Fusion Gene–Negative Alveolar Rhabdomyosarcoma Is Clinically and Molecularly Indistinguishable From Embryonal Rhabdomyosarcoma

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ABSTRACT

Purpose
To determine whether the clinical and molecular biologic characteristics of the alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS) subtypes have relevance independent of the presence or absence of the PAX/FOXO1 fusion gene.

Patients and Methods
The fusion gene status of 210 histopathologically reviewed, clinically annotated rhabdomyosarcoma samples was determined by reverse transcriptase polymerase chain reaction. Kaplan-Meier analysis was used to assess event-free survival and overall survival in fusion gene–negative ARMS (ARMSn; n = 39), fusion gene–positive ARMS (ARMSp; n = 94), and ERMS (n = 77). A total of 101 RMS samples were also profiled for whole-genome expression, and 128 were profiled for genomic copy number imbalances. Profiling data were analyzed by supervised and unsupervised methods to compare features related to histopathology and fusion gene status. Results were also projected by meta-analysis techniques across three separate publically available data sets.

Results
Overall and event-free survival, frequency of metastases, and distribution of site at initial presentation were not significantly different between ARMSn and ERMS. Consistent with this, analysis of gene expression signatures could not reproducibly distinguish ARMSn from ERMS whereas fusion gene–positive cases were distinct. ARMSn and ERMS frequently show whole-chromosome copy number changes, notably gain of chromosome 8 with associated high levels of expression of genes from this chromosome.

Conclusion
The clinical behavior and molecular characteristics of alveolar cases without a fusion gene are indistinguishable from embryonal cases and significantly different from fusion-positive alveolar cases. This implies that fusion gene status irrespective of histology is a critical factor in risk stratification of RMS.

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INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and is broadly divided into two main subgroups—alveolar (ARMS) and embryonal (ERMS)—on the basis of morphological characteristics. ARMS is generally associated with a poorer prognosis than ERMS. Histologic subtype has therefore been incorporated into risk stratification in more recent clinical trials: in the Société Internationale d’Oncologie Pédiatrique (SIOP) Malignant Mesenchymal Tumor 1995 (MMT-95) trial, a diagnosis of ARMS placed a patient in the high-risk category, regardless of other staging and grading considerations. In the fifth trial of the Intergroup Rhabdomyosarcoma Study (IRS-V), alveolar histology automatically moved a patient, irrespective of stage, to at least the intermediate-risk group. This approach to risk stratification means that histology dictates increased use of radiotherapy and intensive multidrug chemotherapy in a substantial proportion of patients with RMS. While this approach has contributed to the steady increase in 5-year survival rate from 25% to 70% for children.
with localized RMS, the risk of long-term sequelae following aggressive treatment of children is substantial.5

ARMS is associated with characteristic balanced chromosomal translocations t(2;13)(q35;q14) and t(1;13)(p36;q14), which form a fusion gene between the 5' end of either PAX3 or PAX7 and the 3' end of FOXO1. These alterations are present in ~70% of ARMSs and are associated with a poor prognosis.6,7

According to International Classification of Rhabdomyosarcoma (ICR) and WHO guidelines, differential diagnosis of ARMS (with the exception of solid variant ARMS) involves identifying a histopathologic area of characteristic alveolar spaces.8 These alveolar spaces may be focal, missing in small biopsies, or even absent, in which case diagnosis of ARMS solid variant may be made if the tumor shows the high cellularity and undifferentiated round cell features typical of ARMS.9 Originally, subtype classification was determined by whether alveolar or embryonal histology predominated in the tumor. Post-1995 guidelines were altered so that observing any amount of alveolar pattern is sufficient to result in a diagnosis of ARMS.10 However, since 1995, identification of the PAX/FOXO1 fusion gene by molecular methods has been largely adopted, if not as a definitive test, then as a supportive diagnostic aid. The upshot is that discovery of a PAX/FOXO1 fusion gene is used to confirm diagnosis where ARMS histology was previously understood and may force a reconsideration where ERMS had previously been diagnosed. This fact, not recognized in the current diagnostic guidelines, has blurred the boundaries between traditional histopathology and molecular pathology diagnosis. Consequently, there is a certain subpopulation of patients—those with PAX/FOXO1-negative ARMS (ARMSn)—stranded between a molecular criterion that recognizes their distinctiveness and a histopathologic treatment stratification that does not.

A number of studies have investigated the expression profiles of RMS and the targets of PAX3/FOXO1. Davicioni et al14,16 described three molecular classes that they related to clinical outcome and described a signature consisting of high expression of CNR1, CDH3, and TFAP2B that is closely associated with the presence of PAX/FOXO1. They stated that ARMSn was more similar to ERMS than to ARMSp. In contrast, Wachtel et al13 claimed to find three distinct expression groups relating to ARMSp, ARMSn, and ERMS. They demonstrated consistent high expression of AP2 and P-cadherin protein in ARMSp and endothelial growth factor receptor and fibrillin-2 in ERMS, while ARMSn was shown to not express AP2 and P-cadherin protein and to have levels of endothelial growth factor receptor and fibrillin-2 intermediate between those of ARMSp and ERMS. In this study, we demonstrate a weight of evidence that, in terms of its clinical presentation and outcome and its molecular biology, ARMSn is largely indistinguishable from ERMS.

### Table 1. Clinical Characteristics of Patients

<table>
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<tr>
<th>Characteristic</th>
<th>Used in Survival Analysis</th>
<th>Used in Microarray Analysis</th>
<th>Used in CGH Analysis</th>
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<tr>
<td></td>
<td>ARMSp</td>
<td>ARMSn</td>
<td>ERMS</td>
</tr>
<tr>
<td>No. of patients</td>
<td>94</td>
<td>39</td>
<td>77</td>
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<tr>
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Abbreviations: CGH, comparative genomic hybridization; ARMSp, fusion gene–positive alveolar rhabdomyosarcoma; ARMSn, fusion gene–negative ARMS; ERMS, embryonal RMS; SIOP, Société Internationale d’Oncologie Pédiatrique.

*Favorable sites are defined as genitourinary, non-bladder/prostate, head and neck, non-parameningeal, and orbit.
PATIENTS AND METHODS

Patient Samples
This study was planned and conducted with ethical committee approval. Informed consent was obtained for inclusion in trials, and analysis of tumor samples was performed according to the relevant European laws on the protection of persons taking part in biomedical research.

Primary samples from 101 individuals with a diagnosis of RMS at French and United Kingdom local hospitals were collected by biopsy and then snap frozen. The clinical characteristics of this patient set are listed in Table 1. Clinical data were collected for these 101 individuals plus a further 109 individuals for whom insufficient RNA was available for expression analysis. Patients were registered to, or treated according to, the MMT-89,17 MMT-95,18 and European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) RMS 2005 protocols for localized RMS and according to MMT-TV-89/91 or MMT-98-4,16 for metastatic RMS. All patients were treated with multitarget chemotherapy and surgery, or with or without radiotherapy, for local control. High-dose therapy with bone marrow or stem-cell rescue in first remission was limited to high-risk patients (ie, the MMT-98.2 arm).19 For inclusion criteria, see Data Supplement.

Overall survival (OS) was calculated as the time between the date of diagnosis and the date of death (from disease) or the last known date at which the patient was alive. Event-free survival (EFS) was calculated as the time between date of diagnosis and the first event, be it first relapse, tumor progression, or otherwise. Survival curves were estimated by the Kaplan-Meier method and the log-rank test. Univariate Cox models were calculated using the R function coxph. Samples were tested by reverse transcriptase polymerase chain reaction for presence of PAX3/FOXO1, PAX7/FOXO1, and PAX3/NCOA1 (for primer sequences and methods, see Data Supplement).

Pathology
Since 1984, SIOP protocol requires that RMS samples be reviewed by a panel of pathologists and subclassed as alveolar or embryonal according to international classification guidelines20 or subclassed as RMS-NOS (not otherwise specified) if tumors are not classifiable because of small size of biopsy. All samples that were negative for PAX fusions underwent further central review by an expert in pediatric pathology (M.L., P.F., or K.T). More details about pathology analysis are given in the Data Supplement.

Isolation of RNA/DNA and Microarray Analysis
Total RNAs were isolated using Trizol (Invitrogen, Carlsbad, CA) and then processed using RNasey Minelute columns (Qiagen, Alameda, CA). Affymetrix GeneChip expression analysis was performed using HGU133plus2 arrays, according to manufacturer’s instructions. DNA was extracted and array comparative genomic hybridization (aCGH) was performed using the Institut Curie platform, as previously described.21,22 Microarray data will be submitted to the European Bioinformatics Institute (EBI) array-express repository. All microarray data were normalized using GeneChips robust multi-array average.23 Gene set enrichment analysis (GSEA) was performed using the GSEA v2 software and the MSigDB database (Broad Institute, Boston MA).24,25 Non-negative matrix factorization (NMF), hierarchical clustering, and heatmaps were generated using GenePattern 3.026,27 and/or the metagene projection script by Tamayo et al.28 aCGH data were segmented using DNAcopy and cghMCR R packages (for full details, see Data Supplement).

RESULTS

Clinical Characteristics and Prognosis Are Significantly Less Favorable for ARMSn But Do Not Distinguish ARMSp From ERMS
To gauge whether ARMSn is distinguishable from ARMSp and ERMS, we compared survival characteristics for each of these three RMS groups. The survival curve for ARMSp shows a significantly poorer prognosis (OS: $\chi^2 = 38.3$, $P < .001$, n = 210; EFS: $\chi^2 = 34.6$, $P < .001$, n = 210) than ARMSn and ERMS. By contrast, the OS and EFS of ARMSn and ERMS are not significantly different from one another (OS: $\chi^2 = 0.054$, $P = .816$, n = 116; EFS: $\chi^2 = 0.177$, $P = .67$, n = 116; Figs 1A and 1B).

Using the univariate Cox regression model, the relative risk (RR) of death for fusion–positive RMS is 3.85 (95% CI, 2.5 to 6.0; $P < .001$), for ARMS histology is 3.04 (95% CI, 1.8 to 5.1; $P < .001$), and for stage is 3.55 (1 to 3 v 4; 95% CI, 2.3 to 5.5). A multivariate model containing fusion status, stage, and histology shows that both fusion status and stage are independently prognostic, individually contributing RRs of 2.5 (95% CI, 1.2 to 5.1; $P < .001$) and 2.6 (95% CI, 1.7 to 4.1; $P < .001$), respectively. Importantly, histology is not independently prognostic (RR, 1.3; 95% CI, 0.6 to 2.9; $P = .48$; for full details, see Data Supplement).

The other recorded clinical data indicates that ARMSn is indistinguishable from ERMS and that ARMSp has significantly less favorable clinical characteristics (Table 1). For instance, the fraction of ARMSp occurring at favorable sites is significantly lower in ARMSp than in ARMSn and ERMS ($\chi^2 = 11.94$; $P = .002$) but not significantly different between ARMSn and ERMS (Fisher’s exact $P = .840$). Similarly the proportion of ARMS that are metastatic at presentation is significantly higher in ARMSp than ARMSn and ERMS ($\chi^2 = 19.95$; $P < .001$) but not significantly different between ARMSn and ERMS (Fisher’s exact $P = .428$; Fig 1C and Table 1).

Unsupervised Expression Analysis of Primary RMS Demonstrates Expression Sub-Groups Associated With Presence or Absence of PAX Fusion But Not Histology
Primary samples from 101 individuals with a confirmed diagnosis of RMS were expression profiled. To identify potential expression subgroups consensus, hierarchical clustering was used, and it demonstrated that a two-cluster model best fits the data (Fig 2A). The majority of samples are split by the presence or absence of PAX/FOXO1 translocation, irrespective of histology. One group of 43 patients contains all PAX fusion–positive samples and one ERMS; the other group of 58 patients contains all but one of the PAX/FOXO1-negative samples plus two ARMS with PAX3/FOXO1 and one with PAX3/NCOA1. Expression data from three independent studies13-15 analyzed individually in the same manner also grouped patients largely by the presence or absence of PAX/FOXO1 rather than histology (see Data Supplement).

NMF is an unsupervised method particularly suited to identifying readily interpretable components in multivariate data and can be efficiently projected across different studies and even different platforms.28 NMF was used to determine four metagenes that best summarize the structure of the data which were then subsequently validated by projecting the metagenes across a further 186 RMS expression profiles (Affymetrix HGU133a) from three independent studies.13-15

Metagene F1 is highly associated with ARMSp samples, as can clearly be seen in our samples, and this pattern of high expression is repeated in ARMSp samples in all studies (Figs 2B and 2C). ARMSn and ERMS do not show high expression of this metagene. In contrast, metagene F2 is highly expressed in ERMS and ARMSn but never in ARMSp; importantly, this pattern holds true for the three independent studies validating that result. Metagenes F3 and F4 are expressed in each subset, but high expression appears, as a general rule, to be...
mutually exclusive. ARMSp metagene F1 is most highly correlated with genes such as TFAP2B, CNR1, DAPK1, ALK, and PIPOX, while metagene F2 is highly correlated with SPRED2, EIF4EBP1, HMGA2, and DDEF1 (for full gene lists, see Data Supplement).

Supervised Expression Analysis of Primary RMS Successfully Characterizes ARMSp But Fails to Consistently Distinguish ARMSn From ERMS

The metagenes were analyzed using a support vector machine (SVM) learning algorithm that learns and then predicts the histology of new samples. Four metagenes were used because, overall, this method gave the most accurate SVM result. The SVM was trained using our samples, and it efficiently learned to discriminate the subgroups of RMS with the exception of ARMSn. ARMSp and ERMS samples were trained efficiently: 98% (44 of 45) and 97% (35 of 36), respectively. Three ARMSp patients were originally misclassified by the consensus clustering, but two of them are correctly classified here. The only incorrectly classified ARMSp patient has a PAX3/NCOA1 fusion which may indicate that the biology of PAX3/NCOA1 is different from other PAX fusions. The one misclassified ERMS may harbor an unknown PAX3 rearrangement. (The misclassified ERMS suspected of harboring a cryptic PAX fusion was ultimately found to have a PAX3-NCOA2 fusion identical to those described by Sumegi et al.29) It shows high expression of metagene F1 and typical PAX fusion–specific genes such as ABAT, CNR1, and DAPK1. aCGH analysis indicates a breakpoint at the PAX3 gene.

The SVM cannot correctly train the ARMSn, classifying only 6% of samples (1 of 18) correctly and failing completely to train two samples. Of the 17 incorrectly trained ARMSn samples, 88% (15 of 17) are trained as ERMS and 6% (1 of 17) as ARMSp. Using the independent studies as a test set, ARMSp and ERMS cases were correctly classified in 99% (81 of 82 with one unclassified) and 95% (79 of 83 with two unclassified) of cases, respectively, whereas 100% (18 of 18) of ARMSn were classified as ERMS (for full

Fig 1. Kaplan-Meier curves showing significant poorer prognosis in (A) overall survival and (B) event-free survival for patients with fusion gene–positive alveolar rhabdomyosarcoma (ARMSp). Survival of patients with fusion gene–negative ARMS (ARMSn) and embryonal RMS (ERMS) is not significantly different. (C) Percentage frequency of metastases is significantly higher in ARMSp than in ARMSn or ERMS. Three ERMS patients who survived and did not relapse for 12.3, 16.7, and 17.2 years were left out of the plot for presentational purposes but were included in the calculation of the statistics.
The metagene SVM performs slightly better than the consensus clustering. Significance analysis of microarray (SAM) analysis—a supervised method—was used to find significantly differentially expressed genes between ARMSp, ARMSn, and ERMS. SAM analysis between ARMSp and ERMS finds 1,140 probe sets whose expression is significantly differentially expressed with a q value (for each gene, this is the lowest false discovery rate at which that gene is called significant) less than 10% and a mean fold change of more than three. Of these 1,140 probe sets, 562 were present on the hgu133a chip used in the other studies. Seventy-two percent (403 of 562) of these probe sets were also significantly differentially expressed in the Davicioni et al data.14

Details, see Data Supplement. Fig 2. (A) Consensus clustering of all rhabdomyosarcomas (RMSs). (B) Heatmap of metagenes sorted by class and study. (C) 2D projection of four metagenes (F1, F2, F3, and F4) along two projected axes S1 and S2, for training set (left) and training and test set (right). Red, fusion gene–positive alveolar rhabdomyosarcoma (ARMSp); gold, fusion gene–negative ARMS (ARMSn); blue, embryonal RMS (ERMS).
In contrast, an identical analysis applied to distinguish ARMSn and ERMS showed 14 significant probe sets in our data and 28 significantly differentially expressed probe sets in the Davicioni et al data; however, none of the probe sets overlap. The results of our study show that, unlike ARMSp, genes that distinguish ARMSn from ERMS are few in number and are inconsistent and unsupported by other studies (Fig 3). The same SAM analysis performed on the Davicioni data set did not find significant probe sets that are also significant in our data set.

**Unlike ARMSp, the Pattern of aCGH Changes in ARMSn Is Not Significantly Different in Frequency or Character From ERMS**

aCGH was performed on 128 primary RMS samples of which 84 were also expression profiled (Fig 3A). The general pattern of gain and loss in ERMS and ARMSn involves more whole-chromosome changes than focal alterations and fewer amplifications than ARMSp (Appendix Fig A1).

The frequency of many specific amplifications and gains is significantly different in ARMSp versus ARMSn and ERMS (Figs 3A and 3B). For example, MYCN amplification is present in 20% (10 of 50) of ARMSp but in only 4% (2 of 51) of ERMS and in none (0 of 27) ARMSn (P < .01). aCGH analysis shows that gain of chromosome 8 occurs in 74% (38 of 51) of ERMS samples, 55% (15 of 27) of ARMSn samples, and none (0 of 50) of ARMSp samples (Appendix Fig A1). Using ERMS samples with both expression and aCGH data to plot average expression along chromosome 8, it is evident that no region on chromosome 8 is particularly affected more than any other but rather a general upregulation of genes is observed (Appendix Figs A2(A) and A2(B), online only).
The strong impact of chromosome 8 gain on gene expression is further shown by analyzing the PAX fusion–negative associated metagene F2. The individual components that make up each metagene can be analyzed by calculating the correlation of each probe to the metagene and using this to rank the genes.28 This rank can then be used as the basis of a GSEA to determine whether other preknown sets of genes are ranked more significantly than could be expected by chance. Analysis of metagene F2 shows that gene sets associated with gain of chromosome 8 are the most significantly enriched: AGUIRRE_PANCREAS_CHR8 NES = 2.781 and CHR8Q24 NES = 2.478; P < .001; q < .001 (Appendix Fig A2(C)).

DISCUSSION

Here we demonstrate through analysis of clinical data and expression and genomic profiling a weight of evidence that, apart from the obvious histologic differences, ARMSn is not readily distinguishable from ERMS and that the primary influence on the clinical presentation, outcome, and molecular biology of RMS is the presence or absence of a fusion gene. Analysis of the clinical data indicates that there is no statistically significant difference in the OS or EFS of ARMSn and ERMS or in the initial presentation (ie, distribution between favorable and unfavorable sites, age, or presence of metastases). In comparison, ARMSp patients show significantly poorer survival, higher frequency of metastases and unfavorable sites, and an older age distribution. It should be noted that the ARMSn patients in this study received increased therapy compared with the therapy received by many of the ERMS patients.

Unsupervised consensus clustering groups all ARMSn in the same group as the ERMS. Metagenes calculated to reflect the most important underlying biologic components in the data produce two metagenes that consistently distinguish ARMSp from ARMSn and ERMS, but no metagene has been found that distinguishes ARMS as a whole from ERMS or indeed distinguishes ARMSn as a separate entity. These metagenes can be projected across three separate studies, all of which validate our results and show that ARMSn resembles ERMS. SAM analysis failed to find genes that distinguish ARMSn from ERMS with any consistency among studies but found many significant and consistent genes that distinguish ARMSp.

aCGH data indicate that the frequency and distribution of a number of gains, amplifications, and losses is significantly different between ARMSp and ARMSn or ERMS but that there are no significant differences between ARMSn and ERMS. This is best exemplified by chromosome 8 which is gained at similar high frequencies in ARMSn and ERMS (but not in ARMSp), causing general upregulation of chromosome 8 genes. ARMSp, in contrast to ERMS and ARMSn, demonstrates a pattern of focal imbalance and amplification that matches with its poor prognosis in much the same way as can be observed in neuroblastoma.30 The frequency of 2p24 and 12q13–15 amplicons in a series of ARMSp and ARMSn patients described by Barr et al31 does not differ significantly from our observations (ie, 20% and 24% amplification in ARMSp, respectively, and occasional amplification in ARMSn).

Wachtel et al13 in their expression analysis of RMS claimed to identify distinct expression profiles corresponding to ARMSp, ARMSn, and ERMS, while Davicioni et al14,16 in their expression and loss of heterogeneity analysis claimed to find no signature distinguishing ARMSn tumors from ERMS tumors. Our analysis of all available data favors the latter conclusion. While we cannot rule out some heterogeneity or cryptic PAX fusions within a few cases (one ARMSn case was classified as ARMSp by the SVM), the evidence does not show this to generally be the case. It should be noted that within each individual study using techniques such as SAM analysis, it is always possible to find genes significantly different between ARMSn and ERMS (as has been done by Wachtel et al) but these differences are low-fold change, inconsistent, and not reproducible across the other studies. We would attribute the difference in conclusions among the different studies to there being a relatively small number of samples and to having no second test set to validate conclusions, an issue that we have addressed in this study.

Adding our novel analysis of the clinical outcome to the molecular biologic evidence, we have demonstrated that rather than histology, the key factor in terms of the biology and clinical progression of RMS is the presence or absence of PAX/FOXO1 fusion genes. We believe that this should have important implications for the ongoing risk stratification strategies of current RMS treatment protocols.

REFERENCES


AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Provision of study materials or patients: Gaëlle Pierron, Benedicte Thuille, Gilles Palenzuela, Khin Thway, Daniel Orbach, Marick Laé, Paul Fréneaux, Kathy Pritchard-Jones, Odile Oberlin, Janet Shipley, Olivier Delattre
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Fig A1. Heatmap of significantly discriminatory genes selected by significance analysis of microarray (3-fold change cutoff Q value < 10%) according to our data and shown for the Davicioni and Wachtel data sets. (A) Fusion gene–negative alveolar rhabdomyosarcoma (ARMSn) versus embryonal rhabdomyosarcoma (ERMS) show inconsistent and weak discrimination within and across studies. (B) Fusion gene–positive alveolar rhabdomyosarcoma (ARMSp) versus ERMS show strong and consistent discrimination across studies (only first 100 of 614 shown). Red, ARMSp; orange, ARMSn; blue, ERMS.
Fig A2. Scatterplot of chromosome 8 gene expression. Ratio between (A) mean expression with gain of chromosome 8 or (B) individual samples and mean expression with no gain of chromosome 8, whole gain or region specific chromosome 8. Lines are smoothed moving average. Blue, array comparative genomic hybridization (aCGH); red, expression. (C) Gene set enrichment analysis of the F2 metagene showing significant enrichment of chromosome 8–specific gene set. Black bars indicate the rank of gene set genes within the larger set of genes ranked from left (associated with chromosome 8 gain) to right (associated with no gain of chromosome 8).