Glioblastoma represents the most common primary brain malignancy in adults. The median survival of patients with glioblastoma after optimal treatment continues to be less than 15 months. Several independent prognostic factors in these patients have been established, such as patient age, extent of resection, and KPS score. Patients with glioblastoma who have multifocal or multicentric disease on presentation are particularly challenging to manage and are generally believed to experience a worse outcome. However, multifocal disease at presentation has not been established as an independent prognostic factor in the modern temozolomide era.

There exists much literature investigating the incidence, radiographic workup, and treatment of multifocal glioblastoma. However, studies investigating...
outcomes of patients with newly diagnosed glioblastoma and multifocal disease on presentation are quite sparse. Furthermore, small subanalyses of 2 previous studies lead to contradictory conclusions on the effect of multifocality on survival. Hassaneen et al. reported a significantly shorter median survival of 9.6 months for patients with newly diagnosed multifocal glioblastoma compared with 14.6 months for matched patients with solitary glioblastomas. In contrast, Parsa et al. matched 14 patients with newly diagnosed multifocal glioblastoma with solitary glioblastoma controls and found no survival difference.

Our study’s objective was to rigorously investigate the effect of multifocality on survival through a case-control design, comparing overall survival between patients with multifocal or solitary newly diagnosed primary glioblastoma in the temozolomide era. Patients with multifocal tumors were defined as those having at least 2 separate foci of enhancing tumor, separated by at least 1 cm. Forty-seven patients with glioblastomas and multifocal disease at presentation were matched with an additional 47 patients with unifocal glioblastoma according to age, KPS score, and extent of resection.

Methods

Patient Population

We retrospectively reviewed 368 patients with newly diagnosed glioblastoma treated between 2003 and 2010 at Cedars-Sinai Medical Center. Patient information was extracted concerning age, KPS score, surgery date, extent of resection, expression of glioblastoma molecular markers, radiographic tumor characteristics, day of death, and last follow-up evaluation. Extent of resection was defined as gross-total (all of the enhancing tumor resected), near gross-total (> 95% of the enhancing tumor resected), partial (< 95% tumor resection), and biopsy procedure only. Patients with multifocal tumors were defined as those having at least 2 distinct foci of enhancing tumor, separated by at least 1 cm. Multicentric tumors with widely separated foci and no apparent connection were analyzed together with the multifocal tumors. Complete information required for matching was available in 216 patients, 47 of whom had multifocal tumors. Each multifocal patient was matched with a unifocal control from the 169 patients with a solitary glioblastoma, using the methodology described below. Matching variables included age (years), extent of resection, and KPS score. Radiation and temozolomide treatments were also well matched between the 2 cohorts. Forty-seven pairs of patients with newly diagnosed glioblastoma were included in the analysis. Treatment response to radiation and temozolomide was assessed by reviewing the 2- to 3-month postradiation therapy MRI. Increased enhancement on this MR image compared with the immediate postoperative MR image, together with clinical decline, was defined as tumor progression. Overall patient survival (months) was calculated from the date of surgery to the day of death or the last follow-up date.

Immunohistochemistry and In Situ Hybridization

The following antibodies were used in this study in accordance with manufacturer’s instructions and at the following dilutions: MGMT (1:80, Millipore), phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204; 1:200, Cell Signaling), laminin β1 (1:2000, Santa Cruz Biotechnology), laminin β2 (1:3000, Santa Cruz Biotechnology), and PTEN (1:250, Cascade Biosciences). Tumor tissue was procured from the block obtained for intraoperative frozen section consultation. Half of the tissue block was sent to be made into frozen section for the intraoperative diagnosis that the pathologist conveyed to the surgeon, whereas the remainder of the tissue was immediately fixed in 4% buffered formalin (within 20 minutes of resection or biopsy). The total length of formalin fixation varied from 4 to 12 hours. Subsequently, the tissue was paraffin-embedded using the PELORIS II automated tissue microprocessor (Leica Microsystems). Five micron-thick sections were deparaffinized, hydrated, and treated with an acidic antigen decloaker (Biocare Medical) on a Leica BOND instrument for 30 minutes for MGMT, PTEN, phosphorylated MAPK, and laminin β1 or by protease pretreatment (for laminin β2). The primary antibodies were applied using the Ventana Benchmark Ultra CC1 stainer for 32 minutes (for MGMT, PTEN, and phosphorylated MAPK) or for 120 minutes (for laminin β1 and β2). Detection was conducted using the Ventana Ulytraview DAB Detection Kit, followed by counterstaining with hematoxylin (Biocare Medical). A negative control was made by omitting the primary antibody, which revealed no immunoreactivity. Evaluation of EGFR amplification by fluorescence in situ hybridization was performed using a commercially available kit (Vysis, according to the manufacturer’s instructions). Dual-probe analysis was performed with a locus-specific probe for EGFR (the gene is located on chromosome 7), and compared with second chromosome enumeration probe (CEP7). The tumors with an EGFR/CEP7 signal ratio greater than 2.00 were classified as having EGFR amplification. Tumors with greater than 10% nuclei with 3 or more CEP7 signals were classified as having a gain of chromosome 7. Both gain of chromosome 7 and EGFR amplification frequently occur together in gliomas.

Morphometric Analysis

Evaluation of phosphorylated MAPK immunostaining was performed in at least 3 separate fields of the tumor, each containing no less than 50 tumor cells. The percentage of immunoreactive nuclei was quantified in 3 different fields and a mean percentage of expression was calculated. The mean expression levels of phosphorylated MAPK were categorized in the following manner: low (0%–10%), moderate (11%–40%), and high (41%–100%).

For morphometric analysis of PTEN we evaluated the intensity of staining within the tumor cells. Cells within at least 3 separate fields of the tumor, each containing not less than 50 tumor cells, were observed and their staining intensity was compared with that of the endothelial cells. Level of staining within the cytoplasm of the endothelial cells was assigned a rank of 3+. Negative or weak staining (0+ or 1+) was considered a loss of expression. A moderate, uniform cytoplasmic staining of tumor cells, to a degree lower than that of endothelial cells, was assigned a rank of 2+ intensity and considered to be preserved.
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For quantitative analysis of MGMT expression, at least 3 tumor fields with more than 50 cells each were selected. Expression in endothelial cells was taken as a positive control with a rank of 3+. Staining of glial cells generally was weak with only a few tumors showing 2+ to 3+ intensity. Any positivity within the tumor cell nuclei was considered positive. The percentage of positive nuclei was quantified and 20% positivity was considered MGMT-positive based on previously published data. Of note, the molecular markers we report in this paper are the standard markers used at our institution to evaluate glioblastoma tumors, but they are not necessarily specific to the multifocal glioblastoma hypothesis.

Statistical Analysis

Pair matching was based on propensity scores and conducted using a nearest available pair matching algorithm. A logistic regression model calculated the predicted probability that a patient in the unifocal cohort belonged to the multifocal group while adjusting for potentially confounding covariates. Once propensity scores were assigned to all patients, each multifocal patient was matched to an unmatched unifocal patient according to the closest propensity score. If more than 1 unmatched unifocal patient was matched to a multifocal case, then the unifocal case was selected at random. Overall survival was compared between multifocal and unifocal patients via Kaplan-Meier estimates, and log-rank tests were used to compare the survival distribution among these groups. Matched survival analysis was conducted using the Cox proportional hazards model. A probability value < 0.05 was considered statistically significant.

Results

In our series, the incidence of newly diagnosed glioblastoma with multifocal disease on presentation was 12.8% (47/368). The median age of patients with multifocal disease was 61 years, 76.6% had KPS scores ≥ 70, and 87.2% underwent either a biopsy or partial resection of their tumors. Twenty-seven (57.4%) of the 47 patients with multifocal disease had tumors located in the same cerebral hemisphere. Of the 20 patients who had tumors in both cerebral hemispheres, 13 were noted to cross the corpus callosum. Seven (14.9%) of the 47 multifocal tumors could be further classified as multicentric, with widely separated foci with no apparent route of dissemination. Radiation therapy was administered to 41 (87.2%) of 47 patients with multifocal disease and temozolomide was prescribed to 36 (76.6%) of 47.

The clinical characteristics of the original unmatched and matched solitary and multifocal groups are summarized in Table 1. The 47 multifocal patients were almost perfectly matched on the basis of age (p = 0.97), extent of resection (p = 1.0), and KPS score (p = 0.80) with 47 patients with solitary glioblastomas using the methodology described above. Of note, because the 2 groups were matched on extent of resection and most of the multifocal tumors received a biopsy (or partial resection), the majority of the matched solitary glioblastoma group was composed of patients with tumor location and characteristics that were considered suitable for biopsy only.

Fourteen of the 47 patients in the multifocal group underwent what we categorized as a partial resection (Table 1). Partly this was due to the very broad definition we employed for partial resection, which was defined as any procedure more than a biopsy, up to a 95% volumetric resection. Resection (compared with biopsy) was generally recommended only when image-complete (or near image-complete) gross-total resection was believed to be possible. The exception to this recommendation was in patients who were quite symptomatic from mass effect and most of them had 1 of the tumors located in the temporal lobe. In these patients, partial resection was sometimes used to palliate symptoms and prevent herniation during the patient’s future treatment course. In addition, in some patients less than 95% volumetric resection was achieved due to eloquent areas or tracks identified during intraoperative mapping, while in a few rare cases the operating surgeon misjudged the extent of resection. Gross-total resection was achieved in 4 (8.5%) of 47 patients with multifocal disease. All 4 patients had multifocal disease in nondominant, eloquent frontal or temporal lobes, and none of the tumors were multicentric; in other words, the multifocal enhancing areas, although more than 1 cm apart, were in the same relative vicinity and not, for example, in different hemispheres. Therefore, gross-total resection through 1 larger craniotomy was possible in these 4 very select patients.

Older age (> 65 years), low extent of resection (partial resection or biopsy), and low KPS score (< 70) were significantly associated with lower median survival within the multifocal group (Table 2). Older multifocal patients (age > 65 years) had a median survival of 4 months compared with 12 months in younger patients (age ≤ 65 years; p = 0.0007). Multifocal patients who underwent biopsy or partial resection had a median survival of 5 months compared with the 14-month median survival noted in patients who underwent gross-total or near gross-total resection (p = 0.04). Finally, multifocal patients with a low KPS score (< 70) had a median survival of 3 months compared with patients with a high KPS score (≥ 70) who survived for 9 months (p = 0.04; Table 2).

Treatment and Response

The multifocal and unifocal groups were well matched regarding radiation therapy (p = 1.00) and temozolomide treatment (p = 0.61; Table 1). Radiation therapy was not administered to 6 patients in each group. In the multifocal group the reason for no radiation therapy was rapid decline and transfer to hospice care for 5 patients, and 1 patient was recommended for temozolomide only, due to significant edema/mass effect and concerns that they would not be able to tolerate radiation therapy. In the unifocal group, all 6 patients experienced rapid decline and the patient and/or family chose hospice over any treatment measures.

Regarding temozolomide treatment, the 2 groups were also well matched (p = 0.61). Eleven patients in the multifocal group and 8 patients in the unifocal glioblastoma group did not receive temozolomide treatment.
Of the 11 patients in the multifocal group who did not receive temozolomide, 5 experienced rapid decline and were transferred to hospice care. Radiation therapy only was recommended as initial treatment in 4 patients who were old and had low KPS scores due to concerns that they would not tolerate both therapies concomitantly, and 2 additional patients declined temozolomide. In the unifocal cohort, 6 patients did not receive temozolomide due to decline and were transferred to hospice care; an additional 2 patients refused temozolomide.

Treatment responses were assessed by reviewing the 2- to 3-month postradiation therapy MR images. Six patients in each cohort (multifocal and unifocal) did not receive radiation therapy due to clinical decline, and therefore did not undergo postradiation therapy follow-up MRI.

In the multifocal group, 19 patients (46.3%) experienced tumor progression compared with 11 patients (26.8%) with tumor progression in the unifocal group. This difference approached statistical significance (p = 0.08).

**Survival Analysis**

The median overall survival for the multifocal glioblastoma group was 6 months (95% CI 4–10 months), whereas the median overall survival for the matched unifocal glioblastoma group was 11 months (95% CI 10–19 months). Of note, the median survival of the unmatched 169-patient unifocal glioblastoma cohort was 16 months (95% CI 14–20 months). Figure 1 shows the Kaplan-Meier estimate for overall survival for patients with multifocal and solitary glioblastomas after matching for age, KPS score, and extent of resection. The difference in survival between the multifocal and solitary glioblastoma groups was statistically significant according to the log-rank test (p = 0.02). Survival rates at 12 months for the multifocal and solitary glioblastoma groups were 28.5% (95% CI 16.3%–42.0%) and 49.7% (95% CI 31.8%–65.2%), respectively. Survival rates at 24 months were 4.3% (95% CI 0.4%–17.2%) and 29.0% (95% CI 13.6%–46.3%) for the multifocal and unifocal cohorts, respectively. Patients with newly diagnosed multifocal disease had an almost 2-fold increase in the hazard of death compared with the patients with solitary glioblastoma (hazard ratio 1.8, 95% CI 1.1–3.1; p = 0.02). Patient tumor samples were analyzed for expression of phosphorylated MAPK, PTEN, MGMT, laminin β1 and β2, as well as EGFR amplification, and are presented in Table 3. No significant differences in expression profile of any of the molecular markers were noted between the multifocal and solitary glioblastoma groups.

**Discussion**

In our study, the incidence of newly diagnosed glioblastoma...
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The extent of resection, and patient age. Previous studies report the incidence of multifocal glioblastoma that were multifocal on presentation was 12.8%. Studies report the incidence of multifocal glioblastoma ranging from 0.5% to as high as 20%. Larger, more recent studies document multifocal rates in the 10%–15% range and are consistent with our experience. In the multifocal group, 19 (46.3%) of 41 patients had tumor progression on postradiation therapy MRI compared with 11 patients (26.8%) with tumor progression in the unifocal group. This difference approached statistical significance, supporting the idea that these multifocal patients may be relatively treatment resistant.

After controlling for age, KPS score, treatment, and extent of resection with matching, our study showed that patients with newly diagnosed glioblastoma who have multifocal disease on presentation experience significantly worse survival than patients with solitary glioblastoma (6 months vs 11 months, respectively). Patients with multifocal disease in the temozolomide era had 1-year and 2-year survival rates of only 28.5% and 4.3%, respectively. Finally, patients with multifocal glioblastoma had a 2-fold increase in the hazard of death compared with patients with solitary glioblastoma. Previous studies report a median survival of 2–10 months for patients with solitary glioblastoma. Although it is generally believed that multifocal patients do not do as well, very few studies have systematically controlled for relevant confounders such as age, KPS score, treatment, and extent of resection, as in this study. Two smaller studies that did compare outcomes of patients with multifocal and solitary glioblastomas directly have reported conflicting results. Parsa et al. reported on the prognostic significance of intracranial dissemination of glioblastoma, but the study mainly focused on multifocal disease on recurrence. In a subanalysis, they matched 16 patients with newly diagnosed multifocal glioblastoma to a cohort with solitary glioblastoma and found no survival difference. In contrast, Hassaneen et al. analyzed a select group of patients with multifocal glioblastoma who underwent multiple craniotomies, and in the 11 newly diagnosed multifocal patients, survival was worse compared with a matched cohort with solitary glioblastoma (median survival 9.6 months vs 14.6 months, respectively; p = 0.014). Showalter et al. described the largest previous series of newly diagnosed multifocal tumors and reported a median survival of 8.1 months after whole-brain or conformal radiation therapy. However, they did not directly compare outcomes to a matched cohort with unifocal tumors. Of note, most if not all the patients included in all 3 of these studies were from the era before the use of temozolomide. Interestingly, 11 patients in our multifocal cohort did not receive temozolomide despite a diagnosis of glioblastoma in the temozolomide era. This, we believe, is the reality of how a significant proportion of patients with rapidly progressing multifocal disease are not able to initiate or complete our standard therapies. The lower than expected compliance with treatment initiation and completion has been previously documented in patients with glioblastoma. For example, van Genugten et al. have recently documented a low 40% compliance rate for temozolomide treatment over the standard 6 cycles in patients with glioblastoma.

Multifocal tumors have been defined as multiple synchronous foci, in which a microscopic connection is presumed, and dissemination is along an established pathway, such as a white matter tract. Multicentric tumors are widely separated without an apparent route of

<table>
<thead>
<tr>
<th>Molecular Marker</th>
<th>Multifocal N (%)</th>
<th>Unifocal N (%)</th>
<th>p Value</th>
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<tr>
<td>laminin β1</td>
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<td>16 (35.6)</td>
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* Data on molecular markers were only available for a subset of the matched cohort.

Fig. 1. Kaplan-Meier estimates for overall survival (months) for 94 patients with either newly diagnosed multifocal glioblastoma or unifocal (solitary) glioblastoma. Patients were matched according to KPS score, extent of resection, and patient age.
dissemination. The clinical relevance of this distinction is unclear in the literature. In fact, 2 of the larger series have shown no clinical utility in the distinction between multifocal and multicentric glioblastoma. In our study, 14.9% (7/47) of the tumors were multicentric, but given the small number of these patients and the questionable clinical utility of this distinction, we classified the tumors in these patients as multifocal.

The pathogenesis of multifocality may offer an explanation as to the poor outcomes noted in patients with multifocal glioblastoma. The exact pathogenic mechanisms of multifocality are unknown, but it is presumed that malignant glial cells in multifocal glioblastoma have an increased ability and propensity to disseminate along existing cytoarchitectural structures such as neurons, white matter tracts, and blood vessels. Given the diffuse and invasive nature of multifocal disease, recent advances in focal therapies for glioblastoma have had little impact on outcomes. Furthermore, the biology of multifocal tumors, with their inherent ability to migrate and invade, may portend the poor survival observed in these patients. Systematic mutational or mRNA expression analysis of differences between multifocal and unifocal glioblastomas has not been published. Few studies have examined specific tumor markers in patients with multifocal glioblastoma. For example, Kyritsis et al. reported germline p53 gene mutations in 6 of 19 patients with multifocal glioma. More recently, a greater proportion of c-Met overexpressing glioblastomas were described to have multifocal features. That study showed a significant association between c-Met expression and matrix metalloproteinases 2 and 9, which could explain the increase of invasive and multifocal features. Finally, Lim et al. found that glioblastomas showing contact with the subventricular zone (which harbors neural stem cells) with cortical infiltration were significantly associated with multifocal disease on presentation and recurrence. We analyzed expression of phosphorylated MAPK, PTEN, MGMT, laminin β1 and β2, as well as EGFR amplification, and found no significant differences between the multifocal and unifocal glioblastoma groups. Verhaak et al. have recently described 4 subtypes of glioblastoma (classical, mesenchymal, proneural, and neural) according to EGFR, neurofibromatosis Type 1, platelet-derived growth factor receptor, and isocitrate dehydrogenase-1 status. These glioblastoma subtypes were shown to respond to therapies differently and had differential prognosis. Due to the retrospective nature of our molecular analysis and lack of currently available tissue, we were unable to investigate whether multifocal tumors in our cohort were clustered in 1 of these glioblastoma subtypes. Such a classification in future studies with multifocal glioblastoma would be very valuable.

Conclusions

Patients with newly diagnosed multifocal glioblastoma continue to have very poor outcomes in the temozolomide era. Recent therapeutic advances have not delayed progression or improved survival in this subgroup. A systematic investigation of the unique biology of multifocal glioblastoma that aims to identify the key invasive and migratory mechanisms is greatly needed to develop a new generation of targeted therapeutic.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Patil, Nuño. Acquisition of data: Patil, Yi, Elramisy, Hu, Nuño. Analysis and interpretation of data: Patil, Hu, Nuño. Drafting the article: Patil, Yi, Nuño. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Patil. Statistical analysis: Nuño. Administrative/technical/material support: Nuño. Study supervision: Patil, Nuño.

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