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# Molecular diagnostics in the management of rhabdomyosarcoma

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## Abstract

**Introduction—**A classification of rhabdomyosarcoma (RMS) with prognostic relevance has primarily relied on clinical features and histologic classification as either embryonal or alveolar RMS. The *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions occur in 80% of cases with the alveolar subtype and are more predictive of outcome than histologic classification. Identifying additional molecular hallmarks that further subclassify RMS is an active area of research.

**Areas Covered—**The authors review the current state of the *PAX3-FOXO1* and *PAX7-FOXO1* fusions as prognostic biomarkers. Emerging biomarkers, including mRNA expression profiling, *MYOD1* mutations, *RAS* pathway mutations and gene fusions involving *NCOA2* or *VGLL2* are also reviewed.

**Expert commentary—**Strategies for modifying RMS risk-stratification based on molecular biomarkers are emerging with the potential to transform the clinical management of RMS, ultimately improving patient outcomes by tailoring therapy to predicted patient risk and identifying targets for novel therapies.

### Keywords

Rhabdomyosarcoma; molecular testing; risk stratification; fusion oncogene; gene amplification; *MYOD1*; *RAS*; *FGFR4*; *NCOA2*; *VGLL2*

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**Declaration of Interest**

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## 1. Introduction

In children, rhabdomyosarcoma (RMS) is the most frequent soft tissue sarcoma [1]. While a skeletal muscle phenotype is the hallmark feature of RMS, there is probable heterogeneity in the cell of origin. Evidence in animal model systems demonstrates that RMS arises from skeletal muscle precursors in some cases [2], but in other cases RMS can arise from other mesodermal precursor cells [3]. Irrespective of its origins, the classification of rhabdomyosarcoma has depended on histology. Historically, pediatric RMS has been classified into two groups; alveolar RMS (ARMS) composed of primitive monotonous round cells typically with scant cytoplasm, and embryonal RMS (ERMS) with a range of appearances from small moderately pleomorphic cells to spindle cells that can have abundant eosinophilic cytoplasm with striations. In general, ERMS presents in younger children as tumors arising in the head and neck or trunk and ARMS often presents as tumors arising in the extremities of teens and young adults. In clinical studies of these two subtypes, ARMS is associated with an inferior outcome as compared to ERMS.

Outcomes for patients with RMS have improved over the last several decades. Notably, this improvement has taken place while the backbone of chemotherapy (vincristine, dactinomycin, and cyclophosphamide in North America or ifosfamide, vincristine, and dactinomycin in Europe) have remained largely unchanged. In a series of clinical trials, the intensity of chemotherapy has been adjusted to match the likelihood of poor outcomes based on prognostic features. Risk stratification in North American and European trials has relied on clinical and histologic features, and not prognostic molecular biomarkers [4–8]. The Children's Oncology Group (COG) risk stratification [4] has traditionally relied on histologic classification, tumor location, tumor size, the extent of surgical excision and the presence of regional or distant metastatic disease to group patients into three treatment categories: low-risk, intermediate-risk and high-risk RMS [6, 9]. Low-risk RMS has been defined as tumors with a histologic classification of ERMS without distant metastasis that either occur in favorable sites (orbit, head and neck except parameningeal, genitourinary sites except the bladder or prostate, biliary tract) or are completely excised from any primary site. Intermediate-risk has been defined as either tumors classified as ERMS without distant metastasis that occur at unfavorable sites and are incompletely excised, or tumors classified as ARMS without distant metastasis. High-risk has been defined as any tumor with distant metastasis. In this system, there has been no role for molecular biomarkers in risk stratification. Here, we review emerging prognostic molecular biomarkers for RMS, and the roles of these biomarkers in modifying this risk stratification system by altering the definitions of individual risk groups, or enhancing stratification by creating novel risk strata. The potential to change risk stratification for RMS can improve clinical outcomes for patients with RMS by allowing further tailoring of current therapies to match individual patient risk as well as defining novel targets for new therapeutics.

## 2. *PAX-FOXO1* Fusion Status is a Key Prognostic Biomarker for RMS Patients

The ARMS subtype is associated with t(2;13) or t(1;13) chromosomal translocations resulting in a *PAX3-FOXO1* or *PAX7-FOXO1* fusion, respectively, and henceforth referred to collectively as *PAX-FOXO1* fusions [10, 11]. The diagnosis of ARMS is based on histology; however the histologic classification of ARMS has evolved over time [12–14]. The current histologic definition of ARMS requires that the majority of a tumor show alveolar histology, or solid sheets of cells with monotonous round nuclei characteristic of ARMS. With this current definition, *PAX-FOXO1* fusion-positive ARMS represents approximately 80% of all cases with the histologic classification of ARMS; in particular, approximately 60% of ARMS cases are *PAX3-FOXO1*positive and approximately 20% are *PAX7-FOXO1*-positive [13, 14]. While the presence of the *PAX-FOXO1* fusion is closely correlated with this histologic subtype, classification of RMS as ARMS or ERMS remains a histologic definition. Therefore, approximately 20% of tumors currently classified as ARMS do not contain these fusions (and are referred to as ARMSn). It should also be noted that the *PAX-FOXO1* fusions have been found in rare cases diagnosed as ERMS or mixed RMS, and these cases are generally grouped with the fusion-positive ARMS cases into an overall fusion-positive RMS (RMSp) category.

Recently, outcome analysis evaluating *PAX-FOXO1* fusion status and current RMS histologic classification has recognized *PAX-FOXO1* fusion status as a superior biomarker of a poor event-free survival (EFS) [14–17]. For patients with intermediate-risk RMS (as defined clinically by findings of ERMS histology with unfavorable primary site and incomplete resection or ARMS histology without distant metastasis), RMSp showed a significantly inferior 5-year EFS (50%) compared with 77% 5-year EFS for ERMS and 82% for ARMSn [15]. Similarly, among patients with clinical parameters consistent with the low-risk category (favorable primary site or complete resection without distant metastasis), 5-year EFS was 100% for ARMSn compared with 64% for RMSp [14]. Further, gene expression profiling of RMS shows ARMSn is highly similar to ERMS [16, 17]. In all these studies, the outcome for ARMSn is not statistically different from that of ERMS, but is superior to the outcome of RMSp. This significantly superior EFS outcome for ARMSn compared with RMSp has led to a novel modification of the COG risk stratification system that is currently being evaluated in the ongoing COG clinical trial ARST1431. In this modified risk system, the definitions for the low-risk and intermediate-risk groups are changed to replace histologic classification with fusion status. This modification will allow reduced intensity therapy for patients with ARMSn that would otherwise be considered clinically low-risk, a modification that is justified based on the favorable outcomes of these patients in previous COG trials [14, 15].

## 3. *PAX-FOXO1* Fusion Gene Partner is Associated with Differences in Outcome

While the presence of a *PAX-FOXO1* fusion gene shows significant prognostic value, the partner gene fused to *FOXO1*, either *PAX3* or *PAX7*, may also hold additional prognostic

significance. Analysis of outcomes for patients with RMSp has shown that patients with *PAX7FOXO1*-positive RMS have superior overall survival (OS) compared to patients with *PAX3FOXO1*-positive RMS [15, 18, 19]. For example, in COG studies of clinically intermediate risk RMS, though 5-year EFS was 55% for *PAX7-FOXO1* and 49% for *PAX3-FOXO1*-positive RMS compared with 77% for ERMS, OS was 82% for patients with either *PAX7-FOXO1*-positive RMS or ERMS, while OS was 61% for patients with *PAX3-FOXO1*-positive RMS [15]. The specific fusion partner may therefore serve as an additional tool for identifying patients with higher risks of poor outcomes, and ultimately to further sub-stratify risk groups for patients with RMSp. However, a significant difference in outcome between *PAX3-FOXO1*-positive RMS and *PAX7-FOXO1*-positive RMS was not found in all reported studies [20]. Therefore, these fusion partners are being prospectively evaluated in COG trial ARST1431 to further delineate their potential as prospective prognostic biomarkers.

## 4. *PAX-FOXO1* Fusion Gene Amplification is Associated with Differences in Outcome

Florescent in situ hybridization (FISH) assays can detect *FOXO1* rearrangements in RMS and thus assess *PAX-FOXO1* fusion status. These FISH assays also demonstrate genomic amplification of the rearranged *FOXO1* locus and the associated fusion gene. Strikingly, such amplification occurs in 93% of *PAX7-FOXO1*-positive cases compared to 9% of *PAX3-FOXO1*positive cases [18]. Clinical correlative studies have demonstrated that outcomes are better for patients with the FISH finding of amplification of the rearranged *FOXO1* gene. However, since this amplification is strongly associated with the *PAX7-FOXO1* fusion [18], it is unclear if this more favorable outcome results primarily from the PAX7 fusion partner or the amplification of the fusion gene. Further study of this phenomenon, including prospective tracking of fusion gene amplification in COG study ARST1431 will aid in determining which characteristic of amplified fusion genes drives the more favorable outcomes.

## 5. Surrogate markers of *PAX-FOXO1* fusion status

As *PAX-FOXO1* fusion status emerged as a key driver of outcomes for patients with RMS, alternative methods for identifying fusion-positive cases have become increasingly valuable. Immunohistochemistry (IHC) is a robust and widely available methodology that is more readily available in many pathology departments than FISH or RT-PCR. By comparing gene expression profiles of RMSp and ERMS, panels of IHC markers have been selected as surrogates of *PAXFOXO1* fusion status. The combination of IHC assays for EGFR, fibrillin-2, AP2β (TFAP2B) and P-cadherin can be used to predict fusion status [21]. ERMS is associated with the expression of EGFR and fibrillin-2, while RMSp is associated with the expression of AP2β and P-cadherin. IHC reactivity for EGFR and fibrillin-2 showed 90% specificity and 60% sensitivity for ERMS, and IHC reactivity for AP2β and P-cadherin showed 98% specificity and 64% sensitivity for RMSp [21]. A more recent approach using IHC detection of myogenin (MYOG), AP2β, NOS1, and HMGA2 has also been shown to closely correlate with the presence of a *PAX-FOXO1* fusion gene [22]. This approach for

detecting RMSp is derived from previous observations that this RMS subset consistently demonstrates Myogenin reactivity in greater than 50% of tumor nuclei (scored as 3+ or 4+) [13]. RMSp also shows stronger relative expression of AP2β and NOS1 whereas fusion-negative RMS (including both ERMS and ARMSn, and henceforth referred to as RMSn) shows stronger relative expression of HMGA2. The combination of strong myogenin expression with strong relative expression of AP2β and NOS1 has 96% sensitivity and 91% specificity for detecting RMS cases with *PAX-FOXO1* fusions [22].

## 6. mRNA Expression Signatures Correlate with Outcomes for RMSn

While *PAX-FOXO1* fusion status currently provides the most significant molecular marker of poor outcome, RMSn cases account for 80% of all RMS cases and thus there is a need to further delineate outcome differences within this large group of RMS cases. In particular, nearly half of all RMS patients are classified as intermediate risk RMSn. Therefore improved risk stratification of this cohort represents a significant opportunity to impact outcomes for many RMS patients. In an effort to further risk-stratify patients with RMS, COG investigators and colleagues in Europe investigated mRNA expression profiles in relation to histologic type, *PAXFOXO1* fusion status, and outcome for children with RMS [19, 23, 24]. Comparisons of genomewide mRNA expression reveals that RMSp and RMSn show distinct expression profiles, and that ERMS is indistinguishable from ARMSn within the larger RMSn group [16, 23, 24]. Further, mRNA expression profiles can identify distinct outcome groups within RMSp or within each COG risk strata [23, 25], as well as predicting *PAX-FOXO1* fusion status [26]. Studies of RMSn identified an mRNA expression signature derived from the weighted expression of five genes (*EPHA2, EED*, *NELF, CBS* and *EPB41L4B*) that can distinguish patient outcomes [19]. Increased expression of this so-called MG5 signature correlates with inferior survival for children with RMSn. The value of this signature was further demonstrated by the finding that MG5 expression in an independent and uniformly treated patient cohort correlated with EFS as well as OS [27]. This study was particularly important because the validation cohort was drawn from a single intermediate-risk COG RMS trial (D9803), in which no other clinical or molecular features could predict outcome within the RMSn cases in this uniformly treated cohort. Though the initial studies were performed with microarrays, the MG5 signature can also be measured by the nCounter platform using RNA extracted from formalin-fixed, paraffin-embedded specimens, rather than frozen material [27]. Hence, this approach holds substantial potential to enhance risk stratification by identifying a higher-risk subgroup within the intermediate risk cohort.

## 7. *MYOD1* L112R Mutation is a Marker of Poor Outcomes

*MYOD1* encodes a myogenic basic-helix-loop-helix (bHLH) transcription factor with critical roles in muscle development [28, 29]. A recurrent somatic mutation of the *MYOD1* gene was found in a subset of ERMS cases, resulting in the amino acid substitution L122R [30]. This substitution increases the similarity of the MYOD1 basic domain to the MYC transcription factor basic domain, thereby blocking wild-type MYOD1 function and imparting a gain-of-function MYC-like capability [31]. Within the larger group of ERMS tumors, this *MYOD1* L122R mutation was specifically associated with the subset that shows

spindle cell and/or sclerosing histology. Whereas this mutation was found in 10 of 104 ERMS cases overall (10%), it was found in 22 of 54 cases (41%) in the spindle cell and/or sclerosing histology subset [32, 33]. Though this mutation may be less frequent overall in ERMS in children than in adults (5% versus 21% in one study [30]), the frequency of this mutation was similar in both pediatric and adult cases of the spindle cell and/or sclerosing subset. The presence of the *MYOD1* L122R mutation appears to be a marker of poor RMS outcome. One study of outcome in 70 ERMS cases found a 29% 5year OS in cases with this MYOD1 mutation compared to a 57% 5-year OS in ERMS cases without this mutation. Furthermore, in 13 patients with the *MYOD1* L122R mutation under age 18 at diagnosis, 9 were dead of disease, and none of these patients were reported to be alive beyond three years of follow-up [30, 33]. While the L122R *MYOD1* mutation represents a small proportion of all children with ERMS, the very poor outcome associated with this mutation indicates that *MYOD1* mutation status may represent an important biomarker in the pediatric RMS population.

## 8. FGFR4 Signaling is Commonly Activated in Rhabdomyosarcoma

In accord with the finding that the *FGFR4* signaling pathway plays an essential role in myogenesis [34, 35], *FGFR4* is expressed in the far majority of RMS tumors [23, 36, 37]. Signaling by FGFR4 and other fibroblast growth factor receptors involves tyrosine phosphorylation of the receptor carboxyl terminus along with other substrates, such as FRS2, recruitment of the GRB2 adaptor, and activation of the downstream RAS and phosphoinositide 3-kinase pathways. The *FGFR4* gene is a target of the PAX-FOXO1 fusion proteins (which are potent transcriptional activators) and is thus expressed at high levels in RMSp. In contrast, in RMSn in which FGFR4 is expressed at lower levels, mutations occur in various components of this signaling pathway. In a deep sequencing study of 94 RMSn cases, *RAS* gene mutations were found in 22% of cases (*NRAS*, 12%; *KRAS*, 6%; *HRAS*, 4%), *FGFR4* mutations in 10% of cases, *PIK3CA* mutations in 7% of cases, and *NF1* mutations in 5% of cases. In total, mutations in genes encoding one or more components of the *FGFR4* pathway were found in 37% of RMSn cases [38]. Though mutations of these genes have been found in rare RMSp cases, *FGFR4* transcriptional activation by the PAX-FOXO1 protein provides an explanation for an alternative mechanism of FGFR4 pathway activation in RMSp compared to the frequent occurrence of mutational activation in RMSn. Overall, these findings in RMSp and RMSn indicate that the FGFR4 pathway is commonly activated in RMS. Though the clinical significance of these findings is not yet known, the FGFR4 signaling pathway provides an important new set of therapeutic targets relevant to a large number of RMSp and RMSn cases.

## 9. *NCOA2* and *VGLL2* Fusions in Infantile RMS

Although the far majority of ERMS cases are characterized by recurrent point mutations, a small subset of ERMS cases has novel fusions involving the *NCOA2* and/or *VGLL2* genes. Interestingly, these fusion-positive ERMS cases occur almost exclusively in children under 1 year of age and are associated with spindle cell morphology. In a recent study of 10 such cases, the fusions include *VGLL2-CITED2* in 4 cases, *TEAD1-NCOA2* in 2 cases, *VGLL2-NCOA2* in 2 cases, and *SRF-NCOA2* in one case [39, 40]. Outcome data for such cases is

limited, but favorable outcomes in this group appear to be common. Six patients with available outcome data were reported to be alive and well with a median of 7 years of follow-up [36]. Further evaluation of the clinical significance of these *NCOA2* and *VGLL2* fusions will determine if these fusions represent useful prognostic biomarkers and potential therapeutic targets.

The absence of *PAX-FOXO1* fusions in a subset of cases with the histologic classification of ARMS led to speculation that these tumors contain variant fusion genes. Subsequent studies identified only rare ARMSn cases with variant fusions, including *PAX3-FOXO4* and *PAX3NCOA1* [14, 37]. As described above, the far majority of these ARMSn cases do not contain any functional fusions, and instead have an expression and mutational pattern similar to ERMS tumors.

## 10. Expert Commentary

Improvement in the precision of RMS pre-treatment risk stratification has resulted in significant decreases in mortality. To date, molecular changes have played a limited role as predictive biomarkers, with *PAX-FOXO1* fusion status only recently becoming integrated into the COG system of risk stratification. Several molecular biomarkers are poised to significantly impact pretreatment risk prediction of both RMSn and RMSp in the future. At least one emerging biomarker is likely to apply to nearly every patient diagnosed with RMS.

These molecular biomarkers also represent candidates for targeted therapeutic approaches. Given the inferior prognosis of *PAX3-FOXO1*-positive RMS, novel therapies to directly target the PAX3-FOXO1 fusion protein or inactivate downstream target genes and signaling pathways could have a significant impact on patient outcomes [41]. Experimental findings that PAX3FOXO1 fusion protein is essential to ARMS tumorigenesis provide further support that the fusion protein is an attractive therapeutic target [42]. Several potential approaches have been demonstrated, including RNA interference-mediated suppression of the *PAX3-FOXO1* transcript [43–45], and small molecule inhibition of PAX3-FOXO1 protein phosphorylation and function [46, 47]. Since transcription factors are challenging pharmacologic targets because their activities are regulated by protein-protein interactions rather than catalytic activity [48], pathways downstream of PAX3-FOXO1, such as those involving MET [49], FGFR4 [50], and Hippo [51], may be more amenable to pharmacologic targeting. The prospects of targeting the *FGFR4* signaling pathway are particularly attractive as pathway activation appears to be frequent in both RMSn and RMSp cases.

Additionally, *MYOD1* mutations, altered expression of the MG5 genes, and novel *NCOA2* and *VGLL2* fusions may each represent opportunities for targeted intervention. For example, approaches to block DNA binding of the altered basic domain in the MYOD1 L122R mutant protein could abrogate its MYC-like transcriptional activity, potentially altering the abysmal prognosis for this cohort. Similarly, while the MG5 signature is a marker of a poor prognosis, it remains to be examined if the altered expression of the proteins encoded by this gene signature drives unfavorable outcomes that could be targeted for therapeutic benefit. Finally, the biologic role and clinical significance of the novel fusions in infantile RMS also

remains to be determined, and downstream pathways aberrantly regulated by these fusions may also provide novel targets for therapeutic interventions.

## 11. Five-year view

While emerging molecular biomarkers represent a significant opportunity, the diversity of testing required to evaluate each of these markers also presents a challenge, especially if limited numbers of RMS cases and limited amounts of diagnostic material are available. In addition to individual validation of these markers, a key aspect of implementing these biomarkers in pretreatment risk stratification will be to define the relative significance of each marker for an individual patient. Progress in evaluating and validating individual biomarkers will be slow, since studies of each marker will draw on a subset of patients with RMS, resulting in smaller available cohorts within this rare tumor. International collaboration will likely be necessary to aggregate sufficient cohort sizes to evaluate biomarkers that are useful in limited subsets of RMS patients. While difficult, these studies will allow development of clinical testing approaches that give priority to the biomarkers with the greatest impact on care for individual patients. Careful histologic classification may also prove to be an important tool in this decision-making process. For example, spindle cell tumors in children under one year of age appear far more likely to contain *NCOA2* or *VGLL2* fusion genes instead of *PAX-FOXO1* fusions. It may therefore be reasonable to prioritize testing for specific biomarkers based on patient age and tumor histology.

Emerging biomarkers will enhance the current system of risk-stratification by introducing additional risk strata. The greatest opportunity for adding risk strata is in the intermediate-risk group, which is the largest cohort among the three risk groups. Further refining the existing risk strata would allow further adjustment and/or modification of therapy, making incremental improvements in outcomes possible. However, major advances in improving outcomes for patients with the poorest prognosis will rely on developing novel therapeutic strategies. Much like the emerging biomarkers, future novel therapies will to be targeted to small groups of patients with RMS, such as patients with mutations in *MYOD1*. While these cohorts may represent small subsets of all patients with RMS, very poor outcomes highlight the urgency and opportunity for improving outcomes by developing novel therapies. The small size of targeted patient cohorts will also impact the development of novel targeted therapies, as financial incentives needed to attract investment by pharmaceutical companies will be lacking; development of novel therapies will likely rely heavily on government programs and philanthropic organizations willing to accept the financial risk. Ultimately, biomarkers and the development of corresponding novel therapies will be essential to make significant improvements in outcomes for patients with RMS.

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Reference annotations

*Of interest

**Of considerable interest


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**Key issues**

- *PAX-FOXO1* fusion status is a key marker of poor outcomes for patients with RMS
- Fusion gene amplification correlates with the PAX7-FOXO1 fusion subtype and favorable patient outcomes, potentially identifying a lower risk subset of patients with RMSp
- Immunohistochemical staining for myogenin, AP2β, NOS1, and HMGA2 is an effective surrogate marker of PAX-FOXO1 fusion status
- A five-gene expression signature (MG5) can distinguish higher and lower-risk cohorts among intermediate-risk RMSn
- *MYOD1* L122R mutations identify a cohort of pediatric patients with very poor prognosis among spindle and/or sclerosing RMS
- The *FGFR4* signaling pathway is activated by the PAX-FOXO1 fusion protein in RMSp, and by somatic alterations in RMSn
- Fusions involving *NCOA2* and/or *VGLL2* are associated with spindle cell RMS in patients under one year of age, and need further study to determine their clinical and biologic significance