Published in final edited form as:

J Cancer Res Clin Oncol. 2012 February ; 138(2): 213–220. doi:10.1007/s00432-011-1089-7.

PAX3/7–FOXO1 fusion status in older rhabdomyosarcoma patient population by fluorescent in situ hybridization

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Abstract

Purpose—In pediatric alveolar rhabdomyosarcoma, the *PAX3–FOXO1* and *PAX7–FOXO1* gene fusions are prognostic indicators, while little is known concerning this disease in older patients. To determine whether *PAX3/7–FOXO1* fusion gene status correlates with outcome in adolescent, young adult, and adult rhabdomyosarcoma patients, the histological, immunohistochemical, and clinical characteristics of 105 patients followed at The University of Texas MD Anderson Cancer Center from 1957 to 2001 were evaluated.

Methods—The samples were assembled into a tissue microarray, and fusion gene status was determined by fluorescence in situ hybridization using *PAX3*, *PAX7*, and *FOXO1* loci-specific probes. The disease characteristics and specific gene fusion were correlated with patient outcomes using the log-rank test.

Results—Fifty-two percent of the samples exhibited a *PAX3–FOXO1* fusion, 15% the *PAX7–FOXO1* fusion, and 33% were negative for a rearrangement of these loci. The presence of *PAX3/7–FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. Although a statistically significant correlation between the *PAX3/7–FOXO1* fusion gene status and overall survival was not identified, there was a trend toward better outcomes for patients with fusion-negative RMS.

Conclusions—Therefore, identification of a FOXO1 fusion appears to be an interesting tool for predicting outcomes in older rhabdomyosarcoma patients and is worth further investigations in this rare subgroup of RMS population.

Keywords

Rhabdomyosarcoma; Chromosomal rearrangement; *PAX-FOXO1*; Fluorescent in situ hybridization; Tissue microarray

Introduction

Rhabdomyosarcoma (RMS) is a rare, highly malignant mesenchymal neoplasm. While it is the most common soft tissue sarcoma in children, RMS incidence declines with age, representing less than 0.1% of adult malignancies and about 10% of all adult soft tissue sarcomas in the United States (Parham and Ellison 2006; Jemal et al. 2006). Moreover, the Intergroup Rhabdomyosarcoma Study Group (IRSG) observed a relationship between age and survival in RMS patients (Raney et al. 2001; Maurer et al. 1988; Maurer et al. 1993; Crist et al. 1995; Crist et al. 2001). The 5-year overall survival for adult patients with primary disease is a dismal 20–40% (Little et al. 2002), whereas in children, it is between 60 and 80% (Crist et al. 1995). In adult patients with metastatic disease, 5-year survival is less than 5% (Esnaola et al. 2001; Ferrari et al. 2003; Hawkins et al. 2001).

RMS is divided into different subtypes according to the histologic features. The main subtypes are alveolar RMS (ARMS), embryonal RMS (ERMS), and pleomorphic RMS. The specific histologic subtype is correlated with survival in both children and adults. Classically, ERMS has a better prognosis than either ARMS or pleomorphic RMS (Little et al. 2002; Raney et al. 2010). A recent study demonstrated that ERMS and pleomorphic RMS likely share a same continuum of disease regarding mutational profile (Rubin et al. 2011).

Because of overlapping morphologic features, particularly with ERMS and solid pattern ARMS, molecular analysis is often used as a complementary diagnostic tool (Kohashi et al. 2008; Asmar et al. 1994; Morotti et al. 2006). ERMS is the most common RMS subtype in adults and is characterized by a loss of heterozygosity in 11p15.5 (Scrabble et al. 1989). In contrast, most ARMS are characterized by chromosomal translocations *t*(2;13)(q35;q14), resulting in the *PAX3-FOXO1* fusion protein, or chromosomes 1 and 13, *t*(1;13)(p36;q14), resulting in the *PAX7-FOXO1* fusion (Sorensen et al. 2002; Davis et al. 1994). Furthermore, pediatric patients with fusion-positive (involving either *PAX3* or *PAX7*) ARMS have shorter overall survival than those with fusion-negative ARMS. Lastly, in metastatic pediatric patients, a translocation involving *PAX3* is associated with shorter overall survival than a translocation involving *PAX7* (4-year overall survival: 75% for *PAX7-FOXO1* vs. 8% for *PAX3-FOXO1*; *P* = 0.0015) (Sorensen et al. 2002). Interestingly, according to the European Pediatric Soft Tissue Sarcoma Study Group (EpSSG), the patient outcomes and gene expression signatures of fusion-negative ARMSs are very similar to those of ERMSs (Williamson et al. 2010). Because the IRSG recommendations indicate that the pathology subtype may influence the aggressiveness of the treatment choice according to risk stratification guidelines (Raney et al. 2001), one of which is the *PAX3/7-FOXO1* translocation in ARMSs, the translocation status is commonly assessed for pediatric patients with RMS (Barr et al. 2006).

The diagnosis and treatment strategy for Adolescent and Young Adult (AYA) as well as adult patients with RMS is challenging (Bleyer 2005; Van Gaal et al. 2011) and often emulated from the childhood RMS guidelines (Miettinen 1988). Indeed, only 27% of patients in IRSG III and IV studies were 10 years old or older. Attempts to justify this

extrapolation frequently cite the unfortunate lack of specific studies for AYA and adults with RMS (Sultan et al. 2009). This leads to several concerns regarding the appropriate management for this patient population. First, there are no data to support the use of the same diagnostic, prognostic, and treatment approaches for these two distinct age-related subgroups, with what may also be distinct biological and clinical disease entities. Second, *PAX3/7-FOXO1* fusion status has not been shown to be an important clinical or biological factor in adult patients; rather, there are limited data on translocation testing in this population and its clinical significance.

To determine whether *PAX3/7-FOXO1* fusion gene status is associated with outcomes in AYA and adult RMS patients, a large panel of specimens of older patients followed at our institution was characterized by fluorescence in situ hybridization (FISH) and these findings were correlated with specific clinicopathologic parameters.

Methods

Patient samples

One hundred and five formalin-fixed, paraffin-embedded tissue samples from a database of 251 patients with RMS followed at The University of Texas MD Anderson Cancer Center between 1957 and 2001 were available for histological, immunohistochemical, and clinical evaluation. Slides of tumor-tissue samples were stained with hematoxylin and eosin at our institution to confirm the diagnosis. Demographic and clinical data were abstracted from the patient records.

Histopathologic analysis

The formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with RMS were assembled into a TMA. Viable tumor was selected according to morphologic features and formatted as two 0.6 mm-diameter tissue cores into a standard 45 × 20 mm recipient TMA paraffin block using a stainless steel stylet (Beecher Instruments, Silver Spring, MD). Finally, 4-μm-thick sections were mounted on poly-L lysine-coated slides.

Fluorescent in situ hybridization

To assess the presence of *PAX3*, *PAX7*, and *FOXO1* rearrangements, break-apart probes for *PAX3*, *PAX7*, and *FOXO1* were employed as previously described (Bridge et al. 2000). Briefly, each probe was labeled by nick translation with either Spectrum-green or Spectrum-orange-deoxyuridine triphosphate which allowed the visualization of two colors by FISH (Fig. 1). Fused or split red and green signals indicate, respectively, absence or presence of *PAX* and *FOXO1* rearrangements.

Statistical analysis

Survival data were retrieved from patient records, and overall survival was measured as the time from diagnosis until death or the date of last contact (censored). The histologic type and specific gene fusion were correlated with patient outcomes using the log-rank test, and comparisons of metastasis frequency and gene fusions were made using the Chi-square test. Prism software was used to generate the Kaplan–Meier curves.

Results

Patients and tumor characteristics

Of the 105 patients included in our study, 85% were older than 10 years at diagnosis, with a median age of 19 years (range 0.3–102 years; Table 1). The alveolar subtype was identified

in 37% of cases, the embryonal subtype in 52% of cases, and the pleomorphic subtype in 11% of cases. Only one specimen remained unclassified. The majority of tumors were localized, with the head and neck being the most common location. The median overall survival was determined for patients with ARMS (26 months), ERMS (31 months), and pleomorphic RMS (18 months) (Fig. 2a). We analyzed separately patients with primary versus meta-static disease. Although the ERMS histology seemed to have a superior overall survival, we found no statistically significant difference in overall survival among the alveolar, embryonal, and pleomorphic subtypes (Fig. 2b, c).

Identification of *PAX3/7-FOXO1* translocations in formalin-fixed paraffin-embedded tumor specimens by tissue microarray

We next determined whether the *PAX7-FOXO1* or *PAX3-FOXO1* translocations could be detected by FISH in the TMA of samples from patients with RMS. From the two analyzed TMA slides, twenty-one (20%) samples were depleted or presented no tumor on the tissue microarray. We were able to assess the result of the FISH experiments in 52 (63%) of the 83 adequate samples. The probes did not hybridize to 31 (37%) of the samples. The *PAX3-FOXO1* fusion was found in 26% of these 52 cases, the *PAX7-FOXO1* fusion in 8%, and no fusion in 65% (Table 2). Among the histologically defined ARMS specimens, 18 (67%) had a gene fusion, mostly *PAX3-FOXO1* (52%). No fusion was detected in the pleomorphic subtype.

Outcomes of patients with fusion-positive RMS

We compared the group of patients with *PAX3/7-FOXO1* fusion-positive RMS to those with fusion-negative RMS. We found that the patients with fusion-positive RMS showed a trend toward worse survival than those patients whose tumors were fusion-negative, although this trend was not statistically significant, even in disease extension subgroups (Fig. 3). When we compared the overall survival of patients with RMS by specific translocation, *PAX3-FOXO1* versus *PAX7-FOXO1*, we found that the type of fusion was not correlated with survival, even when stratified by presence or absence of metastases, but the number of *PAX7-FOXO1* cases was low (Fig. 4).

Patients with ERMS and fusion-negative ARMS have similar outcomes

To determine how fusion-positive ARMSs behave clinically, we compared the overall survival of patients with ERMS, fusion-positive ARMS, and fusion-negative ARMS tumors. We found no significant differences in overall survival between these 3 groups (Fig. 5). However, there was a trend toward shorter survival for those patients with a translocation, but this trend did not reach statistical significance ($P = 0.15$), possibly due to the small numbers. The fusion-negative ARMS and the ERMS groups showed similar outcomes, with several long-term survivors (Fig. 5a, b).

Metastatic disease frequency in fusion-positive patients

To evaluate the effect of *PAX3/7-FOXO1* translocations on risk of metastasis, we calculated the percentage of patients with metastatic disease at diagnosis for patients with fusion-positive, fusion-negative ARMS and ERMS. There was a significant increase in rate of metastatic disease for patients with fusion-positive ARMS (39%), compared with those whose tumor did not have a translocation ($P = 0.0081$, $\chi^2 = 9.6$; 2 degrees of freedom). ERMS and fusion-negative ARMS appeared similar in matter of metastatic disease frequency (22%; Fig. 6).

Discussion

RMS is a rare entity with limited data regarding molecular classification and prognosis for patients over 10 years old (Wolden and Alektiar 2010). Due to the sustained work of the IRSG within the past decade, treatment of childhood RMS is clearly defined according to risk stratification. Both histology subtype and fusion status are important criteria for classifying patients. IRSG guidelines recommend that RMS patients be followed at a specialized center and that the pathology should include the determination of the *PAX3/7-FOXO1* fusion status (Raney et al. 2001; Maurer et al. 1988; Maurer et al. 1993; Crist et al. 1995; Crist et al. 2001).

The present work studied the relevance of the fusion assessment in older patient population including a majority of AYA and adult RMS patients. We found that the presence of *PAX3/7-FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. Additionally, we found that patients with fusion-negative ARMS trended toward better outcomes than those with fusion-positive ARMS. Hence, we found that patients with fusion-negative ARMS tended to have overall survival times that were similar to those of patients with ERMS, which is in accordance with the recent EpSSG conclusions relative to childhood RMS that fusion-negative ERMS is clinically similar to fusion-negative ARMS (Williamson et al. 2010). The type of translocation *PAX3-FOXO1* or *PAX7-FOXO1* did not show any prognostic significance, even in the metastatic disease group.

Due to the rarity of RMS, we had to evaluate patients who had been followed from 1957 to 2001 and only 105 patients fit our criteria. The small number of patients clearly impacted the statistical significance of our analyses. Moreover, the age of the tissue samples appeared to affect the FISH technique resulting, which explains why we were unable to analyze close to one-third of the specimens (Chang et al. 2009). Ideally, less than 2–5 years after fixation in formalin and paraffin embedding is the threshold for a successful FISH analysis; in routine clinical practice, most will be tested within days to weeks of fixation. The majority of our samples were older than this suggested guideline. Performing FISH on a TMA is also technically more demanding than using whole sections from a single case. Regarding a failure rate lower than 30% in these unfavorable specimen conditions, FISH analysis is a good option for identifying the *PAX3/7-FOXO1* fusion in FFPE.

Our study emphasizes both the difficulties with retrospective molecular studies of this rare disease and the need of specific studies regarding the relevance of *PAX3/7-FOXO1* fusion assessment in AYA and adult RMS as a prognostic factor.

Acknowledgments

Institutional Physician-Scientist award (J.C.T.), NIH/NCI 1K23CA109060-05 (J.C.T.), Amschwand Sarcoma Cancer Foundation grant (J.C.T.), Nuovo-Soldati Foundation grant (S.N.D.), AstraZeneca France (S.N.D.) and Association pour la Recherche sur le Cancer (S.N.D.). This research is also supported in part by the National Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672.

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Fig. 1. Representative FISH analysis of the *PAX3/7-FOXO1* fusion **a** FISH analysis with the *FOXO1* break-apart probe set demonstrates split red and green signals indicative of a *FOXO1* rearrangement. **b** The fused red/green signals are consistent with no *FOXO1* rearrangement. **c** FISH analysis using *PAX3-FOXO1* dual spanning probe set shows fused red/green signals indicative of a *PAX3-FOXO1* fusion. **d** FISH analysis with a *PAX7* break-apart probe showing split red and green signals and amplification (common with *PAX7* rearrangements)

Fig. 2. Kaplan–Meier overall survival estimates according to histologic classification

Fig. 3. Kaplan–Meier overall survival estimates for ARMS patients according to PAX3/7–FOXO1 fusion status

Fig. 4. Kaplan–Meier overall survival estimates for ARMS patients with *PAX3–FOXO1* or *PAX7–FOXO1* fusion

Fig. 5. Kaplan–Meier overall survival estimates according to ARMS PAX3/7–FOXO1 fusion status and ERMS

Fig. 6. Frequency of patients with metastatic disease. More patients with fusion-positive ARMS had metastatic disease than those with fusion-negative ARMS or ERMS ($P = 0.0081$)

Table 1
Clinical characteristics of patients with RMS

Characteristics	No. of pts (%)
<i>Age</i>	
10	16 (15)
>10	89 (85)
<i>Sex</i>	
Female	44 (42)
Male	61 (58)
<i>Histology</i>	
ERMS	62 (59)
ARMS	31 (30)
Pleomorphic	11 (10)
Unclassified	1 (1)
<i>Primary site</i>	
Head and neck	47 (45)
Genitourinary	11 (10)
Extremities	23 (22)
Other	24 (23)
<i>Invasion</i>	
Yes	64 (61)
No	35 (33)
Unknown	6 (6)
<i>Size</i>	
5 cm	56 (53)
<5 cm	42 (40)
Unknown	7 (7)
<i>Nodal involvement</i>	
Regional	10 (10)
No	95 (90)
<i>Metastatic</i>	
Yes	31 (30)
No	74 (70)

Table 2

FISH results by histologic subtype

Histology diagnosis	Patient number	Number of FISH analyzed tumor samples	Fusion-negative <i>n</i> (%)	<i>PAX3-FOXP1</i> <i>n</i> (%)	<i>PAX7-FOXP1</i> <i>n</i> (%)
ARMS	39	27	9 (33%)	14 (52%)	4 (15%)
ERMS	54	20	20 (100%)	0 (0%)	0 (0%)
Pleomorphic	11	5	5 (100%)	0 (0%)	0 (0%)
Total	104	52	34	14	4