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# The Inconvenience of Convenience Cohorts: Rhabdomyosarcoma and the *PAX-FOXO1* Biomarker

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## Abstract

"Convenience cohorts" comprise individuals thought to represent the general population, but chosen because they are readily available for evaluation, rather than at random. As such, these methods are subject to bias and may be misleading. Convenience cohorts have been used to investigate the prognostic significance of chromosomal translocations between the *PAX3* or *PAX7* and the *FOXO1* genes in rhabdomyosarcoma, the most common pediatric sarcoma. However, retrospective studies assessing the role of *PAX-FOXO1* translocations have yielded inconsistent results. This review highlights the findings from several clinical correlation studies of the *PAX-FOXO1* biomarker and illustrates the challenges of using such methods to draw clinical conclusions.

### Keywords

rhabdomyosarcoma; PAX3; PAX7; FOXO1; FKHR; convenience cohort; pediatric cancer

## Introduction

Convenience cohorts represent a type of non-probability sampling where study participants thought to represent the general population are selected based on their availability, rather than at random. (1) Because an unknown portion of the population is excluded and the degree of true population representation in convenience cohorts is not known, these methods are subject to bias and may be misleading. (2) While they are essential to generate hypotheses, convenience cohorts are limited in their ability to definitively confirm the role of potential clinical biomarkers.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents (3). RMS is usually divided into two broad histologic groups: embryonal RMS (ERMS), representing approximately 70% of cases and associated with a more favorable prognosis, and alveolar RMS (ARMS), representing 30% of cases, and associated with

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poorer prognosis (4). Clinical factors, including the primary site, completeness of resection prior to chemotherapy, tumor size, regional nodal involvement, and the presence of distant metastases, are also used to define risk groups, with the goal of better stratifying treatment regimens to promote optimal survival with minimal toxicity (5). “Low risk” patients, with localized ERMS, have approximately 90% long-term failure-free survival (FFS), while “high risk” patients, with metastatic RMS, have an expected FFS of less than 20% (6). “Intermediate risk” patients represent a heterogeneous group of both ERMS and ARMS patients with FFS ranging between 50-80% (4, 5, 7-9). To optimize the allocation of therapy intensity based upon the risk of recurrence, further improvement to the stratification of patients is necessary, particularly for those in the heterogeneous intermediate-risk group.

Among patients with ARMS, a translocation between the *PAX3* or *PAX7* gene and the *FOXO1* gene is present in approximately 80% of cases (10-14). These chromosomal translocations generate novel proteins in which the DNA binding portions of PAX are fused to the carboxyl terminus of FOXO1; the PAX-FOXO1 fusion protein acts as a potent transcriptional activator that influences the expression of genes ultimately controlling cell proliferation, apoptosis, differentiation and motility. This “fusion-positive” status leads to expression of a potent transcriptional activator, which effects growth, apoptosis, differentiation and motility (3). Several studies have suggested that fusion status is associated with outcome and should therefore be incorporated into preliminary risk stratification schemata (7-9, 15-19). Each of these studies was based on findings from “convenience samples,” however, and their results are inconsistent. This review aims first to highlight several clinical correlation studies using convenience cohorts to assess the *PAX-FOXO1* translocation as a biomarker, and then to Illustrate the challenges of using such methods to draw clinical conclusions.

## Initial Clinical Studies

Initial clinical reports suggest the *PAX3-FOXO1* and *PAX7-FOXO1* translocations are associated with distinct frequencies and clinical phenotypes. The *PAX3-FOXO1* translocation is more common, present in 60-70% of ARMS cases, in contrast to the *PAX7-FOXO1*, present in 10-20% of cases (8, 10, 13, 18, 20). Patients with the *PAX3-FOXO1* translocation tended to be older, a finding traditionally associated with worse prognosis, and had more aggressively behaving tumors (11, 15, 21, 22). Small case series among patients with known presence of a *PAX-FOXO1* translocation again suggested inferior clinical factors and outcomes [Kelly, 1997 (15); Anderson, 2001 (16)], leading to larger cohort studies to determine the association between *PAX-FOXO1* translocation status and prognosis (Table 1).

The first relatively large analysis of the relationship between *PAX-FOXO1* translocation status and survival used samples from Intergroup Rhabdomyosarcoma Study (IRS) IV (23-25), which enrolled patients from 1991-1997 [Sorensen, 2002 (7)]. Only 141 (11%) of all IRS-IV patients had centrally banked fresh frozen tissue suitable for molecular studies. An additional 27 ARMS cases were identified from local, institutional banks to create a combined cohort of 171 patients, including 78 with ARMS. Potential cases were reviewed by central pathology to confirm alveolar histology, and reverse transcriptase polymerase chain reaction (RT-PCR) was performed by established methods at a single institution. *PAX3-* and *PAX7-FOXO1* fusion transcripts were detected in 55% and 22% of alveolar RMS patients, respectively; 23% were fusion-negative. All other RMS specimens lacked detectable fusion transcripts. Fusion status was not associated with outcome differences in patients with localized ARMS; however, among those with metastatic disease, *PAX3-FOXO1* was associated with inferior 4-year overall survival (OS, 8% versus 75%, p=0.0015).

To confirm these observations, the same investigators analyzed a separate, retrospective cohort from the IRS-III study (open from 1984-1991) (26), including 78 archived formalin-fixed, paraffin-embedded (FFPE) specimens of ARMS tumors [Barr, 2006 (17)]. Satisfactory RT-PCR results were obtained in 59 cases (30% of total ARMS cases). The distribution of fusion types was similar to previous studies: *PAX3-FOXO1*, 59%; *PAX7-FOXO1*, 19%; and fusion negative, 22%. Investigators were unable to detect differences in FFS among assayed ARMS cases using a classical alpha level of 0.05 for statistical significance (p=0.17). However, being a *member* of the cohort (i.e., having FFPE tissue available) was associated with superior outcomes. Those without available fusion data appeared to have inferior outcomes. The hazard ratio for relapse among non-assayed cases was 2.1 (95% CI 1.2-3.5p=0.0075) and the hazard ratio for death was 2.4 (95% CI 1.3-4.1, p=0.00027). Secondary analyses were unable to identify an explanation for this finding. The two groups did not differ significantly with respect to distribution of prognostic clinical variables, arguing that the convenience cohort was, indeed, similar to the larger patient population. Investigators suggested that superior outcomes among assayed patients was due to the fact that these cases were more likely to come from larger institutions (Mantel-Haenszel trend-test p=0.067) They also postulated that factors such as unmeasured socioeconomic status, distance from treating centers, or insurance status may have affected outcomes. Finally, they noted the limitations of retrospective studies of molecular-clinical correlations and underscored the idea that results from convenience samples must be interpreted with caution.

The limitation inherent in a convenience cohort was also seen in a separate, retrospective analysis done within the German Cooperative Soft Tissue Sarcoma Study Group (CWS), reporting results from four consecutive trials open from 1984 to 2004 [Stegmaier, 2011 (18)]. To evaluate the prognostic value of *PAX-FOXO1* fusion status, 121 ARMS specimens were selected for fusion status evaluation (27% of total patient population) based on availability of pre-treatment frozen or FFPE tissue. RT-PCR was performed by established methods at two institutions. Patients with *PAX3-FOXO1*-positive tumors tended to be older than those with *PAX7-FOXO1* (63% versus 17% were older than 10 years, respectively, p=0.0001) and had higher rates of metastatic disease (50% versus 24%, p=0.017). There were no detected differences in 5 year event-free survival (EFS) between patients with localized disease, stratified by fusion status: *PAX3-FOXO1*, 38.9%; *PAX7-FOXO1*, 18.2%; fusion negative, 11.7% (p=0.235). Overall, the 5 year EFS for all localized patients and fusion data was 28.7 %, compared to 65% seen on IRS-III and -IV (4, 26, 27). For patients with metastatic disease, fusion positive status patients tended toward an inferior 5 year EFS compared to fusion negative: *PAX3-FOXO1*, 9.3%; *PAX7-FOXO1*, 14.3%; fusion negative, 60% (p=0.145).

In contrast to the IRS-III series, *members* of the CWS convenience cohort with localized disease had *inferior* EFS compared to those without tissue available for analysis (28.7% versus 50.8%, p=0.009), regardless of fusion status. The distribution of patients and tumor-related parameters was similar in both groups, except that analyzed patients had a greater proportion of unfavorable tumor sites such as extremities. Investigators suggested that tumor site may have contributed to the inferior outcomes seen in analyzed patients. Similarly, they highlighted the substantial differences in EFS among patients with localized disease who did and did not have tissue available and suggested these differences were due to small numbers of patients and limited representativeness of the convenience sample. They again noted the limitations of convenience samples for correlative studies. EFS for patients with metastatic disease was similar regardless of whether or not cases were analyzed.

A recent series studied the impact of RMS histology and fusion status using a convenience cohort of 101 patients with available frozen tissue plus an additional 109 with only clinical

data available (8). RT-PCR was performed by established methods; all samples that were negative for *PAX-FOXO1* fusions underwent further, confirmatory pathological review. ERMS cases were not assessed for fusion status. Patients were treated over several years and with various regimens of multi-agent chemotherapy plus or minus surgery and/or radiation therapy. ARMS fusion-positive patients (either *PAX3*- or *PAX7-FOXO1*) had inferior 5-year EFS compared to ARMS fusion-negative and ERMS patients (20% versus 60% and 55%, respectively, $p<0.001$). The relative risk of death for fusion-positive patients was 2.5 after adjustment for stage and histology (95% CI 1.2-5.1). ARMS fusion-positive patients were more likely to have unfavorable sites of disease (79% versus 53% and 57%, respectively, $p=0.002$) and metastatic disease (43% versus 8% and 12%, respectively, $p<0.001$). The number of cases for which tissue was not available to analyze was not reported, so it is not possible to determine the extent of patient selection in this series. There were no comparisons of outcome by whether or not tissue was available for analysis. The authors concluded that fusion status, rather than histology, be used to risk-stratify patients with RMS.

A retrospective evaluation restricted to older (adolescent and adult) RMS patients treated with various regimens at a single institution between 1957 and 2001 evaluated the role of *PAX-FOXO1* translocations (9). One-hundred and five of 251 consecutively treated patients with either ARMS or ERMS had available FFPE tissue samples and 52 (21% of all potential cases) yielded interpretable FISH results. Among the ARMS specimens, 67% had a detectable fusion gene, and these patients were more likely to have metastatic disease (39%) compared to those with fusion-negative alveolar or embryonal disease (both 22%, $p=0.0081$). No associations were detected between fusion transcript-type and survival; however, variable treatment regimens, stages of disease, and abilities to detect metastatic sites over this 40-year time period may have precluded appropriate survival comparisons.

## Discussion

This review highlights the limitations of using convenience cohorts in clinical correlation studies attempting to define a biomarker for disease-risk. Such studies characterize selected subsets of patients, often treated with different regimens, spanning decades of time. Among RMS patients, this is particularly important as risk criteria and histological classifications have changed over time (5). Nonetheless, some investigators have concluded that fusion status is associated with prognosis (21); for example, studies commonly suggest that fusion-negative patients have better outcomes than fusion-positive, and *PAX7* seems to be associated with less risk than *PAX3*. Other investigators have suggested such conclusions are premature (27) and can only be verified with more robust, prospective studies.

The inconsistent data regarding the prognostic significance of fusion status and type may be explained, in part, by the limitations of convenience sampling methologies. Perhaps the strongest examples stem from the two studies that noted differences in survival based on membership in the convenience cohort alone [Barr, 2006 (17); Stegmaier, 2011 (18)]. Not only did these studies reveal conflicting results, but they reported findings that made little clinical sense. Why should the presence of archived tumor material predict a patient's prognosis? Clearly, the samples epitomize some unrecognized or unstudied selection bias. They are not necessarily representative of the whole population.

Other limitations include the fact that the relatively small number of patients in these studies makes it difficult to detect differences between groups or to fully adjust for potential covariance. For example, while patients with *PAX3-FOXO1* tend to be older, it is unclear if age and translocation status are independent or interacting factors of adverse outcomes. Similarly, the variable association with primary disease site and stage preclude consistent

conclusions. Interpreting differences in survival status is limited by the fact that each study assessed different populations. Some included both ARMS and ERMS, or both metastatic and non-metastatic disease in the same cohort. Other prognostic indicators, such as tumor site or baseline stage were not always included in multivariate models.

"Non-probability sampling" like that used in convenience cohorts involves non-random selection of samples. This does not necessarily mean that convenience samples fail to represent the general population; rather, it implies that convenience samples cannot rely on probability theory and are, therefore, subject to bias. Unfortunately, there are no clear methods to detect or control for such biases and results from such studies must be interpreted carefully. The benefit of convenience cohorts, however, is that they provide efficient and important exploratory information that may be used in larger prospective studies with more uniform assessment of the prognostic factor.

The investigators in aforementioned studies appropriately attempted to characterize if and how their samples might differ from the larger population of patients with ARMS. Likewise, they qualified the limitations of their findings and suggested that future research include baseline tumor tissue for all enrolled patients. Indeed, the current and future Children's Oncology Group RMS clinical trials require tumor specimens from all patients and will ultimately be able to describe the true relationship between fusion-status and clinical outcomes among patients with RMS.

This review underscores the fact that convenience samples are critical for hypothesis generation, but less compelling for confirmatory assessments. Rather, prospective studies which include timely molecular assessments of *all* patients may better elucidate true risk categorization. Ultimately, such studies will enable investigators and clinicians alike to risk-stratify their patients more accurately.

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## Abbreviations

| | |
|---|---|
| **RMS** | Rhabdomyosarcoma |
| **ARMS** | Alveolar RMS |
| **ERMS** | Embryonal RMS |
| **FFS** | Failure Free Survival |
| **OS** | Overall Survival |

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**Table 1**

Studies describing clinical correlates of PAX3- and/or PAX7-FOXO1 translocations among patients with RMS

| | | | Clinical Characteristics Assessed | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Study** | **Total N, by histology** | **Total N, by translocation status** | **Age** | **Gender** | **Primary Site** | **Tumor Size** | **Metastatic Disease** | **IRS Group** | **Survival Status** | **Worse Prognostic Marker** | **Main Findings** |
| Kelly (1997) *Ref* #15 | 27 ARMS 3 ERMS 4 Other | 18 PAX3 16 PAX7 0 F− | √ | √ | √ | | √ | √ | √ | *PAX3-FOXO1* | Four-yr EFS for patients with *PAX3-FOXO1* was 17% v. 43% for those with *PAX7-FOXO1* (p=0.04). Patients with *PAX3-FOXO1* translocation tended to be older (median 13 v. 6 yrs, p=0.01), have primary extremity lesions (82% v. 22%, p=0.001) or metastatic disease (44% v. 19%, p=0.03). |
| Anderson (2001) *Ref* #16 | 38 ARMS 43 ERMS 10 OTHER | 37 PAX3 8 PAX7 46 F− | √ | √ | √ | | √ | | √ | *PAX3-FOXO1* | Four-yr EFS for patients with *PAX3-FOXO1* was inferior (20% v. 70%, p<0.0001).Patients with *PAX3-FOXO1* translocation tended to be older (median age 9 v. 3 yrs, p=0.001), have higher stage disease (63% v. 38% stage 3-4, p=0.006). |
| Sorensen (2002) *Ref* # 7 | 78 ARMS 69 ERMS 24 Other | 43 PAX3 17 PAX7 111 F− | | | | | √ | | √ | *PAX3-FOXO1* (only if metastatic disease) | *PAX3-FOXO1* and *PAX7-FOXO1* fusion transcripts were detected in 55% and 22% of alveolar RMS patients, respectively; 23% were fusion-negative. All other RMS patients lack transcripts. Fusion status was not associated with outcome differences in ARMS patients with localized disease; however, among those with metastatic disease, *PAX3-FOXO1* was associated with inferior overall survival (8% v. 75%, p=0.0015). Fusion negative status suggested an intermediate risk: relative risk of relapse 11.5 (95% CI 5.1-25.6) for *PAX3-FOXO1*, 4.9 (1.4-17.3) for F− and 1.3 (0.2-10.2) for PAX7 patients, respectively. |
| Barr (2006) *Ref* #17 | 59 ARMS | 35 PAX3 11 PAX7 13 F− | √ | √ | √ | √ | √ | √ | √ | Neither | No differences in FFS between groups; however, *patients who had available fusion data for analysis had superior 5-yr FFS (75% v. 45%, p=-.0015).* Neither translocation was associated with FFS or OS in multivariate models. |
| Williamson (2010) *Ref* #8 | 133 ARMS 77 ERMS | 94 F+ 39F− | √ | √ | √ | √ | √ | | √ | Any F+ | ERMS patients were not assessed for fusion status. ARMS F+ patients had inferior 5-yr EFS compared to ARMS F− and ERMS patients (20% v. 60% v. |

| | | | Clinical Characteristics Assessed | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Study** | **Total N, by histology** | **Total N, by translocation status** | **Age** | **Gender** | **Primary Site** | **Tumor Size** | **Metastatic Disease** | **IRS Group** | **Survival Status** | **Worse Prognostic Marker** | **Main Findings** |
| | | | | | | | | | | | 55%, respectively, p<0.001). The relative risk of death for F+ RMS patients was 2.5 after adjustment for stage and histology (95% CI 1.2-5.1). ARMS F+ patients were more likely to have unfavorable sites of disease (79% v. 53% v. 57%, respectively, p=0.002) and metastatic disease (43% v. 8% v. 12%, respectively, p<0.001). |
| Stegmaier (2011) *Ref* #18 | 121 ARMS | 72 PAX3<br>29 PAX7<br>20 F− | √ | √ | √ | √ | √ | √ | √ | Neither | Patients with *PAX3-FOXO1* translocation tended to be older than those with *PAX7-FOXO1* (63% v. 17% older than 10 yrs, p=0.0001) and have higher rates of metastatic disease (50% v. 24%, p=0.017). There was no difference in EFS between patients in the two groups. 5-yr EFS for *PAX3-FOXO1* 3 was 38.9% v. 18.2% for *PAX7-FOXO1* (p=0.235). *Compared to non-analyzed, historical controls, localized patients who had fusion data for analysis had inferior EFS (29% v. 51%, p=0.009), regardless of fusion status.* |
| Dumont (2011) *Ref* #9 | 31 ARMS<br>62 ERMS<br>12 Other | 14 PAX3<br>4 PAX7<br>34 F− | | | | | √ | | √ | Neither | Fifty-two percent of samples identified any fusion status (+ or −). Patients with ARMS and either translocation were more likely to have metastatic disease (39%) compared to fusion-negative ARMS or ERMS patients (both 22%, p=0.0081). No associations were detected between fusion transcripts and survival. |

RMS: Rhabdomyosarcoma; IRS Group: Intergroup Rhabdomyosarcoma Study Group post-surgical stage; ARMS: Alveolar RMS; ERMS: Embryonal RMS; F−: Fusion-negative patients; EFS: Event-Free Survival; F+: Fusion-positive (either PAX3- or PAX7-FOXO1)