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Classification of Rhabdomyosarcoma and Its Molecular Basis

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Abstract

Rhabdomyosarcoma, the most common soft tissue sarcoma of children, has traditionally been classified into embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) for pediatric oncology practice. This review outlines the historical development of classification of childhood rhabdomyosarcoma and the challenges that have been associated with it, particularly problems with the diagnosis of “solid variant” ARMS and its distinction from ERMS. In addition to differences in clinical presentation and outcome, a number of genetic features underpin separation of ERMS from ARMS. Genetic differences associated with rhabdomyosarcoma subclassification include the presence of reciprocal translocations and their associated fusions in ARMS, amplification of genes in ARMS and its fusion subsets, chromosomal losses and gains that mostly occur in ERMS, and allelic losses and mutations usually associated with ERMS. Chimeric proteins encoded from the fusion of *PAX3* or *PAX7* with *FOXO1* are expressed by most ARMS, result in a distinct pattern of downstream protein expression, and appear to be the proximate cause of the bad outcome associated with this subtype. A sizeable minority of ARMS lacks these fusions and shares the clinical and biological features of ERMS. A battery of immunohistochemical tests may prove useful in separating ERMS from ARMS and fusion-positive ARMS from fusion-negative ARMS. Because of limitation of predicting outcome solely based on histological classification, treatment protocols will begin to utilize fusion testing for stratification of affected patients into low-, intermediate, and high-risk groups.

Keywords

rhabdomyosarcoma; classification systems; prognosis; genetic testing

INTRODUCTION

Rhabdomyosarcomas, primitive mesenchymal tumors that recapitulate the process of myogenesis, comprise the most common pediatric soft tissue sarcoma and one of the ten most common childhood malignancies. They arise from a variety of anatomic sites, not limited to skeletal muscle, and show correspondingly diverse clinical presentations, usually related to mass effect and/or obstruction. Overall, the survival of patients with

rhabdomyosarcomas has steadily improved during the past several decades, but a sizeable proportion still dies from advanced disease (1).

Because of the plethora of presentations and variable prognosis, prediction of rhabdomyosarcoma outcome has become a complicated undertaking. Besides adequacy of excision, presence of metastasis, site of origin, and age of the patients histological and genetic properties of the tumor also must be taken into account. A combination of these features serves to stratify rhabdomyosarcomas into low, intermediate, and high risk groups (2).

Rhabdomyosarcomas present a fascinating array of histological aspects that feature the cytological stages of myogenesis and the interaction of neoplastic rhabdomyoblasts with the adjacent connective tissue matrix (1). As with all neoplasms, the surgical pathologist must identify of cytological and histological features that best predict patient outcome. These features have been correlated with a wide variety of genetic abnormalities, with the overall goal of what best predicts patient outcome, guides risk-based therapy, and informs future clinical trials. The goal of this review is to present the history of rhabdomyosarcoma classification, our current understanding of the molecular genetics that underpin it, and the factors that are most informative for current and future risk stratification and therapy.

DIAGNOSIS OF RHABDOMYOSARCOMA

The histologic patterns and cytologic features of rhabdomyosarcomas range from undifferentiated small round blue cell neoplasms to lesions with advanced cytohistologic features reminiscent of rhabdomyoma (3). The *sine qua non* of rhabdomyosarcoma diagnosis is the identification of embryonic myogenesis. This property is usually apparent from standard histological preparations, ultrastructural examination, and/or immunohistochemical assays of myogenic markers such as desmin, muscle specific actin, myosin, or myoglobin. Nuclear transcription factors that initiate myogenesis, such as MyoD (myf3) or myogenin (myf4), have become popular immunohistochemical markers in recent years, as expression of these markers precede later correlates of myogenesis such as cytoplasmic striations, myosin-ribosome complexes, and markers of terminal differentiation such as myoglobin and muscle-specific actin (4, 5). However, these features do not distinguish rhabdomyosarcoma from other potentially myogenic neoplasms, such as Wilms tumor, carcinosarcoma, Sertoli-Leydig cell tumors, or malignant peripheral nerve sheath tumor.

HISTOLOGICAL CLASSIFICATION OF RHABDOMYOSARCOMA AND ITS CLINICAL CORRELATES

Horn-Enterline

Attempts to subclassify rhabdomyosarcoma (RMS) began soon after Riopelle and Theriault described alveolar rhabdomyosarcoma (6) in children and Stout reported pleomorphic rhabdomyosarcoma among adult extremity lesions (7). In 1958 (8), Horn and Enterline published an article proposing that rhabdomyosarcomas could be subdivided into embryonal, alveolar, botryoid, and pleomorphic types (Figure 1). Embryonal

rhabdomyosarcomas (ERMS), the most common subtype, comprised a range of histologies that contained variable degrees of embryonic rhabdomyogenesis (Figure 1A). A common theme was a variably “loose and dense” cellularity within a myxoid matrix. Alveolar rhabdomyosarcomas (ARMS) showed a vague resemblance to fetal alveoli as a result of intersecting fibrous septa with interseptal nesting and discohesion (Figure 1B). Botryoid rhabdomyosarcomas, also called “sarcoma botryoides”, formed grape-like polypoid masses partially lined by epithelium and showing heightened subepithelial cellularity (the “cambium layer”) (Figure 1C). Pleomorphic rhabdomyosarcomas contained intersecting bundles of large, variably myogenic spindle cells with hyperchromatic, irregular nuclei and prominent nucleoli (Figure 1D); the spindle cells were often arranged in storiform whorls resembling “malignant fibrous histiocytoma” (now called pleomorphic sarcoma).

The Horn Enterline system appeared to correlate with clinical presentation. ERMS arose in infants and young children and often involved the head and neck and urogenital tract. ARMS mostly occurred in adolescents and arose in extremities and parameningeal locations. BRMS, by definition, occurred in hollow viscera, usually in young children. PRMS arose from the skeletal muscle of the extremities and mainly affected adults. However, not all pathologists were believers: Stout and Lattes stated in their Armed Forces Institute of Pathology fascicle (9) that “the multiplicity of names seems unnecessary when it is necessary that all of these tumors are extremely malignant and fatal in the large majority of cases”.

Following formation of the Intergroup Rhabdomyosarcoma Study Group (IRSG; now the Soft Tissue Sarcoma Disease Committee of the Children’s Oncology Group), Newton et al (10) performed a univariate analysis of survival vs. RMS histology. This analysis indicated that ARMS patients did badly, BRMS patients did relatively well, and ERMS patients pursued an intermediate course. PRMS occurred so rarely that its existence in children was questioned, as Newton et al. uniformly identified foci of ERMS in the few tumors that otherwise resembled PRMS (10).

Gonzalez-Crussi and Black-Schaffer (11) still noted persistent problems in classification, as many tumors contained mixtures of histological patterns (Figure 1E). IRSG reviewers had similar problems, leading to the somewhat arbitrary decision that the diagnosis of ARMS required the presence of at least 50% alveolar foci in tumors containing histological features typical of other subtypes. This group was selected to receive more aggressive therapy in the third IRSG trial(12).

Palmer-IRSG

Armed with lessons learned from Beckwith and Palmer’s successful subclassification of pediatric renal tumors (13), Palmer et al. applied a completely new paradigm for IRSG rhabdomyosarcomas, based on nuclear and cytological features instead of histology. Palmer et al. (14–16) recognized two cytological types, monomorphous round cell and anaplastic, with an unfavorable prognosis and another cytology, “spindle type A” with a superior prognosis. The majority of the tumors showed other cytologic features that corresponded to an intermediate outcome. These studies resulted in a series of abstracts (14–16) on a “cytohistological classification”, but no published article ever appeared. However, Palmer’s

concepts were subsequently vetted by other publications. Tsokos et al (17) created a new category, “solid variant” ARMS, which had the monomorphous round cell cytology of classical ARMS but lacked fibrous septa (Figure 1F). Like the monomorphous round cell sarcoma of Palmer et al.’s classification, these tumors had an unfavorable outcome that overlapped the survival curve of classical ARMS. Conversely, Cavazzana et al (18) described the spindle cell rhabdomyosarcoma (SCRMS), which tended to arise in the paratesticular region, head, and neck and had a favorable outcome similar to Palmer et al’s “type A” cytology.

International Classification

Using a large cohort of tumors from the first and second IRSG study, Newton et al. (19) subsequently codified a new International Classification of Pediatric Sarcomas (ICPS) based on retrospective review by an international panel of experts. An initial study (20) tested the agreement of these pathologists with four systems, those described by Horn-Enterline, Palmer et al., Tsokos et al., and Caillaud et al. of the International Society of Pediatric Oncology (SIOP) (21). The results indicated that the Horn-Enterline system performed best and that Palmer et al’s results could not be reliably duplicated among a group of pathologists. It also verified the existence of the solid variant ARMS. Along with ERMS concepts formulated by SIOP, these results were incorporated into the ICPS system, a risk based classification (19), (Table 1). The ICPS also promulgated the concept that any focus of ARMS in a lesion was sufficient to make that diagnosis.

Additional modifications

Several modifications have been made to rhabdomyosarcoma subtyping subsequent to the ICPS. Mentzel and Katenkamp (22) and Folpe et al. (23) described a sclerosing RMS (SRMS) that contained abundant desmoplasia rather than delicate fibrous septa (Figure 1G). Within this collagenous milieu, nests of tumor cells formed micro-alveoli without the discohesion of typical ARMS, although ARMS was the initial diagnosis considered in these cases (23). Chiles et al (24) described a series of similar pediatric lesions from a subset of ARMS derived from IRSG and Children’s Oncology Group (COG) sources. The newest WHO classification for myogenic tumors, published in 2013 (25) (Table 2), made SCRMS and SRMS a newly separate subgroup, primarily based on experience with adult tumors. However, adult SCRMS behaves worse than ones in children (26, 27), and descriptions of pediatric SCRMS (18) indicate that foci of ERMS frequently occur.

Although anaplasia as defined by Palmer (15) was not added to the ICPS, several studies indicated its potential value in prognostication. Anaplasia was originally defined as the presence of enlarged hyperchromatic nuclei, three times larger than those of adjacent cells, and multipolar mitoses (Figure 1H), per the Beckwith-Palmer criteria for renal tumors (13). The requirement for multipolar mitoses was relaxed in subsequent studies (28, 29), and anaplasia was separated into focal and diffuse subtypes. Focal anaplasia indicated occasional, sporadic anaplastic cells, whereas diffuse anaplasia indicated clusters with clonal expansion. Univariate analyses showed that this feature had prognostic value in ERMS, but multivariate analysis failed to demonstrate that anaplasia was a significant independent prognostic marker (29), and it has never been used for risk assignment in therapeutic trials.

GENETIC STUDIES OF RMS

Cytogenetics

Karyotypic studies of the ARMS and ERMS subtypes revealed nonrandom chromosomal translocations that distinguish ARMS from ERMS and other solid tumors. The most common chromosomal translocation in ARMS is t(2;13)(q35;q14) (30–32), and a second less frequent variant translocation is t(1;13)(p36;q14) (33, 34). Based on the Mitelman Database of Chromosome Aberrations in Cancer (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>), the 2;13 and 1;13 translocations are present in 56% and 6%, respectively, of 99 ARMS cases. In contrast, these translocations were found in 3% and 0%, respectively, of 77 cases diagnosed as ERMS. Though there are no other recurrent structural chromosome alterations in these RMS subtypes, there are multiple numerical chromosome changes (gains and losses) that are generally more prevalent in ERMS than in ARMS. For example, gains of chromosome 2, 8, 12, and 13 are found in 25%–50% of ERMS karyotypes; the frequency of these chromosome gains in ERMS is higher than the corresponding gains in ARMS. Finally, evidence of genomic amplification, as manifest by the presence of double minute chromosomes or homogeneously staining regions, was found in 10–15% of ARMS and ERMS cases.

Comparative genomic hybridization analyses

The copy number changes in RMS tumors were further elucidated by comparative genomic hybridization (CGH) studies (35–37). A combined analysis of 43 ERMS cases found a high frequency (72%) of chromosome 8 gains as well as gains of chromosomes 2, 7, 11, 12, 13, and 20 in 25–50% of cases. In addition, loss of chromosomes 9 and 10 were found in 20–30% of cases. The overall frequency of chromosome gains and losses was lower in ARMS tumors. More recent DNA-based array studies have confirmed these chromosome gain and loss findings (38–40). The CGH studies also established that genomic amplification occurs more frequently in ARMS than ERMS. Genomic amplification was identified in 7 of 43 ERMS cases (16%) and often involves the 12q13-q15 region (35–37). In one CGH study, these amplified cases were further subdivided into 4 of 6 ERMS cases with anaplasia and 1 of 16 ERMS cases without anaplasia (35). In contrast to ERMS, one or more amplification events were identified in 47 of 84 ARMS tumors (56%) (35–37). The frequent amplification events in ARMS tumors were derived from the 1p36, 2p24, 12q13-q14, 13q14, and 13q31 chromosomal regions.

Microarray analysis of gene amplification

DNA-based microarray and in situ hybridization studies confirmed these amplification findings in ARMS and identified the minimal amplified regions for each amplicon. These studies showed that the 1p36 and 13q14 amplicons generally occur in the same cases and are indicative of amplification of the *PAX7-FOXO1* fusion gene (described below) (41). The targets of the 2p24 and 13q31 amplicons correspond to amplified oncogenes previously identified in other cancers (42, 43). The minimal amplified 2p24 region contains *MYCN*, which is also amplified in neuroblastoma. The minimal amplified 13q31 region contains *MIR17HG*, which encodes the miR-17–92 microRNA cluster and is also amplified in diffuse large B-cell lymphoma and small cell lung carcinoma. In contrast to the small number of

genes within these first four minimal amplified regions, the minimal 12q13-q14 amplicon encompasses 28 genes (42). This cluster of amplified genes includes the *CDK4* oncogene, which is amplified in multiple cancers, including glioblastoma, liposarcoma, and breast carcinoma.

Allelic loss studies

Although ERMS tumors do not contain recurrent rearrangements, they demonstrate frequent allelic loss, which is the selective loss of one of the two parental alleles at one or more contiguous chromosomal loci (44, 45). The chromosomal region that is most frequently affected by allelic loss in ERMS is 11p15.5 (46, 47). Allelic loss of this region has also been detected in fusion-positive ARMS tumors, but with a lower frequency than in ERMS tumors (44, 45). The locus causing the Beckwith-Wiedemann cancer susceptibility syndrome has also been localized to the 11p15.5 region (48, 49). Based on these combined findings, the 11p15.5 region is hypothesized to contain a tumor suppressor gene. Candidate genes include *CDKN1C*, *H19*, and *HOTS*; each of these genes is expressed specifically from the maternal allele and encodes a protein or RNA product with growth inhibitory activity (49, 50). Since ERMS tumors preferentially lose an 11p15.5 region containing the maternal alleles of these genes (51), ERMS tumorigenesis is proposed to involve inactivation of a tumor suppressor gene by allelic loss of the active maternal allele and retention of the inactive paternal allele.

Mutational analysis

Several studies have assayed ERMS and ARMS cases for point mutations of potential oncogenes and tumor suppressor genes (52–54). The largest study utilized Sequenom-based mutation detection and Sanger sequencing to interrogate 29 genes in 60 ERMS and 29 ARMS cases (55). Mutations were detected in 28% of the ERMS cases, and included 7 *RAS* family mutations, 4 *FGFR4* mutations, 3 *PIK3CA* mutations, 2 *CTNNB1* mutations, and single mutations in *BRAF* and *PTPN11*. In contrast, a point mutation (in *KRAS*) was found in only one of the 29 ARMS cases. Therefore, point mutations appear to be significantly more frequent in ERMS than in ARMS tumors.

GENE FUSIONS IN RMS

Molecular genetics of chromosomal translocations

At the genomic level, the 2;13 (or 1;13) translocation results in breaks within specific genes in the 2q35 (or 1p36) and 13q14 chromosomal regions followed by rejoining of portions of these genes to generate fusion genes. The involved genes on chromosomes 2 and 1 are *PAX3* and *PAX7*, respectively, which encode highly related members of the paired box family of transcription factors (56, 57). The fusion partner on chromosome 13 is *FOXO1* (*FKHR*), which encodes a member of the forkhead family of transcription factors (57, 58). The fusion genes generated by these translocations are expressed as fusion transcripts and translated into fusion proteins. Though these translocations generate reciprocal fusions, *PAX3-FOXO1* and *FOXO1-PAX3* (or *PAX7-FOXO1* and *FOXO1-PAX7*), the *PAX3-FOXO1* and *PAX7-FOXO1* genes are more highly and consistently expressed in ARMS tumors with the 2;13 and 1;13 translocations and are presumed to encode the products involved in ARMS pathogenesis (59, 60). The *PAX3-FOXO1* and *PAX7-FOXO1* fusion transcripts encode

chimeric transcription factors containing the PAX3 or PAX7 DNA binding domain and the FOXO1 transcriptional activation domain. These fusion proteins activate transcription from PAX3/PAX7-binding sites but are more potent as transcriptional activators than the wild-type PAX3 and PAX7 proteins (61, 62). In addition to functional changes, the PAX3-FOXO1 and PAX7-FOXO1 fusion products are expressed at higher levels than the corresponding wild-type products (63). These expression and functional changes result in high expression of potent transcription factors that contribute to tumorigenesis by altering growth and apoptotic pathways, modulating myogenic differentiation, and stimulating motility and other metastatic pathways (64).

Recent studies have also identified gene fusions in a small subset of ERMS tumors. In particular, rearrangements of the *NCOA2* gene in the 8q11–13 chromosomal region were found in four ERMS cases (65, 66). Three of the four cases have been subtyped as the spindle cell variant of ERMS and all four cases are congenital or infantile, occurring in children ranging from two weeks to seven months in age. In the three cases with identified fusions, the rearrangement partners are variable - *PAX3* (2q35), *SRF* (8q11), and *TEAD1* (11p15). In addition to these cases, there are reports of congenital/infantile ERMS cases with translocations involving the 8q11–13 region, including t(8;11)(q11.2;p15) and t(8;11)(q12–13;q21) (67, 68). Finally, an unrelated fusion, *EWSR1-DUX4*, was identified in an ERMS case in a 19 year old patient (69).

Fusion subsets in RMS

Multiple studies applied RT-PCR or FISH methodologies to sets of histopathologically diagnosed RMS cases to determine the frequency of the *PAX3-FOXO1* and *PAX7-FOXO1* fusions. In a study of RMS cases from the Intergroup Rhabdomyosarcoma Study (IRS)-IV protocol, 77% of the 78 ARMS cases were fusion-positive whereas 85 cases of ERMS (including botryoid and spindle cell subtypes) and 8 cases of undifferentiated sarcoma were fusion-negative (70). Within the ARMS category, 55% of the cases expressed *PAX3-FOXO1*, 22% expressed *PAX7-FOXO1*, and 23% were fusion-negative. Of note, the *PAX3-FOXO1* frequency is comparable to the frequency of t(2;13) detection in ARMS karyotypes. In contrast, the *PAX7-FOXO1* frequency is higher than the frequency of 1;13 detection because of the high frequency of subsequent amplification, which obscures cytogenetic detection of the translocation (71). Though the relative distribution of fusion subtypes is concordant with a separate analysis of ARMS cases from IRS-III (72), the fraction of *PAX3-FOXO1* to *PAX7-FOXO1* in these two IRS studies is lower than the figure found in several other studies (73, 74), including a study of older patients (75). Since the IRS studies did not enroll patients above 21 years old and *PAX3-FOXO1* is preferentially found in tumors from older patients (discussed below), the *PAX3*- to *PAX7-FOXO1* fraction in the IRS studies is deemed to be an underestimate, and a fraction greater than 3 to 1 is estimated to reflect the full age range of ARMS cases.

An important finding from these molecular pathology studies is the presence of a substantial subset of ARMS cases without the *PAX3-FOXO1* or *PAX7-FOXO1* fusion. In the IRS-III and IRS-IV studies, 22–23% of histologically diagnosed ARMS cases were negative for the two *PAX-FOXO1* fusions (70, 72). Since the IRS studies utilized centralized pathology

review and uniform tissue banking practices, these fusion-negative cases cannot be attributed to inaccurate histopathologic diagnosis or suboptimal tissue samples. Application of several approaches to assay these fusion-negative cases for rearrangements of the *PAX3*, *PAX7*, and *FOXO1* genes identified a small number of cases with variant fusions, such as fusion of *PAX3* to the *FOXO1*-related locus *FOXO4 (AFX)* (76); fusion of *PAX3* to *NCOA1* or *NCOA2*, encoding two similar transcription factors that are unrelated to *FOXO1* (66, 77); and fusion of *FOXO1* to *FGFR1*, encoding a receptor tyrosine kinase (78). Though these variant fusions can explain the absence of a *PAX3-FOXO1* or *PAX7-FOXO1* fusion in a few cases, the far majority of “fusion-negative” cases do not have any rearrangements of *PAX3*, *PAX7*, or *FOXO1* and thus appear to represent true fusion-negative cases with respect to these loci (76).

RNA-based microarray studies

Using microarrays to examine genome-wide RNA expression in RMS tumors, distinct expression patterns were identified among histopathologic and molecular subsets of RMS (40, 44, 77, 79). After obtaining genome-wide expression data for numerous cases of ERMS and ARMS, algorithms were used to cluster the RMS cases based on expression similarities and differences among the cases. Studies involving *PAX3-FOXO1* and *PAX7-FOXO1* cases did not find any clear expression differences between the two fusion-positive ARMS subsets, resulting in these fusion-positive subsets clustering together. In contrast, a striking difference was found between the expression patterns of ERMS and fusion-positive ARMS cases; this difference is consistent with several molecular differences between these categories, as described above. In addition, these unsupervised analyses also found a similarly large difference in expression pattern between fusion-positive and fusion-negative ARMS cases. For the rare ARMS cases with variant fusions, there is an example of one such case that clusters with the fusion-positive tumors and another such case that clusters with the fusion-negative tumors (40, 77). Finally, these analyses did not find consistent differences in expression pattern between ERMS and fusion-negative ARMS cases. Additional multidimensional scaling analyses demonstrated that the fusion-negative RMS cases cluster in a more diffuse pattern whereas fusion-positive RMS cases show a dense clustering (79). These findings suggest that RMS cases can be divided into two major categories based on expression pattern: fusion-positive RMS and fusion-negative RMS. As larger numbers of cases are analyzed, additional expression-based RMS subsets may become evident. For example, one study has proposed that the fusion-positive cases are divisible into two subsets (*PAX7-FOXO1*-enriched and *PAX3-FOXO1*-enriched), and the fusion-negative cases are divisible into three subsets (well-differentiated RMS, moderately differentiated RMS, and undifferentiated sarcomas) (44).

To translate this information for diagnostic purposes, genes differentially expressed between fusion-positive and fusion-negative RMS subsets were identified (40, 77, 79, 80). Various statistical algorithms were used to generate “diagnostic signatures” of the fusion-positive and fusion-negative RMS categories. In one study, four learning algorithms and cross-validation methods were applied to identify a ten gene panel that will accurately distinguish fusion-negative ERMS and fusion-positive ARMS; this ten gene panel was capable of distinguishing the two RMS subsets with 95–96% classification accuracy in both

development and validation cohorts (80). To translate these findings to a microscopic assay applicable to formalin-fixed paraffin embedded samples, protein markers were identified that correspond to differentially expressed genes and have robust antibodies suitable for immunohistochemistry (44, 81). These efforts identified HMGA2, EGFR, and Fibrillin-2 as markers preferentially expressed by the fusion-negative RMS category and TFAP2 β and P-cadherin as markers preferentially expressed by the fusion-positive category. Combinations of two of these markers increase specificity to 90% or greater while providing a sensitivity of 60% or greater (81).

Other genetic approaches to compare fusion subsets

Based on the findings described above of genetic differences between ARMS and ERMS (described above), the fusion subsets of RMS can be distinguished by additional genetic markers. In DNA-based microarray studies, the fusion-negative ARMS cases show a pattern of chromosome gains and losses similar to that found in ERMS cases (40). In addition, a high frequency of chromosome 11p15.5 allelic loss is found in fusion-negative ARMS as well as most ERMS cases (44). Finally, the fusion-positive RMS subset has a higher frequency of genomic amplification than fusion-negative ARMS and ERMS subsets (40). Of note, the high frequency amplicons in fusion-positive tumors have a marked preference for the two fusion-positive subsets (41–43). The 13q14 and 13q31 amplicons occur preferentially in *PAX7-FOXO1*-positive ARMS cases (93% vs. 9% and 75% vs. 8%, respectively) whereas the 12q13-q14 amplicon occurs preferentially in *PAX3-FOXO1*-positive ARMS tumors (24% vs. 4%). In contrast, there is a comparable incidence of 2p24 amplification (20%) in *PAX3-FOXO1* and *PAX7-FOXO1*-positive ARMS tumors.

PROBLEMS, OPPORTUNITIES, AND ISSUES WITH CLASSIFICATION: GENETICS VS. HISTOLOGY

Classification drift

As histological classification evolved, so did the potential for error. A drift in diagnosis became apparent in COG studies. From 1999–2005, the fraction of RMS cases enrolled on COG studies that were diagnosed as ARMS increased from 30% to 41%. Furthermore, the frequency of fusion-negative cases within the ARMS category increased from 20–25% to 40–45% (82).

In a retrospective review of 64 ARMS cases (83), the presence of a completely solid pattern was more frequent in fusion-negative compared to fusion-positive cases. This study revealed that 7 of 9 ARMS (78%) that completely lacked alveolar spaces were *PAX3-FOXO1* or *PAX7-FOXO1* fusion-negative. In comparison, among 55 ARMS that contained at least focal alveolar spaces, only 9 (16%) were fusion-negative. In this study the complete absence of alveolar spaces was the only histological feature that predicted fusion status. However, though the majority of RMS tumors with a solid pattern are fusion-negative, a significant subset of these solid variant tumors does show genetic features akin to classical ARMS (84).

Recognition of the solid variant of ARMS is complicated by criteria for diagnosis that appear to overlap those for diagnosing ERMS. Diagnosis of the solid variant of ARMS relies

heavily on the use of cytological criteria. The difficulty in using cytological features as the sole criteria for separating ARMS from ERMS is reflected by the level of agreement among pathologists in the international panel reviews that led to the ICPS (20). Among four classification systems tested, the Palmer cytohistological system attained a kappa score of only 0.328 (fair agreement), compared with 0.384 (fair agreement) for the NCI system (which introduced solid ARMS), 0.406 (moderate agreement) for the SIOP system, and 0.451 (moderate agreement) for the conventional (Horn-Enterline) system.

In the resulting ICPS system (19), the diagnostic criteria for ERMS permit a wide range in cytology and cellular density, as follows:

“This category of tumors displays a considerable variation in cytology, encompassing the whole range from primitive mesenchymal to highly differentiated muscle tumor cells. The degree of differentiation is approximated by the amount of cytoplasmic development. 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.”

These criteria predict a “dense pattern” subtype of ERMS that contains primitive mesenchymal cells with high cellular density. However, this dense ERMS pattern is reminiscent of the ICPS criteria (19) for solid variant ARMS:

“The ‘solid’ variant of alveolar RMS grows as solid masses of closely aggregated cells, with no or scarcely discernible alveolar arrangement. There may be stroma, as in all tumors, but the stromal bands may surround nests of tumor cells. No marked compartmentalization and no distinct alveolar configuration is apparent.”

The basis for distinction between dense ERMS and solid ARMS thus relies on relatively subtle cytological features (Figure 2). This separation recalls the Palmer classification of “monomorphous round cell tumor” (15) in which dense ERMS should contain “*predominately fusiform or stellate cells with scant cytoplasm*” and solid ARMS should contain cells that are “*round and often similar to each other and usually exhibit a high nucleocytoplasmic ratio*” (19). Because of these subtle distinctions, the diagnosis of solid ARMS is particularly prone to misinterpretation. Inclusion of dense ERMS cases in the ARMS category adds more cases without *PAX3-FOXO1* and *PAX7-FOXO1* fusions, and it thus increases the frequency of the fusion-negative ARMS subset and the overall frequency of ARMS cases.

In our recent review of the histological features of ARMS (82), we found that 84 of 255 cases from the recent D9803 trial could be re-classified as ERMS. Within this group of reclassified cases, 55% of the 84 tumors had cytological features that could be interpreted as “dense pattern” ERMS, based on the above criteria. An additional 20% had features reminiscent of sclerosing RMS, as described by Folpe et al (23), and 12% had mixed histological features. Thus, of the reclassified tumors, a dense pattern appeared the most common source of reclassification, followed by sclerosing RMS and mixed pattern RMS. All of the 84 reclassified dense ERMS tumors were fusion-negative on independent review, compared to only 23 of the 126 (18%) cases for which the diagnosis of ARMS was

confirmed. Of particular note, the outcome of the re-classified patients was similar to that of ERMS patients from the same study, and the tumors tended to originate from sites more typical of ERMS, such as the genitourinary tract.

Mixed pattern RMS also continues to present a diagnostic challenge. In the original ICPS study, any focus of alveolar histology was sufficient for ARMS diagnosis (19). In the COG D9803 study (85), lesions with “[s]eparate, discrete regions of alveolar and embryonal histologies of any histologic pattern” were thus diagnosed as ARMS. In a FISH analysis of 8 mixed histology cases, there was no evidence of PAX3, PAX7, or FOXO1 rearrangements in any of the analyzed regions (86). In a more recent analysis of reclassified D9803 cases, most (9 of 14) of the mixed RMS cases were fusion-negative (unpublished data).

Sclerosing RMS also comprised a portion of the reclassified RMS cases in our recent study (82). Of note, Folpe et al originally classified these lesions as a “microalveolar” form of ARMS (23), but their retrospective study revealed histological features more in keeping with the sclerosing pseudovascular RMS described by Menzel and Katencamp (22). Of note, the single sclerosing RMS tested by Folpe et al was fusion-negative, and similarly, only 1 of 6 of the pediatric cases described by Chiles et al (24) was fusion-positive. Therefore, the previous inclusion of mixed and sclerosing cases in the ARMS category also contributed to an artefactual increase in the frequency of fusion-negative ARMS tumors as well as the overall frequency of ARMS tumors.

Immunohistochemical correlates of histological classification and fusion

Recent papers indicate that immunohistochemistry can be used both as a means of distinguishing ARMS from ERMS and as a surrogate marker of fusion positivity. Dias et al. in 2000 (87) showed that strong myogenin expression typified ARMS, causing diffuse immunostaining that contrasts with the variable, focal, heterogeneous pattern of ERMS (Figure 3). The strong myogenin expression in ARMS likely derives from downstream effects of the *PAX* fusion proteins with regulatory elements in the myogenin gene (88). However, there is considerable overlap in staining, and even botryoid tumors can show relatively strong expression in over 50% of nuclei. On the other hand, fusion-positive ARMS tumors rarely show weak myogenin expression, and even the most undifferentiated ARMS tumors usually show expression in virtually all tumor cell nuclei. In our recent review (82), 91 of 105 ERMS and 16 of 22 fusion-negative ARMS showed relatively weak myogenin expression, compared with only 1 of 86 fusion-positive ARMS. These results are highly significant when fusion-negative ARMS or ERMS is compared with fusion-positive ARMS ($p<0.0001$), but not significant when fusion-negative ARMS is compared to ERMS ($p=0.11$ by Fisher’s exact test).

As noted above, gene expression analyses have revealed other proteins whose expression corresponds to the presence or absence of the *PAX-FOXO1* fusion. Fusion-negative tumors preferentially express HMGA2, EGFR, and Fibrillin-2, whereas fusion-positive-tumors preferentially express TFAP2 β and P-cadherin (44, 77). These findings offer a means of predicting a fusion positive status by standard immunohistochemical means. However, use of surrogate markers for the presence of the *PAX-FOXO1* fusion cannot be strictly equated with a histological diagnosis of ARMS.

Risk stratification and outcome of RMS

The outstanding question of recent years has been the proper risk-based stratification and therapy of fusion-negative ARMS. Following our reclassification of ARMS cases treated on the D9803 COG study, survival analysis was performed comparing fusion-positive and fusion-negative tumors (89). This analysis indicated that failure-free survival of fusion-positive ARMS patients was worse than that of fusion-negative RMS patients, regardless of histologic classification or fusion subtype. On the other hand, there was no difference in treatment failure rate between reclassified fusion-negative ARMS and ERMS. These findings are consistent with previous comparisons of fusion-negative and fusion-positive RMS tumors (40). Of note, the COG study revealed a difference in overall survival between patients with *PAX3-FOXO1* and *PAX7-FOXO1*-positive tumors, with 87% of the *PAX7-FOXO1*-positive patients surviving compared with 64% of *PAX3-FOXO1*-positive patients (89). Unlike their failure-free survival, the overall survival of *PAX7-FOXO1*-positive tumors was similar to that of ERMS and fusion-negative ARMS cases. This study suggests that patients with *PAX7-FOXO1*-positive ARMS tumors exhibit the same tendency for relapse as those with *PAX3-FOXO1*-positive ARMS but have a greater chance of salvage with additional treatment.

A corollary question is whether there are meaningful subsets of fusion-negative RMS including ERMS. As indicated above, anaplastic, botryoid, and spindle cell histology has been correlated with outcome, but the results have never reached a high level of significance. Botryoid RMS is defined by an epithelial membrane, so that it becomes impossible to separate the confounding effect of site of origin (90) from histology. No clear-cut genetic distinctions within this ERMS subset have emerged. Like BRMS, the majority of pediatric SCRMS cases have a relatively limited site distribution associated with a favorable outcome. Initial reports suggested that SCRMS tumors contained more differentiated cells (18), and a recent paper by Rubin et al (91) theorizes that their histology relates to cell of origin and associated constellation of mutations.

Hyperdiploidy was initially suggested to be a prognostic factor in ERMS (92, 93), but subsequent analysis failed to show an effect on survival (94). Clearly more studies are needed, as subsets of ERMS still develop aggressive behavior and drug resistance (95) and reduced chemotherapeutic dosing seems a laudable goal (2).

In spite of strong opinions that the diagnosis of ARMS should be based on genetic testing and not histology (96), there are several reasons that such a move is counterintuitive. Although clinical and biological studies appear to define fusion-positive ARMS as a discrete entity and fail to show distinction between ERMS and fusion-negative ARMS, the names and of definitions of ARMS and ERMS refer to their histological features. Though future treatment may be best predicated on fusion status, maximal clarity is achieved by retaining the terms “fusion-positive” and “fusion-negative” RMS as distinct from “alveolar” or “embryonal” RMS. Sadly, genetic testing is not universally available, so many pathologists must continue to rely on histological distinction for treatment stratification. Also, testing might not be quickly available in dire clinical situations involving large tumors or those arising in critical anatomic regions. Challenges for the pathologist continue to be clear cut

separation of RMS from other neoplasms, selection of cases appropriate for genetic testing, and provision of maximal information when such testing is not available.

Summary

Histological classification of RMS into ARMS and ERMS has been an independent predictor of clinical outcome and a standard component of risk assignment for therapeutic protocols. Genetic testing has revealed that the poor outcome associated with ARMS likely relates to the presence of PAX-FOXO1 fusion genes and not to histological features. Fusion-negative ARMS has now been associated with ERMS in terms of clinical presentation and outcome, so that genetic assessment currently appears to be a more meaningful endeavor for clinical management than histological classification. On the other hand, fusion-positivity is extremely rare in ERMS, so that histological assessment does appear to be effective in selecting cases that benefit most from genetic testing. Categorization of future therapeutic trials will depend on genetic testing for selecting high risk patients. Immunohistochemistry continues to be an integral part of RMS diagnosis, and immunohistochemical expression of certain proteins, such as myogenin, AP2B, and HMGA2, appears to correlate with fusion status. Finally, although an association exists with PAX-FOXO1 fusion genes, the definition ARMS continues to be based on histological and cytological variables, but its distinction from ERMS can be quite subtle, particularly with “solid variant” histology.

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1.

Histological patterns seen with rhabdomyosarcoma. Embryonal rhabdomyosarcoma (ERMS), containing spindle cells with alternating dense and loose cellularity. B. Alveolar rhabdomyosarcoma (ARMS), showing round cells clustered into nests separated by fibrous septa. Note the periseptal rows and peripheral discohesion. C. Botryoid rhabdomyosarcoma, organized into polypoid structures lined by squamous epithelium with underlying cellular aggregates. D. Pleomorphic rhabdomyosarcoma, composed of differentiating neoplastic myocytes with nuclear enlargement and irregularity. E. Rhabdomyosarcoma containing a densely cellular component resembling ARMS and a less cellular component resembling ERMS. F. ARMS with solid cellular pattern, lacking septa but containing monotonous round cells. G. Sclerosing rhabdomyosarcoma, containing dense bands of collagen. Note the microalveolar pattern. H. Anaplastic ERMS containing cells with large hyperchromatic nuclei that contrast with adjacent small cells.

2.

Cytological details of rhabdomyosarcoma. Embryonal rhabdomyosarcoma (ERMS) with densely cellular focus composed of spindle and polygonal cells with irregular nuclear contours (dense ERMS). B. Alveolar rhabdomyosarcoma composed of monomorphous cells with round, even nuclear contours (solid ARMS). For size comparison, this lymph node metastasis also contains an infiltrate of smaller lymphocytes.

3. Myogenin staining patterns in rhabdomyosarcoma. A. Alveolar rhabdomyosarcoma. Almost all tumor cells strongly express myogenin. B. Embryonal rhabdomyosarcoma. Myogenin expression is seen in a minority of cells.

Table 1:

International Classification of Pediatric Sarcomas.

Risk Category	Histology
Superior	Botryoid rhabdomyosarcoma
	Spindle cell rhabdomyosarcoma
Intermediate	Embryonal rhabdomyosarcoma
High	Alveolar rhabdomyosarcoma, including solid variant
	Undifferentiated sarcoma *

* Defined as high grade, round-cell tumors with no myogenic differentiation by histological or ancillary testing. This category excludes Ewing sarcomas and neuroblastomas. Although formerly treated on Intergroup Rhabdomyosarcoma Study Group protocols, these lesions are now treated on non-rhabdomyosarcomatous soft tissue sarcoma protocols by the Children's Oncology Group.(97).

Table 2.

World Health Organization Classification of skeletal muscle tumors

Benign	Rhabdomyoma, adult type
	Rhabdomyoma, fetal type
	Rhabdomyoma, genital type
Malignant	Embryonal rhabdomyosarcoma
	Alveolar rhabdomyosarcoma
	Pleomorphic rhabdomyosarcoma
	Spindle cell/sclerosing rhabdomyosarcoma