Clinical Relevance of DNA Ploidy and Proliferative Activity in Childhood Rhabdomyosarcoma: A Retrospective Analysis of Patients Enrolled Onto the Italian Cooperative Rhabdomyosarcoma Study RMS88

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Purpose: Evaluation of the possible clinical relevance of DNA ploidy and proliferative activity assessed as S-phase fraction (SPF) in childhood rhabdomyosarcoma (RMS).

Patients and Methods: We conducted a retrospective study on 59 RMS patients enrolled onto the ICS-RMS88 protocol (seven botryoid, 35 embryonal, and 17 alveolar RMS), for which formalin-fixed paraffin-embedded (FFPE) tissue was available. Nuclear suspensions for cytometric investigation were obtained using a mechanical disaggregation. Tumors were distinguished according to their DNA index (DI) value as follows: diploid (0.9 < DI < 1.1), hyperdiploid (1.1 ≤ DI < 1.8 or DI ≥ 2.2), and tetraploid (1.8 ≤ DI < 2.2); for analysis of SPF, a cutoff value of 14% was used.

Results: DNA histograms were diploid in 19 (33%) cases, hyperdiploid in 29 (49%), and tetraploid in 10 (32%). One patient showed both a hyperdiploid and a tetraploid peak. The 5-year overall survival (OS) rate

by ploidy status was 73% in hyperdiploid patients as compared with 33% and 25% in diploid and tetraploid patients, respectively (_P_ = .0012). A striking difference emerged when the 5-year OS for the combined diploid and tetraploid RMS groups was compared with survival of the hyperdiploid RMS group: 30% versus 73%, respectively (_P_ = .0006). In addition, the SPF was prognostically relevant: 5-year OS by SPF less than or greater than 14% was 70% and 36%, respectively (_P_ = .009). Multivariate analysis confirmed the importance of DNA content (_P_ = .0006) and SPF (_P_ = .034) in predicting survival.

Conclusion: These findings confirm that ploidy and SPF are important new prognostic factors that are able to identify selected groups of patients at high risk of treatment failure, even if the tumor's presentation is favorable according to standard criteria.

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RHABDOMYOSARCOMA (RMS) is a malignant tumor that differentiates along the skeletal muscle pathways and is the most common pediatric soft tissue sarcoma, accounting for 6% of childhood cancers.[1] Clinical staging, anatomic site, and histologic subtypes have been used in combination to predict outcome in patients with RMS, who can be cured in approximately 60% of cases.[2] Nevertheless, a subgroup of children with favorable features still responds poorly to multimodality therapy (chemotherapy, radiotherapy, and surgery). The accu-

rate identification of patients at high risk for treatment failure would enable appropriate intensification of the therapy, as well as the selection of children at low risk of disease progression, which would allow a reduction of treatment aggressiveness, thus decreasing the long-term toxicity risk.

The relationship between prognosis and abnormalities in DNA content (DNA ploidy) or proliferative characteristics (S-phase fraction [SPF]) has been explored in several tumors. For example, it has been demonstrated that flow cytometric diploid acute lymphoblastic leukemias and neuroblastomas have a worse prognosis than hyperdiploid cases.[3,4] Only a few studies have investigated the prognostic significance of combined flow cytometric evaluation of DNA ploidy and proliferative activity in pediatric RMS, and their results have suggested a correlation between histologic subtypes, DNA ploidy, SPF, and prognosis.[5,6]

We report on a retrospective investigation of the distribution of DNA ploidy and SPF in formalin-fixed and paraffin-embedded (FFPE) tumor specimens from a representative cohort of Italian pediatric patients treated according to the RMS88 protocol. These parameters were compared with clinical and histopathologic characteristics and treatment outcome to verify whether they can add

meaningful prognostic information to support conventional prognostic factors.

PATIENTS AND METHODS

Patients

Clinical data and archival FFPE samples from the primary tumors of 59 patients with a histologic diagnosis of RMS were obtained from the Istituto di Anatomia Patologica and Centro di Emato-Oncologia Pediatrica-Università di Padova, from the Dipartimento di Pediatria, Università La Sapienza di Roma, and from the Dipartimento di Scienze Pediatriche-Università di Torino, Italy. The only selection concerned the availability of paraffin-embedded blocks and treatment according to the RMS88 protocol.

The median age of the children at diagnosis was 57 months (range, 1 to 203). The male-to-female ratio was 1.9:1. The distribution of the patients by primary tumor site was as follows: head and neck (nonparameningeal, n = 14; parameningeal, n = 5), genitourinary tract (bladder and prostate, n = 7; paratestis, vagina, or uterus, n = 6), extremities (n = 5), and others (thorax, abdomen, or trunk, n = 22). All patients were staged according to the Clinical Grouping System proposed by the Intergroup Rhabdomyosarcoma Study (IRS)[9] as follows: three cases in group I, 13 in group II, 40 in group III, and three in group IV.

Children were treated according to the Associazione Italiana Ematologia ed Oncologia Pediatrica-Consiglio Nazionale delle Ricerche (AIEOP-CNR) RMS88 protocol.[7,8] This protocol included primary chemotherapy for patients with tumor size greater than 5 cm in diameter, or with distant metastases at diagnosis, or with bladder, prostate, vagina, uterus, and orbit as primary tumors. Briefly, patients in group I received four cycles of vincristine 1.5 mg/m² on day 1 at weeks 0, 1, 2, and 3, and dactinomycin 1.8 mg/m² on day 1 at weeks 0 and 3 (VA) for a total of 22 weeks. Group II patients were treated with three courses of ifosfamide 3.0 g/m²/d on days 1 and 2 at weeks 0, 3, and 6, vincristine 1.5 mg/m² on day 1 at weeks 0, 1, 2, 3, and 6, dactinomycin 1.5 mg/m² on day 1 at weeks 0, 3, and 6 (IVA II regimen) for a total duration of 27 weeks. Group III patients received one cycle of ifosfamide 2.0 g/m²/d on days 1 to 5 at weeks 0, 3, and 6, vincristine 1.5 mg/m² on day 1 at weeks 0, 1, 2, 3, and 6, dactinomycin 1.5 mg/m² on day 1 at weeks 1 and 6, and doxorubicin 40 mg/m²/d on days 1 and 2 at week 3 (VAIA III regimen) followed by three IVA II cycles for a total duration of 37 weeks. Group IV patients received alternating cycles of carboplatin 500 mg/m²/d on day 1, epirubicin 150 mg/m²/d on day 1, and vincristine 1.5 mg/m²/d on days 1 and 8 (CEV), plus ifosfamide 3.0 g/m²/d on days 1, 2, and 3, dactinomycin 1.5 mg/m²/d on day 1, and vincristine 1.5 mg/m²/d on days 1 and 8 (IVA) and ifosfamide 3.0 g/m²/d on days 1, 2, and 3, vincristine 1.5 mg/m²/d on days 1 and 8, and etoposide 200 mg/m²/d on days 1, 2, and 3 (IVE) for a total of 12 cycles according to the Intergroup European MMT-IV protocol.[10] Surgery was subsequently used to eradicate residual tumor, if feasible. Hyperfractionated and accelerated radiation (40 to 54 Gy) was administrated to all patients with alveolar histology and to patients with embryonal RMS with macro-microscopic residual disease after delayed surgery and/or regional lymph node involvement. It should be noted that protocol guidelines were in favor of the use of preoperative chemotherapy to avoid surgical mutilation, so that group III may include patients in whom radical surgery might have been possible at diagnosis. The median follow-up duration of surviving patients was 35 months (range, 7 to 107).

Histopathologic Diagnosis

Hematoxylin and eosin–stained slides (5 μm) were obtained from each tumor block and subclassified according to the new proposed IRS classification.[11] In all cases, the morphologic diagnosis was supported by a combination of immunohistochemical stains for muscular differentiation (including desmin, muscle-specific actins, and, in selected cases, myoglobin and MyoD1). The initial series included 65 cases, but six specimens that presented abundant necrosis or too little material for adequate flow cytometric assessment were rejected. The 59 patients on whom flow cytometry could be performed were classified as follows: botryoid RMS (n = 7), embryonal RMS (n = 35), and alveolar RMS (n = 17).

Flow Cytometric Analysis

Two 50-μm sections were cut from each FFPE tissue block. An adjacent 5-μm section was obtained for standard hematoxylin and eosin staining and histologic examination. Sections were deparaffinized using xylene, and rehydrated through graded ethanol washes of decreasing concentration and then through deionized water. A nuclear suspension for cytometric investigations, including measurements of ploidy (DNA index [DI]) and proliferative activity (SPF), was obtained using a mechanical disaggregator (Medimachine; Consul-TS, Torino, Italy). This instrument is routinely used at our laboratory to obtain cell suspensions from fresh tissue. Briefly, the rehydrated sections or fresh biopsies, with phosphate-buffered saline (PBS) added, were treated for several minutes in the disaggregator; cell suspensions were collected by aspiration and examined by phase-contrast microscopy. If too many clumps of cells were observed, the suspension was passed through a spinal needle alternating with vortex, washed twice in PBS, then passed through a 30-μm filter (Consul-TS) to remove cell debris. A count of 1×10^6 nuclei/mL was considered optimal. Samples were stained by propidium iodide using an automated DNA-staining device (DNA-Prep reagents; Coulter, Miami, FL), and analyzed after 2 hours by flow cytometer (Epics XL; Coulter) with a 488-nm argon laser. DNA histograms were obtained using a semiautomatic cell-cycle analysis program (Multicycle; Phoenix, San Diego, CA), which compensates for doublets and overlapping nuclei.

Three distinct patterns of cell DNA content were identified.[5] Tumors were classified as diploid when only one G0/G1 peak was present or when the DI was between 0.9 and 1.09; as hyperdiploid when the DI was ≥ 1.10 and less than 1.80; and as tetraploid when the DI was ≥ 1.8 and less than 2.20 (Fig 1A and B). The DI was established from the ratio of the modal channel number of the G0/G1 peak of neoplastic cells to that of normal cells.[12] Samples from thymoma were used as external controls, while normal cells within the tissue sample acted as internal controls. Histograms were considered as valid when (1) at least 10,000 nuclei were analyzed, and (2) the coefficient of variation (CV) was not greater than 7%.

Statistical Analysis

The prognostic significance of clinical parameters was investigated using univariate analysis, ie, the log-rank test and Wilcoxon test. Survival analysis was performed using the Kaplan-Meier method. The approximate χ^2 statistic for the log-rank test was applied to the flow cytometric parameters (DNA content and SPF). Contingency tables were analyzed with the χ^2 test. Student's t test and nonparametric tests were performed for quantitative variables. Multivariate analysis was undertaken with a stepwise method.
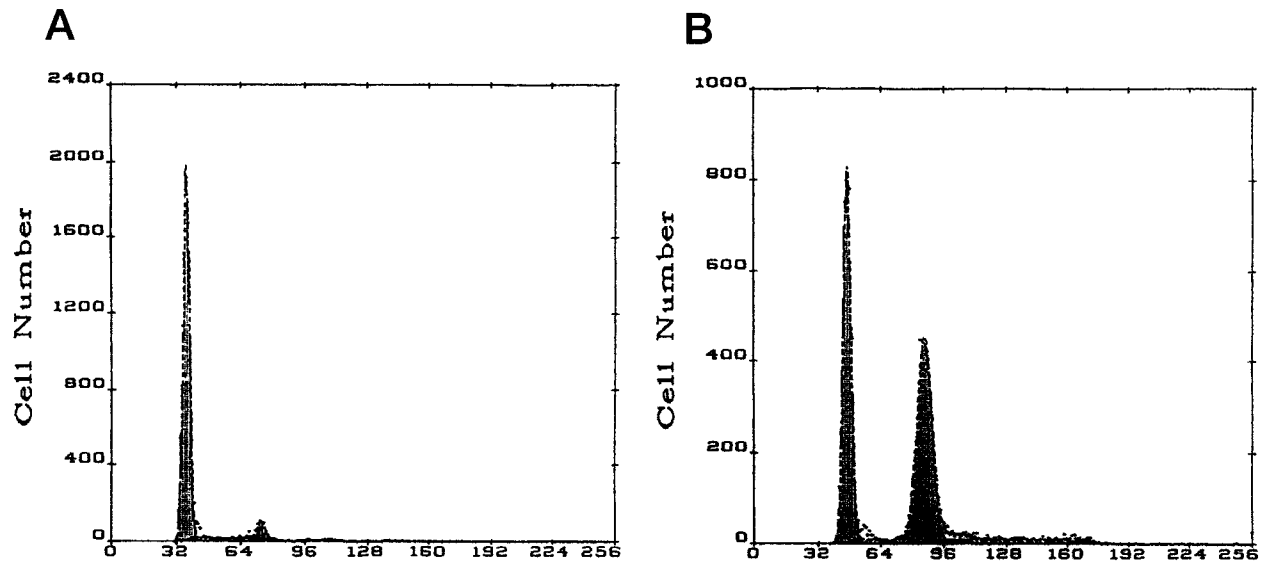
A

B

Fig 1. Representative histograms of distinct patterns of ploidy. (A) Diploid tumor stemline (DI = 1); (B) tetraploid tumor stemline (DI = 1.83).

RESULTS

Among the 65 RMS patients, assessable cytometric findings were obtained from 59. For six tumors, both FFPE and fresh samples were examined to verify the consistency of ploidy values obtained by the two techniques. A consistent DNA content was found in five tumors. In one case, the hyperdiploid cell population detected in the paraffin-embedded material was not confirmed in the fresh tumor sample, which showed a diploid DNA content.

Mechanical disaggregation gave good-quality results, as demonstrated by the low CV (mean, 4%; range, 2.6% to 6.3%). The DNA histograms from the FFPE tissues were diploid in 19 cases (33%), hyperdiploid in 29 (49%), and tetraploid in 10 (17%). Three embryonal tumors had more than one stemline, two with both cell populations hyperdiploid, and so classified in this category, and one with a hyperdiploid and a tetraploid DNA stemline. The latter was not considered in the statistical analysis, because it was the only one with this pattern.

Table 1 compares several clinical variables (sex, age, site, and IRS group) and histopathologic subtypes with the DNA content using the χ^2 test. No significant association was found between ploidy and sex, age at diagnosis ($\leq$ 36 or > 36 months), or IRS group. Primary tumor site was significantly associated with DNA ploidy (P = .038). Most tumors located in the urogenital tract were hyperdiploid (10 of 13, 76%), while a tetraploid pattern was more frequently found in tumors located at the ex-

tremities (three of five, 60%). Regarding the association of ploidy with histologic subclassification, the hyperdiploid pattern was more frequently found in botryoid (four of seven, 57%) and embryonal RMS (21 of 34, 62%), while a tetraploid DNA content was mainly present in alveolar tumors (eight of 17, 47%) (P = .003). In our population, there was a good association between tumor site and histology. Botryoid tumors were mainly located in genitourinary sites (five of seven, 71%), while three of five (60%) tumors at extremities were alveolar RMS (P = .016).

In SPF analysis, a high variability was observed (range, 1.8% to 55%). To separate our population into two groups, several sensitivity and specificity tests were performed and a 14% cutoff value was found to be the most sensitive and specific (92% and 55%, respectively), as in the report by Niggli et al.[5] The samples were therefore divided into two groups of SPF less than or greater than 14%. No significant statistical association was found between SPF and sex, age, or IRS group (Table 1). Conversely, histopathologic subtype and tumor site were significantly associated with SPF. Patients with botryoid and embryonal RMS showed an SPF value less than 14% in six of seven (86%) (range, 3% to 36%) and 16 of 34 (47%) (range, 1.8% to 45%) cases, respectively, whereas those with alveolar RMS more frequently had an SPF greater than 14% (12 of 17, 70%) (range, 3.5% to 52%) (P = .042).

Tumors located in the extremities, head-neck parameningeal regions, and other sites mainly presented a high

Table 1. Distribution of DNA Content and SPF in 58 Cases

| | Total no. | Ploidy Pattern | | | P | SPF | | P |
		Diploid	Hyperdiploid	Tetraploid		≤ 14%	> 14%	
Total no.	58	19	29	10		27	31	
Sex								
Male	35	12	19	4	.38	18	17	.30
Female	23	7	10	6		9	14	
Age, months								
≤ 36	22	7	13	2	.37	13	9	.30
> 36	36	12	16	8		14	22	
Site								
UG not BP tract	6	2	4	0	.038	4	2	.02
UG BP tract	7	0	6	1		5	2	
Extremities	5	1	1	3		0	5	
PM head-neck	5	3	2	0		1	4	
Not PM head-neck	14	6	8	0		10	4	
Others*	21	7	8	6		7	14	
IRS group								
I	3	1	2	0	.26	1	2	.90
II	12	3	6	3		6	6	
III	40	14	21	5		19	21	
IV	3	1	0	2		1	2	
Histopathologic type								
Botryoid RMS	7	3	4	0	.002	6	1	.04
Embryonal RMS	34	11	21	2		16	18	
Alveolar RMS	17	5	4	8		5	12	

Abbreviations: UG, urogenital; BP, bladder-prostate; PM, parameningeal.
*Thorax, abdomen, and trunk.

proliferative activity, with SPF values greater than 14% in 100%, 80%, and 67% of patients, respectively. Tumors in the urogenital tract and head-neck nonparameningeal sites were characterized by a low SPF (70% and 71%, respectively) (P = .02).

A significant association was also found between DNA content and SPF (P = .006). In hyperdiploid tumors, 66% of patients (19 of 29) showed an SPF less than 14%, whereas 63% (12 of 19) of diploid and 90% (nine of 10) tetraploid tumors were characterized by a higher SPF. The median SPF distribution was also significantly related to ploidy (P = .01): hyperdiploid tumors were associated with a lower median SPF (10%; range, 3.5% to 55%) compared with diploid (18.3%; range, 1.8% to 45.7%) and tetraploid tumors (19.7%; range, 11% to 50.7%).

The relationship of clinical, histopathologic, and cytometric parameters to survival was analyzed using the Kaplan-Meier method (Table 2). Ploidy and proliferative activity were found to have a significant impact on survival.

The 5-year overall survival (OS) rate in hyperdiploid RMS was 73% (95% confidence interval [CI], 51% to 95%), as compared with 33% (CI, 6% to 60%) in diploid and 25% (CI, 0% to 54%) in tetraploid tumors (P =

.0012) (Fig 2). Since no significant difference between diploid and tetraploid tumors was demonstrated, these two categories were combined, with a resulting 5-year survival rate of 31%. When the survival rates of diploid and tetraploid patients are compared with that of hyperdiploid RMS patients, the resulting P value is more significant (P = .0006).

The 5-year OS rate in children with an SPF value less than and greater than 14% was 70% (CI, 47% to 93%) and 36% (CI, 16% to 56%), respectively (P = .006) (Fig 3).

Because of the limited number of children with group I and IV RMS in our study, a statistical analysis was performed only for patients in groups II and III. A strong association was found between ploidy and survival, showing that hyperdiploid tumors have a better outcome, ie, 68% 5-year OS versus 30% for diploid and 12% for tetraploid tumors (P = .0004). Moreover, patients with tumors with an SPF less than 14% were confirmed as having a 5-year OS (70%) higher than those with tumors with an SPF greater than 14% (24%; P = .003).

Combining these two variables (DNA ploidy and SPF), and assuming a hyperdiploid DNA content with an SPF less than 14% as favorable and a diploid or tetraploid

Table 2. Univariate Analysis of Survival in 59 Pediatric RMS Patients

Variable	Total	5-Year Survival %	5-Year Survival 95% CI	P Log-Rank Test	P Wilcoxon Test
Total	59	52.5	37.1-68		
Sex					
Male	36	38.2	12.3-64.3	.3	.2
Female	23	60.4	41.4-79.4		
Age, months					
≤ 36	23	37.1	13.6-60.6	.17	.2
> 36	36	64	45-83		
Site					
UG not BP tract	6	100	100-100	.3	.3
UG BP tract	7	33.3	0-83		
Extremities	5	37.5	0-94		
PM head-neck	5	53.3	5-100		
Not PM head-neck	14	47.2	14-80		
Others	22	49.2	26-73		
IRS group					
I	3	100	100-100	.27	.25
II	13	36	6-66		
III	40	52	33-71		
IV	3	0	0-0		
Histopathologic type†					
Botryoid RMS	7	100	100-100	.08	.17
Embryonal RMS	35	58	39-78		
Alveolar RMS	17	33	8-58		
DNA content*					
Diploid RMS	19	33	6-60	.0012	.002
Hyperdiploid RMS	29	73	51-95		
Tetraploid RMS	10	25	0-54		
SPF*					
≤ 14%	27	70	47-93	.009	.007
> 14%	31	36.3	16-56		

*Analysis conducted on 58 samples.

†Univariate analysis considering botryoid-embryonal v alveolar RMS showed the same P value, with an OS of 63% (CI, 45% to 81%) for favorable histology and 33% (CI, 8% to 58%) for the unfavorable one.

DNA content with or without an SPF higher than 14% as unfavorable, we evaluated the 5-year OS rates in groups that presented none, one, or two unfavorable prognostic features (Fig 4), and obtained a significantly different survival rate in the three categories of patients (P = .001).

A multivariate analysis was performed that included sex, age at diagnosis, favorable site (genitourinary not bladder prostate [BP]) versus other sites, groups I and II versus III and IV, histopathologic classification (botryoid RMS and embryonal v alveolar RMS), DNA content (hyperdiploid v diploid/tetraploid), and SPF (≤ 14% v > 14%). Both DNA content (P = .0006) and tumor site (P = .037) proved able to influence survival significantly; the log-rank test confirmed ploidy as the most important
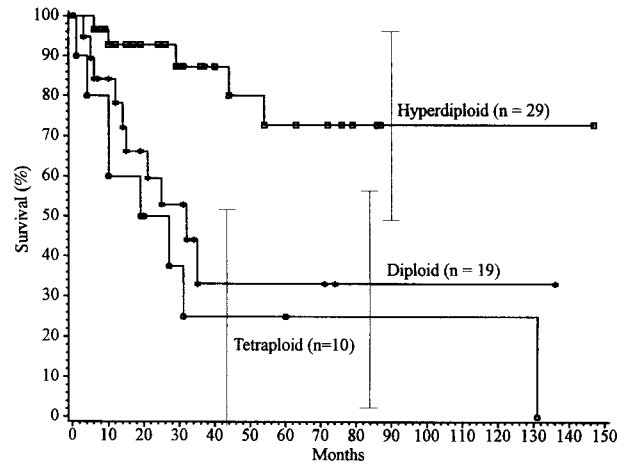
Fig 2. RMS OS by ploidy (P = .0012).

parameter in predicting outcome and in contributing prognostic information (P = .0006) (Table 3).

DISCUSSION

Ploidy has been evaluated and correlated with outcome in several childhood malignancies. It is well recognized that, in acute lymphoblastic leukemia and neuroblastoma, hyperdiploid tumor stemlines are associated with a more favorable outcome than in diploid cases.[3,4] However, conflicting results have been reported for other tumors, such as Wilms' tumor, medulloblastoma, and hepatoblastoma.[13-15] As regards childhood RMS, only a few reports have been published on the prognostic implications of DNA content, and some series concerned included both
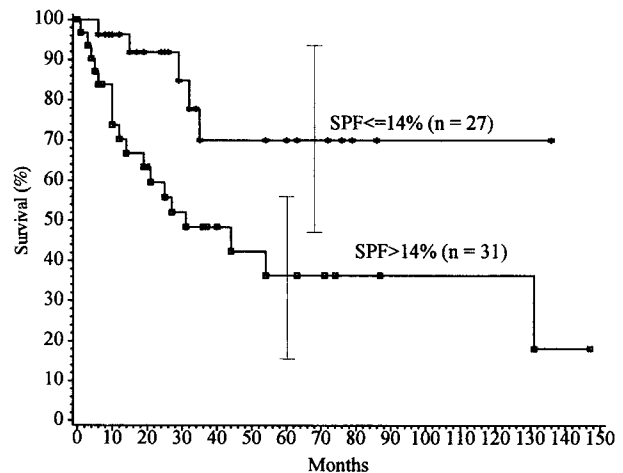
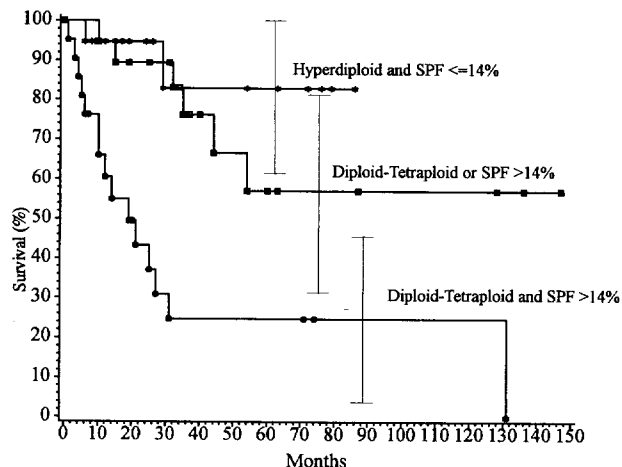

Fig 3. RMS OS by SPF (P = .0006).

Fig 4. Five-year OS analysis combining DNA ploidy and SPF and assuming as favorable prognostic factors a hyperdiploid DNA content or SPF ≤ 14% and as unfavorable, diploid-tetraploid DNA patterns or SPF > 14% (P = .001).

pediatric and adult RMS or only patients with unresectable RMS.[5,6,16-20]

The aim of our study was to evaluate the possible relationships between ploidy, SPF, and several clinical and histopathologic variables, and to verify whether these biologic features have any influence on the outcome of pediatric RMS. We studied a homogeneously treated group of pediatric patients treated according to the RMS88 protocol.[8,9] The availability of FFPE tumor samples was the only selection criteria. The distribution of ploidy in this population showed that 33% of RMS cases were diploid, 17% tetraploid, and 49% hyperdiploid. One patient presented a pattern with both a hyperdiploid and a tetraploid DNA stemline. This last case was rejected from statistical analysis because it was the only one with this pattern. Our results are consistent with those reported by Niggli et al,[5] although we did not separate diploid (DI = 1) and near-diploid (1 < DI < 1.09) tumors and our

Table 3. Multivariate Stepwise Analysis in 58 Pediatric RMS Patients

Variable	P
Log-rank test	
Ploidy	.0006
Ploidy + SPF	.034
Ploidy + SPF + site	.1
Wilcoxon test	
Ploidy	.0006
Ploidy + site	.037
Ploidy + site + IRS group	.086
Ploidy + site + IRS group + SPF	.12

methods of analysis differed slightly. In fact, we performed a mechanical disaggregation while Niggli et al used an enzymatic method according to the Hedley technique.[21] In our hands, the former produced better results, confirmed by the low value of the CV (mean, 4%). Previous studies using enzymatic digestion generally reported a higher CV.[5,16-22]

To confirm the validity of our technique, we compared the DNA ploidy values obtained from fresh and FFPE samples. A valid correlation emerged in five of six cases, while one tumor was hyperdiploid in the paraffin-embedded tissue and diploid in the fresh specimen. This discrepancy might depend on intratumoral heterogeneity[23] or most probably on inadequate sampling of the fresh tissue, which was not controlled before the analysis. In this way, viable tumor cells might not have been adequately included. To avoid the latter possibility, all of the FFPE material submitted for flow cytometric evaluation underwent prior histologic examination of the hematoxylin and eosin–stained slides immediately adjacent to those taken for flow cytometry. As a result, six cases were rejected because of the presence of plentiful necrosis or due to an excessively limited involvement of the sample by the tumor cells, which would mar the results of the analysis.

The present study showed a statistically significant association of ploidy with site (P = .038), histologic subtype (P = .003), and SPF (P = .006), and of SPF with site (P = .02), histologic subtype (P = .04), and ploidy (P = .006). In particular, 80% of tetraploid cases and 70% of cases with an SPF value greater than 14% were strongly associated with alveolar RMS, whereas 70% of hyperdiploid cases and 86% of cases with an SPF value less than 14% were associated with the botryoid and embryonal forms of RMS. These results are consistent with the findings reported by most investigators,[5,6,17,18] but they differ from the report published by Dias et al,[16] who found no correlation between DNA ploidy and histologic subtype.

In the univariate analysis, tumor site, histopathologic subtype, and IRS group, usually considered the most important parameters, had no statistical significant association with OS. This could depend on the availability of FFPE blocks; in fact, patients in groups I and IV are underrepresented. As far as concerned histopathologic types, we may note a clearly decreasing trend in botryoid, embryonal, and alveolar RMS, respectively (100%, 58.5%, and 33%) (P = .08). Ploidy and SPF proved significantly related to OS (P = .002 and P = .007, respectively). In fact, patients with tumors with a diploid/ tetraploid DNA content and/or SPF greater than 14% had

a worse outcome (5-year survival, 25% [CI, 4% to 45%] and 58% [CI, 29% to 84%], respectively) than hyperdiploid patients with an SPF less than 14% (5-year survival, 84% [CI, 60% to 100%]). This is irrespective of histology, as demonstrated by the fact that all dead patients with diploid/tetraploid DNA content and high SPF were equally distributed between embryonal and alveolar histology (53% and 47%, respectively). Moreover, five patients with favorable clinical presentation (site, stage, and histology), but with one or two unfavorable cytometric variables, had a worse outcome. These results are similar to those obtained by Niggli et al,[5] Shapiro et al,[17] and Wijneandts et al,[18] but they differ from the findings published by Dias et al[16] and Kilpatrick et al,[6] in which no correlation between DNA ploidy and OS was found, and also from those described by Alvegard et al,[19] and Kowal-Vern et al,[20] in which DNA hyperdiploidy was an unfavorable prognostic factor. These discrepancies may be explained by various factors such as sample size, stage of disease, age grouping, different cytometric techniques, and different therapeutic regimens. In addition, the distinction of ploidy classes varies in different reports. For example, Dias et al[16] separated diploid, near-diploid, and aneuploid RMS, while we classified our cases as diploid, hyperdiploid, and tetraploid. Finally, when the measurement quality is not optimal (as demonstrated by high values of CV), some hyperdiploid tumors may be misclassified as diploid.

In a multivariate stepwise analysis, ploidy and tumor site (the first and second variables in the Cox model) were significant prognostic factors (P [Wilcoxon] = .0006 considering only ploidy, P = .037 considering both ploidy and site); the same analysis using the log-rank test showed SPF to be the second most important variable after ploidy (P = .034). Patients with RMS occurring in the genitourinary tract had a better outcome than those in whom RMS developed in other sites. Likewise, genitourinary RMS cases are more frequently hyperdiploid (75%) and have a low SPF (68%).

We conclude that DNA content and SPF have a significant prognostic impact in childhood RMS. The biologic behavior of RMS can be divided into three different categories according to DNA content (diploid, hyperdiploid, and tetraploid) and into two groups according to proliferative activity (SPF $\leq$ or $>$ 14%). Diploid-tetraploid tumors and tumors with an SPF greater than 14% have a worse outcome, irrespective of histologic subtype and IRS group. Site is another important prognostic factor.

The relevance of these findings should be kept in mind in planning future clinical trials in childhood RMS, since ploidy and SPF value, as pointed out by the multivariate analysis, could be able to identify selected groups of patients at high risk of treatment failure, even if the tumor's presentation is favorable according to standard criteria. However, further investigations on a larger number of cases should be performed to confirm this important prognostic relevance.

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