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ORIGINAL PAPER

Rhabdomyosarcoma Subtyping by Immunohistochemical Assessment of Myogenin: Tissue Array Study and Review of the Literature

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Abstract Myogenin immunostaining has been described as a useful marker of the alveolar subtype of rhabdomyosarcoma and as a tool for distinguishing it from the more common embryonal subtype. To add to the growing body of literature describing this phenomenon we analysed myogenin immunohistochemical staining in 152 tumors using a rhabdomyosarcoma tissue array. Results were analysed blinded to histological type by two independent investigators. Samples were excluded if any samples failed to stain with desmin and/or myogenin. Mean percentage of myogenin positive cells was significantly greater for ARMS (n=31; mean percentage positivity 59% (95% confidence intervals ± 7%) than ERMS (n=41, mean percentage positivity 16%, 95% confidence intervals ± 4; P<0.0001). This data is consistent with previously published studies identifying strong nuclear myogenin staining in a high proportion of cells as a marker of alveolar histology.

Keywords Rhabdomyosarcoma · Myogenin · Tissue array · Diagnosis

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Introduction

In common with many childhood solid tumors, most cases of rhabdomyosarcoma (RMS) are diagnosed on the basis of morphological and immunohistochemical appearances of small amounts of tissue obtained by biopsy. Several tumor types have a ‘small round blue cell’ appearance and the use of a panel of immunohistochemical markers is now well established in order to distinguish RMS from other tumor types (including lymphoma, neuroblastoma and peripheral primitive neuroectodermal tumor).

Beyond establishing a diagnosis of RMS, it is also important for the pathologist to be able accurately to distinguish its two major different histological subtypes. The majority (approximately 75%) are embryonal rhabdomyosarcoma (ERMS) composed of primitive round, stellate or spindle cells in a myxoid background with botryoid, spindle cell and pleomorphic variants. Alveolar rhabdomyosarcoma (ARMS) classically demonstrates loose aggregates of large, round cells separated by fibrous septae; a solid variant is also recognised. The distinction between ERMS and ARMS is of considerable clinical importance, since the latter confers a worse prognosis. Current treatment regimes for RMS stratify cases into a number of prognostic groups based on factors including histological subtype. However, there may be difficulties in accurately distinguishing subtypes based on morphological examination alone, especially in a limited tissue sample such as a core biopsy. There is therefore growing interest in finding and utilising subtype-specific markers.

Amongst such possible markers are gene fusion products that are specific to ARMS. PAX3-FOXO1A and PAX7-FOXO1A arise from translocations between chromosome 2 (PAX3) or 1 (PAX7) and chromosome 13. These novel fusion genes act as transcription factors but have a higher

potency than the wild type protein and appear to contribute to oncogenesis. PAX fusion transcripts are considered specific for the alveolar subtype and their detection by reverse transcription-polymerase chain reaction, or by fluorescence in situ hybridization applied to biopsies of fine needle aspiration samples, provides a potential means of accurately distinguishing between the two subtypes although about 10% of ARMS cases are fusion negative. [1–4] Such molecular genetic techniques are not, however, routinely available in all laboratories and in practice an immunohistochemical marker would be a more widely applicable and clinically useful diagnostic tool. A potential candidate immunomarker is myogenin, [5] one of a number of transcription factors that regulate the differentiation of skeletal muscle. In the current study, we examined a large series of RMS samples in order to assess the value of myogenin staining in the distinguishing the alveolar from embryonal subtype.

Materials and Methods

The tissue microarray comprising replicate 0.6 mm diameter cores (duplicate or triplicate) from rhabdomyosarcoma tumor blocks of formalin fixed paraffin embedded tissues has been described before. [6] There were 425 cores per slide. (Fig. 1) Data regarding the PAX3-FOXO1A and PAX7-FOXO1A fusion status of the ARMS tumors in the array was provided by Dr. Beat Schaefer in Zurich. Adjacent sections from the array were stained with anti-myogenin (Santa Cruz, CA, USA) and anti desmin (Dako clone D33) antibodies using standard techniques. Briefly tissue sections were microwave heated (20 min in pH 6.0 0.1 M citrate buffer) before staining. Sections were immunostained using the LSAB kit (Dako). All steps were performed at room temperature, and signals were visualised using a diaminobenzidine substrate (Dako).

The tissue arrays were examined and scored by two independent observers who were blinded to the predetermined histological subtype. For each tissue core, positivity for myogenin was determined on the basis of discrete nuclear staining and a semi-quantitative assessment was made of the percentage of positive cells (estimated as <10%, 10–20%, 20–30%, etc). Desmin immunostaining was used as a positive control and assessed in a similar way. In all but a few cases, the tissue arrays included multiple tissue cores from the same tumor biopsy specimen. The results from the two observers for each tissue core were combined to provide a mean percentage myogenin positivity for each tumor specimen. Tissue samples had been collected, fixed and prepared in a number of different ways in different centres prior to incorporation in the tissue array and hence cores were excluded from the analysis if either desmin or myogenin immunostaining were completely negative, or if the results obtained by the two observers varied by more than 30%. Tumor specimens were excluded from analysis only if all relevant cores for that specimen had been excluded. Comparison of the percentage of myogenin positive tumor cells between groups was performed using analysis of variance test.

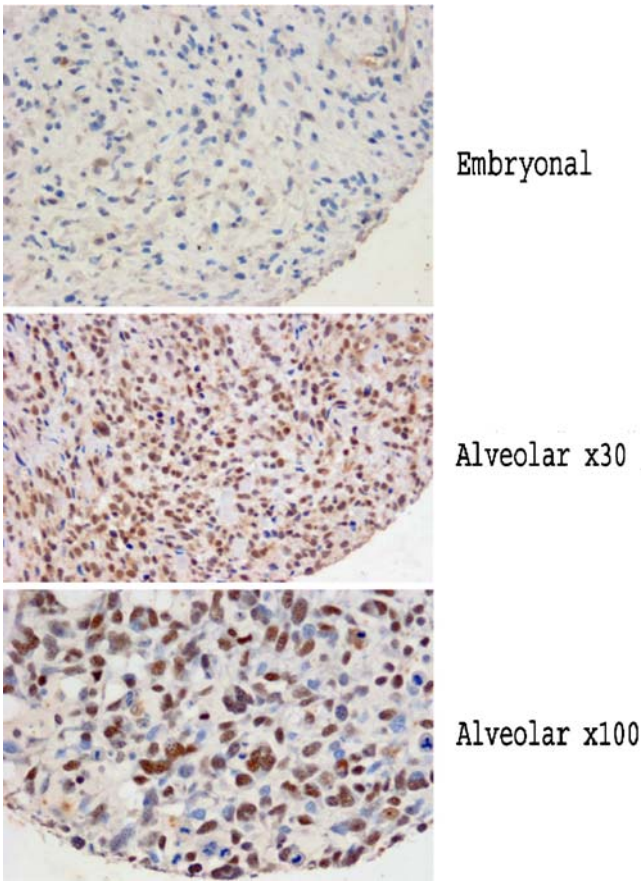

Fig. 1 Representative images of tissue array sections of alveolar and embryonal rhabdomyosarcoma stained with the myogenin antibody

Results

A total of 425 tissue cores were examined, representing 152 tumor biopsy specimens (50 ARMS and 102 ERMS). Of the 50 ARMS tumor specimens, 19 were excluded (38%) and 31 included in the final analysis; for the ERMS specimens, 61 were excluded (60%) and 41 included in the final analysis. For the 72 tumor specimens that formed part of the final analysis, myogenin positivity was determined as the mean of the values given by both observers for all tissue cores relating to that specimen. Typical myogenin staining of ERMS and ARMS is shown in Fig. 1.

Of note, although approximately equal percentages of ARMS and ERMS specimens were excluded due to negative immunostaining for desmin (36% and 38% respectively, NS by chi-square test), this was not the case for myogenin immunostaining (10% and 35% respectively, p=0.001 by chi-square test). This difference is reflected in the significantly larger percentage of the ERMS specimens which were excluded compared to ARMS. It seems likely therefore that at least some of the cases in which myogenin immunostaining was completely absent represent truly negative staining in this small number of samples. All such cases were excluded from further analysis, since it was not possible to distinguish true negative from false negative myogenin staining.

Mean percentage of myogenin positive cells was significantly greater for ARMS (n=31; mean percentage positivity 59% (95% confidence intervals ± 7%) than ERMS (n=41, mean percentage positivity 16%, 95% confidence intervals ± 4; P<0.0001; Fig. 2). Amongst the ARMS cases there was no significant difference in myogenin percent positivity between PAX3-FOXO1A positive (n=18), PAX7-FOXO1A positive (n=15), and fusion negative (n=11) cases. Comparison of the distribution of myogenin positivity between the two histological subtypes indicates two overlapping distributions, with the majority of ERMS samples showing little myogenin positivity; whilst the majority of ARMS samples show myogenin immunostaining in 60–80% of cells (Fig. 3). Although the distributions of positivity are clearly different between subtypes, a number of ARMS samples demonstrated little myogenin positivity and one (of 41) ERMS specimens showed myogenin staining in more than 50% of cells.

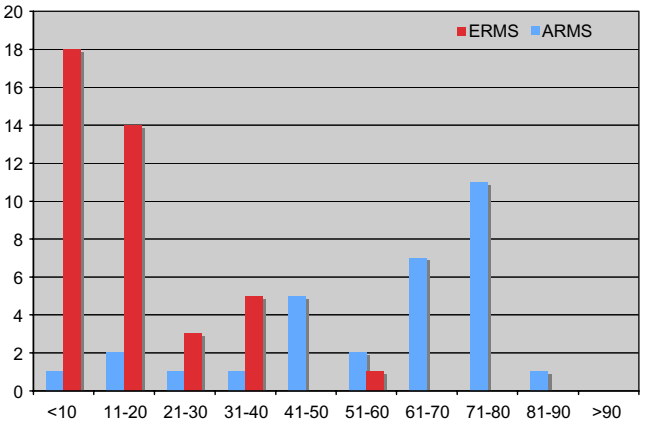

Fig. 3 Distribution of mean percentage of tumor cells demonstrating nuclear myogenin positivity in alveolar (*ARMS*) and embryonal (*ERMS*) rhabdomyosarcoma. There is significantly increased nuclear expression of myogenin in ARMS, but the distributions overlap

Discussion

The findings of this study have confirmed that both ERMS and ARMS express nuclear myogenin detectable by immunohistochemical staining, and that this expression is significantly greater in ARMS. However, the extent of staining, in terms of proportion of tumor cells, demonstrates overlapping distributions between the subtypes.

It has previously been demonstrated that myogenin is sensitive and relatively specific immunomarker for the diagnosis of rhabdomyosarcoma. Examining 150 tissue samples, Cessna et al. [7] demonstrated myogenin expression in all of 32 cases of RMS, but in no other soft tissue tumors (including nodular fasciitis, malignant fibrous

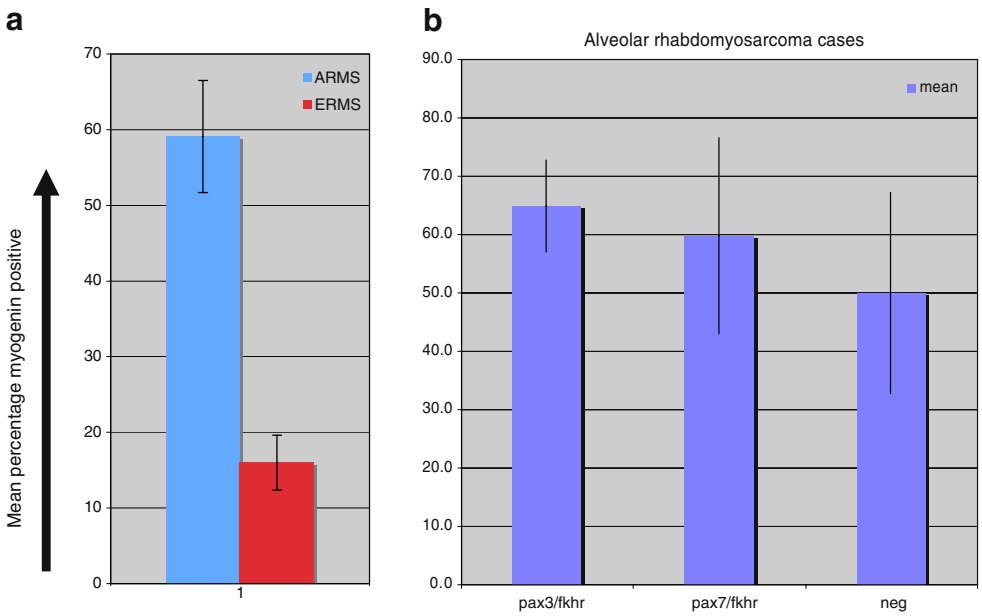

Fig. 2 **a** Mean percentage myogenin positivity, in cases of alveolar (*ARMS*) and embryonal (*ERMS*) rhabdomyosarcoma demonstrating significantly increased nuclear expression of myogenin in ARMS (*error bars*=95% Confidence intervals). **b** Mean percentage positivity for myogenin staining in ARMS according to fusion status PAX3-FOXO1A n=18, PAX7-FOXO1A n=15, fusion negative n=11

histiocytoma, malignant nerve sheath tumor, inflammatory myofibroblastic tumor, myofibrosarcoma, leiomyoma, leiomyosarcoma or alveolar soft part sarcoma), although regenerating, non-neoplastic muscle fibres trapped within other tissue specimens did show occasional myogenin positivity. In an examination of small round blue cell tumors of childhood, Wang et al. [8] confirmed myogenin expression in 30 of 33 RMS specimens, but in none of the Ewing's sarcoma or neuroblastoma samples. Myogenin expression is occasionally seen in rare non-RMS tumors with rhabdomyoblastic differentiation, so although strongly associated with a diagnosis of RMS, cannot of itself be considered pathognomonic. [9]

In the present study, myogenin expression was examined in a series of 425 cores representing 152 RMS tissue samples. Previous studies [10, 11] have reported desmin and myogenin positivity in 100% of RMS samples. By contrast, a rather large number of the cores in the present study (267, 63%) showed no staining for either desmin or myogenin. The reasons for this apparent discordancy include technical issues regarding immunohistochemical staining in these cases, which may have been initially collected, fixed and processed in different ways. Since it was impossible to control for these outside factors, we chose to exclude samples that showed no staining for desmin or myogenin from further analysis. Interestingly, there was a significant difference between ARMS and ERMS in the proportion of samples excluded due to lack of myogenin staining. It is likely, therefore, that some of these cases represent samples which are either truly negative for myogenin, or the small size of the cores included in the tissue array prevented detection of occasional myogenin positive cells that would have been seen had a larger sample size been analysed. As the results presented here and published elsewhere clearly show, myogenin staining is considerably more marked in alveolar than embryonal RMS. In the present study, the exclusion of samples which were entirely myogenin negative, and which were disproportionately ERMS rather than ARMS, would have the effect of reducing any demonstrated difference in myogenin positivity between the two histological subtypes, thus our results may slightly underestimate its true utility in clinical practice.

The finding that ARMS show a significantly greater proportion of myogenin positive nuclear staining than ERMS is consistent with a number of previously published investigations, [6,10–13] summarised in Table 1. The distributions of percentage myogenin positivity for ARMS and EMRS are clearly different but there appears to be an overlap between the two populations; with one of the ERMS specimens showing uncharacteristically high myogenin positivity. Previously published studies have also demonstrated a small population of ERMS with relatively high (greater than 50%) myogenin positivity. However, in a number of cases these samples have been shown to express the PAX-FOXO1A fusion gene that is characteristic of ARMS. Hostein et al. [11] 11 for example, tested 20 cases that had been labelled morphologically as ERMS and had more than 50% myogenin positivity for the expression of fusion genes. Five of 15 informative cases from these 20 were positive for fusion gene expression; whilst no informative ERMS specimens with <50% myogenin positivity tested positive for fusion genes. Similarly, Dias et al. [12] found strong (>50%) myogenin staining in two of

Table 1 Combined data from 5 published studies and the current study on percentage myogenin positivity in embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcoma

Study	Percentage (%)	ARMS	ERMS	Sensitivity	Specificity	+LR	-LR	OR (95% CI)
Dias et al. [12]	>50	9	2	1.00	0.88	8.50	0.00	32.3 (6.5–161)
	<50	0	15					
Kumar et al. [10]	>50	46	0	0.96	1.00	-	0.04	74.8 (23.7–226)
	<50	2	20					
Hostein et al. [11]	>50	45	27	1.00	0.58	2.37	0.00	12.9 (5.8–28.7)
	<50	0	37					
Wachtel et al. [6]	>50	37	26	0.76	0.83	4.36	0.30	14.5 (7.27–29.0)
	<50	12	124					
Morotti et al. [13]	>50	72	42	072	0.58	1.70	0.49	3.33 (1.90–5.83)
	<50	28	57					
This study	>50	21	1	0.68	0.98	28	0.33	20.8 (7.6–56.9)
	<50	10	40					
Combined results	>50	230	98	0.82	0.75	3.25	0.25	9.56 (7.04–13.0)
	<50	52	293					

Sensitivity, specificity and positive and negative likelihood ratios are based on a binary definition of positive and negative tests based on a 50% stain cut off. OR calculated using http://www.hutchon.net/ConfidORnulhypo.htm; Sens/spec/LR calculated using http://www.pennmush.org/cgi-bin/testcalc.pl

17 ERMS specimens, one of which was subsequently found to express the PAX3-FOXO1A fusion gene. Thus, at least a proportion of the cases of apparent ERMS with high myogenin positivity previously reported may represent alveolar RMS with atypical morphology. We did not have data available on the PAX-FOXO1A status of the tumor samples in our study. Of note, there was no apparent correlation between fusion protein status and percent myogenin positivity in the ARMS cases although the numbers of cases with full data was rather small. For five of the 6 previously published papers it was possible to extract data on the proportion of ARMS and ERMS cases with greater or less than 50% nuclear myogenin positivity. These results are summarised in Table 1 (The Cessna study [7] is excluded from this meta-analysis since myogenin expression was categorised only as <30%, 30–70% or >70% and it was therefore not possible to extract data for the 50% cut-off.). Overall, the results are broadly similar and combining the data (from a total of 673 tumors) confirms the clear difference in myogenin expression and its utility as a test to distinguish the two histological subtypes. Two studies [6, 11] reported a rather high number of ERMS samples with high (>50%) myogenin expression but some of these were subsequently reclassified as ARMS on the basis of their expression of PAX-FOXO1A fusion genes.

Having confirmed a significant difference in the distribution of myogenin immunostaining between the groups, it is important to consider how this might be used practically to differentiate alveolar from embryonal RMS in the clinical laboratory setting. Using a cut-off of 50% positivity to differentiate 'high' from 'low' myogenin staining allows the continuous variable of estimated myogenin positivity to be converted to a more practical test with a binary outcome. The data from the present study indicate that such a test has a sensitivity of 0.68 and specificity of 0.98 for distinguishing ARMS from ERMS. The positive likelihood ratio (+LR) is 28. The baseline prevalence of ARMS is approximately 15%, [14] thus following a positive test (i.e. >50% cells positive for myogenin) the probability of ARMS rises to 83%. Conversely, the negative likelihood ratio of 0.33 means that following a negative test (i.e. fewer than 50% of cells myogenin positive), the probability of ARMS falls to 6%.

Distinguishing alveolar from embryonal RMS in clinical practice may benefit from the use of more than one immunohistochemical marker. The recent study by Wachtel et al. [6] examined the expression of four molecular markers that previous analyses had indicated were differentially expressed by ARMS and ERMS. Using Immunohistochemistry, they demonstrated that high levels of expression of P-Cadherin and AP2ß are specific to (translocation-positive) ARMS, whilst expression of epidermal growth factor receptor and Fibrillin-2 is specific to ERMS. By combining two markers, they were able to identify ARMS and ERMS with greater specificity than using myogenin alone, and myogenin may form only one of a larger panel of immunohistochemical markers used in this setting. It will be important, however, to validate any technique used in routine practice to ensure that those tumors labelled as ARMS continue to represent a relevant subgroup with a poorer prognosis and a need for more aggressive chemotherapy. Ultimately, widespread use of molecular markers will both define subgroups and allow a more directed targeting of chemotherapy.

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