Flow Cytometric Phenotype of Rhabdomyosarcoma Bone Marrow Metastatic Cells and its Implication in Differential Diagnosis with Neuroblastoma

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Abstract. *Background: The goal of this study was to develop a flow cytometric (FCM) method for assessing the presence of metastatic cells in bone marrow (BM) and peripheral blood (PB) obtained from rhabdomysarcoma (RMS) patients. Myogenin (Myf4), a specific molecular RMS marker, was also investigated in the same samples. Since neuroblastoma (NB) metastasizes to the BM, the potential application of cytometry in differential diagnosis was explored. Patients and Methods: CD45, CD56, CD90 and CD57 antibodies were used in 7 paired BM and PB samples (from 7 RMS stage IV patients at presentation), 23 BM samples (from 13 RMS stage I and II patients at presentation), and ten paired BM and PB samples taken at presentation and five BM samples taken at recurrence from 13 NB stage 4 patients. Results: All seven BM samples from RMS stage IV (but not those from patients with localized disease) showed both the CD45– CD56+ phenotype and the Myf4 transcript. Four cases also showed CD90 and two CD57 positivity. Neither the CD45– CD56+ phenotype, nor Myf4 were recorded in the BM and PB samples from patients with localized disease. All the NB BM samples (15/15) showed the CD45– CD56+ CD90+ phenotype and 10/15 also showed CD57 positivity. Only 3/10 blood samples from the NB patients revealed tumor cells. Conclusion: CD45, CD56, CD90 and CD57 antibodies can be used in FCM for marrow metastasis detection in both, RMS and NB patients.*

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FCM is a well-established method for diagnosing hematological/lymphoid disease, but FCM methods have rarely been recommended for identifying metastatic cells of solid tumors in BM. An important exception is represented by NB stage 4 patients, in whom FCM has been recommended (1) as an ancillary tool for detecting disease in BM during diagnostic or staging procedures and, an increasing numbers of authors claim that FCM can also be used as an adjunctive tool for the lineage-specific identification of non-hematopoietic neoplasms. FCM works on naive antigens and small samples, it is a rapid and inexpensive quantitative technique but, despite these advantages, no FCM methods have been recommended to date for identifying RMS cells infiltrating BM. RMS is a sarcoma consisting of cells committed to skeletal differentiation and is one of the more common extra-cranial solid tumors of childhood. RMS frequently affects children under 6 years old (in 65% of cases) and metastases to BM are found in 10-15% of patients at presentation (2).

For the first time in the present study, an FCM approach was used to seek metastatic RMS cells in BM and peripheral blood (PB) samples obtained at presentation from RMS patients. In addition, given that, like RMS, NB can also affect children under the age of 6, the diagnostic potential of FCM was also evaluated in BM and PB samples obtained from NB stage 4 patients.

To identify the RMS cells, a panel of antigens, *i.e.* CD45, CD56 (neural cell adhesion molecule, NCAM), CD90 (Thy-1) and CD57 was used, and the FCM findings were supported by performing a molecular detection of the RMS specific transcript *Myf4* (3). CD45 is the leukocyte common antigen and is expressed on virtually all leukocytes, but is lacking in non-hematopoietic tissues. CD56 is a membrane glycoprotein expressed on neural and muscle tissues that is involved in homotypic adhesive interactions (4). Immunocytochemical (ICC) evidence obtained in primary tumors (5) has indicated that CD56 is expressed by RMS. CD90, which is also known

Table I. *FCM evaluation and molecular Myf4 analysis in BM and PB samples from RMS IRS IV patients.*

N	Primary sites	CD45	CD56	CD90	CD57	% of BM-infiltrating cells	*Myf4* transcript in BM	% of circulating cells in PB	*Myf4* transcript in PB
RMS 1	Pr	-	+ low	-	-	60	+	-	-
RMS 2	UE	-	+ bright	-	-	14	+	-	-
RMS 3	Pe	-	+ bright	-	-	40	+	-	-
RMS 4	B	-	+ bright	+ dim	-	1.0	+	-	-
RMS 5	Par	-	+ bright	+ dim	+ bright	0.3	+	-	-
RMS 6	UE	-	+ bright	+ dim	+ bright	1.0	+	-	-
RMS 7	UE	-	+ bright	+ dim	-	4.0	+	-	-

Pr: Prostate, UE: upper extremities, Pe: pelvis, B: breast, Par: parameningeal.

as Thy-1, is a small glycosilphosphatylinositol anchored glycoprotein previously identified in the RMS cell line RD, primary RMS (6) and NB (7) specimens. CD57 is a glycotype epitope expressed on a variety of molecules, such as integrins, proteoglycans and glycolipids. Contrasting ICC results have been published concerning CD57 in RMS: one paper reported 3/4 CD57+ RMS (8), while another 0/8 (5).

Patients and Methods

RMS and NB patients. The study included seven RMS patients in IRS (Intergroup Rhabdomyosarcoma Study) stage IV (five alveolar, one embryonal and one not otherwise specified, all with BM involvement) and a further 13 cases of localized RMS (IRS stages I and II, five alveolar and eight embryonal). Their median age was 13 years (2-20).

The study also included 13 patients with stage 4 NB (11 in stage 4 and two in stage 4s). The primary sites were the adrenal glands (in six cases), the retro peritoneum (in five) and the mediastinum (in two). Their median age was 2.5 years (0.5-6). All the children were treated at the Istituto Nazionale dei Tumori, Milan, Italy, between 2003 and 2006 and their parents' informed consent to participation in the study was obtained in all cases.

Table II. *FCM evaluation in BM and PB samples from stage 4 NB patients.*

N	BM sample timing	CD45	CD56	CD90	CD57	% of cells infiltrating BM	% of circulating cells in PB
NB 1	P	-	+ bright	+ dim	-	13	0.05
NB 2	P	-	+ bright	+ dim	-	1.0	-
NB 3	P	-	+ bright	+ bright	-	0.2	-
NB 4	P	-	+ bright	+ dim	-	10	0.1
NB 5	P	-	+ bright	+ dim	-	0.1	-
NB 6	P	-	+ bright	+ int	+ dim	1.0	-
	R	-	+ bright	+ int	+ dim	0.1	Nd
NB 7	P	-	+ int	+ bright	+ bright	1.0	-
	R	-	+ int	+ bright	+ bright	1.0	Nd
NB 8	P	-	+ bright	+ int	+ int	0.2	-
NB 9	P	-	+ bright	+ dim	+ dim 50%	3.0	0.05
NB 10	P	-	+ bright	+ int	+ dim 50%	0.1	-
NB 11	R	-	+ bright	+ int	+ int	2.0	Nd
NB 12	R	-	+ bright	+ int	+ int	0.1	Nd
NB 13	R	-	+ bright	+ int	+ int	0.1	Nd

P: presentation, R: relapse, Nd: not done.

Morphological evaluation. Where available, aspirates (from the right and left iliac crest) were smeared onto at least three slides and stained. The BM smears were evaluated using the May Grunwald Giemsa (MGG) procedure and by ICC analysis. In particular, immunoreactivities to myogenin were used as ICC specific RMS markers.

FCM evaluation. Fifty μl of mechanically-resuspended whole marrow aspirates or 100 μl of PB samples were placed in a 12×75 mm round-bottom polystyrene tube (Falcon 2054; Becton Dickinson Labware, San Jose, CA, USA), and incubated for 30 minutes at 4˚C with 10 μl of CD90FITC (Pharmingen Clone 5E10), CD56PE (Becton Dikinson clone MY31) and CD45PerCP (Becton Dikinson, clone 2D1), and in a separate tube with CD56PE, CD45PerCP and CD57FITC (Becton Dikinson, clone HNK-1). Then the red blood cells were removed with ammonium chloride solution and the samples were washed and resuspended in 500 μl PBS. The cell analysis was performed on a FACS-Scan flow cytometer (Becton Dickinson) and at least 10^5 events were analyzed. The threshold was set on forward scatter (FSC), and the cell cytogram was verified on an FSC *vs*. side scatter (SSC) dot plot. A capture area (R1) was drawn on a CD56PE *vs*. CD45PerCP dot plot to include all the CD45– CD56+ events. The events coming within R1 were back-gated and evaluated in the FSC *vs*. SSC dot plot. The R1 events were then back-gated and evaluated for CD90FITC/CD56PE and CD57FITC/CD56PE co-expression.

Isotype-matched controls were used to determine non-specific binding and to set the negativity threshold. The median fluorescence intensities of the logarithmic scale (MFI) shown by the antibodies used in this study are indicated in Tables I and II as follows: *bright* 10^3-10^4, *intermediate* (*int*) 10^2-10^3 and (*dim*) 10^1-10^2. The sensitivity of CD45– CD56+ BM phenotype detection had previously been established at 10^{-4} by diluting the NB cell line SK N BE (9).

RNA extraction, reverse-transcription and *Myf4* PCR. The total RNA was extracted from the BM or PB samples (previously analyzed by FCM) and reverse transcribed, as described elsewhere (10).

The primers and PCR conditions for the *Myf4* amplifications have been published elsewhere (3). The sensitivity threshold of the method was established by serial dilutions of RMS cell lines TE 671 (Interlab Cell Line Collection, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, catalogue number: ICLC HTL97021). Briefly, normal EDTA whole blood with a known number of leukocytes obtained from an healthy donor was spiked with graded amounts of TE 671 to represent abnormal cell frequencies ranging from 10^{-1} to 10^{-6}. 10^2 TE671 cells diluted in 10^6 normal leukocytes could be detected (sensitivity threshold :10^{-4}, results not shown). The same leukocytes were used as negative controls in PCR experiments.

Results

Morphological evaluation of BM samples from RMS patients. All seven RMS IRS IV BM samples were judged to be infiltrated by RMS cells at routine morphology and ICC analyses, whereas no infiltrating RMS cells were detected in the 23 BM samples obtained from the 13 localized RMS IRS stage I and II patients. The PB samples obtained from the RMS IRS IV at presentation were evaluated using only FCM and molecular approaches.

Phenotype of RMS cells infiltrating BM. All the BM samples from the RMS IRS IV patients (Table I) revealed the CD45– CD56+ phenotype (Figure 1A and 1B). Although, the CD56 (PE) MFI shown by CD45– cells was *bright* in the majority of the cases, a CD45– CD56+ *dim/int* cells could be also evidenced (Figure 1B, indicated by the arrow). The MFI shown by CD90 and CD57 was *dim* and *int* respectively.

The CD45– CD56+ CD90+ CD57– phenotype was found in the BM of 4/7 RMS stage IV patients (Figure 2A and 2B). Two out of seven of the RMS patients had a BM showing CD45– CD56+ CD90+ CD57+ cells (Figure 3A, 3B).

The analyses of the 23 BM samples from the 13 RMS IRS I and II patients revealed no cells with the CD45– CD56+ CD90+ CD57+ phenotype (Figure 1E). The FSC/SSC properties of the BM-infiltrating RMS cells were FSC intermediate to high and SSC low (Figure 1B).

Correlation with molecular study. In all the BM samples obtained from the RMS IRS IV patients, a transcript corresponding to *Myf4* could be identified after RNA extraction and PCR amplification, whereas molecular analysis of the 23 BM samples obtained from the patients with localized RMS, and of the seven PB samples from the RMS IRS IV patients did not show the *Myf4* transcript (Figure 2D).

NB stage 4 patients. In all the cases, the NB stage 4 BM samples were judged to be infiltrated by NB cells at routine morphology. The PB samples obtained from the 10 patients at presentation were evaluated only by FCM.

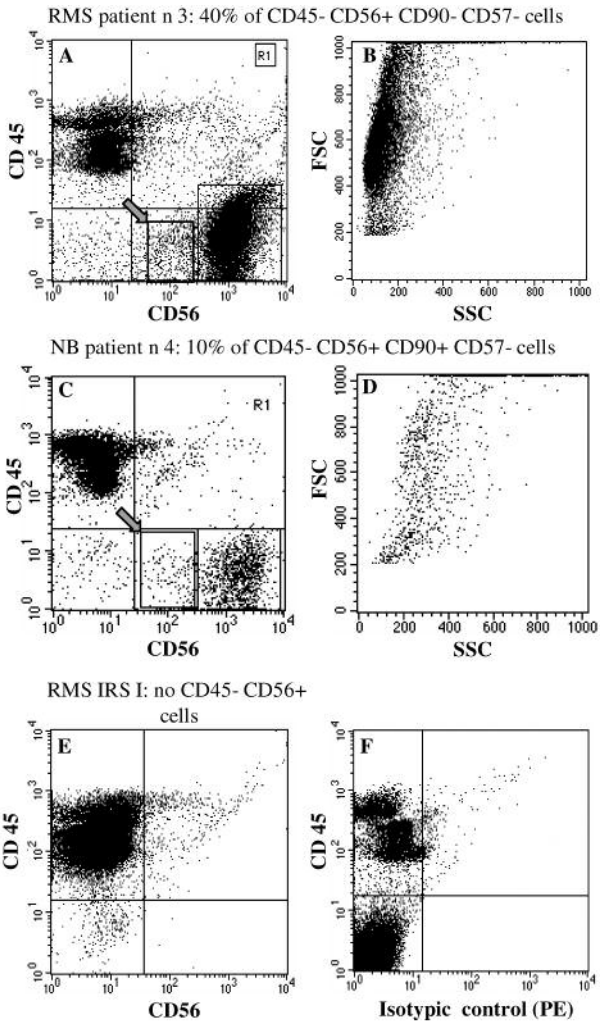

Figure 1. *The CD45– CD56+ phenotype of RMS and NB BM infiltrating cells. The CD45– CD56+ cells were boxed in the R1 region and back-gated in a forwarw scatter/side scatter dot plot. The MFI shown by CD56+ (PE) CD45– cells was bright in both RMS (1A) and NB (1C). The forward scatter was intermediate-high and the side scatter was low in both RMS (1B) and NB (1D). The arrows indicate the CD56+ dim or int expression of CD45– cells. No CD45– CD56+ cells in BM drawn from localized RMS patient (1E). The PE isotype control vs. CD45 PerCP (1F).*

Phenotype of NB cells infiltrating BM. In all the cases (10/10 at presentation and 5/5 at relapse), the NB cells in the BM displayed the CD45– CD56+ CD90+ phenotype. Five of the ten BM samples obtained at presentation and 5/5 taken at relapse showed the CD45– CD56+ CD57+ phenotype (Table II). As in BM drawn from RMS patients, the CD56 (PE) MFI shown by CD45– cells, were *bright* in the majority of cases but a CD45– CD56+ *dim/int* cells could be also evidenced (Figure 1C indicated by the arrow). The MFIs were *dim* (five cases), *int* (seven cases) and *bright* (three cases) for CD90. CD45– 56+ cells co-

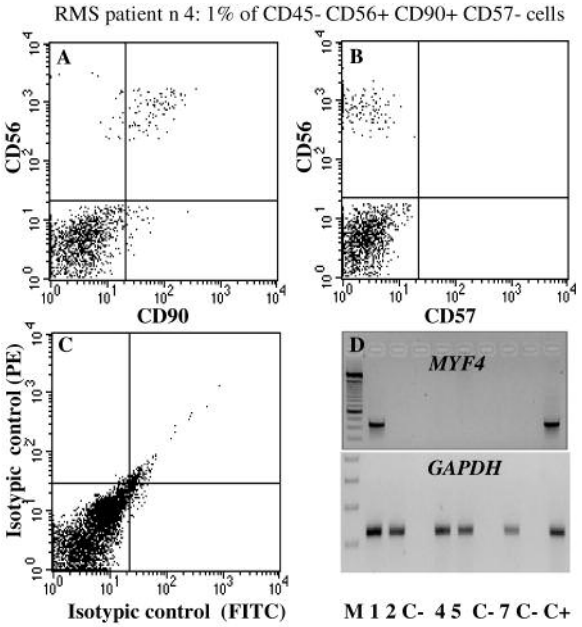

Figure 2. *The CD45– CD56+ CD90+ CD57– phenotype and myf4 expression of BM RMS infiltrating cells. The CD45– CD56+ cells were identified as described in Figure I and then back-gated in a 56PE 57FITC or 56PE 90FITC dot plot. Co-expression of CD90 (2A) but not of CD57 (2B) on CD45– CD56+ cells infiltrating the BM. PE vs. FITC isotype controls (2C). The corresponding myf4 transcript was seen in BM (2D, line 1), but not in PB (2D, line 2). No myf4 transcripts were seen in BM obtained from localized RMS patients (2D lines 4 and 5) or one healthy donor (2D line 7). M, molecular marker; C–: PCR negative controls constituted by a PCR samples without cDNA template; C+: PCR positive control constituted by cDNA obtained from RMS TE 671 cell line.*

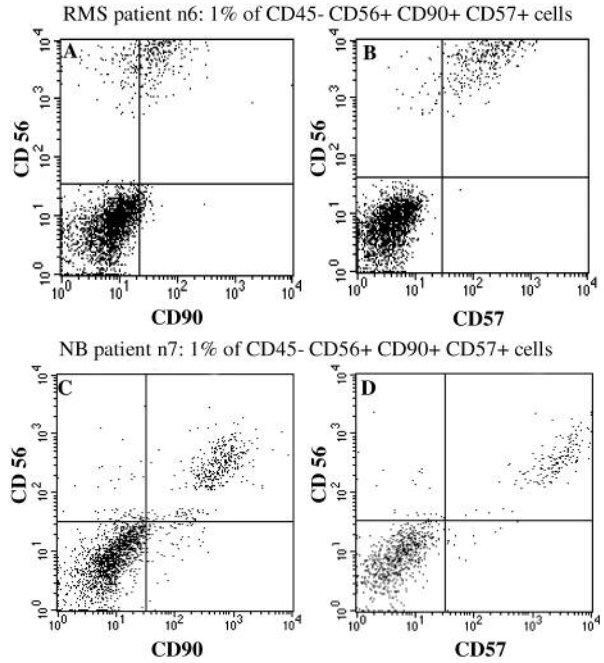

Figure 3. *CD90 and CD57 co expression in RMS and NB BM infiltrating cells. CD90+ and CD57+ co expression of BM infiltrating cells in RMS (3A and 3B) and NB (3C and 3D) patients. Pertinent isotypic controls were used in each experiment (data not shown).*

expressed CD57 with *int* MFI in 4/5 BM samples taken at relapse, and *high* MFI in one. Among the BM samples taken at presentation, CD57 was expressed by all the CD45– CD56+ CD90+ cells in 3/10 cases, while its expression was apparent in only 50% of the CD45– CD56+ CD90+ cells in the other two CD57 positive cases.

Taken together, 15/15 and 10/15 of the NB cells in the BM co-expressed CD90 and CD56, and CD57 and CD56, respectively (Figure 3C and 3D). The FSC/SSC properties of the NB and RMS cells infiltrating the BM were similar in all cases, *i.e.* FSC intermediate to high and SSC low (Figure 1 D).

Circulating RMS and NB tumor cells. None of the PB samples drawn at presentation in the seven RMS IRS IV patients showed circulating cells by FCM analysis, whereas the PB samples drawn from the NB patients at presentation contained circulating tumor cells in 3/10 cases. The phenotype of these circulating cells was the same as in the corresponding BM but the percentage of cells circulating in the PB was 2 log lower (Table II).

Discussion

All the seven RMS IRS IV marrow samples that had previously been judged to be infiltrated by neoplastic cells using standard morphology, displayed the CD45– CD56+ phenotype. In two cases, the CD45– CD56+ cells also showed both CD90+ and CD57+, and two cases only showed CD90+. On the other hand, no CD45– CD56+ CD90+ CD57+ cells were revealed in the 23 BM samples taken from the 13 RMS IRS I and II patients. Moreover, the RMS specific transcript *Myf4* was only revealed in the seven positive BM samples. Our finding that 4/7 RMS BM infiltrating cells showed CD90 positivity could supports the hypothesis that the precursor of RMS might be represented by a skeletal-differentiating CD90+ or CD90- tissue-resident skeletal muscle stem cells precursor (11).

The CD45– CD56+ CD90+ CD57+ phenotype showed by NB BM infiltrating cells could be related to the embryonal origin of this tumor (neural crest). However, some striking differences in phenotype of NB BM infiltrating cells and NB BM derived nonadherent tumorigenic spheres has been reported (12). In particular, the NB sphere lacked CD56 expression and were positves for CD45 and NB84 antigens. Although the CD56 expression in NB BM infiltrating cells is generally high, a sub-populations of CD45– cells expressing CD56 with *dim* or *int* MFI can be evidenced (as

shown in Figure 1A and 1C). Furthermore, it has been reported that the expression of CD90 during early neuronal development is low but, after maturation, the CD90 expression increase (13). Accordingly, the NB BM CD45– CD56+ cells showed a very heterogeneous CD90 expression intensity. Taken together, these results, suggest that the BM microenvironnement could influence the differentiation degree of both, NB and RMS metastatic cells.

It is worth noting that all the BM samples taken at relapse (5/5) showed CD57 positivity, as opposed to only 5/10 of those taken at presentation, suggesting that the aberrant glycosylation might be a common feature of metastatic subclones in NB.

Circulating neoplastic cells in PB are an uncommon feature among patients with solid tumors, but these cells were found by FCM in three PB samples obtained at presentation from ten of the NB patients. Albeit with a lower frequency, this result is consistent with a previously-published report in which a sensitive polymerase chain reaction revealed tyrosine hydroxylase mRNA in 67% of PB samples taken at presentation in NB stage 4 patients (14). No circulating cells were detected at presentation in the seven RMS IRS IV patient samples, even when a very high percentage of BM infiltration was detected. It was only in one PB sample drawn on progression of the disease (RMS patient no. 1 of Table I) that circulating RMS cells could be detected by FCM and by *Myf4* amplification (results not shown). Similar findings have emerged using a more sensitive molecular approach (3) and suggest that (unlike the case of NB) PB cannot be used for FCM disease assessment in RMS patients.

FCM detection of the CD45– CD56+ phenotype in NB stage 4 BM aspirates has been recommended as a staging or diagnostic tool (1, 9). The results of the present work suggest, however, that the CD45– CD56+ phenotype also identified BM-infiltrating cells in RMS patients. This result indicated that, due to the phenotypic characteristics shared by RMS and NB, the FCM detection of CD45– CD56+ cells in BM could be used only for the staging of an already diagnosed RMS patient. For the purposes of differential diagnosis, our results suggest that both CD90 and CD57 are expressed less frequently by RMS cells infiltrating BM (4/7 and 2/7 BM samples respectively) than by NB cells in BM (15/15 and 10/15, respectively) but this difference did not reach statistical significance (p: ns, χ^2 test). That FCM identified circulating cells in the PB from a subset of NB, but not of RMS stage 4 patients suggests that PB might be useful in initial non-invasive diagnostic procedures in such cases.

In conclusion, although ICC remains the gold standard for the lineage-specific identification of BM-infiltrating cells during diagnostic or staging procedures, FCM may be an effective method for the rapid detection of RMS (or NB) cells metastasizing to the BM or PB.

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