbalance) of markers in chromosome regions 19q13 and 22q13, whereas at those sites the second tumor displayed almost complete loss (marker D22S274, Fig. 2). In addition, in the second tumor there were genetic changes at 9p21 and 10p15 which were not present in the primary tumor. At 1p36, the second tumor differed from the primary tumor in that it displayed complete loss of one allele for markers, whereas the primary tumor demonstrated an incomplete loss (allelic imbalance) of the other allele (data not shown).

Genetic profiling of both tumors in the second patient (Case 2) revealed a loss of the same parental alleles at 10p15 (marker D10S249, Fig. 2), 10q23, and 10q25; this finding was suggestive of a complete loss of the same copy of chromosome 10 in the primary as well as the second tumor, and significant EGFR amplification in both tumors. In the second tumor, additional LOH was noted at 1p36 and 22q13, which was not found in the primary tumor. Marker D22S1169 in Fig. 2 represents this finding.
Genetic profiling of a distant second glioblastoma multiforme

Fig. 1. Axial Gd-enhanced T₁-weighted MR images. A: Case 1. Postoperative image revealing the position of the primary tumor in the left frontal region. B: Case 1. Image showing the recurrent tumor in the posterior fossa. C: Case 2. Preoperative image demonstrating the lesion in the left parietal region. D: Case 2. Image showing the recurrent tumor in the right frontal region. Aberrations in the left parietal site of the first tumor were considered stable. Arrows are merely cursors.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor No.</th>
<th>Sample No.</th>
<th>1p36</th>
<th>9p21 (CDKN2A)</th>
<th>10p15</th>
<th>10q23 (PTEN)</th>
<th>10q25 (DMBT1)</th>
<th>17p13.1 (TP53)</th>
<th>19q13</th>
<th>22q13</th>
<th>LOH</th>
<th>Mut of TP53</th>
<th>EGFR Ampl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>primary</td>
<td>VU-11</td>
<td>AI</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>AI</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>second</td>
<td>VU-91</td>
<td>yes†</td>
<td>HD</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>primary</td>
<td>VU-28</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>second</td>
<td>VU-90</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

* AI = allele imbalance; Ampl = amplification; HD = homozygous deletion; Mut = mutation.
† There was LOH for the other allele.
A lasting cure for GBM is very rare, and local disease recurrence develops in nearly all patients. In patients with GBM who undergo stereotactic interstitial radiotherapy or stereotactic radiosurgery, the lesion often recurs immediately outside or distant from the central stereotactic field. The patients in this study had unusual medical histories, in that the residual primary GBMs were relatively small and controlled by surgery and high-dose radiotherapy. The relatively long tumor-free period (almost 2 years), combined with the distant location of the second GBM outside the irradiated target area, raised the question of whether the second tumors should be considered late distant recurrences of disease or second primary tumors.

Considering the large variety of genetic changes that can occur in GBM, it is unlikely that a true second de novo GBM would have a genetic profile so similar to that of the primary tumor. True GBM recurrences are expected to have the same genetic alterations as the primary tumor and possibly even additional genetic alterations.

Both the primary tumor and the second tumors in Case 1 displayed an almost complete loss of the same parental allele of TP53, indicating that these lesions were clonal outgrowths of the same tumor cells (Table 1). As to the genetic profile of the second tumor in this patient, one assumes that this tumor was a recurrence of the portion of the primary tumor that already contained the genetic changes fully displayed by the second tumor. In the primary lesion, tumor cells with these genetic changes were diluted by cells without the changes, resulting in the noted imbalances in the genetic profiles of the two lesions. During development, the recurrent lesion acquired additional genetic changes. The apparent discrepancies in the LOH status at 1p36 between the primary and second tumors can also be attributed to genetic heterogeneity in the primary tumor and outgrowth in the recurrence of a subpopulation of tumor cells with loss of an allele other than that found in the primary tumor. In cases

**Fig. 2.** Microsatellite analysis of DNA extracted from blood (B), primary tumor (T1), and second tumor (T2). **Upper:** Case 1. Complete loss (indicated by the filled arrowhead) of the upper allele of TP53TET is evident in both primary and second tumors. Incomplete loss (indicated by the open arrowhead) of the lower allele of D22S274 is apparent in the primary tumor and complete loss of the lower allele in the second tumor. **Lower:** Case 2. Complete loss of the lower allele of D10S249 in both primary and second tumors. Retention of heterozygosity in the primary tumor, but loss of the lower allele of D22S1169 in the second tumor. A stutter fragment of the upper allele of this marker causes the remaining signal in the latter tumor.
of local glioma recurrence, Hulsebos and colleagues' previously documented genetic heterogeneity in a primary tumor and acquisition of additional genetic changes in the recurrent lesion. In the patient in Case 2 the second tumor was found to have genetic alterations already present in the primary tumor as well as additional genetic changes—a finding consistent with a true distant recurrence (Table 1).

The duration of these patients’ clinical histories (< 2 years) as well as the locations of the second tumors (outside of the previously irradiated target area) make it very unlikely that the second tumors in our patients were produced by radiation damage according to the criteria posed by Cahan and colleagues for radiation-induced tumors. Moreover, another important criterion Cahan, et al., set forth for radiation-induced malignancy is a difference in the histological characteristics of the tumors, which was not the case in our patients.

Based on the similarities in genetic profiles, we conclude that in both of our patients the second GBM was a distant recurrence of the primary GBM and not a second de novo GBM. Our findings contrast with two other reports of distant second GBMs, in which investigators concluded that the second GBM was an independent primary tumor. In the first report, the authors described a patient in whom a second GBM developed in the contralateral frontal lobe 10 years after subtotal resection and 30-Gy whole-brain radiotherapy with a 26-Gy local boost. Discordant patterns of TP53 and PTEN mutations were found, indicating an independent second GBM instead of a recurrence of the primary GBM. In the second report, the patient’s first GBM developed in the right parietal and occipital lobes and was completely resected; postoperatively, the patient received local fractionated radiotherapy and chemotherapy. A second GBM was found in the basal right temporal lobe after a long recurrence-free period of 5 years. Using comparative genomic hybridization, investigators found 19 genetic imbalances in the primary tumor but only two in the second. Moreover, a TP53 mutation was manifested in the primary tumor but not in the second, and the tumors did not have the same type of PTEN mutation. In this case it was also concluded that the second GBM developed independently from the primary GBM.

Given the medical histories of the patients in the present study, we selected a radiation treatment in which a higher dose of radiation was administered than generally given to patients with GBMs. Local control of the disease in each case was obtained at the original tumor site and in the margin area (2–3 cm). Outside the margin area, however, new tumors appeared near the region in which a low dose of radiation had been directed. This raises the question of whether more extensive therapy is needed to control the disease at the primary tumor site. In cases of breast carcinoma, migrating tumor cells can be found 2 to 8 cm from the primary site, and this also applies to small T1 tumors. In cases of breast carcinoma, it is known that after local excision whole-breast irradiation is needed to control all tumor depots.

Perhaps there is a similar necessity in the treatment of GBMs. Despite intensive local treatment, most patients are faced with a recurrence at the primary site. Improvement in control at the primary tumor site and in its vicinity may lead to the finding of tumors far from the original tumor site, which have developed as a result of tumor cell migration from the original lesion. The possible potentiating effect of radiation therapy on tumor cell invasion and migration, which might contribute to the observed phenomenon, is still open to debate. To control these relocation problems, additional therapy such as whole-brain radiotherapy or chemotherapy might be necessary.

Conclusions

Genetic profiling is helpful in discriminating between distant recurrence of the same tumor and a second primary GBM. Our study of two cases raises questions about the biology and regional spread of GBM. The cited cases, in which there was tumor control at the primary tumor site and independent development of a second GBM, are exceptional. Although distant recurrence may be a common feature in GBM, most patients die as a result of a local recurrence at the primary site and do not survive long enough for distant recurrences to manifest themselves.

Acknowledgment

The technical assistance of Ms. J. C. M. Vos is kindly acknowledged.

References

9. Hulsebos TJ, Troost D, Leenstra S: Molecular-genetic characterization of gliomas that recur as same grade or higher grade tumors. J Neurol Neuropsychiatry 75:723–726, 2004


Manuscript received November 24, 2005. Accepted in final form May 17, 2006. This work was supported by Dutch Cancer Society Grant No. VU 2000-2149.

Address reprint requests to: Krista A. van Nifterik, M.Sc., Department of Radiation Oncology, Division of Radiobiology, Vrije Universiteit Medical Center, Faculty of Medicine, Room J-380, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. email: ka.vannifterik@vumc.nl.