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Detection and clinical significance of disseminated tumour cells at diagnosis in bone marrow of children with localised rhabdomyosarcoma

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Abstract

Identification of patients with a poor prognosis for non-metastatic rhabdomyosarcoma (RMS) remains a clinical challenge. Prospective analysis for the presence of disseminated RMS cells in bone marrow at diagnosis, using immunocytochemistry, with MyoD1 and myogenin as markers, was carried out. Thirty-seven patients treated on RMS88 and RMS96 Italian protocols underwent staging investigations, and in addition marrow examination for occult tumour cells. All patients had negative marrow involvement using cytomorphology, but 10/37 were positive with immunostaining. With a median follow-up of 46 months (range, 12–115), 7 patients had died and 30 were disease-free. Overall survival probability was 92% in patients with no occult marrow infiltration, 47% with occult marrow infiltration ($P = 0.001$); event-free survival probability was 89% in the former and 50% in the latter ($P = 0.01$). Disseminated tumour cells are indicative of disease spread and are significantly linked to recurrence at distant sites and poorer outcome. Marrow examination at diagnosis using immunocytochemistry may be an additional tool to modulate treatment.

Keywords: Rhabdomyosarcoma; Disseminated tumour cells; MyoD1; Myogenin; Myogenic regulatory factors

1. Introduction

Improvement in survival of children diagnosed with rhabdomyosarcoma (RMS) has partly been achieved by refining staging and hence clinical grouping, resulting in more tailor-made treatments [1,2]. From a clinical perspective, histological subtype, site and size of the tumour, and patient age are now recognised prognostic factors [2,3]. However, even using these parameters for stratification, there is a population at significantly higher risk which either progress on treatment or relapse, for whom there is no second-line treatment. The inability at diagnosis to adequately assess disease extent and identify early tumour dissemination to secondary sites may partially account for the poor outcome of these high-risk non-metastatic tumours.

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Bone marrow is an accessible and suitable site to evaluate early tumour dissemination. Conventional assessment of marrow involvement at diagnosis is performed by light microscopy, despite the fact that this frequently fails to detect the presence of an infiltrate below 1×10^{-3} cells [4]. Immunocytochemical and polymerase chain reaction (PCR)-based assays enable "unequivocal" detection of individual disseminated tumour cells with a much higher sensitivity and have shown that 20–40% of adults with solid epithelial tumours harbour occult marrow disease even when neither lymph node metastases (stage N0) nor overt distant metastases (stage M0) are detectable [5]. The presence at diagnosis of this occult early marrow involvement is predictive of developing overt metastases at distant sites and poor outcome [5,6]. Similar results have also been reported in localised neuroblastoma [7] and Ewing's sarcoma [8].

RMS has a striking morphological resemblance to foetal skeletal muscle, embryonal RMS (ERMS) most similar to muscle of 7–10 weeks gestation and alveolar RMS (ARMS) to 10–12 weeks gestation [9]. Accordingly, members of the myogenic regulatory factor family, which modulate the commitment of mesenchymal progenitor cells to the myogenic differentiation program [10], are also expressed in RMS and their detection can be a useful tool in identifying the tumour cell phenotype. Two members of this family, MyoD1 and myogenin, have been extensively investigated as diagnostic markers in RMS [11–21] and are expressed with a frequency approaching 100% [21]. Whilst the frequency and clinical significance of morphologically detectable marrow involvement in patients with metastatic RMS have been studied [22], reports focusing on the detection of disseminated tumour cells in morphologically normal marrow have been very limited [20,23,24].

This study investigated prospectively the frequency and clinical significance of occult marrow involvement at diagnosis in a series of 37 children with localised RMS treated according to the Italian protocols RMS88 and RMS96.

2. Patients and methods

2.1. Patients

Thirty-seven consecutive patients, 23 males and 14 females, aged 2–179 months (median, 58), with newly diagnosed localised RMS admitted between 1997 and 2003 in the Divisions of Oncology at Bambino Gesù Children's Hospital and at the Department of Paediatrics of La Sapienza University were prospectively enrolled into the study. Primary site was orbit in 3 patients, head and neck parameningeal in 10, head and neck non-parameningeal in 2, genitourinary bladder or prostate in 8, genitourinary non-bladder/prostate in 6, extremity in 1 and others in 7.

Patients were grouped according to the Intergroup Rhabdomyosarcoma Study (IRS) post-surgical grouping system [25] using conventional techniques of imaging, marrow examination and histological sub-typing. Group I includes patients with total resection and no residue, provided that sampled regional lymph nodes are negative; group II patients with gross resection and microscopic residue and/or totally resected regionally involved lymph nodes; group III patients with incomplete resection and macroscopic residual disease or biopsy only of the primary tumour, with or without regional lymph node involvement. Patients were entered as group I (5), II (3) or III (29). Histological subtype was embryonal in 23 patients and alveolar in 14. Treatment was given according to the RMS88 (7 patients) (Fig. 1(a)) or RMS96 (30 patients) (Fig. 1(b)) protocol of the Italian Association of Paediatric Hematology/Oncology (AIEOP) [26]. Institutional written informed

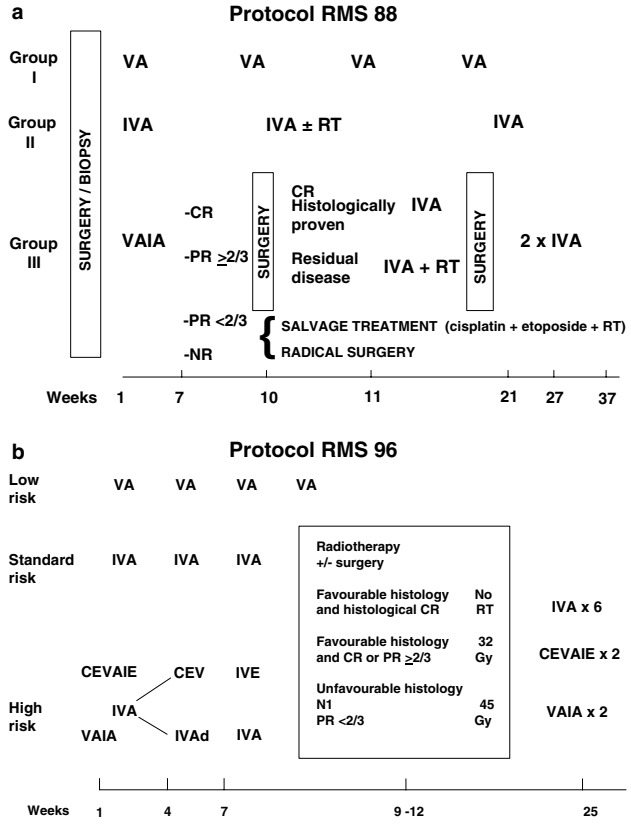

Fig. 1. Diagram of Protocols (a) RMS88 and (b) RMS96 for localised RMS. CEV – carboplatin, epirubicin and vincristine; CEVAIE – carboplatin, etoposide, vincristine, actinomycin D, ifosfamide and epirubicin; CR – complete remission; IVA – ifosfamide, vincristine and actinomycin D; IVAd – ifosfamide, vincristine and adriamycin (doxorubicin); IVE – ifosfamide, vincristine and etoposide; NR – no response; PR – partial remission; RT – radiotherapy; VA – vincristine and actinomycin D; VAIA – vincristine, actinomycin D, ifosfamide and adriamycin (doxorubicin).

consent was obtained from the patient's parents or legal guardians. The study underwent ethical review and approval according to local institutional guidelines.

2.2. Bone marrow analysis

At diagnosis four marrow aspirates were obtained from the iliac crests and were assessed in real time as each patient presented, for tumour involvement using standard cytomorphology and immunocytochemistry. For the latter, mononuclear cells were separated by Ficoll–Paque (Amersham Biosciences Europe GMBH, Milan, Italy) density-gradient centrifugation and multiple cytospins ($\geqslant 10$ per patient) were prepared. Immunocytochemical analysis was performed using monoclonal antibodies against MyoD1 (Dako, Carpinteria, CA) and myogenin (Dako). As detection system, indirect avidin-biotin immunoperoxidase (Vectastain, Vector Laboratories, Burlingame, CA) was carried out. Five separate visual counts on at least five different cytospins were performed, each of which included $\geqslant 500\,000$ cells. For MyoD1 and myogenin, only nuclear immunostaining was scored positive. RMS cell line RH30, established from an alveolar RMS [27], was utilized as a positive control, whilst for a negative control non-immunized mouse serum immunoglobulins (Vector) were used as primary antibody.

The sensitivity of the assay was measured by adding known numbers of cultured RH30 cells to normal marrow mononuclear cells, and then processing and immunostaining the samples. The detection limit was found to be 1×5^{-4} cells.

The specificity of the assay was determined by testing normal marrow samples obtained from 10 healthy volunteer marrow donors and marrow samples obtained from 10 children with neuroblastoma for the presence of cells positive for MyoD1 and/or myogenin. All samples were found to be negative.

The inter-observer reproducibility of the assay was determined by two blinded observers who re-analysed 20 marrow samples without knowledge of the nature of the marrow samples, RMS patients *vs.* normal controls. The concordance rate was found to be 95%.

2.3. Definition of clinical end-points and statistical analysis

The end-points were recurrence and survival. The patterns of recurrence were defined as follows: local (recurrence of disease at the original site of the primary tumour), regional (recurrence of disease in the draining regional lymph nodes with or without local recurrence), or distant (recurrence of disease in a metastatic site such as lung, bone or marrow, with or without loco-regional recurrence) [28]. Both overall survival (OS) and event-free survival (EFS) were considered. OS was calculated from the date of histological diagnosis until time of last follow-up or death from any cause. EFS was calculated from the date of histological diagnosis until time of progression (*i.e.* volumetric increase of any pre-existing lesion and/or appearance of any new lesion), recurrence or death from any cause; patients not experiencing an event of interest were censored at the time of last follow-up.

Clinical variables were categorised as follows: age at diagnosis, 12–120 months *vs.* <12 months or >120 months; sex, male *vs.* female; site of primary, orbit and genitourinary non-bladder or prostate *vs.* head and neck parameningeal and non-parameningeal, genitourinary bladder or prostate, extremity and others; size of primary, $\leqslant 5$ cm *vs.* >5 cm; histological subtype, embryonal *vs.* alveolar; group, I and II *vs.* III; treatment, RMS88 *vs.* RMS96. Correlations between these variables, recurrence rate, patterns of recurrence, and occult marrow involvement were analysed using 2×2 contingency tables and χ^2 test. The impact of the same variables on OS and EFS was assessed using Kaplan and Meier survival curves [29], and log-rank significance tests. The software package SPSS 7.0 for Windows (SPSS Inc, Chicago, IL) was used.

3. Results

Standard cytomorphology on marrow smears did not detect overt tumour involvement in any of the 37 patients. In 10/37 cases (27%) immunocytochemistry demonstrated cells positive for MyoD1 and/or myogenin that were interpreted as disseminated tumour cells (Fig. 2). These cells represented a minimal percentage of the mononuclear cells present in the marrow, ranging from 1×5^{-4} to 5×10^{-3} (median, 6.5×10^{-4}) of the total, well below or near to the threshold of 1×10^{-3} cells conventionally regarded as the detection limit for light microscopy [4]. In 7/10 cases, cells were positive for both

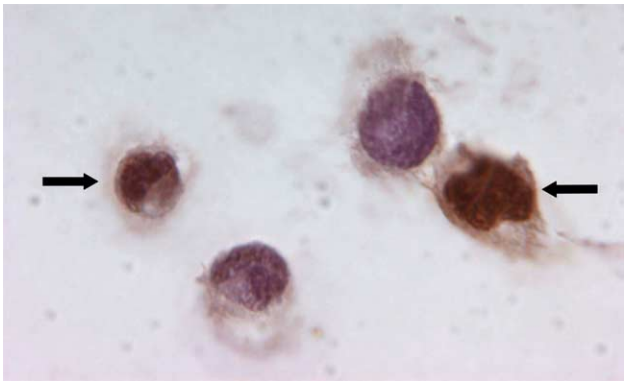

Fig. 2. Immunocytochemical analysis of disseminated tumour cells in bone marrow: two tumour cells show positive nuclear staining for myogenin whilst two surrounding normal marrow cells are negative (original magnification 1000×).

proteins, in 2 cases only for MyoD1 and in 1 case only for myogenin.

Correlations between clinical variables and occult marrow involvement are shown in Table 1. Marrow disease was not significantly associated with any variable, although there was a trend for disseminated tumour cells to be found more frequently in association with unfavourable primary sites (head and neck parameningeal and non-parameningeal, genitourinary bladder or prostate, extremity and others), size of primary >5 cm and group III (Table 1).

As of the end of March 2005, the median follow-up for the 37 patients was 46 months (range: 12–115), and their status was disease-free (DF) in 30 or dead of disease (DOD) in 7 (at 10, 11, 14, 21, 21, 23 and 27 months from diagnosis). Disease recurrence occurred in 8/37 (22%) patients: 2 (25%) had local relapse (at 9 and 13 months from diagnosis), 1 (12.5%) regional relapse (at 14 months from diagnosis), and 5 (62.5%) distant relapse (at 2, 6, 8, 8 and 9 months from diagnosis).

Occult marrow involvement and the other clinical variables were then analysed with regard to the rate and pattern of recurrence (Table 2). Only occult marrow involvement and RMS88 treatment were shown to be associated with a significantly higher risk of global recurrence ($P = 0.01$). We further analysed the correlations of these two variables with pattern of recurrence: because of the small number of cases, local and regional recurrences were grouped together ($n = 3$) and compared to distant recurrences ($n = 5$). Occult marrow involvement was found to be associated with a significantly higher risk of distant recurrence ($P = 0.014$).

The impact of occult marrow involvement and the other variables on survival was evaluated using univariate analysis (Table 3). The presence of disseminated tumour cells and RMS88 treatment were associated with significantly shorter OS and EFS; size of primary >5 cm was only associated with shorter OS. For occult marrow involvement in particular, OS probability was 92% in patients with no marrow infiltration at diagnosis compared to 47% in those with marrow infiltration ($P = 0.001$) (Fig. 3(a)); EFS probability was 89% in the former compared to 50% in the latter ($P = 0.01$) (Fig. 3(b)). Multivariate analysis of variables independently associated to survival was not feasible because of the small number of cases.

4. Discussion

This is the first study which prospectively assessed at diagnosis a clinical series of localised RMS for occult disseminated tumour cells in the marrow and whether their presence impacts on recurrence and survival. Cells with positive immunostaining for MyoD1 and/or myogenin, interpreted as disseminated tumour cells, were detected in 27% of cases. Patients with detectable occult marrow involvement showed a significantly higher rate of recurrence, particularly at distant sites, with significantly shorter OS and EFS.

Two main approaches have been developed to detect disseminated tumour cells, either immunocytochemical or PCR-based assays [6]. Immunocytochemistry utilizes monoclonal or polyclonal antibodies that recognise

Table 1
Correlations between conventional clinical variables and occult marrow involvement

Clinical variable	Levels	Patients (n)	Bone marrow		P
			Positive	Negative	
Age (months)	<12 or >120	12	2 (16.7%)	10	n.s.
	12–120	25	8 (32.0%)	17	
Sex	Female	14	6 (42.9%)	8	n.s.
	Male	23	4 (17.4%)	19	
Histology	Embryonal	23	5 (21.7%)	18	n.s.
	Alveolar	14	5 (35.7%)	9	
Primary site	Favourable[a]	9	1 (11.1%)	8	n.s.
	Unfavourable[b]	28	9 (32.1%)	19	
Size	≤5 cm	19	3 (15.8%)	16	n.s.
	>5 cm	18	7 (38.9%)	11	
Group	I and II	8	1 (12.5%)	7	n.s.
	III	29	9 (31.0%)	20	
Treatment	RMS88	7	2 (28.6%)	5	n.s.
	RMS96	30	8 (26.7%)	22	

[a] Favourable primary site includes orbit and genitourinary non-bladder/prostate.
[b] Unfavourable primary site includes head and neck parameningeal and non-parameningeal, genitourinary bladder or prostate, extremity and others.

Table 2
Correlations between clinical variables (including occult marrow involvement) and recurrence rate

Clinical variable	Levels	Patients (*n*)	Recurrences (*n*)	Recurrence %	*P*
Age (months)	<12 or >120	12	2	16.7	n.s.
	12–120	25	6	24.0	
Sex	Female	14	3	21.4	n.s.
	Male	23	5	21.7	
Histology	Embryonal	23	4	17.4	n.s.
	Alveolar	14	4	28.6	
Primary site	Favourable[a]	9	1	11.1	n.s.
	Unfavourable[b]	28	7	25.0	
Size	⩽5 cm	19	2	10.5	n.s.
	>5 cm	18	6	33.3	
Group	I and II	8	1	12.5	n.s.
	III	29	7	24.1	
Treatment	RMS88	7	4	57.1	0.011
	RMS96	30	4	13.3	
Bone marrow	Negative	27	3	11.1	0.011
	Positive	10	5	50.0	

[a] Favourable primary site includes orbit and genitourinary non-bladder/prostate.
[b] Unfavourable primary site includes head and neck parameningeal and non-parameningeal, genitourinary bladder or prostate, extremity and others.

Table 3
Correlations between clinical variables (including occult marrow involvement) and survival probability

Clinical variable	Levels	Patients (*n*)	Event-free survival			Overall survival		
			Events (*n*)	Survival %	*P*	Events (*n*)	Survival %	*P*
Age (months)	<12 or >120	12	2	83.3	n.s.	2	80.2	n.s.
	12–120	25	6	76.0		5	78.9	
Sex	Female	14	5	78.3	n.s.	5	76.2	n.s.
	Male	23	3	76.0		2	83.9	
Histology	Embryonal	23	4	82.4	n.s.	3	85.3	n.s.
	Alveolar	14	4	71.4		4	69.3	
Primary site	Favourable[a]	9	1	88.9	n.s.	0	100.0	n.s.
	Unfavourable[b]	28	7	74.8		7	73.0	
Size	⩽5 cm	19	2	89.5	n.s.	1	94.7	0.039
	>5 cm	18	6	66.2		6	63.3	
Group	I and II	8	1	87.5	n.s.	0	100.0	n.s.
	III	29	7	75.7		7	73.3	
Treatment	RMS88	7	4	42.9	0.004	4	42.9	0.0091
	RMS96	30	4	86.4		3	88.5	
Bone marrow	Negative	27	3	88.7	0.012	2	91.9	0.0015
	Positive	10	5	50.0		5	46.7	

[a] Favourable primary site includes orbit and genitourinary non-bladder/prostate.
[b] Unfavourable primary site includes head and neck parameningeal and non-parameningeal, genitourinary bladder or prostate, extremity and others. *P*-log rank comparison of Kaplan–Meier curve.

specific tumour markers. These assays preserve the morphology of suspect cells, are inexpensive and easy to carry out where routine facilities for clinical pathology are available, and can detect a single disseminated tumour cell among 1×10^{-5} normal cells [7]. PCR-based assays utilize appropriate primers to identify DNA- or RNA-based tumour markers. This approach is highly sensitive detecting a single disseminated tumour cell among 1×10^{-6} or even 1×10^{-7} normal cells [5,30]. However, it is laborious, imposes stringent requirements for sampling, and requires skills and equipment not always available in clinically oriented laboratories.

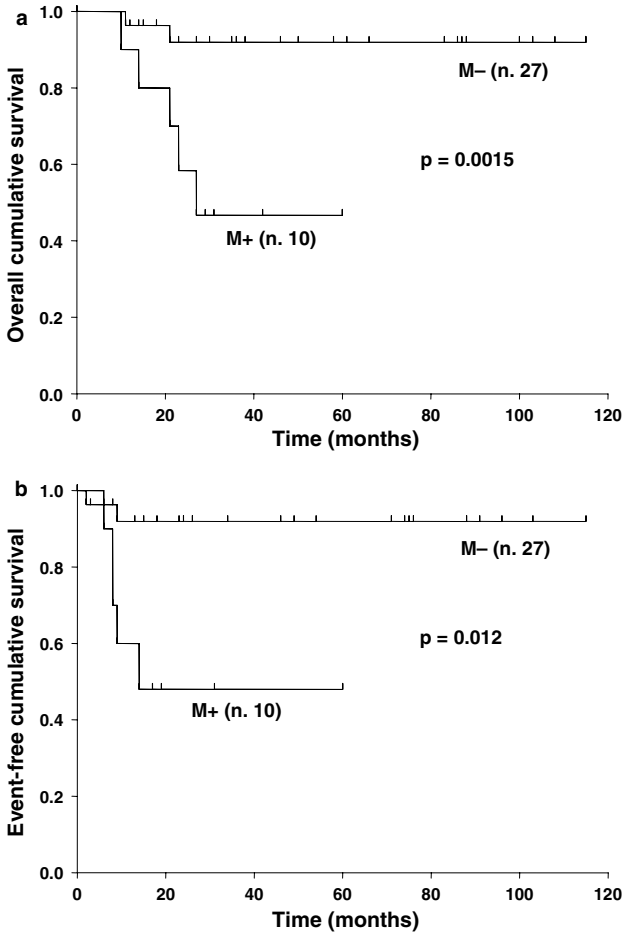

Fig. 3. Kaplan Meier survival analysis for patients with (M+) or without (M−) occult marrow disease at diagnosis. (a) Overall survival was 92% in M− compared to 47% in M+ ($P = 0.001$). (b) Event-free survival was 89% in M− compared to 50% in M+ ($P = 0.01$).

A further limiting factor with regard to using PCR-based assays for occult disease detection is that only two genetic markers are available for RMS, resulting from the translocations t(2;13)(q35;q14) and t(1;13)(p36;q14) which juxtapose the *PAX3* or *PAX7* genes on chromosomes 2 and 1, and the *FKHR* gene on chromosome 13 [31]. In ARMS, 75–80% carry one or other of these translocations, but none of ERMS, which represents 80% of RMSs [32]. Therefore, at present there is no specific genetic marker for 85% of RMSs.

However, ERMS and ARMS do show an overlapping expression of markers related to muscle differentiation such as actins, myosins, creatine kinases and myogenic regulatory factors [12]. MyoD1 and myogenin, two myogenic regulatory factors whose expression determines the commitment and differentiation of mesenchymal progenitor cells into skeletal muscle [10], have extensively been investigated in RMS cell lines and primary tumours using immunohistochemical [11,13,14,16–19] or molecular [12,15,20] techniques. These studies have shown that they are both expressed at variable levels in 80–100% of RMS tumours [16,19,20], with a tendency for higher levels of myogenin expression in ARMS [18,19,21]. There were also suggestions that myogenin may be a more informative tissue marker than MyoD1 and more reliable in detecting occult disseminated tumour cells [20]. Furthermore, transcripts for MyoD1 and myogenin were not detected in normal peripheral blood and bone marrow using RT-PCR [15,20]. This was also confirmed in the present study where immunostaining for both markers was not detected in normal marrow samples obtained from healthy volunteer marrow donors or in marrow samples obtained from children with neuroblastoma.

Therefore, it seems reasonable to assume that the immunophenotype of the cells detected in some of the marrow samples is highly suggestive that these are disseminated tumour cells. The propensity of RMS to metastasize to marrow is well known and morphologically detectable tumour marrow involvement is present at diagnosis in about 30% of patients with metastatic disease [22]. The molecular determinants of this behaviour are not fully understood but it is supposed that specific chemoattractants released by stromal marrow cells can direct tumour cells to marrow where these can find a favourable microenvironment for survival and eventual expansion [33]. Despite this clinical and experimental evidence, until recently the workup for RMS did not routinely require extensive marrow assessment at diagnosis with either standard cytomorphology or more sensitive immunocytochemical or molecular assays. We could only find three previous reports investigating the presence of disseminated tumour cells in morphologically normal marrow from patients with RMS at diagnosis: all of them utilised a RT-PCR assay evaluating the expression of either *PAX3–FKHR* and *PAX7–FKHR* [23,24] or MyoD1 and myogenin [20]. In two of these studies, occult marrow involvement was detected in 14% (1/7) [23] and 22% (2/9) [20] of samples, whilst no tumour involvement was demonstrated in the other study [24]. These percentages, although obtained in smaller series by using a molecular detection technique, are quite similar to the percentage of patients with localized disease whose marrow was found positive in the present study (27%). This range of frequency seems also comparable to the frequencies reported in other localised childhood solid tumours such as neuroblastoma (34%) [7] and Ewing's sarcoma (19%) [8]. However, since the heterogeneous and focal distribution that tumour rhabdomyoblasts can have in marrow [22] and/or since in primary RMSs small tumour cell populations can exist which do not express detectable levels of either MyoD1 or myogenin proteins [16–19], the present findings could underestimate to a certain extent the actual number of disseminated tumour cells in marrow of patients with localised RMS. To clarify this aspect, further results from the ongoing prospective studies employing more sensitive RT-PCR assays will be needed

[34]. The same is true regarding which one between MyoD1 and myogenin may be the most informative RMS marker in detecting occult disease. In this series the detection rate for disseminated tumour cells was not significantly different using either MyoD1 (9/10 samples) or myogenin (8/10). We cannot however exclude that the lower sensitivity of immunocytochemistry compared to RT-PCR may have hidden possible marginal differences.

Regarding the biological significance of disseminated tumour cells in marrow of patients with localised RMS and their possible relationship with the development of overt metastases, current consensus recommendations for identifying metastases set a minimum size threshold of 2 mm for nests of tumour cells detectable with hematoxylin–eosin stain [6]. Smaller clusters, isolated cells or cells only detectable by immunocytochemical or RT-PCR-based assays fall into a category for which the neutral definition "disseminated tumour cells" has been recommended [6]. This was suggested since until recently very little was known about the genotypic and phenotypic characteristics of these cells, with regard in particular to their actual potential to evolve into overt metastases [5,6]. To successfully colonise a distant site, a tumour cell must complete a series of sequential steps that includes invasion through surrounding tissues, entry and survival in blood or lymphatic vessels, arrest in a distant site, extravasation through surrounding tissues, survival in the foreign microenvironment through evasion from apoptotic death and immune response, proliferation, and induction of angiogenesis [35]. Due to the low survival rate of tumour cells throughout the post-intravasation steps, neither the presence of circulating tumour cells in peripheral blood nor their arrest in a distant site by itself is a reliable predictor of overt metastasis. Experimental evidence suggests instead that the different ability of disseminated tumour cells to go successfully through the stages following extravasation may be the most important determinant of metastatic efficiency [36]. Although the number of circulating tumour cells can be high, the estimated success rate for metastasis is extremely low, on the order of 1×10^{-5} or 1×10^{-6} cells [37]. Despite the inefficiency of the metastatic process however, the higher the number of disseminated tumour cells, the greater is the probability that one of these might acquire the full metastatic phenotype and produce an overt metastasis. A substantial body of evidence shows that the presence at diagnosis of occult marrow disease is predictive of overt metastases at distant sites and poor outcome in adult patients with localised (N0M0) breast cancer, colon cancer, lung cancer and prostate cancer [5,6]. RMS should now be included among the childhood solid tumours for which similar results have been reported [7,8].

One of the key elements in improving treatment for RMS has been the ability to define prognostic factors, which has led to the re-grouping of patients either to less intensive treatment, as in the case of paratesticular tumours, or to intensified treatment, as in alveolar tumours [38–40]. In localised RMS, analysis of previous studies has also allowed predictions of failure patterns leading to appropriate alterations in treatment regimens [41]. This study has determined the extent of occult disseminated tumour cells that can be detected using immunocytochemistry, a simple and inexpensive procedure, to better define disease extent at diagnosis. Although the numbers are small and the present results need to be confirmed in a larger series, the presence of these cells, although not overtly metastases, was significantly linked to relapse at distant sites and to poor outcome. A similar trend for disseminated tumour cells in marrow to be associated with disease progression and poor outcome in patients with RMS has also been described in both the previous studies carried out using RT-PCR assays that showed early occult involvement [20,23]. Taken together, these findings strongly suggest that patients with localised RMS and disseminated tumour cells in marrow actually represent a subset at high risk of progression that might benefit from an early intensification of their treatment. If so, this strategy could result in a possible improvement in outcome as it has already been documented by the increased survival of patients treated on RMS88 and RMS96, the latter used more intensified regimens, and by other working groups such as IRS [42].

Furthermore, recent data suggest that the identification in localised RMS of early disseminated tumour cells in marrow could possibly enable us to select systemic treatments and monitor their efficacy more effectively. The widely accepted model for tumour progression hypothesise that metastases are derived from small cell subpopulations in primary tumours that develop relatively late during cancer development [43]. Recent studies on gene expression profile investigating molecular signature of poor prognosis in breast cancer however have challenged this model by identifying the signature of metastatic phenotype in most tumour cells of primary tumours [44]. It has been suggested that in breast cancer and maybe in many other tumour types, the proclivity to metastasis is acquired very early during tumour development, although it manifests much later [45,46]. According to this model, tumour cells may disseminate to distant sites in a far less genotypic advanced stage than previously thought and may evolve to metastatic cells independently from the primary tumour, after additional genetic changes have taken place [45,46]. These findings and consequent assumptions would have important clinical implications. Systemic treatments should not only target primary tumour cells but also disseminated tumour cells, otherwise treatment will be ineffective against metastatic tumour cells that have evolved independently at distant sites. The identification of specific targets in disseminated tumour cells could poten-

tially provide a powerful tool to tailor systemic treatment aimed at preventing these cells from evolving to micrometastases and therefore to improve cure rate. Large prospective multicenter studies including gene expression profiling of both primary tumour cells and disseminated tumour cells will be needed in order to validate the feasibility and clinical utility of this approach.

Conflict of interest statement

The authors indicate no financial and personal relationships with people or organizations that could have inappropriately influenced their work.

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