

Molecular-Based Recursive Partitioning Analysis Model for Glioblastoma in the Temozolomide Era

A Correlative Analysis Based on NRG Oncology RTOG 0525

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IMPORTANCE There is a need for a more refined, molecularly based classification model for glioblastoma (GBM) in the temozolomide era.

OBJECTIVE To refine the existing clinically based recursive partitioning analysis (RPA) model by incorporating molecular variables.

DESIGN, SETTING, AND PARTICIPANTS NRG Oncology RTOG 0525 specimens (n = 452) were analyzed for protein biomarkers representing key pathways in GBM by a quantitative molecular microscopy-based approach with semiquantitative immunohistochemical validation. Prognostic significance of each protein was examined by single-marker and multivariate Cox regression analyses. To reclassify the prognostic risk groups, significant protein biomarkers on single-marker analysis were incorporated into an RPA model consisting of the same clinical variables (age, Karnofsky Performance Status, extent of resection, and neurologic function) as the existing RTOG RPA. The new RPA model (NRG-GBM-RPA) was confirmed using traditional immunohistochemistry in an independent data set (n = 176).

MAIN OUTCOMES AND MEASURES Overall survival (OS).

RESULTS In 452 specimens, MGMT (hazard ratio [HR], 1.81; 95% CI, 1.37-2.39; $P < .001$), survivin (HR, 1.36; 95% CI, 1.04-1.76; $P = .02$), c-Met (HR, 1.53; 95% CI, 1.06-2.23; $P = .02$), pmTOR (HR, 0.76; 95% CI, 0.60-0.97; $P = .03$), and Ki-67 (HR, 1.40; 95% CI, 1.10-1.78; $P = .007$) protein levels were found to be significant on single-marker multivariate analysis of OS. To refine the existing RPA, significant protein biomarkers together with clinical variables (age, Karnofsky Performance Status, extent of resection, and neurological function) were incorporated into a new model. Of 166 patients used for the new NRG-GBM-RPA model, 97 (58.4%) were male (mean [SD] age, 55.7 [12.0] years). Higher MGMT protein level was significantly associated with decreased MGMT promoter methylation and vice versa (1425.1 for methylated vs 1828.0 for unmethylated; $P < .001$). Furthermore, MGMT protein expression (HR, 1.84; 95% CI, 1.38-2.43; $P < .001$) had greater prognostic value for OS compared with MGMT promoter methylation (HR, 1.77; 95% CI, 1.28-2.44; $P < .001$). The refined NRG-GBM-RPA consisting of MGMT protein, c-Met protein, and age revealed greater separation of OS prognostic classes compared with the existing clinically based RPA model and MGMT promoter methylation in NRG Oncology RTOG 0525. The prognostic significance of the NRG-GBM-RPA was subsequently confirmed in an independent data set (n = 176).

CONCLUSIONS AND RELEVANCE This new NRG-GBM-RPA model improves outcome stratification over both the current RTOG RPA model and MGMT promoter methylation, respectively, for patients with GBM treated with radiation and temozolomide and was biologically validated in an independent data set. The revised RPA has the potential to contribute to improving the accurate assessment of prognostic groups in patients with GBM treated with radiation and temozolomide and to influence clinical decision making.

TRIAL REGISTRATION clinicaltrials.gov Identifier: [NCT00304031](#)

JAMA Oncol. 2017;3(6):784-792. doi:10.1001/jamaoncol.2016.6020
Published online January 12, 2017.

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Glioblastoma (GBM), the most aggressive primary brain tumor, has a current 5-year survival rate of 5% in the United States.¹ However, a small subset of patients do experience longer survival, suggesting underlying heterogeneity; therefore, development of better prognostic classification models is crucial. The first effort to comprehensively analyze GBM patient survival by prognostic grouping was published in 1993, using recursive partitioning analysis (RPA), a nonparametric statistical technique that creates distinct prognostic groups based on combinations of variables.² This initial RPA analysis included both GBM and anaplastic astrocytoma patients, who received radiation with and without chemotherapy or a radiation sensitizer. Based on clinical and histological characteristics, this analysis identified 6 prognostic classes (I–VI) with distinct survival outcomes.² Subsequently, a follow-up study involving only patients with GBM revised the original RPA model into 3 classes (III, IV, and V/VI),³ and this has been applied to many recent GBM clinical trials. Although the current RPA appears to accurately stratify patients in the temozolomide era,⁴ the original classes were not identified using a training set of temozolomide-treated patients and may not reflect the most accurate prognostic classes. Moreover, as recent studies have revealed key molecular pathways associated with pathogenesis of GBM, it was hypothesized that inclusion of corresponding proteins could enhance the discriminatory power of the current, RPA model. Therefore, we incorporated potential protein-based variables using specimens from the NRG Oncology (RTOG) 0525 trial, a phase 3 trial that compared standard adjuvant temozolomide vs a dose-dense schedule in patients with newly diagnosed GBM.⁵ No statistical difference in survival outcomes was found between the 2 arms of NRG Oncology RTOG 0525.

Analyzing the relative expression levels, subcellular distributions, and posttranslational modifications of biomarker proteins may be a powerful approach to generate information to predict outcome in GBM. In this regard, signal transduction proteins that are commonly deregulated in GBM along with MGMT were analyzed. Multiple signal transduction pathways are deregulated in GBM, including RTK/PI3K/Akt, RTK/RAS/MAPK, and Stat3 among others,^{6–10} which promote cell proliferation, survival, invasion, angiogenesis, and resistance to radiation therapy and chemotherapy.¹¹ In the present study, 452 of 833 randomized patients (NRG Oncology RTOG 0525) were used to measure the expression of 22 proteins associated with GBM pathogenesis to assess whether these were associated with outcome and whether the addition of proteins to the current clinically based GBM RPA could strengthen the prognostic classification. To accurately assess protein expression at subcellular levels, quantitative fluorescence immunohistochemistry (AQUA) was used, a method that previously showed high reproducibility and accuracy similar to enzyme-linked immunosorbent assays.¹² Furthermore, to enhance the clinical applicability, these findings were confirmed in an independent data set using traditional semiquantitative immunohistochemistry (IHC).

Key Points

Question Do molecular variables improve prognostic classification of glioblastomas treated with radiation and temozolomide?

Findings In this secondary analysis of a randomized clinical trial, 22 proteins were analyzed by quantitative immunohistochemistry using RTOG 0525 specimens and assessed for prognostic significance of overall survival. Significant proteins with currently used clinical variables were included to develop a newly refined model (NRG-GBM-RPA), composed of age, c-MET and MGMT protein, and confirmed in an independent cohort.

Meaning Incorporation of c-MET and MGMT protein data enhances prognostic classification of patients with glioblastoma over and above clinical variables and MGMT methylation and provides further insight into underlying resistance to radiation and temozolomide treatment.

Methods

Quantitative Fluorescence Immunohistochemistry

A total of 452 patients from NRG Oncology RTOG 0525 had available specimens and were included for preparation of 4 tissue microarrays. These tissue microarrays were analyzed for 12 high priority protein biomarkers (based on literature^{6–10}): epidermal growth factor receptor (EGFR), NFκBp65, pNFκBp65, pAKT, pERK, pmTOR, IGF1R, O⁶-methylguanine DNA methyltransferase (MGMT), phosphatase and tensin homolog (PTEN), survivin, Ki-67, and Src. Additionally, 294 patients had remaining tissue available for further protein analysis and were stained for VEGFR1, VEGFR2, pSRCY419, pSRCY529, CD24, CD44, p16, p53, PARP-1, and c-Met. RTOG patients participating in this study provided informed consent based on an institutional review board-approved protocol at the enrollment site. The secondary analysis was conducted with an institutional review board-approved waiver of consent from The Ohio State University due to the retrospective nature of the study (eMethods in the [Supplement](#)).

Validation Studies

Four tissue microarrays comprising patients with GBM (n = 176) with known survival outcomes and clinical characteristics treated at the University Medical Center of Utrecht were analyzed by traditional IHC for c-Met (Abcam-EP1454Y; 1:500) and MGMT (Millipore-clone MT3.1; 1:100) protein. Patients were scored manually using the Allred method¹³ by 2 independent pathologists (A.L.S. and A.P.B.). Institutional samples were used under an institutional review board-approved waiver of consent due to the retrospective nature of the study.

Statistical Analysis

Cox proportional hazards regression analysis was used to explore the relationship between marker expression and overall survival (OS).¹⁴ All models were forced to retain age, Karnofsky performance status (KPS), resection status, and treatment to control for possible confounding marker effects.¹⁵

Table 1. Pretreatment Characteristics of the 166 Patients From NRG Oncology RTOG 0525 Included in the Biomarker Study With Data for All 5 Protein Markers Included for the NRG Glioblastoma Recursive Partitioning Analysis (NRG-GBM-RPA)

| Characteristic | No. (%) | | P Value for χ^2 |
|--|---------------------------------------|---|----------------------|
| | Arm 1, Standard Temozolomide (n = 82) | Arm 2, Dose-Dense Temozolomide (n = 84) | |
| Age, y | | | |
| <50 | 21 (25.6) | 23 (27.4) | .80 |
| ≥50 | 61 (74.4) | 61 (72.6) | |
| Sex | | | |
| Male | 47 (57.3) | 50 (59.5) | .77 |
| Female | 35 (42.7) | 34 (40.5) | |
| Karnofsky Performance Status | | | |
| 60-80 | 27 (32.9) | 32 (38.1) | .49 |
| 90-100 | 55 (67.1) | 52 (61.9) | |
| Surgery | | | |
| Biopsy | 3 (3.7) | 3 (3.6) | .99 |
| Partial resection | 28 (34.1) | 28 (33.3) | |
| Total resection | 51 (62.2) | 53 (63.1) | |
| Neurologic function (no vs minor vs moderate/severe) | | | |
| Symptoms | | | |
| None | 25 (30.5) | 24 (28.6) | .29 |
| Minor | 33 (40.2) | 43 (51.2) | |
| Moderate | 24 (29.3) | 15 (17.9) | |
| Severe | 0 (0.0) | 2 (2.4) | |
| RPA class | | | |
| III | 18 (22.0) | 17 (20.2) | .40 |
| IV | 42 (51.2) | 51 (60.7) | |
| V | 22 (26.8) | 16 (19.0) | |

Nonnested models were compared with the Akaike information criterion (AIC)¹⁶ and were limited to patients with non-missing covariates in the models being compared. The AIC uses maximum likelihood and the number of parameters to assess the relative quality of statistical models with the superior model having the lower AIC. Overall survival rates were estimated using the Kaplan-Meier method,¹⁷ and differences were tested using the log-rank test.¹⁸ Means were compared using the *t* test. The RPA included only randomized patients treated on NRG Oncology RTOG 0525 with data available for all 6 significant proteins; therefore, a reduced sample size, *n* = 166, was used in the RPA. Variables in the clinical RPA, age, KPS, resection status, and neurofunction status, were also considered for inclusion in this RPA. The resulting RPA class was biologically validated in a separate data set. To determine the best cut points for markers with continuous values significantly associated with survival for inclusion in the RPA model, the technique of using receiver operating characteristic curves was applied.¹⁹ Because the area under the receiver operating characteristic curve for all markers was 0.65 or less, limiting the ability to determine optimal cut points, methods using quartiles, tertiles, and medians were used. For RPA class determination, each class was chosen based on minimizing the

conditional probability standard error of the pruned tree. Two classes had overlapping survival curves and were combined into a single class. SAS/STAT software and R Statistical Software were used for all analyses, and the “rpart” package in R Statistical Software was used for the RPA class determination. Explanation of variance,²⁰ specifically the Schemper-Henderson predictive measure, the concordance index, and net classification improvement (NRI) were used to compare the effect of OS between each RPA class within the framework of the Cox proportional hazards model.²¹⁻²⁴ For explanation of variance, the predictive inaccuracy of the model is used to determine the percent of variance explained. For interpretation, the smaller the predictive inaccuracy, the better the prediction. A 2-sided α = .05 was used to determine statistical significance.

Results

Single-Marker and Multimarker Modeling

All 22 proteins were quantified (eTable 1 in the [Supplement](#)) and correlated with OS in randomized patients. No significant differences were detected between OS for patients with and without tissue samples (eTable 2 in the [Supplement](#)). Single-marker Cox regression modeling was performed and identified 6 significant proteins (pAKT, MGMT, Ki-67, pmTOR, survivin, and c-Met) that were associated with OS when represented as a continuous variable (eTable 3 in the [Supplement](#)). Pretreatment characteristics of NRG Oncology RTOG 0525 randomized patients with data from all significant proteins with identified cutoff points are provided in **Table 1**. When investigated as discrete categorical variables formed by division at specific quantiles, 5 of the 6 proteins yielded cutoff points that were significantly associated with OS: MGMT, Ki-67, pmTOR, survivin, and c-Met (**Table 2**).

In **Figure 1**, high MGMT protein level within the tumor (hereafter MGMT tumor) measured by AQUA and split by the median was shown to significantly correlate with decreased OS (**Figure 1A**) (hazard ratio [HR], 1.73; 95% CI, 1.32-2.27; *P* < .001). High c-Met protein level within the cytoplasm (hereafter c-Met cytoplasm) when split by the top quartile was significantly correlated with decreased OS (**Figure 1B**) (HR, 1.56; 95% CI, 1.08-2.24; *P* = .02). A higher than median cytoplasmic/nuclear ratio of survivin protein had nonsignificantly decreased OS (eFigure 1A in the [Supplement](#)) (HR, 1.29; 95% CI, 1.00-1.67; *P* = .05). Higher than median Ki-67 protein level within the nucleus (hereafter Ki-67 nuclear) was also significantly associated with decreased OS (eFigure 1B in the [Supplement](#)) (HR, 1.34; 95% CI, 1.05-1.70; *P* = .02). Conversely, a high nuclear/cytoplasmic ratio of pmTOR protein when split by the median had nonsignificantly increased OS (eFigure 1C in the [Supplement](#)) (HR, 0.81; 95% CI, 0.63-1.03; *P* = .08).

Due to involvement of MGMT in response to temozolomide treatment,²⁵ MGMT protein levels were evaluated in relation to MGMT promoter methylation. As shown in eTables 4.1 and 4.2 and eFigure 2 in the [Supplement](#), MGMT tumor and MGMT nuclear protein expression are significantly different between MGMT promoter methylated vs unmethylated

samples (1425.1 vs 1828.0 for MGMT tumor and 2195.1 vs 2917.1 for MGMT nuclear mask; $P < .001$). However, MGMT protein expression within the tumor (HR, 1.84; 95% CI, 1.38-2.43; $P < .001$) demonstrated a stronger prognostic effect compared with MGMT promoter methylation (HR, 1.77; 95% CI, 1.28-2.44; $P < .001$) on OS on single-marker modeling based on AIC, and thus protein expression was the only MGMT marker incorporated into the revised RPA.

Multimarker Cox regression modeling was performed on the protein biomarkers that were statistically significant under single-marker Cox regression modeling (when evaluated as discrete variables; for biomarkers with multiple candidate cut points, the representation with higher significance level was used). Thus, 5 proteins, pmTOR, MGMT, Ki-67, c-Met, and survivin, were tested in a multimarker model, with stepwise selection. As detailed in Table 2, MGMT tumor (at least median vs less than median [HR, 1.91; 95% CI, 1.27-2.88; $P = .002$]), Ki-67 nuclear (at least median vs less than median [HR, 1.50; 95% CI, 1.01-2.22; $P = .04$]), and c-Met cytoplasm (at least top quartile vs less than top quartile [HR, 1.65; 95% CI, 1.10-2.48; $P = .02$]) were all found to be significant.

MGMT and c-Met Protein Expression Strengthens Recursive Partitioning Analysis for Glioblastoma

Protein biomarkers that were significant on single-marker modeling were incorporated into an RPA model consisting of the same variables of the current RTOG RPA classification to determine whether protein biomarkers can help stratify patients into prognostic groups. The 166 patients used for RPA modeling, which required patients to be randomized and have nonmissing data for all of the biomarkers considered for inclusion, was stratified by the 3 current RPA² classes (based on age, KPS, resection status, neurofunction status) relative to OS (Figure 2A and eTable 5 in the Supplement). The newly developed NRG-GBM-RPA classes are shown in Figures 2B-D: class I (MGMT tumor less than median or MGMT tumor at least median and age younger than 50 years), class II (MGMT tumor at least median and age at least 50 years and c-Met cytoplasm less than top quartile), and class III (MGMT tumor at least median and age at least 50 years and c-Met cytoplasm at least top quartile).

The median OS times for these 3 classes are 21.9 (95% CI, 16.4-29.9), 16.6 (95% CI, 13.3-20.0), and 9.4 (95% CI, 5.6-11.6) months, respectively, demonstrating that these classes are significantly different (I vs II: HR, 1.83; 95% CI, 1.21-2.76; $P = .004$; I vs III: HR, 5.19; 95% CI, 3.07-8.79; $P < .001$). Survival estimates and confidence intervals are presented in eTable 6 in the Supplement. Explanation of variance, concordance index, and NRI were computed to compare the 2 RPAs. The NRG-GBM-RPA explains a higher percent of the variance (11.09% vs 3.78%) and has a lower value of predictive inaccuracy (0.33 vs 0.36) as compared with the currently used clinical RPA for OS. Although the concordance index and NRI were in favor of the model with NRG-GBM-RPA compared with the clinical RPA, there was no significant difference between the 2 models with respect to con-

Table 2. Cox Models of Protein Biomarkers by Cutoff Points in NRG Oncology RTOG 0525 Specimens

| Characteristic | Hazard Ratio (95% CI) | P Value |
|---|-----------------------|---------|
| Single-Marker Models^a | | |
| Maximum pmTOR nuclear/cytoplasm ratio (at least median vs less than median ^b) | 0.76 (0.60-0.97) | .03 |
| MGMT Tumor mask | | |
| At least median ^b vs less than median | 1.81 (1.37-2.39) | <.001 |
| At least top tertile ^b vs less than top tertile | 1.57 (1.17-2.10) | .003 |
| At least top quartile ^b vs less than top quartile | 1.55 (1.14-2.11) | .005 |
| Maximum survivin cytoplasm/nuclear ratio (at least median ^b vs less than median) | 1.36 (1.04-1.76) | .02 |
| Average Ki-67 in nuclear mask | | |
| At least median ^b vs less than median | 1.40 (1.10-1.78) | .007 |
| At least top tertile ^b vs less than top tertile | 1.40 (1.09-1.79) | .008 |
| At least top quartile ^b vs less than top quartile | 1.32 (1.01-1.72) | .05 |
| Minimum c-Met cytoplasm mask | | |
| At least top tertile ^b vs less than top tertile | 1.48 (1.04-2.09) | .03 |
| At least top quartile ^b vs less than top quartile | 1.53 (1.06-2.23) | .02 |
| Multimarker Model^c | | |
| Treatment arm (arm 1 ^b vs arm 2) ^d | 0.72 (0.50-1.05) | .09 |
| Age (continuous) | 1.03 (1.01-1.05) | <.001 |
| KPS (60-80 vs 90-100) | 1.38 (0.90-2.11) | .14 |
| Surgery (biopsy/partial resection vs total resection) | 1.00 (0.67-1.51) | .99 |
| MGMT tumor mask (at least median ^b vs less than median) | 1.91 (1.27-2.88) | .002 |
| Mean Ki-67 in nuclear mask (at least median ^b vs less than median) | 1.50 (1.01-2.22) | .04 |
| Minimum c-Met cytoplasm mask (at least top quartile ^b vs less than top quartile) | 1.65 (1.10-2.48) | .02 |

Abbreviation: KPS, Karnofsky Performance Status.

^a For single-marker models: All models are adjusted by radiation treatment, age, KPS, and surgery. Only markers with $P < .05$ are listed.

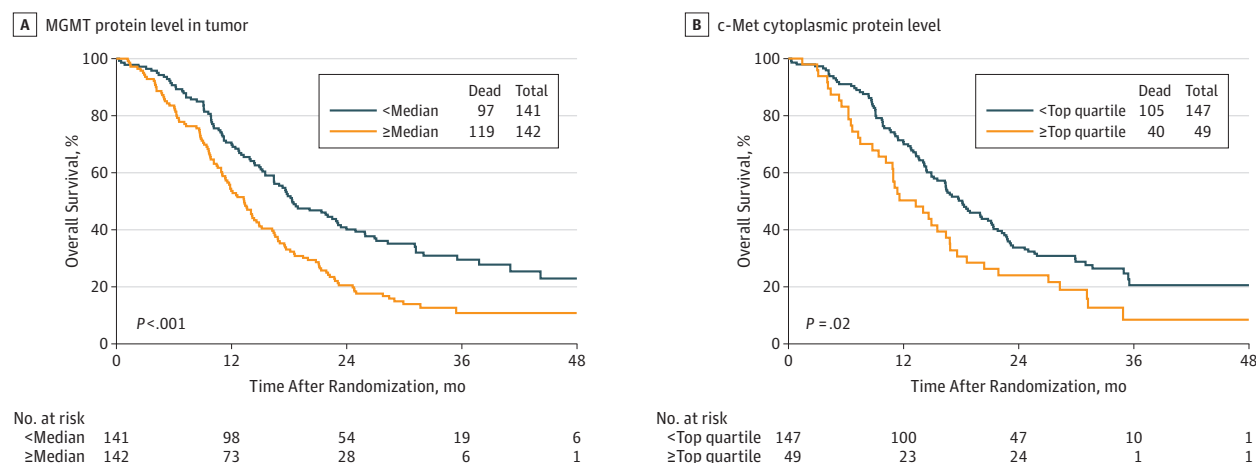
^b Unfavorable outcome.

^c For multimarker model: model derived from stepwise selection by forcing radiation treatment, age, KPS, and surgery to be included in the model. During stepwise regression, pmTOR, maximum nuclear/cytoplasm ratio, survivin, and cytoplasm/nuclear mask ratio were dropped out.

^d Arm 1 indicates standard temozolomide, and arm 2, dose-dense temozolomide.

cordance index (0.64; 95% CI, 0.55-0.72 for clinical RPA; 0.70; 95% CI, 0.63-0.77 for NRG-GBM-RPA; $P = .93$) or NRI (7.89%; 95% CI, -11% to 49%). Importantly, KPS and extent of resection did not add any additional information to the NRG-GBM-RPA. In addition, the NRG-GBM-RPA (I vs II: HR, 1.59; 95% CI, 1.00-2.54; $P = .05$; I vs III: HR, 4.56; 95% CI, 2.55-8.17; $P < .001$) demonstrated a stronger prognostic effect compared with MGMT promoter methylation (HR, 1.58; 95% CI, 1.01-2.47; $P = .046$) on OS on single-marker modeling based on AIC (eTable 7.1 in the Supplement). The

Figure 1. MGMT and c-Met Levels and Overall Survival (OS) in Randomized NRG Oncology RTOG 0525 Study Participants



A, High MGMT tumor protein staining when split by the median was significantly associated with decreased OS. B, High levels of c-Met cytoplasmic protein staining when split by the top quartile was significantly associated with decreased OS.

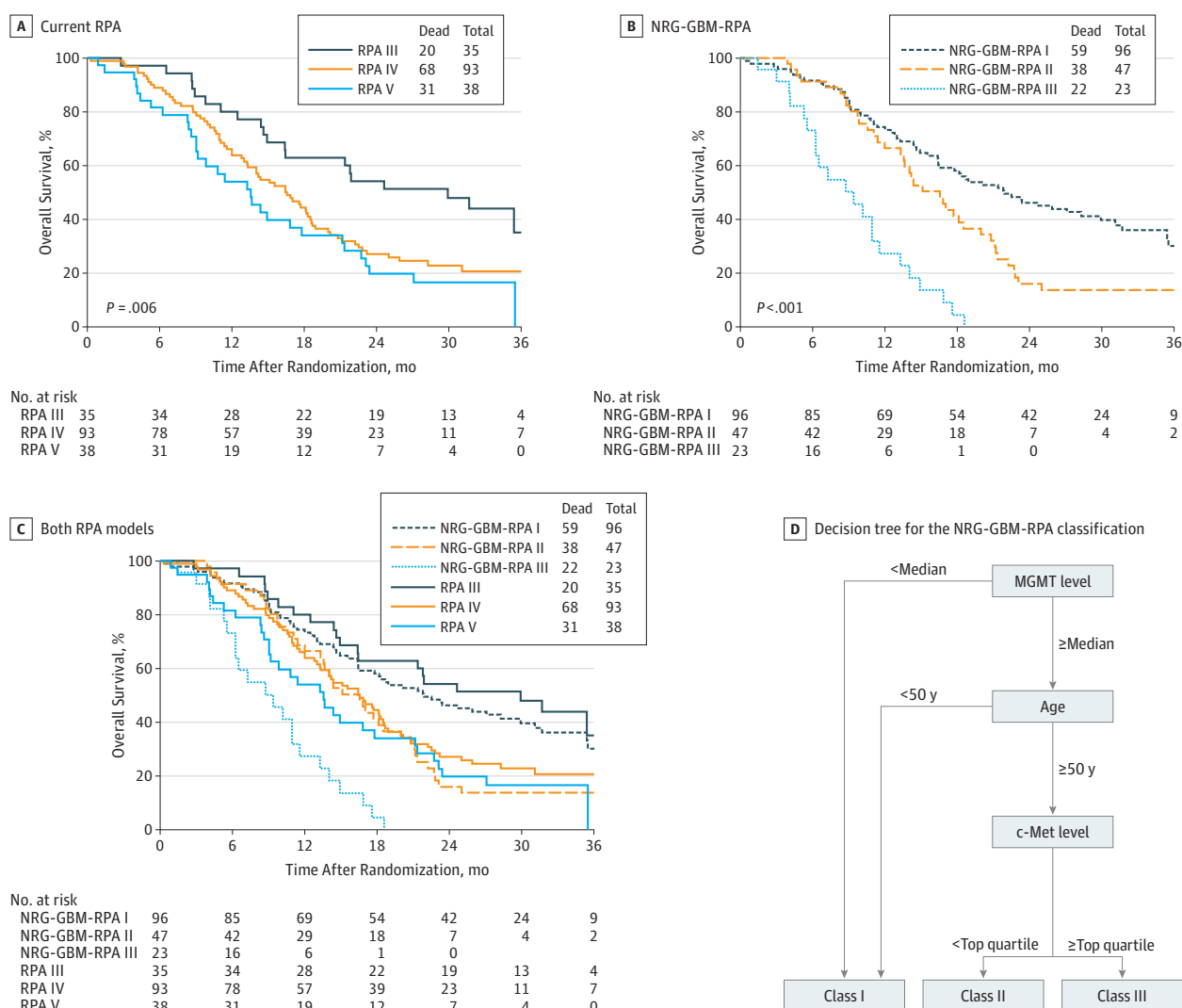
NRG-GBM-RPA also explains a higher proportion of the variance (11.11% vs 1.68%) compared with *MGMT* methylation (eTable 7.2 in the Supplement). Furthermore, class I (which represents the best prognostic group) in the NRG-GBM-RPA comprises both patients with methylated and unmethylated *MGMT* (eTable 7.3 in the Supplement).

To validate the biological relevance of the NRG-GBM-RPA, samples from 176 patients treated at the University of Utrecht were examined on tissue microarrays using traditional IHC staining. Pretreatment characteristics of the validation cohort (87 [49%] received radiation therapy and temozolomide) are provided in eTable 8 in the Supplement. As shown in Figure 3, the NRG-GBM-RPA was confirmed to be a statistically significant prognostic classifier using traditional IHC for all patients (Figure 3A) ($n = 176$; I vs II: HR, 1.46; 95% CI, 1.01-2.11; $P = .04$; I vs III: HR, 1.88; 95% CI, 1.20-2.96; $P = .006$) and for patients who received radiation therapy and temozolomide (Figure 3B) ($n = 87$; I vs II: HR, 1.91; 95% CI, 1.10-3.32; $P = .02$; I vs III: HR, 3.68; 95% CI, 1.84-7.35; $P < .001$). Classification of patients who received radiation therapy and temozolomide based on the current RPA is shown in Figure 3C. Concordance between pathologists' analyses for the Allred score (0-8) was moderate and varied from c-Met (weighted $\kappa = 0.4$) to MGMT (weighted $\kappa = 0.6$).²⁶⁻²⁹ Effects of isocitrate dehydrogenase (*IDH*) were then analyzed in the validation cohort as *IDH* mutation status was unavailable for NRG Oncology RTOG 0525. Removing known *IDH*-mutant glioblastomas ($n = 9$) from the analysis did not affect these findings; the new RPA remained statistically significant in some treatments but not in others ($n = 167$; I vs II: HR, 1.37; 95% CI, 0.94-1.97; $P = .10$; I vs III: HR, 1.87; 95% CI, 1.18-2.98; $P = .007$). The new RPA remained statistically significant in patients treated with radiation and temozolomide ($n = 82$; I vs II: HR, 1.65; 95% CI, 0.95-2.87; $P = .07$; I vs III: HR, 3.24; 95% CI, 1.62-6.48; $P < .001$) as shown in eFigure 3 in the Supplement.

Discussion

The current RPA classification system for GBM was created using trials conducted in the pretemozolomide era.^{2,3} The goal of this study was to refine the current RPA by incorporating both clinical and protein parameters using radiation- and temozolomide-treated patients with GBM. The findings of this study have important implications for patients with GBM because a new RPA was identified based on underlying molecular markers, some putatively involved in GBM pathogenesis. Importantly, these newly identified prognostic risk groups may help guide decision making, as well as yielding insights into possible underlying resistance mechanism(s) to radiation and temozolomide treatment. Most notably, the NRG-GBM-RPA classification (Figure 2) improved the separation among prognostic groups relative to the current system, as well as *MGMT* promoter methylation, and therefore this could potentially serve as a superior stratification variable in clinical trials. Prognostic biomarkers identified to be significant on single-marker modeling (pAKT, pmTOR, MGMT, Ki-67, survivin, and c-Met) validated previous findings with regards to their respective prognostic values. Prognostic protein biomarkers identified to be significant after multimarker modeling for GBM were Ki-67, c-Met, and MGMT. Each of these protein biomarkers has been previously associated with worse outcome in GBM,³⁰⁻³² but most of these studies have failed to determine whether these proteins are independent prognostic factors through comprehensive multivariate analysis. High c-Met protein expression (detected by traditional IHC) has been previously shown to be significantly associated with poor OS.³⁰ Our study further validates these findings in an independent, larger cohort of 196 patients all treated with radiation and temozolomide in NRG Oncology RTOG 0525. Notably, c-Met inhibitors are currently in multiple clinical trials for solid tumors including GBM, and high-expressing c-Met GBM patients may

Figure 2. Protein Biomarker Data Strengthen Current Glioblastoma Recursive Partitioning Analysis (RPA) Classification



Current and new RPA classification of NRG Oncology RTOG 0525 study participants. A-C, The cohort of 166 randomized patients used is shown stratified by the 3 current RPA classes relative to OS, for the current RPA and the new NRG-GBM-RPA, and both RPA models. D, A decision tree for the NRG-GBM-RPA classification. Current RPA class III (age <50 years and Karnofsky Performance Status [KPS] 90-100) vs class IV (age <50 years and KPS <90 or age ≥50 years and partial or total resection with no worse than minor neurofunction impairment) vs class V (age ≥50 years and partial or total

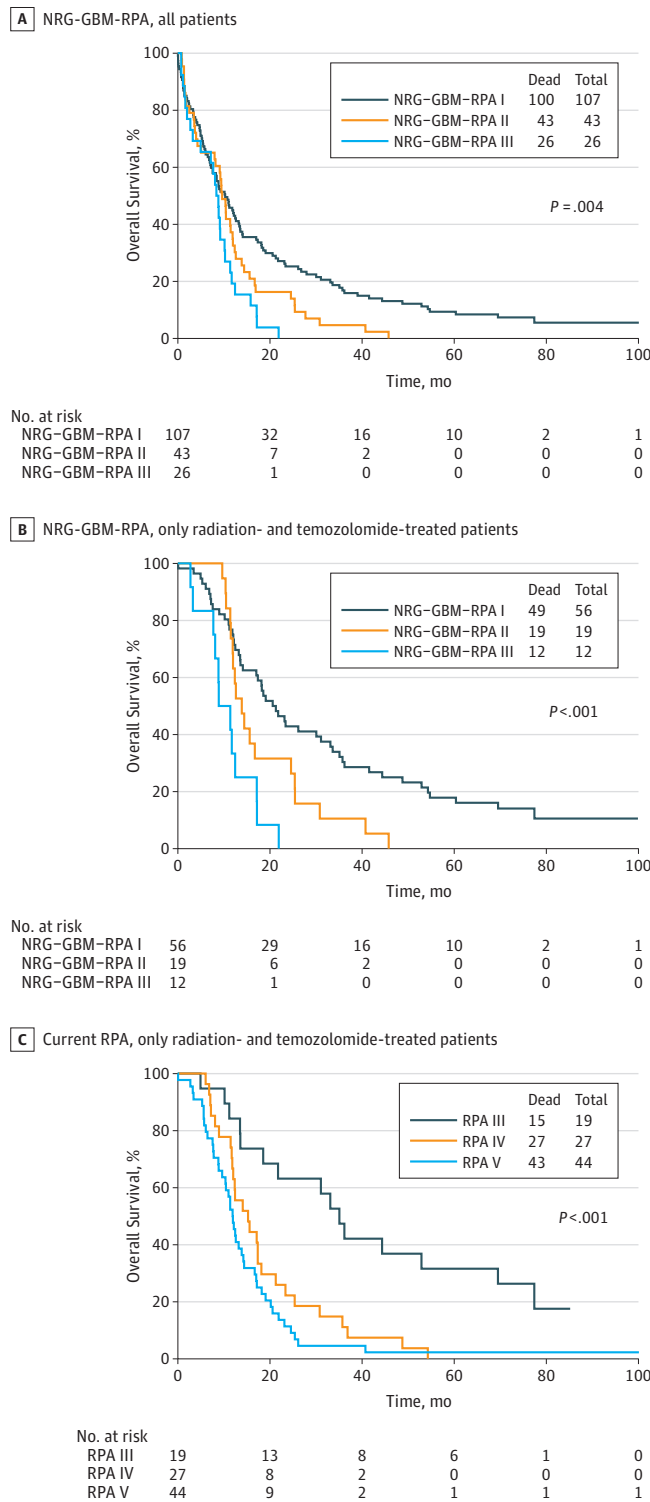
resection with worse than minor neurofunction impairment, or age ≥50 years and biopsy only, or age ≥50 years, KPS ≥ 60, and normal mental status). NRG-GBM-RPA class I: MGMT tumor level less than median or MGMT tumor level median or greater and age younger than 50 years vs class II: MGMT tumor at least median and age 50 years or older and c-Met cytoplasm less than top quartile vs class III: MGMT tumor at least median and age at least 50 years and c-Met cytoplasm at least top quartile.

be good candidates for this targeted therapy as evidenced by in vitro and in vivo models,^{33,34} as well as a single case report.³⁵ Although the upper quartile cutoff for c-Met appears to be clinically relevant in the NRG-GBM-RPA, it may not be the best cut-off point for selection of patients who may be treated with and respond to c-Met inhibition.

Furthermore, *MGMT* promoter methylation has been one of the most studied prognostic biomarkers in patients with GBM; however, *MGMT* protein expression has not been well characterized in large data sets and there are conflicting results regarding expression level of the protein and its prognostic significance.^{32,36} Therefore, we sought to deter-

mine whether *MGMT* protein expression levels using a quantitative fluorescence IHC approach could determine prognostic significance similar to that of *MGMT* promoter methylation. Importantly, *MGMT* protein tumor expression appeared to be of greater prognostic significance for OS than *MGMT* promoter methylation even after multimarker modeling. Further, *MGMT* protein expression in tumor was found to be significantly associated with *MGMT* promoter methylation. This result confirms a previous publication in which we demonstrated that decreased *MGMT* protein expression was correlated with increased sensitivity to radiation and temozolomide in vitro.³⁷ Intriguingly, *MGMT*

Figure 3. Biological Validation of the NRG-GBM-RPA (Recursive Partitioning Analysis) Classification in an Independent Glioblastoma (GBM) Cohort



A, NRG-GBM-RPA classification was correlated with overall survival (OS) in all patients with GBM with heterogeneous treatments.

B, NRG-GBM-RPA classification was correlated with OS in patients with GBM treated with radiation and temozolomide. C, Current RPA classification was correlated with OS in patients with GBM treated with radiation and temozolomide.

protein appeared to have greater prognostic value vs *MGMT* promoter methylation. This is likely due to *MGMT* protein expression being a better surrogate of *MGMT* activity as there were multiple instances of tumors with methylated

MGMT expressing higher levels of *MGMT* protein, as well as multiple tumors with unmethylated *MGMT* expressing lower levels of *MGMT* protein (eFigure 2 in the Supplement).

Of importance, both c-Met and MGMT demonstrated statistical significance on multimarker modeling and in the RPA, providing evidence that both proteins are necessary in the newly developed RPA model and add independent value specifically for patients older than 50 years. Our biological validation of the NRG-GBM-RPA (including MGMT protein, c-Met protein, and age) using traditional semiquantitative IHC with an independent patient cohort shows that the NRG-GBM-RPA has the potential to be implemented as a routine histopathological test accessible to the majority of routine clinical pathology laboratories. Furthermore, the NRG-GBM-RPA displayed greater prognostic value relative to both MGMT promoter methylation and the existing clinically based RTOG RPA in NRG Oncology RTOG 0525.

Limitations

The prognostic significance of MGMT protein expression and the NRG-GBM-RPA identified warrants further studies on its clinical applicability using large sample sizes because it is possible that the small sample size limited the ability to find a statistically significant difference compared with the current RPA using NRI and concordance index. Further validation will also determine whether semiquantitative or quantitative methods will be required to overcome reproducibility issues and the subjectivity of traditional IHC.^{38,39}

However, our approach for traditional IHC differed from previous studies as we used a sophisticated scoring method¹³ to assess proportion and intensity of protein expression (eFigure 4 in the Supplement). Furthermore, protein analysis by traditional IHC may potentially be more accessible and cost-effective to community-based practices world-wide. The validation study, which comprised patients treated with radiation and temozolomide, radiation alone, or surgery alone, further demonstrated the validity of the NRG-GBM-RPA in patients treated with radiation and temozolomide compared with the heterogeneous treated group and the ability of the refined RPA to separate out the poor and intermediate prognostic classes.

Conclusions

By validating the known signal transduction proteins and MGMT protein expression as independent prognostic factors, as well as deriving a new RPA (NRG-GBM-RPA) incorporating MGMT protein and c-Met protein expression, the present study has the potential to contribute to improving the accurate assessment of prognostic groups in patients with GBM treated with radiation and temozolomide and also influence clinical decision making.

ARTICLE INFORMATION

Accepted for Publication: November 2, 2016.

Published Online: January 12, 2017.

doi:10.1001/jamaoncol.2016.6020

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Author Contributions: Dr Bell had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of Interest Disclosures: Dr Mehta reports consulting honoraria from Caviton and Novocure; research funding from Cellectar and Novocure; Data Safety Monitoring Board for Monteris; and previously served on the Board of Directors of Pharmacyclics (with options). No other disclosures are reported.

Funding/Support: This project was supported by grants U10CA21661, U10CA180868, U10CA180822, U10 CA37422, UG1CA189867, RO1CA108633 (to A.C.), 1RC2CA148190 (to A.C.) U10CA180850-01 (to A.C.), and RO1CA169368 (to A.C.) from the National Cancer Institute, a Brain Tumor Funders Collaborative Grant (to A.C.), Ohio

State University Comprehensive Cancer Center Award (to A.C.), and Merck & Co.

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Additional Contributions: We thank S. Jaharul Haque, PhD, Department of Radiation Oncology, The Ohio State University, for assistance in preparation of the manuscript. No compensation was received from a funding sponsor for his contributions.

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