Dense Pattern of Embryonal Rhabdomyosarcoma, a Lesion Easily Confused With Alveolar Rhabdomyosarcoma

A Report From the Soft Tissue Sarcoma Committee of the Children's Oncology Group

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Key Words: Alveolar rhabdomyosarcoma; Embryonal rhabdomyosarcoma; Histologic classification; Rhabdomyosarcoma; Myogenin

DOI: 10.1309/AJCPA1WN7ARPCMKQ

Abstract

Objectives: *To examine whether the frequency of fusion-negative alveolar rhabdomyosarcoma (ARMSn) increased coincident with changes in the definition of alveolar histology.*

Methods: *We re-reviewed alveolar rhabdomyosarcoma (ARMS) in the Children's Oncology Group study D9803, comparing histopathology with fusion status.*

Results: *Our review of 255 original ARMS cases (compared with a control group of 38 embryonal rhabdomyosarcomas [ERMS] cases) revealed that many had an ARMS-like densely cellular pattern with cytologic features and myogenin expression more typical of ERMS. Following re-review, 84 (33%) cases of original ARMS were rediagnosed as ERMS. All reclassified ERMS, including dense ERMS, were fusion negative, whereas 82% of confirmed ARMS cases were fusion positive. Total ARMS diagnoses returned to historic rates of 25% to 30% of all rhabdomyosarcomas, and ARMSn decreased from 37% to 18% of ARMS cases. The outcome of reclassified ERMS was similar to confirmed ERMS.*

Conclusions: *To address the role of fusion status in risk stratification, pathologists should include both a histologic diagnosis and an evaluation of fusion status for all new ARMS diagnoses.*

Pediatric rhabdomyosarcoma (RMS)[1] traditionally has been classified by histologic appearance into 2 major subtypes, alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS). Stage- and group-matched ARMS typically behaves more aggressively than does ERMS.[2-4] The International Classification of Rhabdomyosarcoma (ICR),[5] published in 1995, divided RMS into 4 histologic types comprising 3 prognostic groups: botryoid and spindle cell variants of ERMS associated with superior prognosis, conventional ERMS associated with intermediate prognosis, and ARMS associated with poor prognosis. As defined by the ICR, ARMS includes both classic and solid-variant tumors; moreover, the presence of any alveolar histology (classic or solid variant) is sufficient for classification of a tumor as ARMS. The ICR criteria represent a significant departure from those used in prior Intergroup Rhabdomyosarcoma Studies (IRS) I to III, in which classic alveolar histology defined ARMS, and the presence of more than 50% alveolar histology was required for classification of a tumor with varying histologies as ARMS.

Subsequent to adoption of the ICR, the Children's Oncology Group (COG) Soft Tissue Sarcoma (STS) Committee observed an increase in both the number of cases classified as ARMS and the frequency of fusion-negative ARMS (ARMSn) in the Intergroup Rhabdomyosarcoma Study Group (IRSG) and its successor COG RMS studies. From 1999 through 2005, the COG intermediate-risk rhabdomyosarcoma study D9803 enrolled 616 patients with diagnoses of both ARMS and ERMS.[6] Although application of the ICR improved the recognition of the solid variant of ARMS, total ARMS diagnoses on central pathology review increased from 30% to 41% during the D9803 enrollment period. In addition, the frequency of ARMSn doubled, increasing from 20% to

25% of ARMS in IRS III and IV studies to 40% to 45% of ARMS in later studies.[5,7,8]

Although the expanded criteria were expected to result in an increase in ARMS, the observed upward drift, particularly in the frequency of ARMSn, called into question the application of the ICR criteria by COG central pathology reviewers. Importantly, COG central pathology review did not routinely or consistently incorporate the results of myogenin staining into the classification of IRSG and COG RMS cases. Strong, diffuse myogenin expression by immunoperoxidase stains has been shown to correlate with alveolar histology and poor outcome.[9-11] These observations prompted our reexamination of criteria for the diagnosis of ARMS and the role of myogenin in classifying RMS. Our initial re-review of a subset of ARMSn diagnosed during this period demonstrated that up to 61% of these cases were misclassified.[12] ARMSn comprised a morphologically heterogeneous group. Particularly notable was a dense pattern of ERMS mimicking the solid variant of ARMS.

As a result of our preliminary study, we hypothesized that a similar reduction of ARMS would occur in a controlled population of patients treated in a single clinical trial. Therefore, we re-reviewed all ARMS patients enrolled in a single well-defined and uniform treatment group (D9803). We herein report the features of this group, particularly focusing on the dense variant of ERMS that resembles ARMS.

Materials and Methods

Pathology Review

In total, 277 patients with a diagnosis of ARMS were enrolled in the D9803 study.[6] Diagnostic pathology material archived at the Biopathology Center in Columbus, Ohio, was available for re-review from 255 (92%) patients. Of these, 213 had original central review diagnoses, whereas the remaining 42 had institutional histologic classification. In the latter cases, material was not available for central review at enrollment but was submitted at a later time. A control group of 38 randomly selected patients with an original diagnosis of ERMS was also reviewed. In total, we re-reviewed the histopathology of 293 of the 616 patients enrolled in D9803. Available material included H&E slides and select immunohistochemical stains (including myogenin for 250 of 293 cases).

Two pathologists (E.R.R. and D.M.P.) conducted the re-review. Reviewers were not in complete agreement but reached a consensus diagnosis for all cases. Cases were classified as ARMS, mixed RMS, or ERMS. Patterns of ARMS included classic and solid variants. ERMS patterns included botryoid, spindled, dense, sclerosing,[13,14] epithelioid,[15] and typical variants **Appendix 1** and **Image 1**. A diagnosis of RMS–not otherwise specified (NOS) was made if the biopsy sample was too small, crushed, or necrotic for a definitive classification.

Nuclear myogenin expression was scored from 0 to 4+ based on the following percentages of tumor cells : 0 (absent expression), 1+ (<10%), 2+ (10%-50%), 3+ (>50%-90%), and 4+ (>90%). A diagnosis of ARMS (classic or solid variants) was supported by strong, diffuse (3 or 4+) myogenin expression.[9-11] In the 43 cases lacking myogenin stains, a diagnosis of ARMS was based on typical histologic and cytologic features.

Determining Fusion Status

For 119 cases, we used a quantitative reverse transcription–polymerase chain reaction (RT-PCR) assay to assess expression of a PAX3-FOXO1 (P3F) or PAX7-FOXO1 (P7F) fusion transcript from formalin-fixed, paraffin-embedded material as described previously.[7] Following reverse transcription from a FOXO1-specific primer, the assay incorporated consensus 5¢ PAX3/PAX7 and 3¢ FOXO1 primers and gene-specific PAX3 and PAX7 probes, thus determining both the presence and subtype of the fusion. In a second reaction, expression of the GAPDH gene was quantified to assess the quality of the RNA. A GAPDH expression level equivalent to that found in 0.5 ng of a control RMS cell line was the minimum cutoff for a satisfactory result in a sample with a negative fusion result.

For 128 cases, a fluorescence in situ hybridization (FISH) strategy was employed to detect rearrangements of FOXO1 (13q14), PAX3 (2q35), and PAX7 (1p36) loci on representative formalin-fixed, paraffin-embedded tissue sections. Studies were conducted using a FOXO1 dual-color break-apart probe (Abbott Molecular, Des Plaines, IL), per slight modification of the manufacturer's instructions, and PAX3 (2q35) and PAX7 (1p36) custom-designed break-apart probes as previously described.[16] Hybridization signals were assessed in interphase nuclei with strong, well-delineated signals and distinct nuclear borders. A cell specimen was interpreted as abnormal if a split of flanking probe signals was detected in more than 10% of the cells evaluated (more than 2 standard deviations above the average false-positive rate). Seventy-two cases were examined by RT-PCR and FISH with concordance for all cases in which satisfactory material was available for both tests.

Statistics

Event-free survival (EFS) was defined as the time from study enrollment to the first occurrence of progression, relapse after response, or death from any cause. Overall survival (OS) was defined as the time from study enrollment to death from any cause. Patients not experiencing an event were censored at their last follow-up time. Estimates of time-to-event distributions were calculated using the Kaplan-Meier method, and distributions were compared using log-rank tests.

DOI: 10.1309/AJCPA1WN7ARPCMKQ

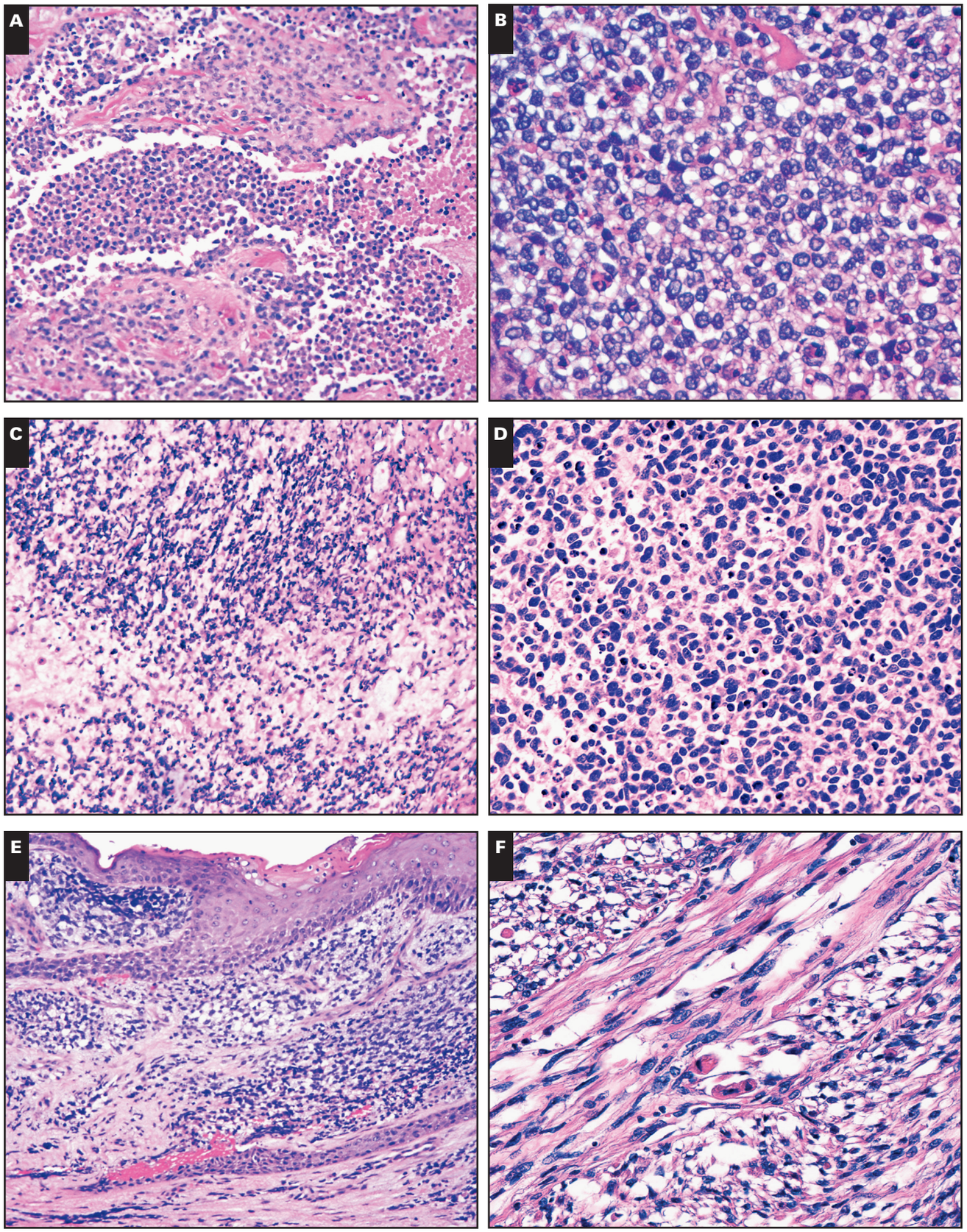
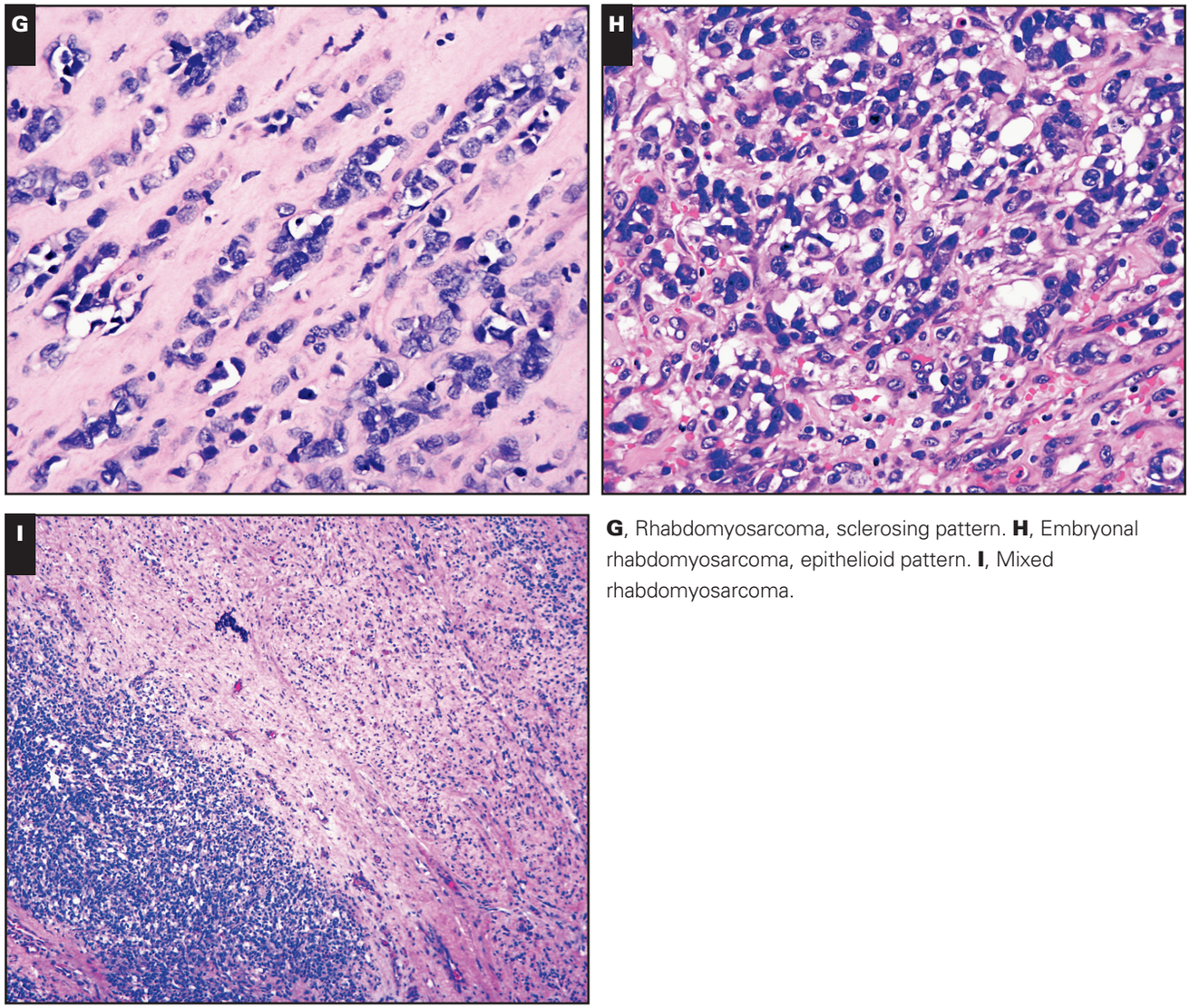


Image 1 Rhabdomyosarcoma histologies (see Appendix 1 for a description). **A**, Alveolar rhabdomyosarcoma, classic pattern. **B**, Alveolar rhabdomyosarcoma, solid pattern. **C**, Embryonal rhabdomyosarcoma, typical pattern. **D**, Embryonal rhabdomyosarcoma, dense pattern. **E**, Embryonal rhabdomyosarcoma, botryoid pattern. **F**, Embryonal rhabdomyosarcoma, spindle cell pattern.

DOI: 10.1309/AJCPA1WN7ARPCMKQ



G, Rhabdomyosarcoma, sclerosing pattern. **H**, Embryonal rhabdomyosarcoma, epithelioid pattern. **I**, Mixed rhabdomyosarcoma.

Results

Re-review of D9803 Cases Previously Classified as ARMS

Of the 255 cases originally classified as ARMS, 84 (33%) were classified as ERMS on re-review **Table 1**. A diagnosis of ARMS was confirmed in 148 cases (58%), whereas 18 (7%) were reclassified as mixed RMS and 5 (2%) were called RMS-NOS.

Cases reclassified as ERMS showed heterogeneous histology. Botryoid morphology was predominant in 3 of 84 (5%) cases of ERMS, and typical ERMS morphology accounted for 5 of 84 (6%) cases. A dense pattern of ERMS was the most commonly reclassified group and the primary reason for review discrepancy. Fifty-five percent (46/84) of reclassified ERMS had uniformly dense cellularity superficially resembling solid ARMS; however, their angulated nuclei, variably prominent nucleoli, and moderate (2-3+) myogenin expression were more consistent with a revised diagnosis of ERMS **Image 2**. An additional 17 of 84 (20%) cases had a partial or complete sclerosing pattern with weak (1-2+) myogenin expression.[16,17] Ten of 84 (12%) ERMS cases exhibited codominant patterns with a combination of sclerosing/dense or spindled/sclerosing patterns. Finally, 2 of 84 (2%) cases were reclassified as

Table 1
Re-review Diagnoses for Original ARMS Cases Enrolled in Study D9803

Method of Prior Histologic Diagnosis	Re-review Diagnosis, No. (%)			
	Alveolar	Embryonal	Mixed	NOS
Central path review (n = 213)	126 (59)	68 (32)	15 (7)	4 (2)
Institution (n = 42)	22 (52)	16 (38)	3 (7)	1 (2)
Total (n = 255)	148 (58)	84 (33)	18 (7)	5 (2)

ARMS, alveolar rhabdomyosarcoma; NOS, not otherwise specified.

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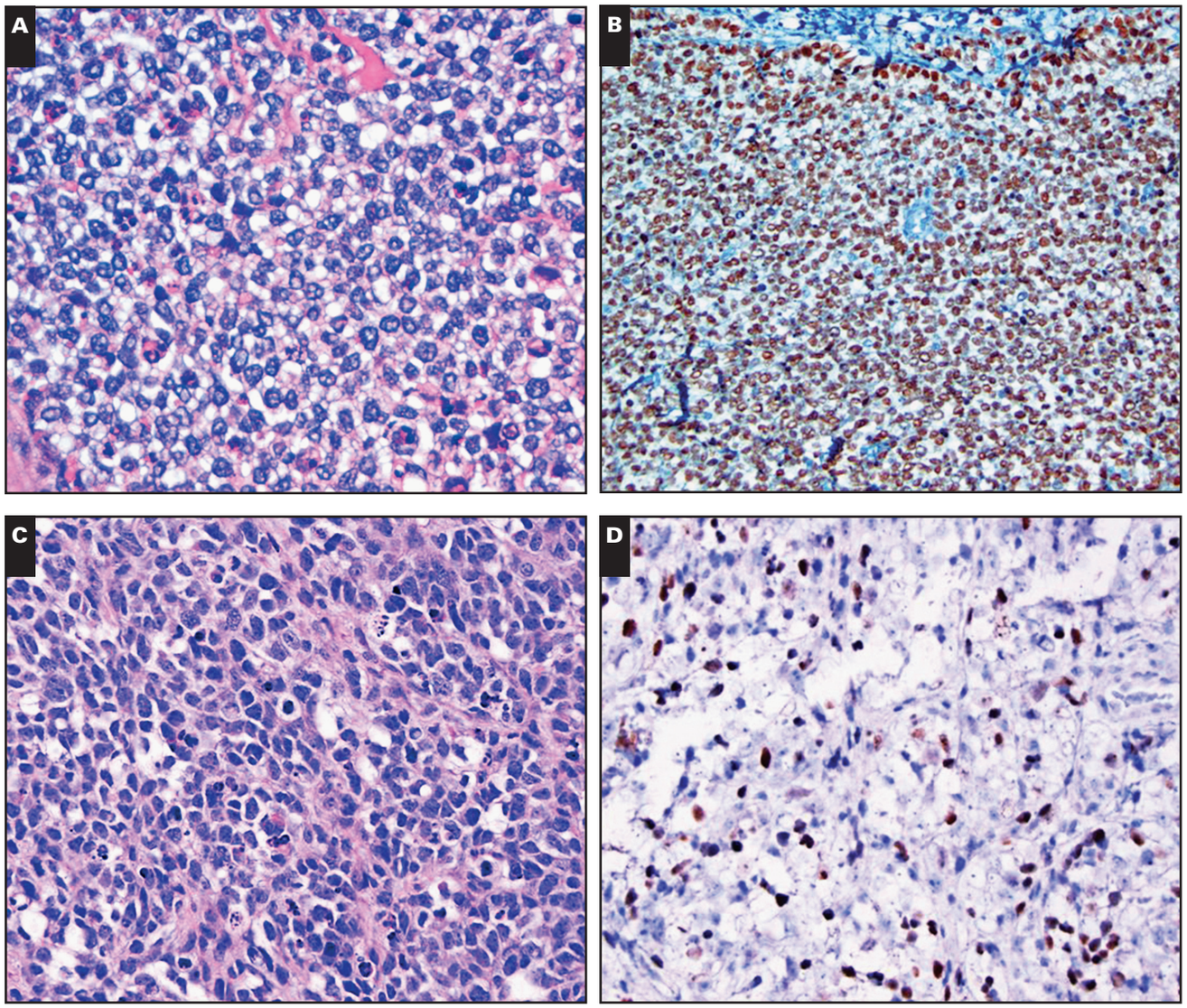

Image 2 **A**, The solid pattern of alveolar rhabdomyosarcoma (ARMS) shows monomorphic round cell cytology with vesicular chromatin and inconspicuous nucleoli (H&E, ×400). **B**, Myogenin expression is strong and diffuse in solid-variant ARMS (×200). **C**, The dense pattern of embryonal rhabdomyosarcoma (ERMS) shows uniformly dense cellularity superficially resembling solid ARMS; however, the angulated nuclei and variably prominent nucleoli allow diagnosis of ERMS (H&E, ×400). **D**, Dense ERMS shows moderate myogenin expression (×400).

spindle cell RMS, and 1 (1%) was reclassified as epithelioid RMS18 **Figure 1**.

Of control ERMS cases, 25 of 38 (66%) showed typical histology and 4 of 38 (11%) showed botryoid morphology. Only 6 of 38 (16%) had a dense pattern, 1 of 38 (3%) had a sclerosing pattern, and 2 of 38 (5%) had a combination of spindled/sclerosing or dense/sclerosing patterns.

Histology and Primary Site

When categorized by primary tumor site, genitourinary (GU) tract tumors not arising from the bladder or prostate and perineum, retroperitoneum, and trunk tumors were overrepresented among reclassified ERMS **Table 2**. Dense ERMS showed no obvious association with sites of higher reclassification, however. In fact, the GU non–bladder/prostate site showed the highest rates of reclassification but the lowest incidence of dense ERMS. Most GU non–bladder/prostate primary tumors had a focal or diffuse sclerosing pattern.

Histology and Fusion Status

Fusion data were available for 173 cases **Table 3** but not for 23 re-review cases. Fifty-seven (34%) original ARMS cases classified as ERMS or ARMS on re-review were fusion negative. After our second review, 83 (66%) ARMS cases

DOI: 10.1309/AJCPA1WN7ARPCMKQ

showed a P3F fusion, and 20 (16%) confirmed ARMS cases showed a P7F translocation. In the final analysis, only 23 (18%) confirmed ARMS cases were fusion negative. Importantly, no reclassified ERMS case, including all dense variants, contained a gene fusion.

Myogenin Expression by Histology and Fusion Status

Myogenin expression data were available for 231 ARMS and ERMS cases. The distribution of myogenin scores for 71 of 84 reclassified ERMS cases was as follows: 6%, 0+ expression; 24%, 1+ expression; 59%, 2+ expression; and 13%, 3+ expression. None had 4+ expression. Myogenin stains were not available for evaluation for 13 reclassified ERMS cases. This distribution was similar for the 34 original ERMS cases with myogenin stains: none showed 0+ expression, 41% had 1+ expression, 44% had 2+ expression, and 15% had 3+ expression. None had 4+ expression. In total, 87% of original and reclassified ERMS cases showed 0 to 2+ myogenin expression, whereas only 5% of ARMS cases had 0 to 2+ myogenin expression **Table 4**. Cases with 3+ myogenin expression were nearly equally divided between ARMS and ERMS. The majority (79%) of ARMS cases showed 4+ myogenin expression, but no original or reclassified ERMS cases showed 4+ myogenin expression. For the 54 dense ERMS cases, 50% (27/54) showed 2+ myogenin expression, 17% (9/54) showed 1+ myogenin expression, and 19% (10/54) showed 3+ myogenin expression. Myogenin stains were not available for the remaining 8 dense ERMS cases.

Fusion status was available for a subset of 126 ARMS cases with myogenin expression data (Table 4). The fraction of ARMSn increased with decreased myogenin expression: 13% of ARMS cases with 4+ myogenin expression, 30% of ARMS cases with 3+ myogenin expression, and all ARMS cases with 2+ myogenin expression were fusion negative. Weak (0-1+) myogenin expression was found in a single ARMS case that harbored evidence of gene fusion; however, the weak staining appeared to be secondary to poor staining quality. Five of the 20 ARMS cases with 3+ myogenin expression and 15 of 99 ARMS cases with 4+ myogenin expression had unknown fusion status.

Outcome of Reclassified ERMS

Analysis of outcome for D9803 cases was restricted to patients with stage 2/3, group III disease since these patients were eligible for the D9803 study irrespective of histology subtype. The estimated 5-year EFS was 77% (95% confidence interval [CI], 71%-82%) for original and confirmed ERMS (n = 238), 69% (95% CI, 50%-82%) for reclassified ERMS (n = 34), and 55% (95% CI, 44%-64%) for confirmed ARMS (n = 88) (P < .001) **Figure 2A**. The estimated 5-year OS was 81% (95% CI, 75%-85%) for original and confirmed ERMS, 81% (95% CI, 63%-91%) for reclassified ERMS, and 68% (95%

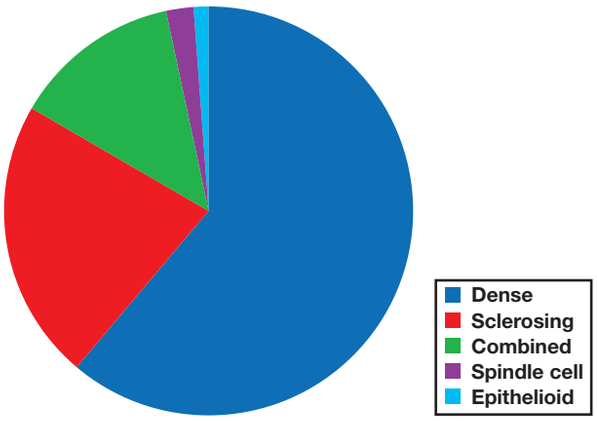

Figure 1 Distribution of histologic patterns in reclassified embryonal rhabdomyosarcoma. Combined histology indicates tumors with codominant patterns, including sclerosing/dense or sclerosing/spindled patterns.

Table 2
Percentage of Reclassified and Dense ERMS by Primary Site

Primary Site	No. (%) of Reclassified ERMS Among Previous ARMS	No. (%) of Dense ERMS Among Reclassified ERMS
Parameningeal	16/65 (25)	10/16 (63)
Extremity	14/62 (23)	9/14 (64)
Orbit	6/15 (40)	3/6 (50)
Head and neck	8/24 (33)	5/8 (63)
GU, bladder/prostate	3/9 (33)	2/3 (66)
GU, non–bladder/prostate	18/29 (62)	8/18 (44)
Retroperitoneum	6/13 (46)	6/6 (100)
Perineum, trunk	6/18 (33)	1/6 (16)
Other	2/10 (20)	2/2 (100)

ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; GU, genitourinary.

Table 3
Fusion Status of Original Alveolar Rhabdomyosarcoma by Re-review Diagnosis

Re-review Diagnosis	Fusion Status, No.		
	Negative	*PAX3*	*PAX7*
Embryonal (n = 34)	34	0	0
Alveolar (n = 126)	23	83	20

Table 4
Myogenin Expression vs Histology and Fusion Status in Re-reviewed Rhabdomyosarcoma

Myogenin Score	ERMS Total, No. (%)	ARMS Total, No. (%)	Fusion Status in ARMS-Negative *PAX3*/*PAX7*, No.		
0	4 (4)	0			
1+	30 (29)	3 (2)	2	0	1
2+	57 (54)	4 (3)	4	0	0
3+	14 (13)	20 (16)	5	11	0
4+	0	99 (79)	11	58	16

ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma.

DOI: 10.1309/AJCPA1WN7ARPCMKQ

CI, 57%-77%) for confirmed ARMS (P = .018) **Figure 2B**. The outcome for patients with ARMS was poorer than that for those with ERMS, and cases reclassified from ARMS to ERMS had an outcome more similar to ERMS than ARMS.

Discussion

The histologic features of classic ARMS as described by Riopelle and Theriault[17,18] are well recognized and usually allow distinction from ERMS, including the botryoid and spindle cell subtypes. In contrast, the distinction between solid-variant ARMS, described by Tsokos et al[3] in 1992, and dense ERMS[5] is, in our experience, more challenging. The solid variant is recognized in the ICR by the following statement: "The 'solid' variant of alveolar RMS grows as solid masses of closely aggregated cells, with no or scarcely discernible alveolar arrangement."[5] In contrast to solid-variant ARMS, dense ERMS is characterized by variation in cellular and nuclear size and shape within a tumor; however, this feature may be subtle. In the ICR, this pattern is recognized by the following statement: "The histologic pattern of embryonal RMS is predominantly that of a moderately cellular tumor with loose myxoid stroma, although some dense areas may occur frequently. Some tumors may consist exclusively of fields of closely packed cells."[5] Reflecting this diagnostic difficulty, our re-review of ARMS in the D9803 study resulted in the reclassification of 33% of "original" ARMS cases as ERMS.

Alveolar rhabdomyosarcomas lacking P3F or P7F gene fusions comprise a heterogeneous group consisting of tumors with alternate fusions, fusions without production of detectable RNA, tumors with only rare fusion-positive cells, and those with no molecular evidence of fusion.[19,20] The latter category constitutes the bulk of this group, and it is likely that a significant portion of these cases is accounted for by misclassification of histologic variants of ERMS. Following our re-review of D9803 ARMS cases, the number of ARMSn decreased from 37% prior to reclassification to 18% following reclassification, approximating historical rates of 22% to 23% ARMSn in previous IRSG studies.[7]

In part, this drift in fusion status reflects differences in the criteria used to classify tumors as ARMS.[5] In IRS III, tumors composed of more than 50% ARMS were classified as ARMS, whereas in IRS IV and recent COG protocols, the presence of any ARMS was sufficient for classification as ARMS. This definition for classification of tumors as ARMS persisted until February 2007, when COG protocols were amended to require at least 50% ARMS. Another confounding factor was recognition and inclusion of the solid variant in the definition of ARMS.[3,5] Although this resulted in appropriate classification of a group of tumors without classical alveolar histology but with similar biological behavior and cytogenetics, it also led to erroneous inclusion of some dense ERMS. In a previous report, we found that solid alveolar tumors were more likely to be fusion negative.[21] In the present study, the majority of fusion-negative tumors reclassified as ERMS showed a dense pattern, either focally or diffusely, that was originally interpreted as solid-variant ARMS.

Our results further confirm that the use of myogenin aids in the classification of RMS, as suggested by earlier studies.[9-11] ARMSs, including the solid variant, typically show strong, robust, homogeneous expression with myogenin immunoperoxidase stains, as compared with the relatively weak to heterogeneous pattern seen with ERMS. Even with myogenin immunohistochemistry, the most diagnostically difficult category remains the dense pattern of ERMS, as it typically shows moderate (2-3+) myogenin expression, overlapping that of solid ARMS. For this group, cytology remains the only distinguishing morphologic feature, as suggested by Meza et al.[2]

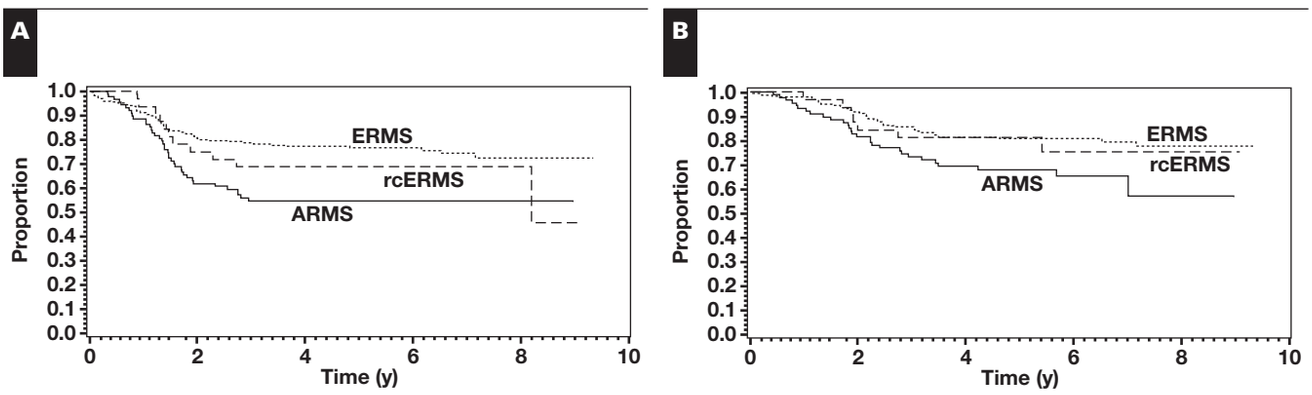

Figure 2 Event-free survival (*P* < .001) (**A**) and overall survival (*P* = .018) (**B**) of stage 2/3, group III reclassified embryonal rhabdomyosarcoma (rcERMS) cases vs original and confirmed alveolar rhabdomyosarcoma (ARMS) and ERMS cases enrolled in the D9803 study.

DOI: 10.1309/AJCPA1WN7ARPCMKQ

Recognizing the importance of myogenin expression and histologic variants of ERMS improves our classification of RMS, but myogenin immunohistochemistry remains imperfect as a single test. Reclassification of D9803 cases using current histologic criteria and myogenin expression suggests that the distribution of RMS subtypes in the D9803 intermediate-risk group should be 26% ARMS and 62% ERMS, with 3% mixed RMS, 6% RMS-NOS, and 3% undifferentiated sarcoma. These data suggest that some cases previously classified as ARMSn may be reclassified as ERMS, and drift in the classification of ARMS, at least in IRS IV and COG studies prior to the February 2007 amendment, accounts for some of the biological similarities between ARMSn and ERMS in previous expression array studies.[22,23]

Although a large proportion of RMS cases can be accurately classified by histology alone, the distinction between tumors such as solid-variant ARMS and dense ERMS may be particularly difficult in the absence of genetic testing, which may not be available in some settings. Studies are thus ongoing to evaluate additional immunohistochemical markers that further enhance prognostication of RMS without the need for molecular analysis. To address the role of fusion status in risk stratification, pathologists should include both a histologic diagnosis and an evaluation of fusion status for all new ARMS diagnoses.

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Research support was provided by research grant 179772 from St Baldrick's Foundation, Monrovia, CA (E.R.R.); NIH-RC2-CA148216 (S.X.S., J.M., J.A.B., F.G.B., and J.M.G.-F.), NIH-R01-CA104896 (F.G.B., J.M.G.-F.), NIH-U10-CA098412 (J.R.A.), and NIH-U10-CA98543 (COG Chair's grant) from the National Institutes of Health, Bethesda, MD; and grants (F.G.B.) and U-10-CA98543-07 (J.A.B.) from the Joanna McAfee Childhood Cancer Foundation, Warner Robins, GA.

Portions of this article were presented at the Society for Pediatric Pathology Interim Meeting; September 30, 2011; Milwaukee, WI.

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Appendix 1

Working Definitions of the Histologic Variants of Rhabdomyosarcoma

Alveolar rhabdomyosarcoma, classic pattern: Tumor cells contain small amounts of eosinophilic cytoplasm and round, uniform nuclei with multiple or inconspicuous nucleoli. The cells are arranged in nests separated by delicate fibrous septae. Floating islands of tumor cells (groups of dyshesive tumor cells within the center of the nests and separated from the periphery) are often present (Image 1A).

Alveolar rhabdomyosarcoma, solid pattern: Monomorphic cells with round nuclei arranged in sheets, without intervening fibrous septae. Cytologic features are identical to the classic pattern, and nucleoli are inconspicuous (Image 1B).

Embryonal rhabdomyosarcoma, typical pattern: Alternating regions of loose, myxoid mesenchymal-appearing tissues and densely cellular regions. Cellular regions may contain primitive mesenchymal cells or show rhabdomyoblasts in varying stages of differentiation (Image 1C).

Embryonal rhabdomyosarcoma, dense pattern: Sheets of primitive cells predominate. These primitive cells are typically stellate with scant cytoplasm and central, ovoid, or angulated nuclei. Occasional tumors may have a prominent central nucleolus. This pattern typically lacks evidence of myogenic differentiation (Image 1D).

Embryonal rhabdomyosarcoma, botryoid pattern: Linear aggregates of tumor cells cluster beneath an epithelial surface (ie, cambium layer) (Image 1E).

Embryonal rhabdomyosarcoma, spindle cell pattern: Elongate spindled cells with oval, blunted central nuclei arranged in whorls or fascicles, often resembling smooth muscle (Image 1F).

Rhabdomyosarcoma, sclerosing pattern: Primitive round to ovoid cells with scant cytoplasm are separated by abundant hyalinized stroma that may resemble osteoid or cartilage. The cells may be arranged in small cords or nests ("microalveoli") that resemble classic alveolar rhabdomyosarcoma (ARMS). Floating islands of tumor cells are rare, however. Classic immunophenotypic findings of strong (4+) MyoD1 and weak (1-2+) myogenin are helpful in distinguishing sclerosing rhabdomyosarcoma from classic ARMS (Image 1G).

Embryonal rhabdomyosarcoma, epithelioid pattern: Sheets of cells with eccentric, round nuclei and an abundant eosinophilic cytoplasm. Also referred to as a "rhabdoid" pattern of rhabdomyosarcoma (Image 1H).

Mixed rhabdomyosarcoma: Separate, discrete regions of alveolar and embryonal histologies of any histologic pattern. These tumors resemble nested or collision tumors, with the various patterns showing differential myogenin expression (Image 1I).

Rhabdomyosarcoma, not otherwise specified: Reserved for cases in which the tumor sample is too small or crushed for definitive subclassification, but a diagnosis of rhabdomyosarcoma can be made by ancillary testing or obvious myogenesis.

DOI: 10.1309/AJCPA1WN7ARPCMKQ

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DOI: 10.1309/AJCPA1WN7ARPCMKQ

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