Impact of Fusion Gene Status versus Histology on Risk-Stratification for Rhabdomyosarcoma: Retrospective analyses of patients on UK trials

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Abbreviations

MMT	Malignant Mesenchymal Tumour
PCR	Polymerase Chain Reaction
EpSSG	European Paediatric Soft Tissue Sarcoma Study Group
RMS	Rhabdomyosarcoma
ACCIS	Automated Childhood Cancer Information System
ERMS	Embryonal rhabdomyosarcoma
ARMS	Alveolar rhabdomyosarcoma
FFPE	Formalin fixed paraffin embedded
SIOP	Society of Paediatric Oncology
TMA	Tissue microarray
FISH	Fluorescence *in situ* hybridisation
BAC	Bacterial artificial chromosome
DNA	Deoxyribonucleic acid
DIG	Digoxygenin
FITC	Fluorescein isothiocyanate

51	RT-PCR	Reverse transcription coupled polymerase chain
52		reaction
53	RNA	Ribonucleic acid
54	cDNA	complementary DNA
55	OS	Overall survival
56	EFS	Event free survival
57	HR	Hazard ratio
58	MG5	Metagene-5
59	RMS-NOS	Rhabdomyosarcoma (not otherwise specified)

Abstract

Background

Long-term toxicities from current treatments are a major issue in pediatric cancer. Previous studies, including our own, have shown prognostic value for the presence of *PAX3/7-FOXO1* fusion genes in rhabdomyosarcoma. It is proposed to introduce *PAX3/7-FOXO1* positivity as a component of risk stratification, rather than alveolar histology, in future clinical trials.

Procedure

To assess the potential impact of this reclassification, we have determined the changes to risk category assignment of 210 histologically reviewed patients treated in the UK from previous MMT (Malignant Mesenchymal Tumor) clinical trials for non-metastatic rhabdomyosarcoma based on identification of *PAX3/7-FOXO1* by fluorescence *in situ* hybridization and/or reverse transcription PCR.

Results

Using fusion gene positivity in the current risk stratification would re-assign 7% of patients to different EpSSG (European Paediatric Soft Tissue Sarcoma Study Group) risk subgroups. The next European trial would have 80% power to detect differences in event free survival of 15% over 10 years and 20% over 5 years in reassigned patients. This would decrease treatment for over a quarter of patients with alveolar histology tumors that lack *PAX3/7-FOXO1*.

Conclusions

Fusion gene status used in stratification may result in significant numbers of patients benefitting from lower treatment associated toxicity. Prospective testing to show this reassignment maintains current survival rates is now required and is

shown to be feasible based on estimated recruitment to a future EpSSG trial. Together with developing novel therapeutic strategies for patients identified as higher risk, this may ultimately improve the outcome and quality of life for patients with rhabdomyosarcoma.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, with ~450 children and adolescents newly diagnosed each year in Europe (countries which report data to the Automated Childhood Cancer Information System, ACCIS[1,2]). The substantial improvement in survival rate for RMS patients that occurred from 1960 to 1996 with the advent of chemotherapeutic agents has largely stagnated with an estimated 5 year survival rate of 72%[3,4]. The reality remains that while the majority of children suffering from cancer will survive to adulthood, more than 80% of these will develop a serious or life threatening chronic health condition by the age of 45 as a result of their curative treatment[5]. Accurate risk determination in RMS patients is a priority to enable safe reduction of treatment intensity for those at lower risk and identify those at highest risk of succumbing to their disease who could benefit from treatment intensification and/or novel therapeutic strategies.

Current clinical trials for RMS in Europe and the US use histological subtype alongside other clinical parameters including age at diagnosis, site and size of primary tumour, extent of residual disease after surgery, node involvement, and metastases to allocate patients to a risk group which will determine treatment intensity [6]. Two main histological subtypes are recognised, embryonal (ERMS) which typically has a better prognosis than the alveolar (ARMS) “unfavourable histology” subtype. The majority (70-80%) of ARMS cases have translocations resulting in fusion of the *PAX3* or *PAX7* gene with *FOXO1* [7,8]. The resultant fusion proteins are novel transcription factors and considered key drivers of tumorigenesis[9].

Previous studies including large-scale expression profiling have revealed that ARMS tumours lacking characteristic fusion genes are molecularly and clinically indistinguishable from ERMS tumors[10,11]. This is consistent with several studies, including a recent prospective assessment, that show a prognostic value for the fusion genes [12–15] although some issues with the representativeness of sample cohorts are also reported[16,17]. Based on the consensus view from these studies, that fusion gene presence rather than alveolar histology *per se* contributes to poorer outcome, it is proposed to incorporate fusion-gene status, rather than histology, into risk stratification of RMS. In order to address the impact of such a change in non-metastatic patients, we used the current EpSSG RMS2005 trial framework for risk stratification and applied this to a large cohort of well-annotated RMS cases enrolled in the series of Malignant Mesenchymal Tumour (MMT) trials, which we subjected to histopathological re-review. The treatment and outcome for patients in these trials were similar [18,19] and therefore were considered suitable for analysis as a single cohort.

Here we report the impact of adopting fusion gene status in place of histology as part of RMS risk stratification. This has allowed us to estimate the proportion of patients that would change risk group and the power of future clinical trials to assess any adverse changes in patient outcome.

Materials and Methods

Pathology and tissue microarray construction

Formalin fixed paraffin embedded (FFPE) samples from UK patients enrolled on the MMT89, MMT95 and MMT98 trials from the International Society of Paediatric Oncology (SIOP) were collected from multiple UK centres (Local Research Ethics Committee protocol 1836 and Multi-Regional Research Ethics Committee/98/4/023). Our cohort was subjected to histological re-review (A.K.) to apply current histological classification criteria[20]. Cases with mixed histologies but containing true alveolar histology (classical and solid variant patterns) were considered to be ARMS. Clinical parameters were accessed from trial databases[21]. These and updated histological subtypes of samples from cases non-metastatic at diagnoses (stage I-III) are summarised in Table 1, and were representative of other RMS cohorts[12]. A smaller cohort of metastatic cases (summarised in Supplemental Table S1) was used separately for additional analyses. Moreover, outcomes from MMT89 and MMT95 cases used in this study were representative of their respective trials (MMT89; Overall survival (OS) 74.4%, Event Free survival (EFS) 62.6%, MMT95; OS 74.3%, EFS 64% at 5 years)[18,19] (Outcome data shown refers to the cohort used in this study). The histopathologic diagnoses of the cases studied are also considered largely representative of the cases on the MMT89, MMT95 and MMT98 trials.

Haematoxylin and eosin stained slides were marked for regions of tumour and a tissue microarray (TMA) constructed containing 1,863 cores representing RMS tumour from 329 patients. This involved taking 0.6mm cores from tumour-containing regions of donor blocks and insertion into a recipient array block.

There was an average of 6 cores per sample (range 1-24). RMS cell lines negative and positive for each fusion gene (RD (negative)[22], RH30 (PAX3-FOXO1)[23], RMZ-RC2 (PAX7-FOXO1)[24]) were formalin fixed, paraffin embedded and cores inserted into each array block to act as controls. Sources and culturing conditions for cell lines have been previously described [25].

Fusion gene status assessment by fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) was performed on the TMA slides to determine whether samples carried a *PAX3-FOXO1* or *PAX7-FOXO1* fusion gene or neither. Bacterial artificial chromosome (BAC) DNA probes were identified that hybridize to the 5' end of *PAX3* and *PAX7* and to the 3'end of *FOXO1.* BAC DNA was amplified and subsequently purified using the Genomiphi Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturers instructions. BACs used for *PAX3* were RP11-81I8, RP11-16P6 and RP11-612G6 (labelled with Digoxygenin (DIG) (Roche, Basel, Switzerland) by random priming and indirectly detected using fluorescein isothiocyanate (FITC)-conjugated anti-DIG antibodies (Thermo Fisher Scientific, Waltham, MA, USA)). BACs used for *PAX7* were RP11-468NG, CTD-2009F7 and RP11-121A23 (directly labelled using FISH*Bright*® Aqua and the FISH*Bright*® Nucleic Acid Labelling Kit (Leica Microsystems, Wetzlar, Germany)) and BACs used for *FOXO1* were RP11-452K11, RP11-805F18 and RP11-350A18 (labelled with biotin by random priming and indirectly detected using Cy3-conjugated Streptavidin (Thermo Fisher Scientific)). All labelled BACs were individually hybridized to normal metaphase chromosomes to ensure their correct chromosomal location. FISH was carried out on TMA sections as previously described[26]. Slides were scanned using an Ariol

slide scanner (SL-50) (Leica Microsystems) and each core was independently scored for fused red/green and red/aqua signals in a minimum of 50 non-overlapping tumour nuclei by 2 independent observers. Fused signals, less than a signal width apart, were required to be present in at least 10% of scorable nuclei for a core to be considered fusion gene positive.

Fusion gene status assessment by reverse transcription-PCR

In addition to preparing TMAs, we also cut 10-micron FFPE sections for a subset of samples where sufficient material was available. These were assessed for fusion gene status by reverse transcription (RT)-PCR. RT-PCR results were used to confirm FISH results or provide a result in the event that FISH hybridisation for a patient was not successful. RNA was extracted from the FFPE rolls using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific) according to manufacturers' instructions. Reverse transcription was subsequently carried out on up to 1 mg of total RNA using the High Capacity Reverse Transcription Kit (Thermo Fisher Scientific). cDNA was then amplified in triplicate by real-time quantitative RT-PCR using Taqman (Thermo Fisher Scientific) reagents for *PAX3-FOXO1*, *PAX7-FOXO1* and *Beta-2-microglobulin (B2M)* expression, the latter acting as a reference gene. The primer sequences used in these assays have been previously described [27]. Each assay was performed separately and cDNA from control cell lines (as indicated above) (no fusion gene, *PAX3-FOXO*- and *PAX7-FOXO1*-positive) was included in each run. Samples were designated fusion gene positive if amplification occurred for the relevant assay whereas samples were only designated fusion gene negative if no

amplification was seen for either fusion gene assay and the signal from the *B2M* assay was not reached in less than or equal to 30 cycles.

Survival analysis was evaluated using the Mantel-Cox log rank test, Mantel-Haenszel Hazard Ratio and Kaplan-Meier plots.

Results

Ascertainment of fusion gene status in TMA cohort

Using FISH and/or RT-PCR analysis, fusion gene status was successfully determined in 210 patients with non-metastatic disease and a smaller cohort of 50 patients with metastasis that were treated on MMT clinical trials and had full clinical follow up data. 155 samples were assigned using FISH results only, 17 using PCR results only and 88 were assigned using both methods with complete concordance. The results are included in Table 2. We identified one patient described as having embryonal histology yet was found to harbour a *PAX3-FOXO1* fusion gene (0.64% of all ERMS patients). 20 patients with ARMS histology were found to be fusion gene negative (37.7% of all ARMS patients), 5 of which had mixed histology with only areas of true alveolar histology[20].

Comparison between risk determined using histology or molecular fusion gene status

Within the non-metastatic setting, Kaplan-Meier analysis demonstrated that there was no significant difference in overall (OS) or event free survival (EFS) between patients with ERMS and fusion negative ARMS in contrast to the fusion positive cases that showed a significantly poorer overall survival outcome than fusion

negative (log rank test, chi square value 21.9, p<0.0001, HR 6.047 (95% CI 2.845-2.85)) (Fig 1). This is consistent with previous studies, including our own[11,12]. The Kaplan-Meier plots for fusion positive cases divided into *PAX3-FOXO1* and *PAX7-FOXO1* (Supplemental Fig S1) shows no significant difference in survival between *PAX7-FOXO1* cases and any other subgroup, although the numbers are low. In the metastatic cohort, the outcome of patients with fusion negative alveolar disease appeared to be as poor as fusion positive cases (Supplemental Fig S2a) although there is no statistical significance between ERMS and fusion negative ARMS groups, but the numbers of these metastatic cases are very low. We also assessed outcome of our non-metastatic cohort according to the current non-metastatic EpSSG risk groups (Supplemental Table S2, treatment protocol associated with risk groups is outlined in Supplemental Table S3) and showed that the survival rates for each risk group were as expected (Supplemental Fig S2b).

In order to assess the impact of using fusion status rather than histology on patient risk stratification, we stratified all patients using i) histopathology, according to the EpSSG 2005 trial regimen using the re-reviewed histology (ERMS as favourable, ARMS as unfavourable) and ii) fusion status in place of histopathology (fusion negative as favourable, fusion positive as unfavourable). The risk group of each patient from each analysis was then compared. Using fusion gene status, 14 patients with fusion gene negative ARMS (26.4% of all patients with ARMS, 70% of fusion negative ARMS patients) changed risk group (5 moved from very high to high, 8 moved from high to standard, 1 moved from high to low). A summary of these changes using fusion gene status is shown in

for risk groups in Table 3 and for subgroups in Supplemental Table S4. Note in Supplemental Table S4, that although 6 patients changed risk subgroup from G to E, there was no change in overall risk group (high) and therefore no change in treatment strategy for those particular patients. These changes would result in reducing treatment intensity for 14/20 fusion negative ARMS.

It is vital to assess the patients receiving less intense treatment as a result of the change in stratification in forthcoming trials to ensure that their clinical outcome is not compromised. Assuming a null hypothesis that patients with fusion negative ARMS with downgraded risk will have an identical EFS rate to patients with ERMS of 70%, we performed power calculations to estimate the total patient number needed to have 80% power to identify decreases in EFS in this group (Table 4). Based on the previous trial, we predict that the next EpSSG trial is expected to recruit ~125 patients with non-metastatic paediatric RMS per year. Using the frequencies found in this study, we estimate that the next trial will have 80% power to detect differences in EFS of 15% over 7 years and 20% over 5 years.

Discussion

Assessment of the molecular features of tumours is increasingly required for accurate diagnoses, risk stratification and precision approaches to treatment decisions for patients. Previous studies, including our own, have shown a prognostic value for the presence of the fusion gene in RMS and it is proposed to introduce this as a molecularly unfavourable category, in place of alveolar histology, into future clinical trials. Here, our assessment of 210 samples from

previous clinical trials, that are representative of the trials as a whole, shows that overall this would affect assignment of patients to specific risk subgroups, reducing treatment for over a quarter of patients with alveolar histology and 7% of all non-metastatic RMS (it is noteworthy that the next European trial plans to intensify chemotherapy for the High and Very High risk groups, which is likely to increase treatment associated morbidity). This has potential to reduce long-term toxicities in these patients, which is important as such toxicities are a major issue in the majority of RMS patients that are cured of their disease[28].

Changes in the histopathological criteria used to discriminate between embyronal and alveolar histology have been updated over time, with the introduction in 1995 of a prognostically relevant classification system which determined that even focal alveolar histology should confer an ARMS diagnosis[29] resulting in an increasing proportion of ARMS cases. More recently, a re-examination of these criteria noted that certain histological patterns may be mimicking ARMS[30], leading to an artificially high rate of ARMS diagnosis. Despite our cohort being re-reviewed using current criteria, we observed a relatively high proportion of fusion negative ARMS (37.7%). However, including patients with metastasis in our cohort reduced this proportion to 26.9% similar to other studies and may reflect the more metastatic behaviour of ARMS driven by the fusion protein [11,31]. The range of proportions of fusion negative ARMS reported is underpinned by diagnostic uncertainty using histopathological criteria in challenging cases, where informal use of the fusion gene status and other clinical parameters is guiding histological diagnoses. Standardizing use of molecular criteria in future trials is therefore highly desirable.

We identified 1 out of 157 patients with ERMS to be *PAX3-FOXO1* positive by both FISH and RT-PCR. Fusion positive ERMS cases have been reported before[27] where PCR detection was used, notably all of these cases demonstrated diffuse myogenin staining, a feature associated with ARMS[32]. This suggests that there is a rationale to screen for fusion genes in all patients, as these patients may move from low to high-risk groups. Previous studies have reported that patients with tumours harbouring a *PAX7-FOXO1* gene have a superior outcome compared to *PAX3-FOXO1*[11,12,33] however numbers are limited and this may be stage-dependent[13]. We only had 6 patient samples with a *PAX7-FOXO1* gene in our cohort and therefore could not address this question adequately in this study. Rarer fusion gene variants are reported such as *PAX3-NCOA1* and *PAX3-NCOA2*[34] in ARMS and ERMS, however the clinical significance of these are unclear.

Stratifying RMS patients according to molecular rather than histopathological criteria will result in a proportion of fusion negative alveolar patients (26.4% of patients with ARMS in this study) receiving less intense treatment, being perceived to be at lower risk. It is important to establish that these patients will have a similarly favourable outcome as patients with ERMS when treated on the same protocol. Using data from our patient population, we have estimated that the expected number of patients recruited to the next EpSSG trial will be sufficient to detect changes in event free survival of 15% over 7 years and 20% over 5 years with 80% power. Patients with ERMS have an EFS of 70% at 5 years compared to fusion positive ARMS with 36.1% at 5 years. It is anticipated that

molecular features of RMS will be increasingly incorporated into risk stratification as there is evidence that *MYOD1* mutations in sclerosing/spindle RMS[35–37], CDK4 amplification[38] and the MG5 gene signature in fusion negative RMS[25,39] can all impact survival.

Here we have determined the potential impact of using fusion gene status rather than the histopathological definition of alveolar histology as an adverse indicator in the risk-stratification of RMS that is proposed for use in the next clinical trials. We show that a significant proportion of patients with non-metastatic RMS (7%) will be assigned to a different risk group and treatment protocol as a consequence of this change. It is expected that this will result in children being spared some of the considerable toxicities and late effects of intense therapy without compromising their chance of cure, in addition to the possibility of identifying fusion positive patients presenting with ERMS or RMS-NOS that will benefit from being considered as high-risk.

Acknowledgements

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Conflict of interest statement

None declared.

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Figure Legends

Fig 1. Overall survival (A) and event free survival (B) in non-metastatic RMS patients grouped into ERMS fusion negative (ERMS FN), ARMS fusion negative (ARMS FN) and fusion positive patients (FP).

Supplemental Figure Legends

Supplemental Figure S1. Overall survival (A) and event free survival (B) in non-metastatic RMS patients grouped into ERMS fusion negative (ERMS FN), ARMS fusion negative (ARMS FN), *PAX3-FOXO1* and *PAX7-FOXO1*.

Supplemental Figure S2. (A) Overall survival in metastatic (stage IV) RMS grouped into ERMS fusion negative (ERMS FN), ARMS fusion negative (ARMS FN) and fusion positive patients (FP). (B) Overall survival in non-metastatic RMS patients stratified into risk groups according to the current EpSSG RMS2005 clinical trial criteria.

Supplemental Table Legends

Supplemental Table S1. Clinical and molecular characteristics of the metastatic cohort.

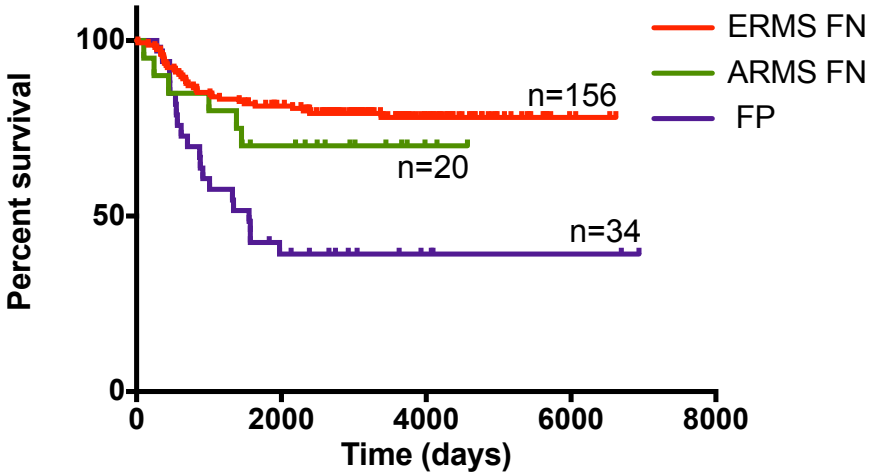
Supplemental Table S2. Risk Stratification for the EpSSG non-metastatic RMS study. Pathology: Favourable indicates embryonal histology including botryoid and spindle cell subtypes; Unfavourable indicates alveolar histology. Post

surgical stage (IRS group): I indicates complete primary resection; II indicates microscopic residual or primary complete resection but N1; III indicates macroscopic residual. Site: Favourable indicates Orbit, Genitourinary (non bladder/prostate), Head and neck (non-parameningeal); Unfavourable indicates parameningeal, extremities, Genitourinary bladder/prostate and all other sites. Node Stage: N0 indicates no clinical or pathological node involvement; N1 indicates pathological node involvement. Size and Age: Favourable indicates tumour size less than or equal to 5 cm and age less than 10 years; Unfavourable indicates all other options (i.e. Size greater than 5 cm and/or age greater than or equal to 10 years).

Supplemental Table S3. Treatment protocol for EpSSG RMS risk groups. Tumour assessment carried out between first and second course of frontline therapy. VA = Vincristine/Actinomycin;
IVA = Ifosfamide/Vincristine/Actinomycin; RT = radiotherapy; IVADo = Ifosfamide/Vincristine/Actinomycin/Doxorubicin
*only given if patient shows complete response (CR) to first course and has favourable age and tumour size.
**If patient shows stable disease (SD) after first course, second line treatment (usually Carboplatin, Cyclophosphamide, Topotecan or Doxorubicin) with radiotherapy will be given.
***Randomised trial arms.

Supplemental Table S4. Summary of changes in subgroup between histological and molecular categorization of pathology. Hist. = Histology; Mol. = Molecular. Note that grey boxes indicate patients that remain in the same risk group using either histological or molecular categorization.

A


Percent survival
100
50
0
0
2000
4000
6000
8000
Time (days)
ERMS FN
ARMS FN
FP
n=156
n=20
n=34

B

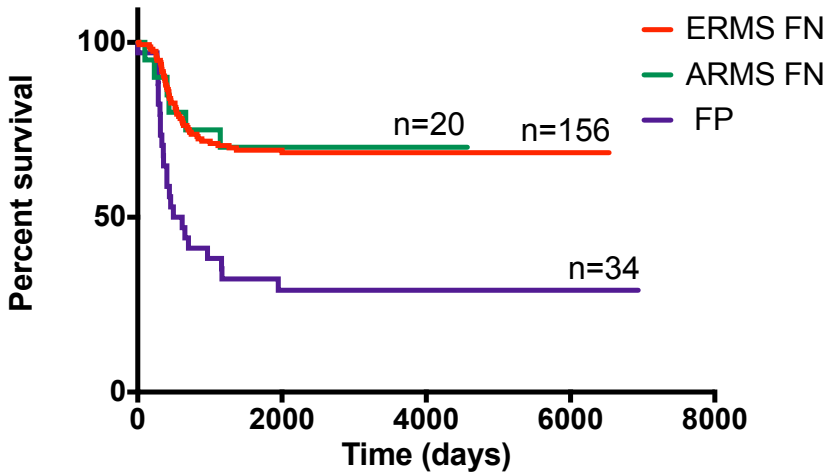

Percent survival
100
50
0
0
2000
4000
6000
8000
Time (days)
ERMS FN
ARMS FN
FP
n=20
n=156
n=34

TABLE 1 Clinical characteristics of the non-metastatic cohort

Histology	ERMS	157
	ARMS	53
Median age at diagnosis (years)		4.5
Age at dx	<10	173
	>=10	37
IRS group	1	28
	2	40
	3	142
Size of primary tumour	<=5cm	90
	>5cm	115
	unknown	5
Site of primary tumour	Favourable	83
	Unfavourable	127
Median follow up time (years)		8.1
Patient Survival	Alive	151
	Dead	59
Total no of patients		210

TABLE 2 Fusion gene status of the non-metastatic patient cohort, grouped by histology

	ERMS	**ARMS**	**Total**
Negative	156	20	176
PAX3-FOXO1	1	27	28
PAX7-FOXO1	0	6	6
Total	157	53	210

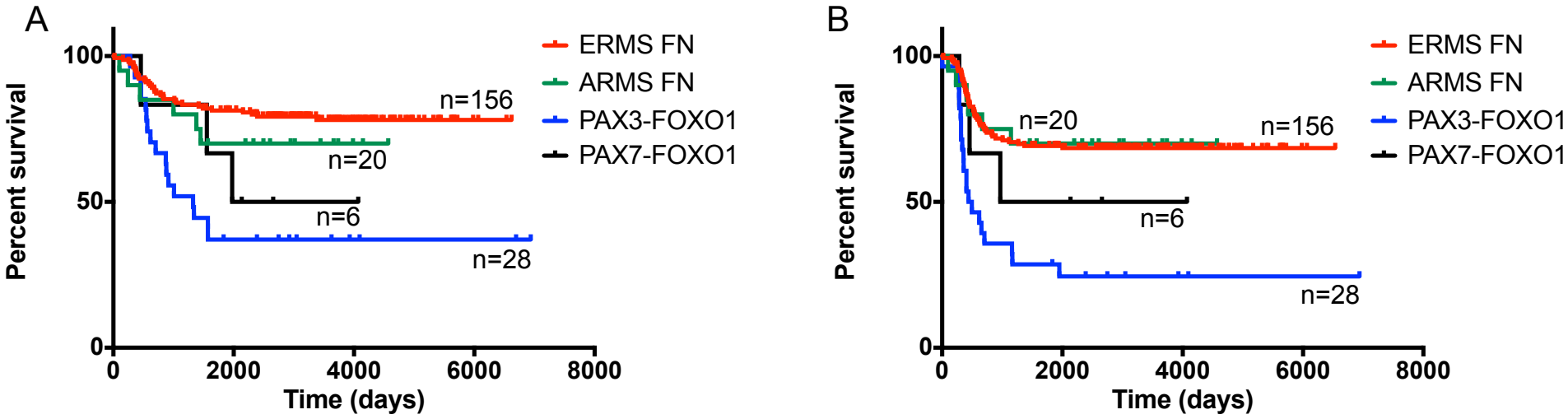
TABLE 3 Summary of changes in EpSSG risk group between histological and molecular categorization of pathology

Risk Group	Subgroup	Histology Risk group	Molecular Risk group	% change
Low	A	9	10	+11.1
Standard	B	70	78	+11.4
	C			
	D			
High	E	117	113	-3.4
	F			
	G			
Very High	H	14	9	-35.7

TABLE 4 Estimation of the number of patients needed for 80% power to detect decreased EFS rate in fusion gene negative alveolar patients with downgraded risk

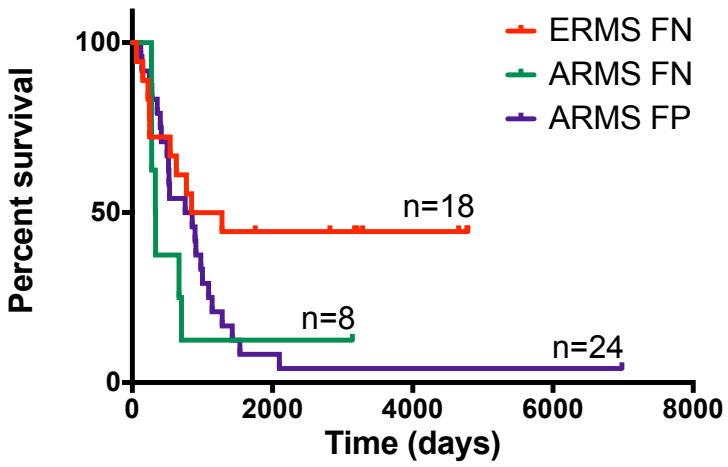
Change in EFS rate	No of downgraded patients with ARMS FN	Total patient number
10%	141	2,015
15%	63	900
20%	36	515
25%	23	329

Supplemental Fig S1



Supplemental Fig S2

A



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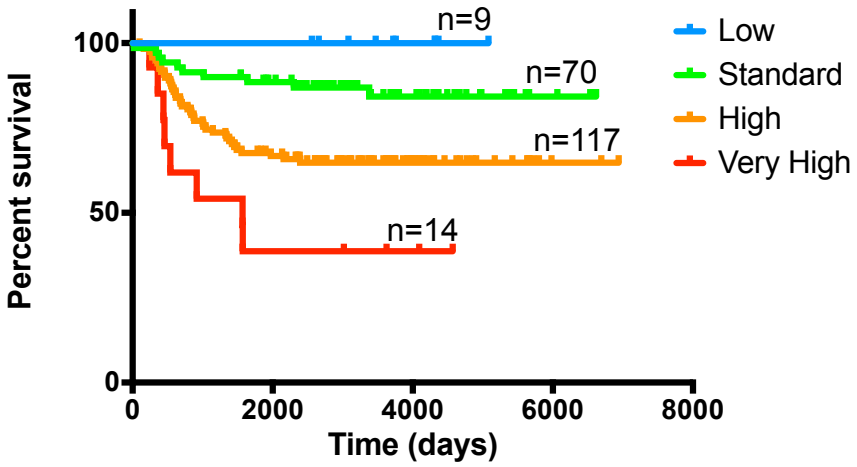

Supplemental Table S1 Clinical and molecular characteristics of the metastatic cohort

Histology	ERMS	18
	ARMS	32
Median age at diagnosis (years)		8.2
Fusion gene status	ERMS Negative	18
	ARMS Negative	8
	PAX3-FOXO1	20
	PAX7-FOXO1	4
Median follow up time (years)		2
Patient Survival	Alive	10
	Dead	40
Total no of patients		50

Supplemental Table S2 Risk Stratification for the EpSSG non-metastatic RMS study.

Risk Group	Subgroup	Pathology	IRS Group	Site	Node Stage	Size & Age
Low	A	Favourable	I	Any	N0	Favourable
Standard	B	Favourable	I	Any	N0	Unfavourable
	C	Favourable	II, III	Favourable	N0	Any
	D	Favourable	II, III	Unfavourable	N0	Favourable
High	E	Favourable	II, III	Unfavourable	N0	Unfavourable
	F	Favourable	I, II, III	Any	N1	Any
	G	Unfavourable	I, II, III	Any	N0	Any
Very High	H	Unfavourable	I, II, III	Any	N1	Any

Pathology: Favourable indicates embryonal histology including botryoid and spindle cell subtypes; Unfavourable indicates alveolar histology. Post surgical stage (IRS group): I indicates complete primary resection; II indicates microscopic residual or primary complete resection but N1; III indicates macroscopic residual. Site: Favourable indicates Orbit, Genitourinary (non bladder/prostate), Head and neck (non-parameningeal); Unfavourable indicates parameningeal, extremities, Genitourinary bladder/prostate and all other sites. Node Stage: N0 indicates no clinical or pathological node involvement; N1 indicates pathological node involvement. Size and Age: Favourable indicates tumour size less than or equal to 5 cm and age less than 10 years; Unfavourable indicates all other options (i.e. Size greater than 5 cm and/or age greater than or equal to 10 years).

Supplemental Table S3 Treatment protocol for EpSSG RMS risk groups

Risk group	1st Course	2nd course	Maintenance
Low	VA (8 cycles, 24 weeks)	-	-
Standard (Subgroup B)	IVA	VA	-
Standard (Subgroup C)	IVA	IVA/No RT*	-
		IVA/VA with RT	-
Standard (Subgroup D)	IVA	IVA with RT**	-
High	IVA or IVADo***	IVA with RT**	None or Vinorelbine/Cyclophosphamide (6 months)***
Very High	IVADo	IVADo/IVA**	Vinorelbine/Cyclophosphamide (6 months)

Tumour assessment carried out between first and second course of frontline therapy. VA = Vincristine/Actinomycin; IVA = Ifosfamide/Vincristine/Actinomycin; RT = radiotherapy; IVADo = Ifosfamide/Vincristine/Actinomycin/Doxorubicin

*only given if patient shows complete response (CR) to first course and has favourable age and tumour size.

**If patient shows stable disease (SD) after first course, second line treatment (usually Carboplatin, Cyclophosphamide, Topotecan or Doxorubicin) with radiotherapy will be given.

***Randomised trial arms.

Supplemental Table S4 Summary of changes in subgroup between histological and molecular categorization of pathology

			Molecular Risk Group								Total
			Low	Standard			High			Very High	
			A	B	C	D	E	F	G	H	
Histology Risk Group	Low	A	9								9
	Standard	B		11							11
		C			40						40
		D				19					19
	High	E					57				57
		F						23			23
		G	1	2	3	3	6		22		37
	Very High	H						5		9	14
	Total		10	13	43	22	63	28	22	9	210
% change (Hist. to Mol.)			+11.1	+18.2	+7.5	+15.8	+10.8	+21.7	-40.5	-35.7	

Hist. = Histology; Mol. = Molecular. Note that grey boxes indicate patients that remain in the same risk group using either histological or molecular categorization.