Original Article

Fusion Status in Patients With Lymph Node-Positive (N1) Alveolar Rhabdomyosarcoma Is a Powerful Predictor of Prognosis: Experience of the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG)

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BACKGROUND: Alveolar rhabdomyosarcoma (aRMS) with lymph node involvement (N1 classification) accounts for up to 10% of all cases of RMS. The prognosis is poor, and is comparable to that of distant metastatic disease. In the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) RMS2005 protocol, patients with a histologic diagnosis of aRMS/N1 received intensified chemotherapy with systematic locoregional treatment. **METHODS:** Patients with aRMS/N1 were enrolled prospectively after primary surgery/biopsy and fusion status was assessed in tumor samples. All patients received 9 cycles of induction chemotherapy and 6 months of maintenance therapy. Local treatment included radiotherapy to the primary site and lymph nodes with or without secondary surgical resection. **RESULTS:** A total of 103 patients were enrolled. The clinical characteristics of the patients were predominantly unfavorable: 90% had macroscopic residual disease after initial surgery/biopsy, 63% had locally invasive tumors, 77% had a tumor measuring >5 cm, and 81% had disease at unfavorable sites. Fusion genes involving forkhead box protein O1 (*FOXO1*) were detected in 56 of 84 patients. Events occurred in 52 patients: 43 developed disease recurrence, 7 had disease that was refractory to treatment, and 2 patients developed second neoplasms. On univariate analysis, unfavorable disease site, tumor invasiveness, Intergroup Rhabdomyosarcoma Study group III, and fusion-positive status correlated with worse prognosis. The 5-year event-free survival rate of patients with fusion-positive tumors was 43% compared with 74% in patients with fusion-negative tumors ($P = .01$). On multivariate analysis, fusion positivity and tumor invasiveness proved to be unfavorable prognostic markers. **CONCLUSIONS:** Fusion status and tumor invasiveness appear to have a strong impact on prognosis in patients with aRMS/N1. Fusion status will be used to stratify these patients in the next EpSSG RMS study, and treatment will be intensified in patients with fusion-positive tumors. ***Cancer*** **2018;124:3201-9.**

KEYWORDS: alveolar rhabdomyosarcoma, lymph node involvement, paired box (*PAX*)-forkhead box protein O1 (*FOXO1*) fusion, prognostic factors, rhabdomyosarcoma.

INTRODUCTION

Rhabdomyosarcoma (RMS) is one of the most frequent extracranial solid tumors diagnosed in children and the most common form of soft-tissue sarcoma diagnosed in children and young adults.[1] The prognosis of patients with localized RMS has improved considerably over time thanks to numerous clinical trials conducted by collaborative groups working in North America (Children's Oncology Group [COG]) and Europe (International Society of Pediatric Oncology [SIOP] Malignant Mesenchymal Tumor Group [MMT], Italian Soft Tissue Sarcoma Committee [STSC], and German Cooperative Soft Tissue Sarcoma Study Group [CWS]). The presence of disseminated disease at the time of diagnosis is

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the most powerful prognostic factor in RMS. Although the probability of cure in pediatric patients with localized disease is >70%, the prognosis of those with distant metastatic disease remains poor.[2-8] In patients with localized disease, clinical and tumor characteristics have been used to classify RMS into different risk categories and to determine treatment intensity. Unfavorable characteristics include alveolar histology, invasive tumor (T2 classification), tumor location, lymph node involvement, tumor size >5 cm, and patient age ≥10 years[9-11] and constitute the basis for the risk stratification system used in the recent European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) RMS2005 study. Previous experience has suggested that patients with alveolar RMS (aRMS) and regional lymph node involvement represent a group with a particularly poor prognosis.[11]

Approximately 70% of patients with aRMS present with the fusion genes paired box 3 (*PAX3*)-forkhead box protein O1 (*FOXO1*) or paired box 7 (*PAX7*)-*FOXO1* as a consequence of the reciprocal chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14).[12] Recent data have suggested that the *PAX3/7-FOXO1* fusion genes have prognostic significance.[13,14] This observational study reports on the results obtained in this very high-risk population, and focuses on the prognostic role of fusion gene status.

MATERIALS AND METHODS

Patients

The RMS2005 protocol was initiated in October 2005 and opened in 14 countries. Eligibility criteria for inclusion in the RMS2005 protocol were age >6 months to <21 years, a pathologically proven diagnosis of RMS, no evidence of distant metastatic lesions, tumor previously untreated except for primary surgery, no preexisting illness preventing treatment, no previous malignant tumors, and an interval between diagnostic surgery and treatment of ≤8 weeks. Patients with localized aRMS and regional lymph node involvement (N1 classification) were assigned to the very high-risk group according to the EpSSG stratification system. This group is the focus of the current analysis, with particular attention to the group of patients who underwent molecular analysis of *PAX3/7-FOXO1* fusions. Only patients enrolled before December 31, 2013 were included in this analysis to ensure an adequate follow-up. The cutoff date for the analysis was April 4, 2017.

Staging

Disease was staged according to the TNM classification and the Intergroup Rhabdomyosarcoma Study Group (IRS) postsurgical grouping system.[15] Regional lymph node involvement was indicated as N0 or N1 and distant metastases at the time of onset as M0 or M1 based on histologic or clinical/radiologic assessments.

Tumor location was considered favorable if arising from the orbit, genitourinary region other than the bladder or prostate (ie, paratesticular and vagina/uterus), and nonparameningeal head and neck, and was considered unfavorable when arising from any other site.

Regional lymph nodes were defined as those appropriate to the site of the primary tumor. Any evidence of distant lymph node involvement other than these was considered metastasis and patients were treated according to the protocol for those with metastatic disease at the time of diagnosis. Surgical exploration of regional lymph nodes was mandatory in cases of RMS arising in the limbs. In tumors originating in other locations, regional lymph node involvement was determined clinically and by imaging, including magnetic resonance imaging and/or positron emission tomography (PET)-computed tomography scan. In doubtful cases, a lymph node biopsy was recommended. Systematic sentinel lymph node examination was suggested but implemented only at a small number of centers.

Treatment

Patients received intensified initial chemotherapy and additional maintenance chemotherapy with systematic local treatment to the primary and lymph node sites. Induction chemotherapy comprised 4 cycles of 21 days each of ifosfamide at a dose of 3 g/m^2 on days 1 to 2 with mesna; vincristine at a dose of 1.5 mg/m^2 (maximum, 2 mg) on days 1, 8, and 15 in the first 2 cycles and day 1 in cycles 3 and 4; actinomycin D at a dose of 1.5 mg/m^2 (maximum, 2 mg) on day 1; and doxorubicin at a dose of 30 mg/m^2 on days 1 to 2 (IVADo) followed by 5 cycles of 21 days each of ifosfamide at a dose of 3 g/m^2 on days 1 to 2 with mesna, vincristine at a dose of 1.5 mg/m^2 on day 1, and actinomycin D at a dose of 1.5 mg/m^2 on day 1 (IVA) and 6 cycles of 28 days each of maintenance chemotherapy comprising continuous daily oral cyclophosphamide at a dose of 25 mg/m^2 and intravenous vinorelbine at a dose of 25 mg/m^2 on days 1, 8, and 15 of each cycle.[16]

Local treatment after the initial 4 cycles of IVADo (week 13) included delayed (secondary) surgery to remove macroscopic residual tumor and radiotherapy (RT). External beam RT was scheduled to be given to the primary tumor area and the affected lymph node region. Doses varied according to chemotherapy response and surgical results and were administered in 1.8-gray (Gy)

daily fractions. The total dose to the primary tumor in postsurgical IRS group II and group III patients with complete remission after secondary surgery was 41.4 Gy. For patients in IRS group III with incomplete secondary resection or when secondary surgery was not feasible, the total dose was 50.4 Gy with an optional additional boost of 5.4 Gy in 3 fractions for large tumors with poor responses to chemotherapy. RT to the involved lymph nodes was recommended at a dose of 41.4 Gy regardless of surgical resection. Treatment was delivered with megavoltage photons at 1 fraction per day for 5 days per week.

Response was evaluated after initial chemotherapy (week 9) and at the end of treatment by 3-dimensional volumetric assessment using the formula: tumor volume (cm^3) = 0.52 × length (cm) × width (cm) × thickness (cm). Responses were defined as complete response (clinically or histologically confirmed complete disappearance of disease), partial response (at least a two-thirds reduction in tumor volume), minor response (a reduction in tumor volume greater than one-third but less than two-thirds), stable disease (a modification in tumor volume of less than one-third), and progressive disease (an increase in tumor size >30% or the detection of new lesions).

The site of first disease recurrence was defined as local if the tumor recurred at the site of primary disease, lymph node if regional lymph nodes were involved, locoregional in cases of local and lymph node disease recurrence, distant in cases with the appearance of metastatic disease, and combined when locoregional plus metastatic disease recurrence were evident.

Pathology and Biology

Histologic analysis was performed locally at participating EpSSG centers using routine hematoxylin and eosin staining. Following protocol guidelines, a panel of appointed pathologists reviewed 2 to 12 tumor slides from each patient and confirmed the diagnosis of aRMS.

The molecular characterization of aRMS was part of several translational studies to be implemented in the RMS2005 protocol. The analysis was strongly recommended and should be conducted at a single laboratory for each participating national group. However, fusion status data were not available for the entire population because of a shortage of suitable or fresh biologic material. Molecular analysis of the *PAX3/7-FOXO1* fusion was performed by fluorescent in situ hybridization (FISH) in paraffin blocks and/or by reverse transcriptase-polymerase chain reaction (RT-PCR) in frozen tissue. Interphase and metaphase FISH studies for RMS translocations were performed using chromosome 13 cosmids flanking the *FOXO1* gene using a commercial break-apart probe as described.[12] RNA from snap-frozen tumor was assayed by single-round RT-PCR using the primer pairs and conditions as described.[17] Only samples with a sufficient number of tumor cells (>50%) were considered for the analysis. Alternative *PAX3* fusions with partners other than *FOXO1* were not analyzed. Samples with *FOXO1* gene disruption (ie, positive *PAX3-FOXO1, PAX7-FOXO1*, or *FOXO1* with an unknown gene partner) were considered fusion status positive.

Statistical Analysis

Data were collected via a Web-based system and analyzed at Veneto Oncologic Institute (Padova, Italy). Continuous variables were summarized with the median, minimum, and maximum, whereas categorical variables were reported as counts and percentages.

Survival was calculated from the date of diagnosis to the time of the event or last follow-up. Tumor progression, disease recurrence, occurrence of a second malignancy, or death due to any cause were considered for event-free survival (EFS). Overall survival (OS) was measured from the date of diagnosis to the date of death from any cause. Patients who still were alive at the end of the study were censored at the date of the last observation.

Survival probability was computed using the Kaplan-Meier method and heterogeneity in survival among strata of selected variables was assessed with the log-rank test. The 5-year EFS and OS rates of the patient subgroup with available molecular data were reported along with their 95% confidence intervals (95% CIs), computed using the Greenwood formula.

The Cox proportional hazards regression method was used to ascertain whether fusion-positive status may have prognostic significance in this cohort of patients. A stepwise variable selection procedure was applied to the covariates with a P value $\geq .25$ in the univariate analysis. Hazard ratios (HRs) with 95% CIs according to the Wald method were reported for independent selected variables. All data analyses were performed using the SAS statistical package (release 9.4; SAS Institute Inc, Cary, North Carolina).

Ethics

The EpSSG RMS2005 treatment protocol was submitted to the institutional and national review boards of each participating country for review and approval before the enrollment of patients. Written informed consent for participation was obtained from patients, parents, or legal guardians in all cases. The study was conducted in

TABLE 1. Clinical Characteristics of Patients With aRMS and Lymph Node Involvement (N1 Classification)

Characteristic	Molecular Biology Not Performed N=18		Molecular Biology Performed N=85		Total N=103		*P*
	No. of Patients	%	No. of Patients	%	No. of Patients	%	
Age at diagnosis							
<10 y	5	27.8	47	55.2	52	50.5	.0339
≥10 y	13	72.2	38	44.8	51	49.5	
Sex							
Female	6	33.3	35	41.2	41	39.8	.5369
Male	12	66.7	50	58.8	62	60.2	
Postsurgical IRS group							
II	1	5.6	9	10.6	10	9.7	.5124
III	17	94.4	76	89.4	93	90.3	
Tumor invasiveness (T classification)							
T1	5	27.8	33	38.8	38	36.9	.3776
T2	13	72.2	52	61.2	65	63.1	
Tumor size							
a: ≤5 cm	3	16.7	20	23.5	23	22.3	0.7224
b: >5 cm	15	83.3	64	75.3	79	76.7	
x: Not evaluable	-	-	1	1.2	1	1.0	
Site of origin of primary tumor							
Favorable site	1	5.6	19	22.4	20	19.4	0.1017
Unfavorable site	17	94.4	66	77.6	83	80.6	
Fusion status							
PAX3-FOXO1 positive	-	-	31	36.5	31	30.1	
PAX7-FOXO1 positive	-	-	8	9.4	8	7.8	
FOXO1 positive	-	-	17	20.0	17	16.5	
FOXO1 negative	-	-	28	32.9	28	27.2	
Sample inadequate	-	-	1	1.2	1	0.9	
Not analyzed	18	100.0	-	-	18	17.5	

Abbreviations: aRMS, alveolar rhabdomyosarcoma; FOXO1, forkhead box protein O1; IRS, Intergroup Rhabdomyosarcoma Study; PAX3, paired box 3; PAX7, paired box 7.

accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines (European Union Drug Regulating Authorities Clinical Trials EUDRACT No. 2005-000217-35).

RESULTS

Patient and Tumor Characteristics

From December 2005 to December 2013, a total of 103 patients with aRMS/N1 were included, accounting for 8.1% of all patients (1272 patients) enrolled in the EpSSG RMS2005 protocol. *PAX3/7-FOXO1* fusion was analyzed in 85 patients (82.5%). *FOXO1* gene disruption was detected by FISH or RT-PCR in 56 patients, 31 of whom had *PAX3-FOXO1*, 8 of whom had *PAX7-FOXO1*, and 17 of whom were *FOXO1* positive with an unknown gene partner. Twenty-eight patients had fusion-negative tumors and 1 sample was inadequate for analysis. Molecular study was not performed in 18 patients.

The clinical characteristics of the entire cohort (Table 1) demonstrated a predominance of unfavorable prognostic factors: 90% of patients had IRS group III tumors, 81% of tumors were located at unfavorable sites, 77% of tumors measured > 5 cm, invasive (classified as T2) tumors represented approximately 63% of all cases, and approximately 50% of patients were aged >10 years. No significant differences were found with regard to the prognostic factors considered in the current study between patients with or without biologic data, with the exception of the predominance of age ≥10 years in the group without molecular study ($P = .0339$). For this reason, inferential statistical analyses were performed in patients with available biological data.

Treatment

Chemotherapy

Of the 103 enrolled patients, 73 received chemotherapy as per protocol and 30 received treatment with modifications. Of these 30 patients, 19 had interrupted chemotherapy before completing treatment (18 because of progressive disease or disease recurrence and the parents of 1 patient refused to continue treatment) and in 11 patients chemotherapy was modified because of (CTCAE v4.03) toxicity in 2 patients (septic shock and hemorrhagic cystitis, respectively), a lack of tumor response in 2 patients, and by the attending physician's decision in 7 patients. All patients presented with at least 1 episode of

TABLE 2. Association Between Potential Prognostic Factors and Outcome in Patients With Fusion Status Analyzed

		EFS			OS		
	No. of Patients	Failed	5-Year (95% CI)	*P*	Failed	5-Year (95% CI)	*P*
Age							
<10 y	47	18	60.4 (44.7-73.0)	.0596	16	60.6 (43.4-74.0)	.0797
≥10 y	37	22	44.0 (27.3-59.5)		19	47.9 (30.2-63.6)	
Tumor size							
≤5 cm	20	7	64.3 (39.3-81.2)	.3475	7	59.8 (32.9-78.8)	.6395
>5 cm	63	33	49.9 (36.8-61.6)		28	52.8 (38.6-65.1)	
Tumor invasiveness (T classification)							
T1	33	10	67.3 (47.3-81.1)	.0137	7	71.5 (48.6-85.5)	.0040
T2	51	30	44.8 (30.8-57.8)		28	45.2 (30.8-58.5)	
Fusion status							
Positive	56	33	43.0 (29.5-55.7)	.0101	28	45.5 (30.8-59.2)	.0548
Negative	28	7	74.4 (53.6-87.0)		7	73.7 (52.4-86.6)	
IRS group							
II	9	1	88.9 (43.3-98.4)	.0367	1	87.5 (38.7-98.1)	.0533
III	75	39	49.0 (37.0-60.0)		34	51.0 (38.1-62.6)	
Site of primary tumor							
Favorable site	19	4	75.7 (46.9-90.3)	.0177	3	81.2 (51.9-93.6)	.0293
Unfavorable site	65	36	46.9 (34.2-58.5)		32	48.2 (34.7-60.4)	

Abbreviations: 95% CI, 95% confidence interval; EFS, event-free survival; IRS, Intergroup Rhabdomyosarcoma Study; OS, overall survival.

grade 3 to 4 hematologic toxicity. The most frequent nonhematologic toxicity was gastrointestinal (mucositis) and neurologic (peripheral neuropathy and ileus) (see Supporting Table 1).

Surgery

Ten patients (10%) underwent primary surgery: 6 in IRS group IIb (primary complete resection without microscopic residual disease and lymph node involvement) and 4 in IRS group IIc (primary complete resection with microscopic residual disease and lymph node involvement). A total of 48 patients underwent secondary surgery (resection of the primary tumor in 29 patients, combined resection of the tumor and lymph nodes in 15 patients [1 bilateral lymphadenectomy, 7 unilateral lymphadenectomies, and 7 biopsies], and surgery to the lymph nodes alone in 4 patients [2 biopsies and 2 unilateral lymphadenectomies]). Among the 44 patients who underwent delayed surgical resection of the primary tumor, complete local resection (R0) was performed in 29 patients, with microscopic residual disease (R1) noted in 8 patients, macroscopic residual disease (R2) noted in 4 patients, and no residual tumor noted in 3 patients.

Radiotherapy

Overall, 92 of 103 patients (89.3%) were irradiated. RT was not administered because of progressive disease in 4 patients, amputation in 2 patients, parental refusal in 2 patients, and physician decision in 3 patients. Eight patients received irradiation to the primary tumor area alone, 81 to the primary tumor and lymph nodes, and 3 to lymph nodes alone (2 patients after limb amputation and 1 with a completely resected primary tumor at the time of diagnosis). The median dose to the primary tumor for the overall population was 50.4 Gy (range, 36.0-59.4 Gy) and that to the lymph nodes was 41.4 Gy (range, 24.0-54.4 Gy). Fifteen of 103 patients were aged ≤3 years: 11 received RT and 4 did not receive RT because of parent refusal in 1 patient, physician decision in 1 patient, and tumor progression before the initiation of RT in 2 patients.

Outcome

With a median follow-up of 64.9 months (range, 19.8-116.3 months), 52 patients developed an event and 47 died of disease. Seven patients had refractory disease (no response or disease progression at week 9) and presented with early disease progression (median time to disease progression of 6.2 months [range, 2.1-9.7 months]), 2 patients developed secondary neoplasms, and 43 patients developed disease recurrence. The site of the first recurrence was local in 10 patients, lymph node in 6 patients, and locoregional in 2 patients. Seventeen patients had distant disease recurrence and 8 patients had combined disease recurrence. Local and locoregional events (18 of 43 patients) accounted for approximately 42% of the cases of disease recurrence and lymph node recurrences were present in 13 patients as the first event (33%). The median

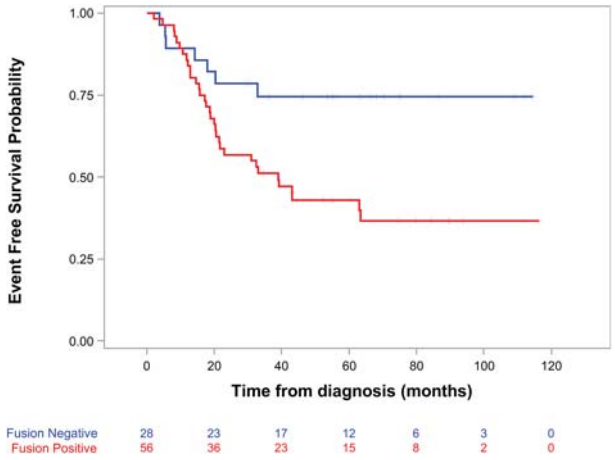

Figure 1. Kaplan-Meier curves representing 5-year event-free survival (EFS) by fusion status. The EFS rate for patients with fusion-positive tumors was 43% compared with 74.4% for those with fusion-negative tumors ($P = .01$).

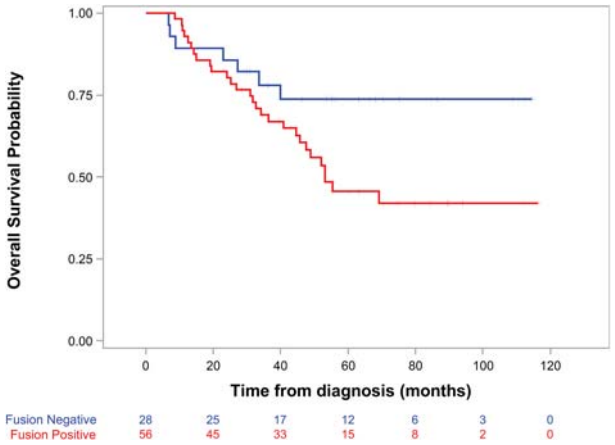

Figure 2. Kaplan-Meier curves representing 5-year overall survival (OS) by fusion status. The OS rate for patients with fusion-positive tumors was 45.5% compared with 74.7% for those with fusion-negative tumors ($P = .05$).

time from diagnosis to disease recurrence was 16.4 months (range, 2.1-63.5 months). At the time of last follow-up, among the 5 patients surviving tumor recurrence, 3 were alive with disease and 2 were in complete response after second-line chemotherapy and RT. The 5-year EFS rate for the entire population was 50.1% (95% CI, 39.8%-59.5%) and the 5-year OS rate was 50.6% (95% CI, 39.7%-60.5%). The median time from first event to death was 8.8 months (range, 0-41.0 months). In the univariate analysis performed in the group of patients for whom fusion status data were available (Table 2), the following factors were found to be associated with an increased risk of disease recurrence or death: unfavorable primary site, invasive tumor (T2 classification), the presence of the *FOXO1* translocation, and classification into IRS group III. Significant variables ($P < .25$) emerged from univariate analysis (patient age at the time of diagnosis, primary tumor site, tumor invasiveness, fusion status, and IRS group) and were included in the Cox model. Only fusion gene status and tumor invasiveness remained as independent prognostic factors for the risk of disease recurrence. Fusion-positive aRMS was associated with EFS with an HR of 2.6 (95% CI, 1.1-5.9; $P = .0226$) and tumor invasiveness (T2 classification) was associated with an HR of 2.2 (95% CI, 1.1-4.6; $P = .0296$). Fusion gene status and tumor invasiveness also remained as independent prognostic factors for the risk of death with an HR associated with fusion-positive tumors of 2.5 (95% CI, 1.1-5.6; $P = .0300$) and an HR related to tumor invasiveness (T2 classification) of 2.2 (95% CI, 1.1-4.6; $P = .0298$). The 5-year EFS rate in patients with fusion-positive tumors was 43.0% (29.6%-55.7%) compared with 74.4% (53.6%-86.9%) in those with fusion-negative tumors ($P = .0101$) (Fig. 1). The 5-year OS rate for patients with fusion-positive tumors was 45.5% (95% CI, 30.8%-59.2%) compared with 74.7% (95% CI, 52.4-86.6) for patients with fusion-negative tumors ($P = .0548$) (Fig. 2).

DISCUSSION

The results of the current study provide evidence of the prognostic impact of fusion status and tumor invasiveness in patients with aRMS and lymph node involvement. Results from previous European and North American cooperative studies have demonstrated very poor survival in patients with aRMS and lymph node involvement, who account for up to 10% of all patients with RMS. In the CWS/RMS86 study, the 3-year EFS rate was 28% and the OS rate was 29%.[18] Results in the SIOP experience were only slightly better, with a 5-year EFS rate of 39% in the SIOP MMT84 study,[19] which is comparable to that of stage IV disease.

The impact of lymph node involvement on prognosis in patients with RMS remains a matter of controversy. Rodary et al[20] evaluated a cohort of 951 international patients with nonmetastatic RMS and identified tumor invasiveness, tumor size, primary tumor site, and N1 disease as prognostic factors. Similarly, in their analysis of patients with nonmetastatic RMS enrolled in American IRS protocols, Meza et al[10] demonstrated that only stage of disease and IRS group were significantly associated with EFS for the majority of patients with aRMS. However, for patients in group III with aRMS, N1 disease was

associated with poorer EFS and OS. These observations influenced the development of the current EpSSG treatment protocol, which assigned patients with aRMS of N1, but not embryonal N1 RMS, to the very high-risk group, for whom a more intensive treatment was recommended.[21]

Rodeberg et al[22] investigated the contribution of regional lymph node disease to the prognosis of patients enrolled in the IRS-IV study. They included 125 patients with localized RMS and lymph node involvement. Patients with alveolar histology and positive lymph nodes were found to have significantly worse 5-year failure-free survival compared with those with alveolar histology without lymph node involvement (43% and 73%, respectively). Moreover, in patients with alveolar histology and N1 disease, outcomes were more similar to those of patients with solitary metastatic disease compared with patients with N0 disease. These results are consistent with the results of the current study. The main difference between the aforementioned study and the current report is that the former included both alveolar and embryonal tumors with lymph node involvement; however, as in the current study, patients with tumors located at unfavorable sites, those with disease at advanced stages, and those with large and invasive tumors were predominant. All these characteristics have been associated with an increased risk of distant metastatic disease.[3,5,23,24] Conversely, involvement of regional lymph nodes in patients with embryonal tumors did not prove to have any negative effect on outcome in the study by Rodeberg et al[22] or in the more recent report by Rogers et al.[25] This could be due at least in part to the intensified treatment with RT and chemotherapy administered, suggesting that patients with lymph node-positive embryonal tumors can attain equivalent outcomes when given intensified treatment. To the best of our knowledge, the overall outcome of the current study cohort was better than the historical series reported to date. The reasons for the apparent improvement in outcome among these patients could be due in part to better risk stratification, more adequate treatment with intensified chemotherapy, systematic local treatment, and improvements in supportive care.

In the current study, a significant number of patients had tumors that did not respond to initial chemotherapy and these individuals presented with progressive disease shortly after diagnosis, thereby representing 14% of those patients who developed disease recurrence. A recent report from Vaarwerk et al[26] demonstrated the lack of correlation between early radiologic response and outcome in patients enrolled in the MMT95 protocol, even though patients with progressive disease were excluded from the analysis. It must be emphasized that the patients with progressive disease in the current study failed to respond to further treatment and the chance of cure after disease recurrence was very low (5% of the entire cohort), which suggests that patients with refractory disease or disease recurrence could be offered experimental therapy immediately after tumor events. Nevertheless, even with the implementation of combined local therapy with delayed surgery and systematic RT to the primary tumor site and lymph nodes in the current study protocol, locoregional disease recurrences were frequent and accounted for approximately 42% of the initial events. Furthermore, lymph node failures occurred in approximately 33% of the disease recurrences. Some authors have recommend that the in-transit lymphatics be imaged at the time of diagnosis.[27] The involvement of in-transit lymph nodes could be better assessed by performing systematic [^{18}F]fludeoxyglucose (FDG) positron emission tomography-computed tomography at the time of diagnosis, a procedure that was not performed routinely in the cohort of patients in the current study. Moreover, the question of whether in-transit lymph nodes should be irradiated routinely remains unsolved, given the risk of significant toxicity associated with extensive irradiation in pediatric patients.[28,29]

In the current series, we identified some variables found to have prognostic significance on univariate analysis (unfavorable site of tumor origin, tumor invasiveness, *FOXO1* fusion, and IRS group III). However, on multivariate analysis, only tumor invasiveness and the presence of a characteristic fusion gene associated with aRMS resulted in independent predictors of disease recurrence or death. This is consistent with several studies that correlated the presence of a fusion gene with poorer outcome; however, to the best of our knowledge, the real contribution of the presence of *PAX3/7-FOXO1* fusions to the outcome of aRMS remains to be elucidated.[30-32] In the current series, approximately 66% of tumors were fusion positive. These figures are lower than the rate of 70% to 75% reported in the literature, which could be due in part to the fact that fusions involving *PAX3* with partners other than *FOXO1* were missed in the current analysis.[33] We will attempt to avoid these false-negative results in the future EpSSG protocol: in an alveolar tumor that is negative for *PAX3/7-FOXO1* by RT-PCR and for *FOXO1* rearrangement by FISH, additional FISH assessments for the disruption of *PAX3* will be made.

In the current study, fusion status appeared to identify the "real" very high-risk population, thereby

highlighting the importance of performing biologic studies in all patients. We did not attempt to analyze outcome according to the type of fusion because of the limited number of patients with the *PAX7-FOXO1* fusion.

Survival in this newly defined very high-risk group is comparable to results observed in patients with metastatic aRMS treated in the high-risk COG studies D9802 and ARST0431.[34] In those studies, fusion status was not found to be an independent prognostic factor, despite better EFS noted in patients with fusion-negative aRMS. Poorer outcomes for patients with metastatic disease in the COG report were most closely related to other clinical risk factors, including age, primary tumor site, and number of metastatic sites.

The clinical implications of the current study will include a new stratification for patients with aRMS/N1 disease according to fusion status in the future EpSSG RMS study. Patients with fusion-negative N1 tumors will be treated with a strategy similar to that for those with eRMS/N1 disease, with no reduction in treatment intensity. Patients with fusion-positive N1 disease will be treated in the same group as patients with metastatic tumors. For patients with refractory disease or disease recurrence, the EpSSG is working to establish an effective, innovative strategy for the study of new agents and the inclusion of patients in phase 1 and 2 clinical trials.

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CONFLICT OF INTEREST DISCLOSURES

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AUTHOR CONTRIBUTIONS

Soledad Gallego: Conception and design, collection and assembly of data, and data analysis and interpretation. **Ilaria Zanetti**: Data analysis and interpretation. **Gian Luca de Salvo**: Conception and design and data analysis and interpretation. **Gianni Bisogno**: Conception and design, collection and assembly of data, and data analysis and interpretation. **All authors**: Article writing and final approval of article.

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