Improving Individualized Rhabdomyosarcoma Prognosis Predictions Using Somatic Molecular Biomarkers

Mark Zobeck, MD, MPH[1,2]; Javed Khan, MD[3]; Rajkumar Venkatramani, MD[1,2]; M. Fatih Okcu, MD, MPH[1,2]; Michael E. Scheurer, PhD, MPH[1,2]; and Philip J. Lupo, PhD, MPH[1,2]

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ABSTRACT

PURPOSE Molecular markers increasingly influence risk-stratified treatment selection for pediatric rhabdomyosarcoma (RMS). This study aims to integrate molecular and clinical data to produce individualized prognosis predictions that can further improve treatment selection.

METHODS Clinical variables and somatic mutation data for 20 genes from 641 patients with RMS in the United Kingdom and the United States were used to develop three Cox proportional hazard models for predicting event-free survival (EFS). The Baseline Clinical (BC) model included treatment location, age, fusion status, and risk group. The Gene Enhanced 2 (GE2) model added *TP53* and *MYOD1* mutations to the BC predictors. The Gene Enhanced 6 (GE6) model further included *NF1*, *MET*, *CDKN2A*, and *MYCN* mutations, selected through least absolute shrinkage and selection operator regression. Model performance was assessed using likelihood ratio tests and optimism-adjusted, bootstrapped validation and calibration metrics.

RESULTS The GE6 model demonstrated superior predictive performance compared with the BC model ($P < .001$) and GE2 model ($P < .001$). The GE6 model achieved the highest discrimination with a time-dependent area under the receiver operating characteristic curve of 0.766. Mutations in *TP53*, *MYOD1*, *CDKN2A*, *MET*, and *MYCN* were associated with higher hazards, while NF1 mutation correlated with lower hazard. Individual prognosis predictions varied between models in ways that may suggest different treatments for the same patient. For example, the 5-year EFS for a 10-year-old patient with high-risk, fusion-negative, *NF1*-positive disease was 50.0% (95% CI, 39 to 64) from BC but 76% (64 to 90) from GE6.

CONCLUSION Incorporating molecular markers into RMS prognosis models improves prognosis predictions. Individualized prognosis predictions may suggest alternative treatment regimens compared with traditional risk-classification schemas. Improved clinical variables and external validation are required before implementing these models into clinical practice.

ACCOMPANYING CONTENT

Data Sharing Statement

Data Supplement

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INTRODUCTION

Rhabdomyosarcoma (RMS) is an aggressive soft tissue sarcoma with highly variable survival outcomes. RMS is the most common soft tissue sarcoma in children in the United States. Approximately 350 children and 150 adults develop RMS each year.[1] Successful treatment in most cases may require any combination of surgical resection of the primary tumor, radiation to the primary and metastatic sites, and multiagent chemotherapy that can last anywhere between 24 and 48 weeks. Prognostic clinicopathologic features determine the intensity of the treatment regimen. These features stratify cases into three primary risk groups in the United States with widely varying survival reported within each group. Low-risk (LR) RMS consistently demonstrates 5-year event-free survival (EFS) ≥90%, intermediate-risk (IR) RMS has a 5-year EFS between 50% and 85%, and high-risk (HR) RMS has a 5-year EFS between 5% and 45%.[2]

Molecular biomarkers have been shown to improve RMS prognostic stratification. *FOXO1* gene fusions with either *PAX3* [t(2;13)] or *PAX7* [t(1;13)] have significant prognostic implications and have been incorporated into standard risk-stratified treatment assignments in the United States.[3,4] More recently, tumors with mutations in *MYOD1* or *TP53*

CONTEXT

Key Objective

Can incorporating additional molecular biomarkers beyond *PAX::FOXO1* fusion status and *TP53* and *MYOD1* mutations improve individualized prognosis predictions for patients with pediatric rhabdomyosarcoma (RMS)? This study evaluates novel gene mutations to enhance risk stratification beyond current clinical practices.

Knowledge Generated

Mutations in *CDKN2A*, *MET*, and *MYCN* were associated with worse prognosis, while *NF1* mutations correlated with improved outcomes. Individual patient survival predictions changed when these new molecular markers were included in prediction models in ways that suggest different risk categories and treatment assignments compared with current classification methods.

Relevance

These new biomarkers can be used to improve risk stratification and treatment assignment for RMS and may serve as targets for precision therapies.

genes have been shown to have worse prognoses.[5] These have been incorporated into the current Children's Oncology Group (COG) LR clinical ARST2032 to increase the treatment intensity for patients with these mutations and to enrich a subset of very LR patients who are negative for the mutations to test the effect of treatment de-escalation.[6] These molecular markers have improved the accuracy of prognosis classification, thereby enabling progress in studying treatments.

Despite these successes, improvements in RMS risk stratification are still needed. The prevalence of mutations in either *MYOD1* or *TP53* among *PAX::FOXO1* fusion-negative (FN) RMS has been reported to be around 3% and 13%, respectively.[5] No other biomarkers have been clearly identified among FN RMS. Nor are there established biomarkers for *PAX::FOXO1* fusion-positive (FP) RMS. The most common alterations among FP RMS are amplifications of *MYCN* at 10% prevalence and *CDK4* at 13%. Studies suggest these lesions have negative prognostic significance, while other analyses have been equivocal.[5,7,8] Given the wide range of survival outcomes between and within risk strata, new biomarkers can enhance risk-adapted treatment selection by identifying patients who may safely receive dose-reduced therapy or may benefit from treatment intensification.

The aim of this exploratory study is to identify new predictive biomarkers that can improve RMS risk stratification. To accomplish this aim, we leveraged a large data set of over 600 pediatric patients for whom a panel of somatic mutation data were available to develop statistical models that combine clinical and genetic variables to predict 5-year EFS. By focusing on prediction, we quantified the amount of prognostic information for promising biomarkers and evaluated how prognoses change according to mutation status.

METHODS

Study Population and Data Collection

We conducted a retrospective analysis of pediatric RMS cases from the United Kingdom and the United States. Case data were obtained from a publicly available data set hosted by the National Institutes of Health OncoGenomics data portal.[5,9] Case-level clinical data for age at diagnosis, risk group, fusion status, and treatment location (United Kingdom or United States) were available. Several variables were modified to harmonize the data sets (see the supplement by Shern et al[5] for full details). A dedicated *FOXO1* fusion assay was unavailable for 40 of the 126 cases designated as alveolar histology after central pathology review. These cases were presumed to be positive for *FOXO1* fusions with either *PAX3* or *PAX7* (hereafter *PAX::FOXO1*). The risk group designation was modified to accommodate slight differences in risk-adapted treatment assignment between the treatment contexts in the United Kingdom and the United States.[10,11] LR was defined as nonmetastatic embryonal or FN RMS with an orbital, paratesticular, female genitourinary, or head/neck (nonparameningeal) primary site. HR was defined by the presence of any metastatic disease. IR was defined as not meeting the definition of HR or LR.

Somatic Mutation Data

Somatic mutation data for 39 genes were generated for tumor samples using next-generation sequencing as described in the supplement by Shern et al.[5] Only mutations with a frequency of five or more in the cohort were retained for final analysis, resulting in 20 total genes evaluated. Nineteen genes had four or fewer mutations.

Model Development

The primary outcome to predict was 5-year EFS where relapse, disease progression, second malignant neoplasm, and death were classified as events. All events, even those occurring beyond 5 years, were included in the analysis. Patients without events were censored at their last follow-up time. We developed three Cox proportional hazard models that built on each other (Table 1).

Predictor Selection and Model Specification

The variables in the Baseline Clinical (BC) model were chosen to reflect the standard variables used in prognostic stratification for treatment assignment. Tumor stage and clinical group were unavailable in the complete data set. The modified risk grouping designed to harmonize the data sets from the United States and the United Kingdom also functioned as a proxy variable to reflect standard prognostic categories. Age was evaluated using a restricted cubic spline with four knots. All genetic variants were dichotomous variables specifying whether the variant was present or absent. *TP53* and *MYOD1* were included in the Gene Enhanced 2 (GE2) model to evaluate the added predictive value of these mutations that have been integrated into risk-stratification schemas in the ongoing COG trial ARST2032 after these data were collected. Predictors for the Gene Enhanced 6 (GE6) model were selected using least absolute shrinkage and selection operator (LASSO) Cox regression, a form of regularized regression. Details for these model development steps are provided in the Data Supplement (Sections S1.1-S1.4 and Figs S1 and S2).

Internal Validation, Model Comparison, and Analysis

Model performance for all three models was assessed and compared across a range of metrics. The likelihood ratio test and the percent of added information (one minus the ratio of the variance of outcome predictions for the smaller model over the variance of predictions from the larger model) were used for comparisons of the overall improvements in predictive performance with the addition of the molecular variables to the models. Time-dependent area under the receiver operating characteristic curve (AUROC) at 5 years, Harrel's C-indices, Nagalkerke's R^2, calibration slopes, and Gini's mean difference measures were calculated and corrected for optimism (a form of internal validation) using the bootstrap procedure. CIs for each metric were also produced using bootstrap methods. Apparent and bias-corrected calibration curves for 5-year EFS were also produced through bootstrap resampling. The final model performance was described by reporting the hazard ratios for the predictors, individual survival predictions, and the change in predicted survival probabilities for the same observation between two models. All statistical analyses were performed using R version 4.3.1 using the tidyverse, gtsummary, survival, survminer, rms, and survivalROC packages.[12-18]

TABLE 1. Predictors Included in Each Model

Model Name	Predictors
BC	Age,[a] risk group,[b] fusion status, treatment location
GE2	Age,[a] risk group,[b] fusion status, treatment location, *TP53*, *MYOD1*
GE6	Age,[a] risk group,[b] fusion status, treatment location, *TP53*, *MYOD1*, *CDKN2A*, *MET*, *MYCN*, *NF1*

Abbreviations: BC, Baseline Clinical; GE2, Gene Enhanced 2; GE6, Gene Enhanced 6.
[a]Age modeled using a cubic spline term with four knots.
[b]Risk group is a modified grouping to accommodate differences between the US and UK treatment contexts.

RESULTS

Of the 641 patients in the data set, nine were excluded because of missing values in age, fusion status, or risk group, and 632 were eligible for analysis. Table 2 lists the demographic and clinical variables for the entire data set and by treatment cohort (COG or United Kingdom). Two hundred thirty-six (36%) patients in the data set experienced an event. The median time to event was 445 days (IQR, 309-654 days). The median follow-up time among censored patients was 2,691 days (1,897-3,642 days).

The GE6 model demonstrated the best overall predictive performance ability. By the likelihood ratio test, the GE6 model demonstrated superior predictive performance compared with the BC (χ^2 = 71.4 on 6 *df*; P < .001) and GE2 (χ^2 = 29.3 on 4 *df*; P < .001) models. The GE2 model similarly demonstrated superior predictive ability compared with BC (χ^2 = 42.2 on 2 *df*; P < .001). The GE6 model provided 39% more predictive information than the BC model and 15% more than the GE2 model. GE2 provided 28% more information than the BC model.

Across all optimism-corrected performance metrics, the GE6 model performed better than GE2, which performed better than BC (Table 3). GE6 achieved the highest discrimination of all the models with a time-dependent AUROC of 0.766. All three models demonstrated good bias-corrected calibration curves for 5-year EFS, with only slight tendencies toward regression to the mean for each model upon bias correction (slope of the blue line <1; Data Supplement, Section S2 and Fig S3; regression slope metric <1; Table 3), demonstrating that the addition of the genetic biomarkers does not induce overfitting. Performance characteristics were similar when predicting overall survival (OS; time-dependent AUROC 0.763; Data Supplement, Section S3, Table S1 and Figs S4 and S5 for complete results) and when predicting EFS by treatment context (Data Supplement, Section S4, Tables S2 and S3 and Figs S6-S9).

For the GE6 model, all variables, including the nonlinear component for age, were significantly associated with EFS by

TABLE 2. Demographic Characteristic of the Patient Cohort Overall and by Treatment Context

Characteristic	All Patients (N = 632)	Treatment Context	
		COG (n = 344)	United Kingdom (n = 288)
Sex, No. (%)			
Male	416 (66)	232 (67)	184 (64)
Female	216 (34)	112 (33)	104 (36)
Age, median (Q1-Q3)	6.0 (3.1-11.4)	6.4 (3.4-12.2)	5.3 (2.8-9.5)
Risk group, No. (%)			
Low	220 (35)	124 (36)	96 (33)
Intermediate	298 (47)	147 (43)	151 (52)
High	114 (18)	73 (21)	41 (14)
PAX::FOXO1 fusion, No. (%)			
Negative	508 (80)	275 (80)	233 (81)
Positive	124 (20)	69 (20)	55 (19)
Total mutations, No. (%)			
0	184 (29)	88 (26)	96 (33)
1	247 (39)	136 (40)	111 (39)
2	144 (23)	85 (25)	59 (20)
3	57 (9.0)	35 (10)	22 (7.6)
TP53 mutation, No. (%)	74 (12)	37 (11)	37 (13)
MYOD1 mutation, No. (%)	17 (2.7)	11 (3.2)	6 (2.1)
CDKN2A mutation, No. (%)	23 (3.6)	17 (4.9)	6 (2.1)
NF1 mutation, No. (%)	79 (13)	41 (12)	38 (13)
MYCN mutation, No. (%)	13 (2.1)	10 (2.9)	3 (1.0)
MET mutation, No. (%)	9 (1.4)	6 (1.7)	3 (1.0)
Event,[a] No. (%)	236 (37)	121 (35)	115 (40)
Death, No. (%)	187 (30)	90 (26)	97 (34)

Abbreviations: COG, Children's Oncology Group; Q1, quartile 1; Q3, quartile 3.
[a]Events were defined as disease progression or relapse, second malignant neoplasm, or death.

the Wald test (Data Supplement, Tables S4 and S5 and Fig S10). The hazard ratios for the categorical variables are presented in Table 4. Mutations were associated with a higher hazard for all genes except for *NF1*, which was associated with a lower hazard. The hazard for age showed a U-shaped relationship (Data Supplement, Fig S10), with younger and older ages showing higher log relative hazard compared with around 5 years. The variables in BC and GE2 showed similar patterns to GE6 (Data Supplement, Section S5 and Tables S6-S9). Variable Wald tests, coefficient values, logarithmic hazard, and hazard ratios are provided in the Data Supplement (Tables S6-S9). Results were similar when predicting OS (Data Supplement, Section S2) and on cohort-specific analysis (Data Supplement, Section S3).

Survival predictions for each patient in the cohort varied significantly across models. Figure 1 demonstrates how survival predictions changed for the patients from BC compared with GE2 (left panel) and GE6 (right panel). The figure shows that including genetic information results in

TABLE 3. Bootstrapped Optimism-Corrected Performance Metrics for Each Prediction Model

Metric	BC, Median (95% CI)	GE2, Median (95% CI)	GE6, Median (95% CI)
Time-dependent AUROC, 5-year EFS	0.706 (0.654 to 0.751)	0.723 (0.679 to 0.766)	0.766 (0.729 to 0.805)
Concordance index	0.658 (0.618 to 0.695)	0.673 (0.634 to 0.710)	0.714 (0.684 to 0.747)
Nagalkerke's R^2	0.137 (0.084 to 0.194)	0.161 (0.108 to 0.207)	0.220 (0.168 to 0.294)
Calibration slope	0.946 (0.909 to 0.977)	0.941 (0.906 to 0.967)	0.915 (0.884 to 0.942)
Gini's mean difference	0.678 (0.511 to 0.853)	0.702 (0.565 to 0.856)	0.908 (0.776 to 1.103)

Abbreviations: AUROC, area under the receiver operating characteristic curve; BC, Baseline Clinical; EFS, event-free survival; GE2, Gene Enhanced 2; GE6, Gene Enhanced 6.

TABLE 4. Hazard Ratios for the Categorical Variables in the Gene Enhanced 6 Model

Variable	Estimate	95% CI	*P*
Risk group (reference: low)			
Intermediate	1.31	0.91 to 1.91	.130
High	3.20	2.12 to 4.82	<.001
PAX::FOXO1 fusion (+)	2.43	1.72 to 3.43	<.001
Treated in the United Kingdom	1.49	1.14 to 1.95	.007
MYOD1 mutation	4.61	2.44 to 8.70	<.001
TP53 mutation	2.66	1.83 to 3.85	<.001
CDKN2A mutation	3.22	1.74 to 5.93	<.001
MET mutation	5.98	2.55 to 13.98	<.001
MYCN mutation	2.01	1.02 to 3.95	.048
NF1 mutation	0.56	0.34 to 0.92	.020

very different survival predictions for some patients. Table 5 provides survival predictions for specific types of patients according to the BC and GE6 models. Predictions from BC, where genetic information is ignored, may be compared with predictions from GE6 for patients with and without the specified mutations. For all predictions, 95% CIs are provided to demonstrate the range of survival predictions compatible with the data and model.

DISCUSSION

Our results demonstrate that known and new molecular markers can improve prognosis predictions for RMS. We showed how mutation information *TP53* and *MYOD1*, known to be associated with survival, can improve prognosis predictions over predictions using only age, risk category, fusion status, and treatment location. We also described the added predictive utility of a new set of genes, *CDKN2A*, *MET*, *MYCN*, and *NF1*. We showed that each of these genes is significantly associated with 5-year EFS on multivariable modeling and that this set of genes improves the discrimination of predictions while maintaining adequate calibration. These associations were consistent when predicting OS and when predicting EFS within each treatment context (United States or United Kingdom). If the predictive potential of these new molecular markers is validated in an external cohort, they can be used to make risk stratification more accurate, as with *TP53* and *MYOD1*, and improve risk-adapted therapy assignment.

By predicting 5-year EFS for individual patients in Table 4, we demonstrated that expected survival can change dramatically in ways that may suggest alternative therapies when molecular markers are included. For LR disease, mutations in *TP53*, *MYOD1*, *CKDN2A*, and *MET* were associated with a decreased mean 5-year EFS that more closely resembled the prognosis of IR patients of around 50%-70%. *MYCN* amplification, observed exclusively in FP patients, was associated with a lower mean survival of <50%, similar to that of HR patients. By contrast, an *NF1* mutation in a HR patient was associated with an improved survival of 76%. In each scenario, the mean predicted survival changed to suggest an alternative risk category for the patient. This change in risk category, in turn, suggests that a change in treatment intensity may also be appropriate.

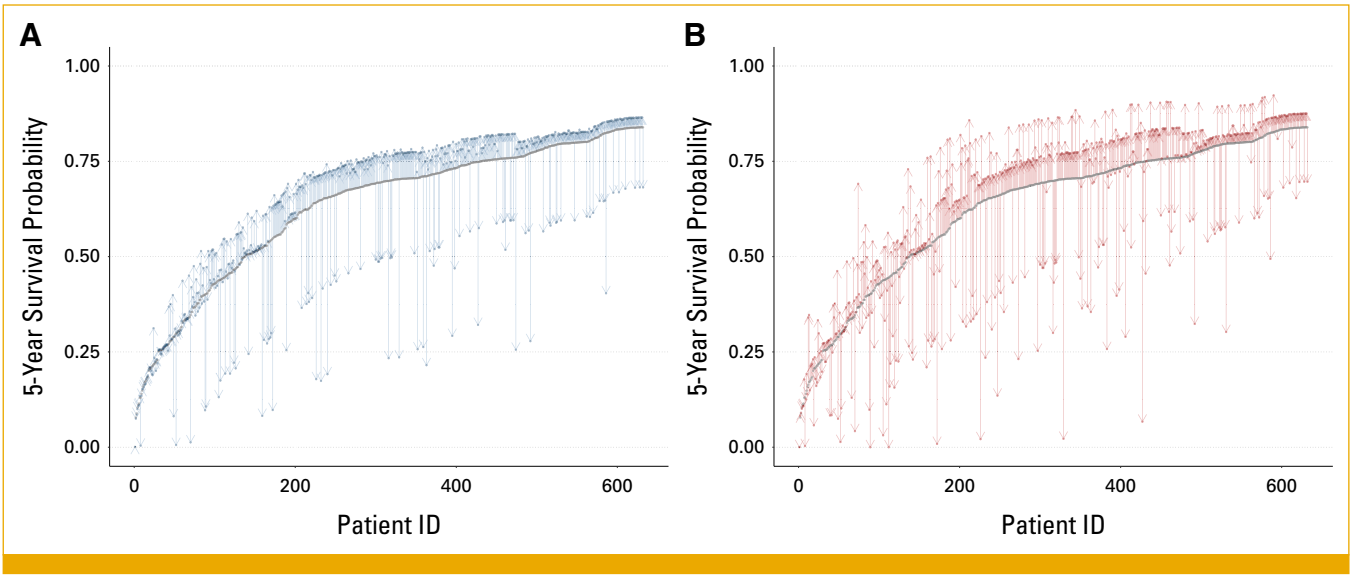

FIG 1. Predictive probability of surviving 5 years without an event for each patient in the data set for (A) the GE2 model (blue) compared with BC (gray) and (B) the GE6 model (red) compared with BC (gray). Points represent the point estimate for 5-year survival. Patients are aligned by lowest to highest estimated 5-year survival according to the BC model. Vertical arrows at the same patient ID represent the difference in the estimated 5-year survival between the models. Both the GE2 and GE6 models estimate worse and improved survival chances relative to the BC model (panels A and B, colored compared with gray points). BC, Baseline Clinical; GE2, Gene Enhanced 2; GE6, Gene Enhanced 6; ID, Identifier.

TABLE 5. Predicted 5-Year EFS From the BC and GE6 Models for Different Types of Patients

	BC, Mean % (95% CI)	GE6, Mean % (95% CI)	
	Mutation Ignored	Mutation-Negative	Mutation-Positive[a]
Patient Characteristic	5-Year EFS	5-Year EFS	5-Year EFS
7 years, LR, FN			
TP53	84 (78 to 89)	87 (83 to 92)	69 (58 to 82)
MYOD1	84 (78 to 89)	87 (83 to 92)	53 (34 to 84)
CDKN2A	84 (78 to 89)	87 (83 to 92)	65 (49 to 86)
MET	84 (78 to 89)	87 (83 to 92)	36 (22 to 90)
7 years, IR, FP			
MYCN	59 (49 to 71)	64 (54 to 76)	42 (23 to 75)
10 years, HR, FN			
NF1	50 (39 to 64)	60 (49 to 74)	76 (64 to 90)

Abbreviations: BC, Baseline Clinical; EFS, event-free survival; FN, *PAX::FOXO1* fusion-negative; FP, *PAX::FOXO1* fusion-positive; GE6, Gene Enhanced 6; HR, high-risk; IR, intermediate-risk; LR, low-risk.

[a]Mutations for all other genetic variables in GE6 are negative except for the gene indicated corresponding row.

CKDN2A, *MET*, *MYCN*, and *NF1* all participate in a common genetic pathway characterized by the activity of several receptor tyrosine kinases (RTKs), and *RAS* and *PIK3CA* genes.[19] *PAX::FOXO1* translocations represent important driver mutations in this pathway that upregulate the expression of MYCN, which increases the malignant transformation of RMS precursor cells.[20] *CKDN2A*, *MET*, and *NF1* are commonly observed as driver mutations among FN disease.[19] Although the mutation panel of the primary study emphasized genes within the RTK/*RAS*/*PIK3CA* axis, the identification of independently prognostic mutations lends support to the hypothesis that derangements along this axis lead to different clinical phenotypes of RMS beyond the primary FN/FP divide.

NF1 (neurofibromin 1) plays an inhibitory role among genes in the *RAS* pathway and has been implicated in multiple cancers and cancer predisposition syndromes.[21] *NF1* was the most commonly mutated gene among the candidate predictors, with 79 cases. It was associated with improved survival in this study (hazard ratio, 0.55 [0.34-0.91]) and resulted in a large improvement in predicted 5-year EFS from 50% (BC model) to 76% (GE6) for a 10-year-old patient with HR, FN RMS. Among *TP53* mutation-negative but *NF1* mutation-positive cases, the observed 5-year OS was 96% for the 22 LR cases (compared with 88% for the 173 *TP53*-negative, *NF1*-negative cases) and 86% for the 37 IR cases (compared with 70% for 224 *TP53*-negative, *NF1*-negative cases). Similar survival outcomes were recently reported in a cohort of 14 *TP53*-negative, *NF1*-positive cases, which had a 5-year OS of 90%.[22] Taken together, this is compelling evidence that *NF1* mutation could be a positive prognostic marker that warrants further evaluation.

MET (mesenchymal epithelial transition) is an oncogene that codes for a RTK that affects cellular survival, migration, and invasion, which has been implemented as a pathogenic driver in a variety of cancer types.[6,8] Expression of the RTK that it encodes, c-MET, has been previously associated with OS in a small case series of patients with ERMS,[23] and downregulation of the MET receptor has been shown in vitro to result in decreased migration and metastatic behavior of RMS cells.[24] Importantly, multiple therapies are available that target the c-MET protein.[25] *MET* mutations yielded a hazard ratio of 5.91 (2.53-13.83) and resulted in a large decrease in predicted survival from 84% (BC model) to 36% (GE6) for a 7-year-old LR, FN patient, although the CI for this estimate was large because of the small number of cases in the data set. Nonetheless, these results suggest that *MET* mutations may be a rare but important targetable prognostic marker that deserves further evaluation.

CDKN2A (cyclin-dependent kinase inhibitor 2A) is a tumor suppressor gene that regulates cell cycle progression.[21] It has been previously associated with poor prognosis for soft tissue sarcomas.[26] The adjusted hazard ratio from the GE6 model for a mutation was 3.18 (1.72-5.87), and the decrease in the predicted survival was comparable with *MYOD1* and *TP53* mutations, although there was a large amount of residual uncertainty in the CIs. These results support the continued evaluation of *CKDN2A* as a prognostic biomarker.

MYCN codes for a transcription factor that regulates a variety of cellular processes. Amplification of this gene has been implicated in the development of neuroblastoma and other cancers.[21,27] *MYCN* is most commonly amplified in FP disease and was observed exclusively in FP disease in this cohort.[5,19] Our results indicated that *MYCN* is associated with 5-year EFS among FP cases independently from the other variables in GE6 with an adjusted hazard ratio of 1.97 (1.00-3.88). However, this finding should be considered more tenuous than the other identified genes, as the association was comparatively smaller, the *P* value was larger, and the selection of this gene in the final model on bootstrap

evaluation of the LASSO procedure was the most inconsistent (selected in 65% of bootstrap samples).

This study provides supportive evidence to reinforce the importance of *TP53* and *MYOD1* mutations as molecular markers of poor prognosis. Survival rates for *MYOD1* reported in small case series range between 0% and 30%, even for LR disease.[28-30] In the primary analysis of this cohort, Shern et al demonstrated that the *MYOD1* mutation had an overall 5-year EFS of 18%, and had a hazard ratio after adjusting for risk group of 5.58 (95% CI, 2.80 to 11.2),[5] which decreased to 4.57 (2.41 to 8.62) after adjustment with GE6. Our analysis complements the original analysis by quantifying how survival predictions change when *MYOD1* mutation status is considered. Of note, the mean prognosis predictions for patients classified as LR are higher than the survival for LR diseases reported in the literature. However, the 95% CI for the predictions is wide and includes values in the low 30%. Direct comparison between results from other studies and this study is difficult because the predictions are adjusted for age and treatment cohort, and the risk groupings in this study are nonstandard to harmonize the US and UK data. If there is miscalibration, it may be due to the small number of cases preventing precise risk estimation after adjusting for age, risk group, and other covariates. Alternatively, *MYOD1* mutation may differentially confer a negative prognosis across risk groups. Therefore, interactions between risk groups and mutation status should be assessed in future modeling efforts. Nevertheless, this study supports the clear associations between *MYOD1* and poor prognosis.

In the primary analysis of *TP53* mutations in this cohort, Shern et al reported a hazard ratio after adjusting for risk group for *TP53* of 1.97 (1.13-3.44) and an overall 5-year EFS of 49%, which varied by risk group when stratified by treatment cohort.[5] After adjustment in the GE6 model, the hazard ratio rose to 2.72 (1.88-3.93). The calibration of predictions from the models compares favorably to the observed risk-stratified survival rates in the primary analysis. Like *MYOD1*, these results quantify how survival predictions change due to including information about *TP53* mutation in predictions, reinforcing the evidence of its importance as a prognostic marker.

Although the prediction models we developed performed well, the limitations of this study entail that further development is required before clinical implementation. The risk grouping variable used in the model represents a consensus grading to facilitate comparison between UK and US treatment contexts. Although this variable enabled adjustment as a covariate to assess the predictive utility of the genetic biomarkers, to use these models in specific clinical contexts, the risk grouping variables must reflect the actual clinicopathologic criteria used for prognostic stratification, such as the current staging system used in COG clinical trials. These limitations also impaired comparing the models with the standard risk-adapted treatment assignment schema. Treatments are also not included in predictions. Although this does not impair inference about the predictive value of the biomarkers, treatment is an important variables when predicting prognosis in specific contexts and when using the model to make decisions about treatment assignment.[31,32] Using the LASSO procedure to select the molecular predictors for GE6 can induce overfitting, making the predictors appear more predictive than they will be in a general population. Although the bias-corrected calibration slope did not demonstrate major overfitting, the prognostic value of these predictors must be verified in a prespecified model in an external cohort. If the performance of models with these improvements can be validated, then they will be fit for use to assist with prognosis-informed clinical decision making. Finally, the ability to demonstrate that genetic loci contain predictive information depends upon the mutation frequency and effect size. Thus, lack of inclusion in the model is not evidence of no association for these loci, but rather insufficient evidence in our data set to meet the inclusion criteria.

In conclusion, in a large cohort of pediatric patients with RMS, we identified several promising prognostic biomarkers that may improve risk-adapted treatment assignment. Mutations in *NF1* are associated with a substantially decreased risk of treatment failure, and mutations in *CDNK2A*, *MET*, and *MYCN* are associated with an increased risk. We also quantified the risk of treatment failure in the known prognostic markers *MYOD1* and *TP53*. Finally, we demonstrated that clinical prediction models that combine clinicopathologic and molecular prognostic markers can adequately predict survival. With further development and external validation, these models could be implemented into clinical practice to improve how providers care for pediatric patients with RMS.

AFFILIATIONS

[1]Department of Pediatrics, Division of Hematology/Oncology, Baylor College of Medicine, Houston, TX
[2]Texas Children's Cancer and Hematology Centers, Texas Children's Hospital, Houston, TX
[3]Center for Cancer Research, National Cancer Institute, Bethesda, MD

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CORRESPONDING AUTHOR

Mark Zobeck, MD, MPH; Twitter: @MarkZobeck; e-mail: Mark.Zobeck@bcm.edu.

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AUTHOR CONTRIBUTIONS

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Financial support: Mark Zobeck
Administrative support: Philip J. Lupo
Provision of study materials or patients: Mark Zobeck, Javed Khan
Collection and assembly of data: Mark Zobeck, Javed Khan, Philip J. Lupo
Data analysis and interpretation: Mark Zobeck, Javed Khan, Rajkumar Venkatramani, M. Fatih Okcu, Michael E. Scheurer, Philip J. Lupo
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