6-1-2017

Molecular-Based Recursive Partitioning Analysis Model for Glioblastoma in the Temozolomide Era: A Correlative Analysis Based on NRG Oncology RTOG 0525.

Erica Hlavin Bell
The Ohio State University

Stephanie L Pugh
NRG Oncology Statistics and Data Management Center

Joseph P. McElroy
The Ohio State University

Mark R. Gilbert
National Institutes of Health

Minesh Mehta
University of Maryland Medical Systems; Miami Cancer Institute

See next page for additional authors

Authors
Erica Hlavin Bell, Stephanie L Pugh, Joseph P. McElroy, Mark R. Gilbert, Minesh Mehta, Alexander C Klimowicz, Anthony Magliocco, Markus Bredel, Pierre Robe, Anca L. Grosu, Roger Stupp, Walter Curran, Aline P. Becker, Andrea L. Salavaggione, Jill S. Barnholtz-Sloan, Kenneth Aldape, Deborah T. Blumenthal, Paul D. Brown, Jon Glass, Luis Souhami, R. Jeffrey Lee, David Brachman, John Flickinger, Minhee Won, and Arnab Chakravarti

Published in final edited form as:

JAMA Oncol. 2017 June 01; 3(6): 784–792. doi:10.1001/jamaoncol.2016.6020.

Molecular-Based Recursive Partitioning Analysis (RPA) Model for Glioblastoma in the Temozolomide Era: A Correlative Analysis Based Upon NRG Oncology RTOG 0525

Erica Hlavin Bell, PhD[1], **Stephanie L. Pugh, PhD**[2], **Joseph P. McElroy, PhD**[3], **Mark R. Gilbert, MD**[4], **Minesh Mehta, MD**[5], **Alexander C. Klimowicz, PhD**[6], **Anthony Magliocco, MD**[7], **Markus Bredel, MD, PhD**[8], **Pierre Robe, MD, PhD**[9], **Anca-L. Grosu, MD**[10], **Roger Stupp, MD**[11], **Walter Curran Jr., MD**[12], **Aline P. Becker, MD, PhD**[1], **Andrea L. Salavaggione, MD**[1], **Jill S. Barnholtz-Sloan, PhD**[13], **Kenneth Aldape, MD**[14], **Deborah T. Blumenthal, MD**[15], **Paul D. Brown, MD**[14], **Jon Glass, MD**[16], **Luis Souhami, MD, FASTRO**[17], **R. Jeffrey Lee, MD**[18], **David Brachman, MD**[19], **John Flickinger, MD**[20], **Minhee Won, MA**[2], and **Arnab Chakravarti, MD**[1]

[1]The Ohio State University Comprehensive Cancer Center-Arthur G. James Cancer Hospital Columbus, OH [2]NRG Oncology Statistics and Data Management Center, Philadelphia, PA [3]The Ohio State University Center for Biostatistics, Columbus, OH [4]National Institutes of Health, Bethesda, MD [5]University of Maryland Medical Systems, Baltimore, MD (during trial) - Miami Cancer Institute, Coral Gables, FL (current) [6]University of Calgary, Calgary, AB, Canada [7]Moffitt Cancer Center, Tampa, FL [8]University of Alabama, Birmingham, AL [9]Utrecht Cancer Center, Utrecht, Netherlands [10]University of Freiburg, Freiburg, Germany [11]University Hospital Zurich, Zürich, Switzerland [12]Emory University, Atlanta, GA [13]Case Comprehensive Cancer Center, Case

Corresponding Author: Arnab Chakravarti, The Ohio State University Comprehensive Cancer Center and Richard L. Solove Research Institute, Arthur G. James Cancer Hospital, The Ohio State University Medical School, 460 W. 10th Avenue, Room D252F, Columbus, Ohio 43210, Tel# 614-293-0672, Fax# 614- 293-1943, arnab.chakravarti@osumc.edu.

Author Contributions:

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

Study concept/design: Chakravarti

Acquisition, analysis, or interpretation of data: Bell, Pugh, McElroy, Gilbert, Mehta, Klimowicz, Magliocco, Bredel, Robe, Grosu, Stupp, Curran, Becker, Salavaggione, Barnholtz-Sloan, Aldape, Blumenthal, Brown, Glass, Souhami, Lee, Brachman, Flickinger, Won, Chakravarti

Drafting of the manuscript: Bell, Pugh, Chakravarti

Critical revision of the manuscript for important intellectual content: Bell, Pugh, McElroy, Gilbert, Mehta, Klimowicz, Magliocco, Bredel, Robe, Grosu, Stupp, Curran, Becker, Salavaggione, Barnholtz-Sloan, Aldape, Blumenthal, Brown, Glass, Souhami, Lee, Brachman, Flickinger, Won, Chakravarti

Statistical analysis: Pugh, McElroy, Barnholtz-Sloan, Won

Obtained funding: NRG Oncology RTOG, Chakravarti

Administrative, technical, or material support: Bell, Pugh, McElroy, Gilbert, Mehta, Klimowicz, Magliocco, Bredel, Robe, Grosu, Stupp, Curran, Becker, Salavaggione, Barnholtz-Sloan, Aldape, Blumenthal, Brown, Glass, Souhami, Lee, Brachman, Flickinger, Won, Chakravarti

Study supervision: Bell, Chakravarti

Conflicts of Interest:

Dr Mehta reports consulting honoraria from Cavion and Novocure; research funding from Cellectar and Novocure; DSMB for Monteris, and previously served on the Board of Directors of Pharmacyclics (with options).

Trial Registration: NCT00304031 (RTOG 0525)

Additional Contributions:

The authors thank S. Jaharul Haque for assistance in preparation of the manuscript.

Western Reserve University School of Medicine, Cleveland, Ohio [14]MD Anderson Cancer Center, Houston, Texas [15]Tel-Aviv Sourasky Medical Center, Tel Aviv, Israel [16]Thomas Jefferson University Hospital, Philadelphia, PA [17]McGill University Health Centre, Montreal, Québec, Canada [18]Intermountain Medical Center, Murray, UT [19]Arizona Oncology Services Foundation, Tucson, AZ [20]UPMC-Shadyside Hospital, Pittsburgh, PA

Abstract

Importance—The need for a more refined, molecularly-based classification model for glioblastoma (GBM) in the temozolomide era.

Objective—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 semi-quantitative immunohistochemical validation. Prognostic significance of each protein was examined by single-marker and multi-marker Cox-regression analyses. In order to reclassify the prognostic risk groups, significant protein biomarkers upon single-marker analysis were incorporated into a RPA model consisting of the same clinical variables (age, KPS, 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 dataset (n=176).

Main Outcomes and Measures—Overall survival (OS)

Results—MGMT (HR=1.81, 95% CI(1.37, 2.39), p<0.001), survivin (HR=1.36, 95% CI(1.04, 1.76), p=0.02), c-Met (HR=1.53, 95% CI(1.06,2.23), p=0.02), pmTOR (HR=0.76, 95% CI(0.60,0.97), p=0.03), and Ki-67 (HR=1.40, 95% CI(1.10, 1.78), p=0.007), were found to be significant upon single-marker multivariate analysis of OS. To refine the existing RPA, significant protein biomarkers together with clinical variables (age, performance status, extent of resection, and neurological function) were incorporated into a new model. Higher MGMT protein was significantly associated with decreased *MGMT* promoter methylation and vice-versa. Further, MGMT protein expression had greater prognostic value for OS compared to *MGMT* promoter methylation. The refined NRG-GBM-RPA consisting of MGMT protein, c-Met protein, and age revealed greater separation of OS prognostic classes compared to 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 dataset (N=176).

Conclusions and Relevance—The new NRG-GBM-RPA model significantly enhances outcome stratification over both the current RTOG RPA model and *MGMT* promoter methylation, respectively, for GBM patients treated with radiation and temozolomide and was biologically validated in an independent dataset. The revised RPA has the potential to significantly contribute to improving the accurate assessment of prognostic groups in GBM patients treated with radiation and temozolomide and also influence clinical decision making.

Introduction

Glioblastoma (GBM), the most aggressive primary brain tumor, has a current five-year survival rate of 5% in the United States.[1] However, a small subset of patients do experience longer survival, suggesting underlying heterogeneity, and 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 non-parametric 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 six prognostic classes (I – VI) with distinct survival outcomes.[2] Subsequently, a follow-up study involving only GBM patients revised the original RPA model into three 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 NRG Oncology's (RTOG) 0525 trial, a phase III trial that compared standard adjuvant temozolomide versus a dose-dense (dd) schedule in newly diagnosed GBM.[5] No statistical difference in survival outcomes was found between the two arms of NRG Oncology RTOG 0525.

Analyzing the relative expression levels, subcellular distributions, and post-translational 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 and chemotherapy.[11] In the current 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 if 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 ELISA assays.[12] Further, to enhance the clinical applicability, these findings were confirmed in an independent dataset using traditional semi-quantitative immunohistochemistry (IHC).

Methods

Quantitative Fluorescence Immunohistochemistry

A total of 452 patients from NRG Oncology RTOG 0525 had available specimens and were used for preparation of four tissue microarrays (TMAs). These TMAs were analyzed for 12

high priority protein biomarkers (based on literature[6–10]): EGFR, NFKBp65, pNFKBp65, pAKT, pERK, pmTOR, IGF1R, MGMT, 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 utilized in this study provided informed consent based on an institutional review board (IRB)-approved protocol. See the Supplemental Appendix for additional methods.

Validation Studies

Four TMAs comprised of GBM patients (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 method[13] by two independent pathologists. Institutional samples were used under an IRB-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] Non-nested models were compared with Aikaike's Information Criteria (AIC)[16] and were limited to patients with non-missing covariates in the models being compared. 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. OS 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 six 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 dataset. In order to determine the best cut-points for markers with continuous values significantly associated with survival for inclusion in the RPA model, the technique of utilizing receiver operating characteristic (ROC) curves was applied.[19] Because the area under the ROC curve for all markers was ≤ 0.65 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 was used for all analyses and the 'rpart' package in R Statistical Software was used for the RPA Class determination .[20] Explanation of variance[21], 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 [22–25]. 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 indicates better prediction.

Results

Single- and Multi-marker Modeling

All 22 proteins were quantified (eTable 1) and correlated with OS in randomized patients. No significant differences were detected between OS for patients with and without tissues (eTable 2). Single-marker Cox-regression modeling was performed and identified six significant ($p<0.05$) proteins (pAKT, MGMT, Ki-67, pmTOR, survivin, and c-Met) that associated with OS when represented as a continuous variable (eTable 3). Pre-treatment characteristics of NRG Oncology RTOG 0525 randomized patients with data from all significant proteins with identified cut-off points are shown in Table 1. When investigated as discrete categorical variables formed by division at specific quantiles, five of the six proteins yielded cut-off points that were significantly associated with OS: MGMT, Ki-67, pmTOR, survivin, and c-Met (Table 2).

In Figure 1 and eFigure 1, high MGMT protein within the tumor (MGMT tumor) measured by AQUA and split by the median was shown to significantly correlate with decreased OS (Fig. 1A; Hazard Ratio [HR] =1.73, 95% Confidence Interval [CI] (1.32, 2.27), $p<0.001$). High c-Met protein within the cytoplasmic (c-Met cyto) when split by the top quartile significantly correlated with decreased OS (Fig. 1B; HR=1.56, 95% CI (1.08, 2.24), p=0.02). A high cytoplasmic/nuclear ratio of survivin protein when split by the median trended toward significance with decreased OS (eFig. 1A; HR=1.29, 95% CI (1.00, 1.67), p=0.05). High Ki-67 protein within the nucleus (Ki-67 nuc) when split by the median also significantly associated with decreased OS (eFig. 1B; HR=1.34, 95% CI (1.05, 1.70), p=0.02). Conversely, a high nuclear/cytoplasmic ratio of pmTOR protein when split by the median also trended toward significance with increased OS (eFig. 1C; HR=0.81, 95% CI (0.63, 1.03), p=0.08).

Due to involvement of MGMT in response to temozolomide treatment,[26] MGMT protein levels were evaluated in relation to *MGMT* promoter methylation. As shown in eTables 4.1–4.2 and eFigure 2, MGMT tumor and MGMT nuclear protein expression are significantly different by *MGMT* promoter methylated vs unmethylated (1425.1 vs. 1828.0 for MGMT tumor and 2195.1 vs. 2917.1 for MGMT nuclear mask, $p<0.001$). However, MGMT protein expression within the tumor (HR=1.84, 95% CI (1.38, 2.43); $p<0.001$) demonstrated a stronger prognostic effect compared to *MGMT* promoter methylation (HR=1.77, 95% CI (1.28, 2.44); $p<0.001$) on OS upon single marker modeling based on AIC, and thus protein expression was the only MGMT marker incorporated into the revised RPA.

Multi-marker Cox-regression modeling was performed on the protein biomarkers that were statistically significant ($p<0.05$) 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, five proteins, pmTOR, MGMT, Ki-67, c-Met, and survivin, were tested in a multi-marker model, with stepwise selection. As shown in Table 2, MGMT tumor ((≥median vs <median) HR=1.91; 95% CI (1.27, 2.88); p= 0.002,), Ki-67 nuclear ((≥median vs <median) HR=1.50; 95% CI (1.01, 2.22); p= 0.04), and c-Met cytoplasmic ((≥top quartile vs <top quartile) HR=1.65; 95% CI (1.10, 2.48); p=0.02) were all found to be significant.

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

Protein biomarkers that were significant upon single-marker modeling were incorporated into a RPA model consisting of the same variables of the current RTOG RPA classification to determine if protein biomarkers can help stratify patients into prognostic groups. The 166 patients used for RPA modeling, which required patients to be randomized and have non-missing data for all of the biomarkers considered for inclusion, was stratified by the three current RPA[2] classes (based on age, KPS, resection status, neurofunction status) relative to OS (Figure 2A; eTable 5). The newly developed NRG-GBM-RPA classes are shown in Figures 2B–D: Class I (MGMT tumor < median or (MGMT tumor ≥ median & age < 50)), Class II (MGMT tumor ≥ median & age ≥ 50 & c-Met cyto < top quartile), and Class III (MGMT tumor ≥ median & age ≥ 50 & c-Met cyto ≥ top quartile).

The median OS times for these three classes are 21.9 (95% CI: 16.4, 29.9), 16.6 (95% CI: 13.3, 20.0), and 9.4 months (95% CI (5.6, 11.6)), respectively, demonstrating that these classes are significantly different (I vs. II: HR=1.83, 95% CI (1.21, 2.76), p=0.004, I vs. III: HR = 5.19, 95% CI (3.07, 8.79), p<0.001). Survival estimates and confidence intervals are shown in eTable 6. Explanation of variance, concordance index, and NRI were computed to compare the two 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 to the currently used clinical RPA for OS. Although the concordance index and NRI were in favor of the model with NRG-GBM-RPA compared to the clinical RPA, there was no significant difference between the two models with respect to concordance 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=0.93) or NRI (7.89%, 95% CI: -0.11, 0.49). 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=0.05; I vs III; HR=4.56 95% CI (2.55, 8.17); p<0.0001) demonstrated a stronger prognostic effect compared to *MGMT* promoter methylation (HR=1.58, 95% CI (1.01, 2.47); p=0.05) on OS upon single marker modeling based on AIC (eTable 7.1). The NRG-GBM-RPA also explains a higher percent of the variance (11.11 vs 1.68) as compared to *MGMT* methylation (eTable 7.2). Furthermore, Class I (which represents the best prognostic group) in the NRG-GBM-RPA is comprised of both methylated and unmethylated *MGMT* patients (eTable 7.3).

To validate the biological significance of the NRG-GBM-RPA, 176 patients treated at the University of Utrecht were examined on TMAs utilizing traditional IHC staining. Pre-treatment characteristics of the validation cohort (49% received radiation and temozolomide) are shown in eTable 8. As shown in Figure 3, the NRG-GBM-RPA was confirmed to be a statistically significant prognostic classifier using traditional IHC for all patients (Fig. 3A; n=176; I vs. II: HR=1.46, 95% CI (1.01, 2.11), p=0.04, I vs. III: HR = 1.88, 95% CI (1.20, 2.96), p=0.006) and for patients who received radiation and temozolomide (Fig. 3B; n=87; I vs. II: HR=1.91, 95% CI (1.10, 3.32), p=0.02, I vs. III: HR = 3.68, 95% CI (1.84, 7.35), p<0.001). Classification of patients who received radiation and temozolomide based on the current RPA is shown in Fig. 3C. Concordance between Pathologists' analyses for the Allred score (0–8) were moderate and varied from c-Met (weighted kappa = 0.4) to MGMT

(weighted kappa = 0.6)[27–30]. Effects of IDH were then analyzed in the validation cohort as IDH 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 trended or remained statistically significant in all treatments (n=167; I vs. II: HR=1.37, 95% CI (0.94, 1.97), p=0.10, I vs. III: HR = 1.87, 95% CI (1.18, 2.98), p=0.007) and in patients treated with radiation and temozolomide (n=82; I vs. II: HR=1.65, 95% CI (0.95, 2.87), p=0.07, I vs. III: HR = 3.24, 95% CI (1.62, 6.48), p<0.001) as shown in eFigure 3.

Discussion

The current RPA classification system for GBM was created using trials conducted in the pre-temozolomide 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 GBM patients. The findings of this study have important implications for GBM patients as a new RPA was identified based upon underlying molecular markers, some putatively involved in GBM pathogenesis. Importantly, these newly-identified prognostic risk groups may help guide decision-making as well as yield insights into possible underlying resistance mechanism(s) to radiation and temozolomide treatment. Most notably, the NRG-GBM-RPA classification (Figure 2) dramatically 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 upon single marker modeling (pAKT, pmTOR, MGMT, Ki-67, survivin, and c-Met) with a p-value < 0.05 validated previous findings, with regards to their respective prognostic values. Prognostic protein biomarkers identified to be significant (p< 0.05) after multi-marker modeling for GBM were Ki-67, c-Met, and MGMT. Each of these protein biomarkers has been previously associated with worse outcome in GBM,[31–33] 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 .[31] Our study further validates these findings in an independent, larger cohort of 196 patients all treated with radiation and temozolomide on 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 be good candidates for this targeted therapy as evidenced by *in vitro* and *in vivo* models[34,35] as well as a single case report.[36] 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 GBM patients; 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.[33,37] Therefore, we sought to determine 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 with OS than *MGMT* promoter methylation even after multi-marker modeling.

Further, MGMT protein expression in tumor was found to be significantly associated with *MGMT* promoter methylation. This result confirms a previous publication where we demonstrated that decreased MGMT protein expression was correlated with increased sensitivity to radiation and temozolomide *in vitro*.[38] Intriguingly, MGMT protein appeared to have greater prognostic value versus *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).

Of importance, both c-Met and MGMT demonstrated statistical significance upon multi-marker 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 semi-quantitative 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. The prognostic significance of MGMT protein expression and the NRG-GBM-RPA identified warrants further studies on its clinical applicability using large sample sizes as it is possible that the small sample size limited the ability to find a statistically significant difference compared to the current RPA using NRI and concordance index. Further validation will also determine if semi-quantitative or quantitative methods will be required to overcome reproducibility and subjectivity of traditional IHC.[39,40] However, our approach for traditional IHC differed from previous studies as we used a sophisticated scoring method[13] to assess proportion and intensity of protein expression (eFigure 4). Further, protein analysis by traditional IHC may potentially be more accessible and cost-effective to community-based practices world-wide. The validation study, which was comprised of 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 to the heterogeneous treated group and the ability of the refined RPA to separate out the poor and intermediate prognostic classes. 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, our current study has the potential to significantly contribute to improving the accurate assessment of prognostic groups in GBM patients treated with radiation and temozolomide and also influence clinical decision making.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by grants U10CA21661, U10CA180868, U10CA180822, U10 CA37422, UG1CA189867, RO1CA108633 (To AC), 1RC2CA148190 (To AC) U10CA180850-01 (To AC), R01CA169368 (To AC) from the National Cancer Institute (NCI), Brain Tumor Funders Collaborative Grant (To AC), Ohio State University Comprehensive Cancer Center Award (To AC) and Merck & Co.

Role of the Funder/Sponsor:

The funding organizations 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.

References

1. Ostrom QT, Gittleman H, Liao P, et al. CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2007–2011. Neuro Oncol. Oct; 2014 16(Suppl 4):iv1–iv63. [PubMed: 25304271]
2. Curran WJ Jr, Scott CB, Horton J, et al. Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. J Natl Cancer Inst. May 5; 1993 85(9): 704–710. [PubMed: 8478956]
3. Li J, Wang M, Won M, et al. et al. Validation and simplification of the Radiation Therapy Oncology Group recursive partitioning analysis classification for glioblastoma. Int J Radiat Oncol Biol Phys. Nov 1; 2011 81(3):623–630. [PubMed: 20888136]
4. Mirimanoff RO, Gorlia T, Mason W, et al. Radiotherapy and temozolomide for newly diagnosed glioblastoma: recursive partitioning analysis of the EORTC 26981/22981-NCIC CE3 phase III randomized trial. J Clin Oncol. Jun 1; 2006 24(16):2563–2569. [PubMed: 16735709]
5. Gilbert MR, Wang M, Aldape KD, et al. Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. J Clin Oncol. Nov 10; 2013 31(32):4085–4091. [PubMed: 24101040]
6. Brennan CW, Verhaak RG, McKenna A, et al. The somatic genomic landscape of glioblastoma. Cell. Oct 10; 2013 155(2):462–477. [PubMed: 24120142]
7. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. Oct 23; 2008 455(7216):1061–1068. [PubMed: 18772890]
8. Chakravarti A, Zhai G, Suzuki Y, et al. The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. J Clin Oncol. May 15; 2004 22(10):1926–1933. [PubMed: 15143086]
9. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res. Jan 1; 2002 62(1):200–207. [PubMed: 11782378]
10. Chakravarti A, Chakladar A, Delaney MA, Latham DE, Loeffler JS. The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. Cancer Res. Aug 1; 2002 62(15):4307–4315. [PubMed: 12154034]
11. Sunayama J, Matsuda K, Sato A, et al. Crosstalk between the I3K/mTOR and MEK/ERK pathways involved in the maintenance of self-renewal and tumorigenicity of glioblastoma stem-like cells. Stem Cells. Nov; 2010 28(11):1930–1939. [PubMed: 20857497]
12. Moeder, C., Giltnane, J., Moulis, SP., Rimm, D. Quantitative, Fluorescence-Based In-Situ Assessment of Protein Expression. In: Tainsky, MA., editor. Tumor Biomarker Discovery. Vol. 520. Humana Press; 2009. p. 163-175.
13. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. Feb; 1998 11(2):155–168. [PubMed: 9504686]
14. Cox DR. Regression Models and Life Tables. Journal of the Royal Statistical Society. 1972; (34): 187–229. Series B.

15. Lacroix M, Abi-Said D, Fourney DR, et al. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. J Neurosurg. Aug; 2001 95(2):190–198.
16. Akaike H. A new look at the statistical model identification. IEEE Transactions on Automatic Control. 1974; 19(6):716–723.
17. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Amer Stat Assoc. 1958; 53:457–481.
18. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep. Mar; 1966 50(3):163–170. [PubMed: 5910392]
19. Obuchowski NA. Receiver operating characteristic curves and their use in radiology. Radiology. Oct; 2003 229(1):3–8. [PubMed: 14519861]
20. Therneau, T., Atkinson, B., Ripley, B. rpart: Recursive Partitioning and Regression Trees. R package version 4.1–8. 2004. http://CRAN.R-project.org/package=rpart
21. Schemper M, Henderson R. Predictive accuracy and explained variation in Cox regression. Biometrics. Mar; 2000 56(1):249–255. [PubMed: 10783803]
22. Harrell FE Jr, Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med. Feb 28; 1996 15(4):361–387. [PubMed: 8668867]
23. Pencina MJ, D'Agostino RB. Overall C as a measure of discrimination in survival analysis: model specific population value and confidence interval estimation. Stat Med. Jul 15; 2004 23(13):2109–2123. [PubMed: 15211606]
24. Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. Stat Med. Jan 15; 2011 30(1):11–21. [PubMed: 21204120]
25. Haibe-Kains B, Desmedt C, Sotiriou C, Bontempi G. A comparative study of survival models for breast cancer prognostication based on microarray data: does a single gene beat them all? Bioinformatics. Oct 1; 2008 24(19):2200–2208. [PubMed: 18635567]
26. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. Mar 10; 2005 352(10):997–1003. [PubMed: 15758010]
27. Cohen J. A coefficient of agreement for nominal scales. Educational and Psychological Measurement. 1960; 20:37–46.
28. Cohen J. Weighted kappa: Nominal scale agreement with provision for scaled disagreement or partial credit. Psychological Bulletin. 1968; 70:213–220. [PubMed: 19673146]
29. Fleiss JL, Cohen J, Everitt BS. Large sample standard errors of kappa and weighted kappa. Psychological Bulletin. 1969; 72:323–327.
30. Viera AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. Fam Med. May; 2005 37(5):360–363. [PubMed: 15883903]
31. Kong D-S, Song S-Y, Kim D-H, et al. Prognostic significance of c-Met expression in glioblastomas. Cancer. 2009; 115(1):140–148. [PubMed: 18973197]
32. Torp SH. Diagnostic and prognostic role of Ki67 immunostaining in human astrocytomas using four different antibodies. Clin Neuropathol. Nov-Dec;2002 21(6):252–257. [PubMed: 12489673]
33. Cao VT, Jung T-Y, Jung S, et al. The Correlation and Prognostic Significance of Mgmt Promoter Methylation and Mgmt Protein in Glioblastomas. Neurosurgery. 2009; 65(5):866–875. DOI: 10.1227/1201.NEU.0000357325.0000390347.A0000357321 [PubMed: 19834398]
34. Navis AC, Bourgonje A, Wesseling P, et al. Effects of dual targeting of tumor cells and stroma in human glioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2. PLoS One. 2013; 8(3):e58262. [PubMed: 23484006]
35. Rath P, Lal B, Ajala O, et al. In Vivo c-Met Pathway Inhibition Depletes Human Glioma Xenografts of Tumor-Propagating Stem-Like Cells. Transl Oncol. Apr; 2013 6(2):104–111. [PubMed: 23556031]
36. Chi AS, Batchelor TT, Kwak EL, et al. Rapid radiographic and clinical improvement after treatment of a MET-amplified recurrent glioblastoma with a mesenchymal-epithelial transition inhibitor. J Clin Oncol. Jan 20; 2012 30(3):e30–33. [PubMed: 22162573]

37. Melguizo C, Prados J, Gonzalez B, et al. MGMT promoter methylation status and MGMT and CD133 immunohistochemical expression as prognostic markers in glioblastoma patients treated with temozolomide plus radiotherapy. Journal of Translational Medicine. 2012; 10(1):250. [PubMed: 23245659]

38. Chakravarti A, Erkkinen MG, Nestler U, et al. Temozolomide-mediated radiation enhancement in glioblastoma: a report on underlying mechanisms. Clin Cancer Res. Aug 1; 2006 12(15):4738–4746. [PubMed: 16899625]

39. Jaeckle KA, Eyre HJ, Townsend JJ, et al. Correlation of tumor O6 methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bis-chloroethylnitrosourea: a Southwest Oncology Group study. J Clin Oncol. Oct; 1998 16(10):3310–3315. [PubMed: 9779706]

40. Preusser M, Charles Janzer R, Felsberg J, et al. Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker. Brain Pathol. Oct; 2008 18(4):520–532. [PubMed: 18400046]

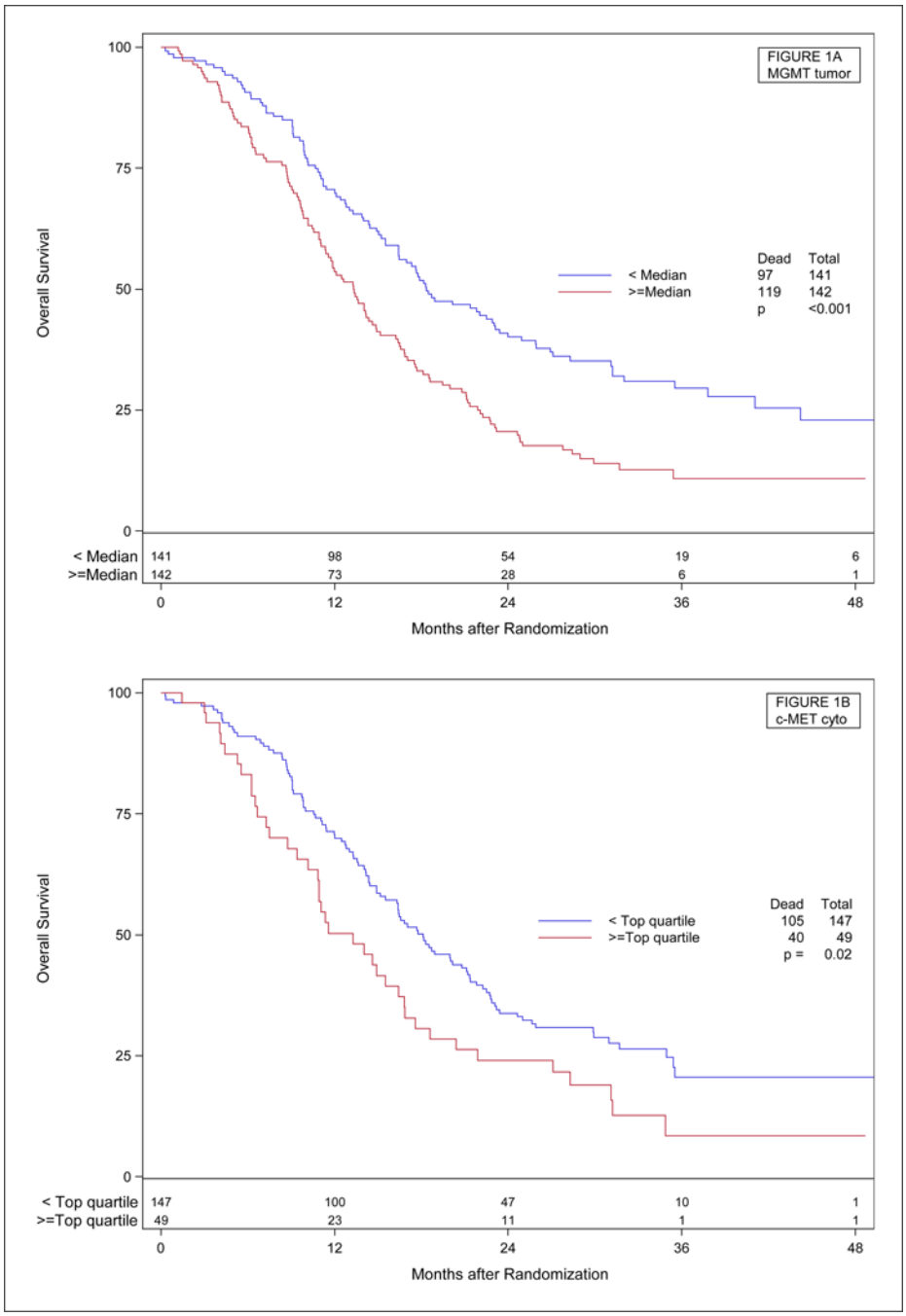
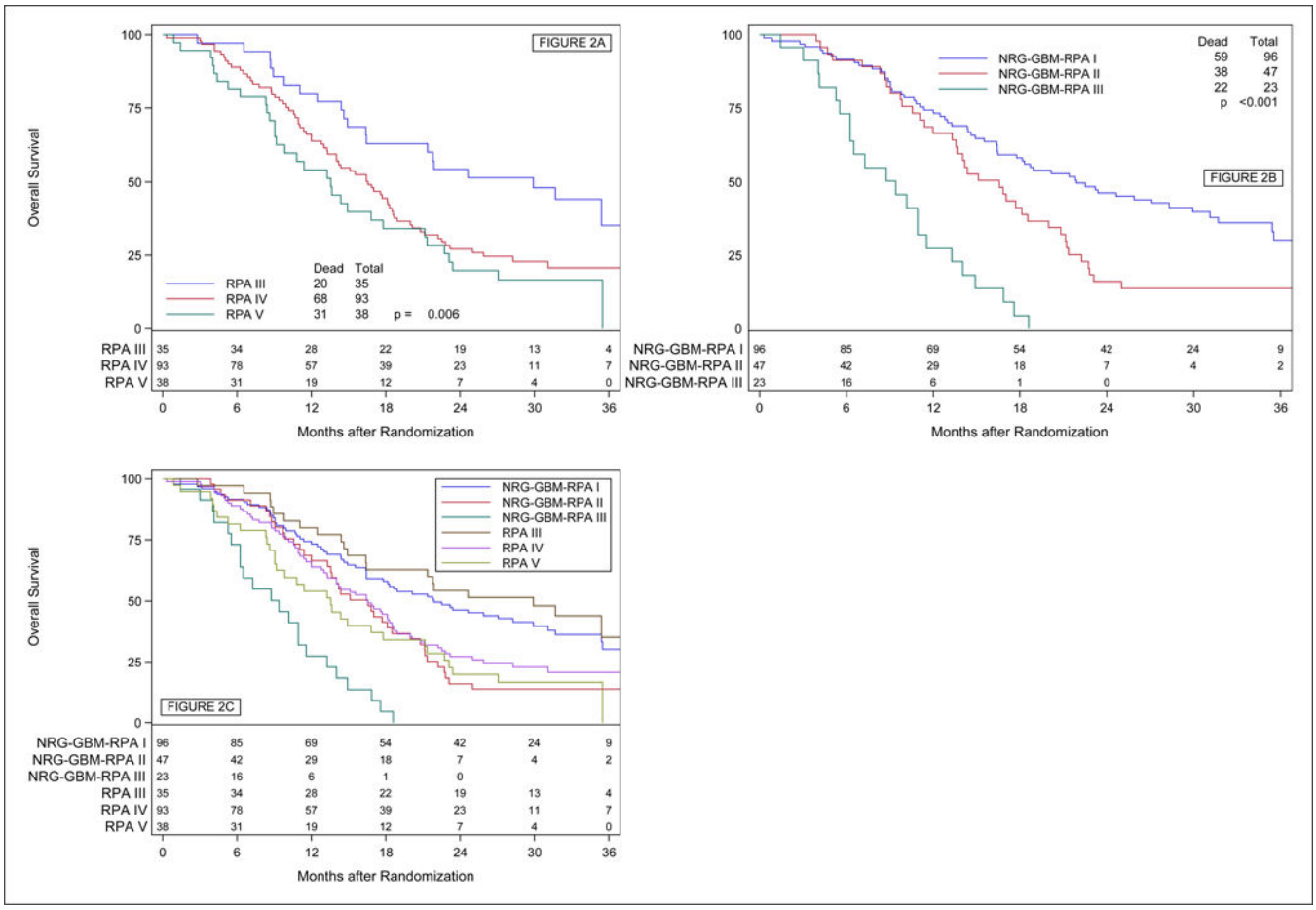


Figure 1. MGMT and c-Met correlate with OS in randomized NRG Oncology RTOG 0525 study participants

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



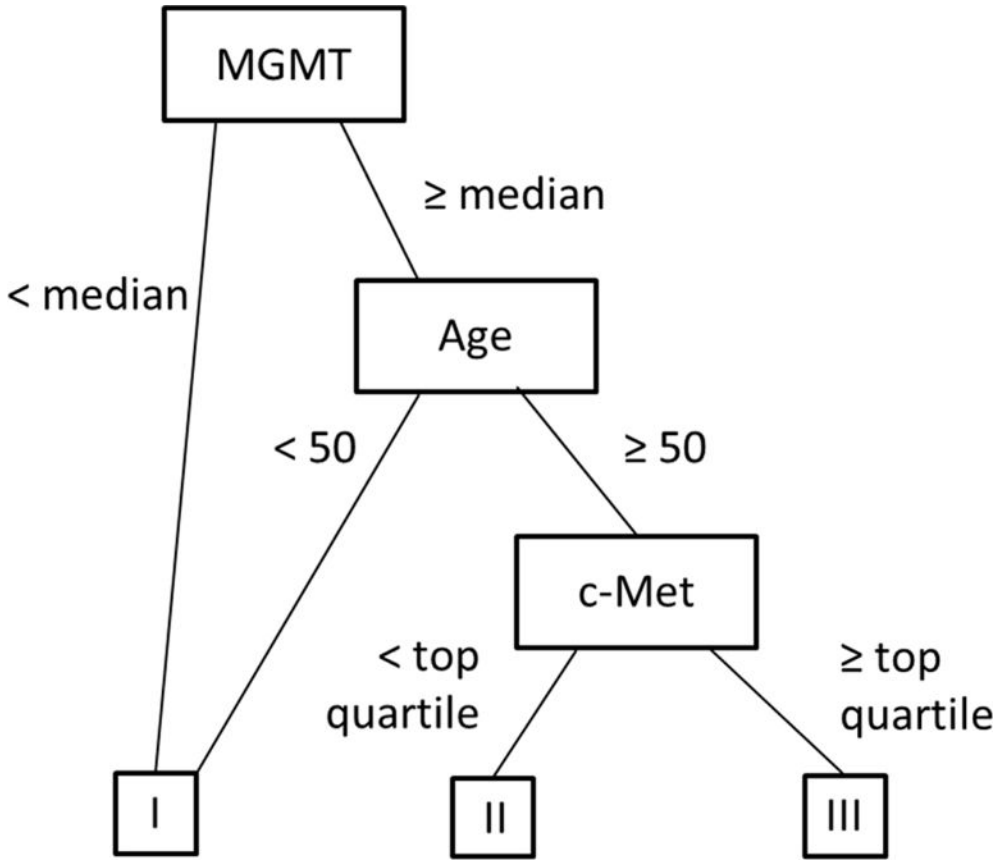


Figure 2. Protein biomarker data strengthens current GBM RPA classification

Current and New RPA classification of NRG Oncology RTOG 0525 study participants. The cohort of 166 randomized patients used is shown stratified by the three current RPA classes relative to OS, respectively for the current RPA (A) the new NRG-GBM-RPA (B) and both RPA models overlayed (C). A decision tree for the NRG-GBM-RPA classification (D). Current RPA Class III (age <50 and KPS 90–100) vs. Class IV (age <50 and KPS <90 OR age ≥ 50 and partial or total resection with no worse than minor neurofunction impairment) vs Class V (age ≥ 50 and partial or total resection with worse than minor neurofunction impairment, OR age ≥ 50 and biopsy only, OR age ≥ 50, KPS ≥ 60, and normal mental status). NRG-GBM-RPA Class I: MGMT tumor < median or (MGMT tumor ≥median & age <50) vs Class II: MGMT tumor ≥ median & age ≥ 50 & c-Met cyto < top quartile vs. Class III: MGMT tumor ≥ median & age ≥ 50 & c-Met cyto ≥ top quartile.

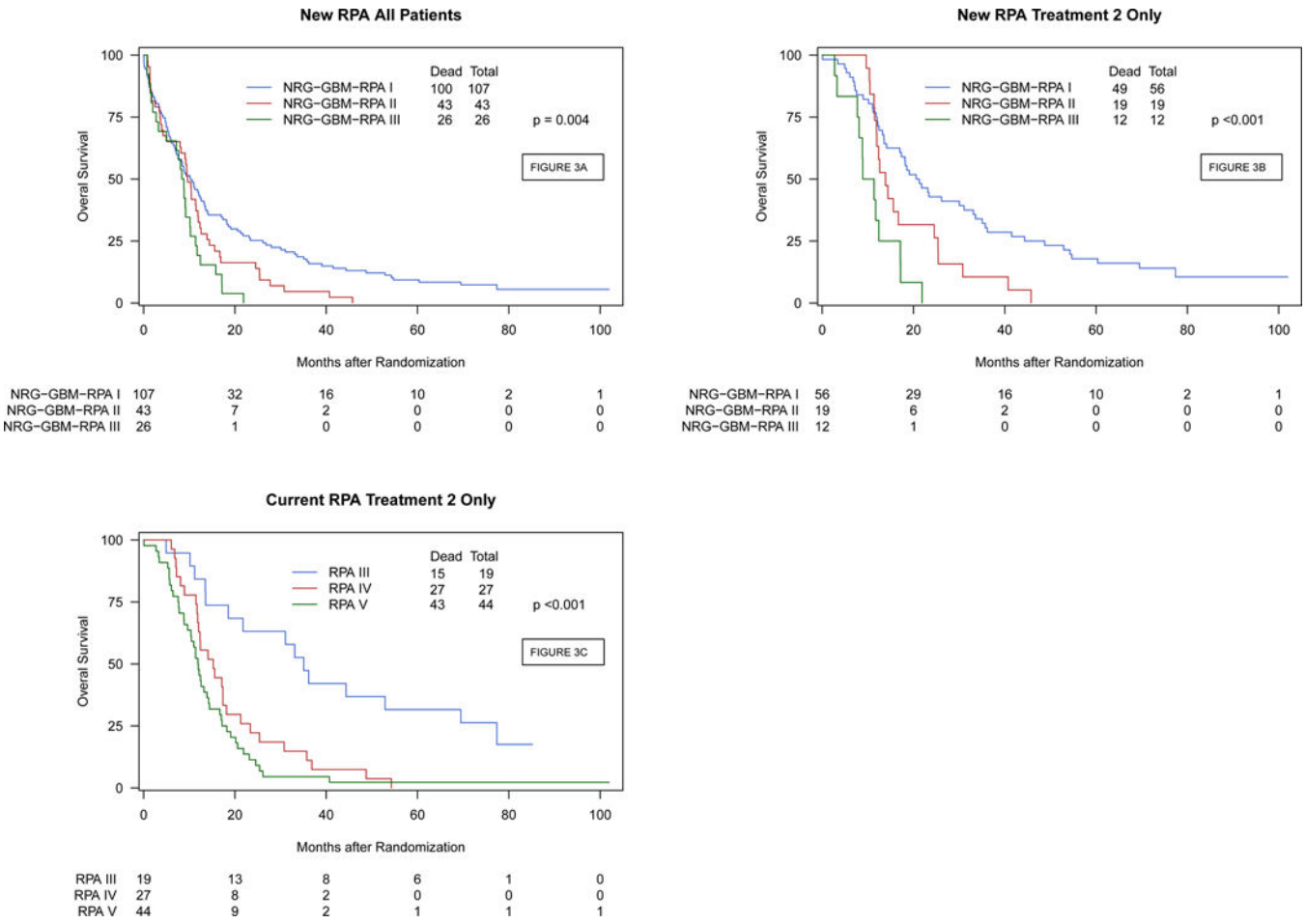


Figure 3. Biological validation of the NRG-GBM-RPA classification in an independent GBM cohort

A) NRG-GBM-RPA classification correlated to OS in all GBM patients with heterogeneous treatments, B) NRG-GBM-RPA classification correlated to OS in GBM patients treated with radiation and temozolomide, and C) Current RPA classification correlated to OS in GBM patients treated with radiation and temozolomide.

Table 1

Pre-treatment Characteristics of the 166 patients from NRG Oncology RTOG 0525 included in the biomarker study with all 5 protein marker data included for the NRG-GBM- RPA.

	ARM 1 (Standard TMZ) (n=82)	ARM 2 (Dose-dense TMZ) (n=84)	Chi-square p-value
Age (years)			0.80
<50	21 (25.6%)	23 (27.4%)	
≥ 50	61 (74.4%)	61 (72.6%)	
Gender			0.77
Male	47 (57.3%)	50 (59.5%)	
Female	35 (42.7%)	34 (40.5%)	
KPS			0.49
60–80	27 (32.9%)	32 (38.1%)	
90–100	55 (67.1%)	52 (61.9%)	
Surgery			0.99
Biopsy	3 (3.7%)	3 (3.6%)	
Partial Resection	28 (34.1%)	28 (33.3%)	
Total Resection	51 (62.2%)	53 (63.1%)	
Neurologic Function	No vs. Minor vs. Moderate/Severe		0.29
No symptoms	25 (30.5%)	24 (28.6%)	
Minor symptoms	33 (40.2%)	43 (51.2%)	
Moderate symptoms	24 (29.3%)	15 (17.9%)	
Severe symptoms	0 (0.0%)	2 (2.4%)	
RPA class			0.40
III	18 (22.0%)	17 (20.2%)	
IV	42 (51.2%)	51 (60.7%)	
V	22 (26.8%)	16 (19.0%)	

RPA = Recursive Partitioning Analysis.

Table 2

Cox models of protein biomarkers by cut-off points in NRG Oncology RTOG 0525 specimens.

(Bolded value has unfavorable outcome)		**p-value**	**Hazard Ratio (95%CI)**
Single Marker	Maximum pmTOR Nuclear/Cytoplasm Ratio (≥median vs <**median**)	0.03	0.76 (0.60, 0.97)
Models	MGMT Tumor Mask (≥**median** vs <median)	<0.001	1.81 (1.37, 2.39)
	MGMT Tumor Mask (≥**top tertile** vs <top tertile)	0.003	1.57 (1.17, 2.10)
	MGMT Tumor Mask (≥**top quartile** vs <top quartile)	0.005	1.55 (1.14, 2.11)
	Maximum Survivin Cytoplasm/Nuclear Ratio (≥**median** vs <median)	0.02	1.36 (1.04, 1.76)
	Average Ki-67 in Nuclear Mask (≥**median** vs <median)	0.007	1.40 (1.10, 1.78)
	Average Ki-67 in Nuclear Mask (≥**top tertile** vs <top tertile)	0.008	1.40 (1.09, 1.79)
	Average Ki-67 in Nuclear Mask (≥**top quartile** vs <top quartile)	0.05	1.32 (1.01, 1.72)
	Minimum c-Met Cytoplasm Mask (≥**top tertile** vs <top tertile)	0.03	1.48 (1.04, 2.09)
	Minimum c-Met Cytoplasm Mask (≥**top quartile** vs <top quartile)	0.02	1.53 (1.06, 2.23)
Multi-Marker	Treatment Arms (**Arm 1** vs Arm 2)	0.09	0.72 (0.50, 1.05)
Model	Age (Continuous)	<0.001	1.03 (1.01, 1.05)
	KPS (**60–80** vs 90–100)	0.14	1.38 (0.90, 2.11)
	Surgery (biopsy/partial resection vs total resection)	0.99	1.00 (0.67, 1.51)
	MGMT Tumor Mask (≥**median** vs <median)	0.002	1.91 (1.27, 2.88)
	Average Ki-67 in Nuclear Mask (≥**median** vs <median)	0.04	1.50 (1.01, 2.22)
	Minimum c-Met Cytoplasm Mask (≥**top quartile** vs <top quartile)	0.02	1.65 (1.10, 2.48)

For single marker models: All models are adjusted by RX, age, KPS, and surgery. Only markers with p-value less than 0.05 are listed in the above table.

For multi-marker model: Model derived from stepwise selection by forcing RX, age, KPS, and surgery included in the model. pmTOR - maximum nuclear/cytoplasm ratio, survivin - cytoplasm/nuclear mask ratio were dropped out during stepwise.