Mesenchymal Neuroblastoma Cells Are Undetected by Current mRNA Marker Panels: The Development of a Specific Neuroblastoma Mesenchymal Minimal Residual Disease Panel

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PURPOSE Patients with neuroblastoma in molecular remission remain at considerable risk for disease recurrence. Studies have found that neuroblastoma tissue contains adrenergic (ADRN) and mesenchymal (MES) cells; the latter express low levels of commonly used markers for minimal residual disease (MRD). We identified MES-specific MRD markers and studied the dynamics of these markers during treatment.

PATIENTS AND METHODS Microarray data were used to identify genes differentially expressed between ADRN and MES cell lines. Candidate genes were then studied using real-time quantitative polymerase chain reaction in cell lines and control bone marrow and peripheral blood samples. After selecting a panel of markers, serial bone marrow, peripheral blood, and peripheral blood stem cell samples were obtained from patients with high-risk neuroblastoma and tested for marker expression; survival analyses were also performed.

RESULTS *PRRX1*, *POSTN*, and *FMO3* mRNAs were used as a panel for specifically detecting MES mRNA in patient samples. MES mRNA was detected only rarely in peripheral blood; moreover, the presence of MES mRNA in peripheral blood stem cell samples was associated with low event-free survival and overall survival. Of note, during treatment, serial bone marrow samples obtained from 29 patients revealed a difference in dynamics between MES mRNA markers and ADRN mRNA markers. Furthermore, MES mRNA was detected in a higher percentage of patients with recurrent disease than in those who remained disease free (53% v 32%, respectively; *P* = .03).

CONCLUSION We propose that the markers *POSTN* and *PRRX1*, in combination with *FMO3*, be used for real-time quantitative polymerase chain reaction–based detection of MES neuroblastoma mRNA in patient samples because these markers have a unique pattern during treatment and are more prevalent in patients with poor outcome. Together with existing markers of MRD, these new markers should be investigated further in large prospective studies.

INTRODUCTION

Despite intensive multimodality therapy, patients with high-risk neuroblastoma often experience a relapse.[1-7] Because the bone marrow (BM) is a common site of recurrence, residual neuroblastoma cells are believed to be the major cause of relapse.[8]

Real-time quantitative polymerase chain reaction (RT-qPCR) provides a highly sensitive means of detecting minimal residual disease (MRD) in peripheral blood (PB), BM, and peripheral blood stem cells (PBSCs).[9,10] Although the detection of MRD markers in high-risk neuroblastoma has been correlated with poor outcome,[11-19] many patients who become negative for these markers still experience relapse.[11]

Epithelial-to-mesenchymal transition (EMT)—the process by which epithelial cells transform to a mesenchymal (MES) phenotype—is associated with tumor progression, metastasis, and therapy resistance in several cancer types.[20-23] For example, in patients with breast cancer, the presence of circulating MES tumor cells is associated with disease progression and poor outcome[22]; moreover, MES neuroblastoma cells are enriched in post-treatment and recurrent tumors and are relatively chemoresistant in vitro.[24-27] Of note, these cells do not express the commonly used neuroblastoma markers *PHOX2B* or *DBH*.[24]

MRD marker selection often is primarily based on expression levels in adrenergic (ADRN) cell lines and primary neuroblastoma tumors.[10,15,28,29] We

CONTEXT

Key Objective

The aim of this study was to identify a panel of markers to detect mesenchymal (MES) neuroblastoma mRNA and to study these markers' dynamics in patients with high-risk neuroblastoma.

Knowledge Generated

We identified a panel of mRNA markers (*PRRX1*, *POSTN*, and *FMO3*) to specifically detect MES mRNA in peripheral blood, bone marrow (BM), and peripheral blood stem cell samples. In contrast to the frequently used adrenergic (ADRN) mRNA markers in real-time quantitative polymerase chain reaction studies, MES mRNA increased during therapy in BM, which supports the idea that MES neuroblastoma cells have a different response to therapy than ADRN neuroblastoma cells. Furthermore, MES mRNA was more frequently detected in BM and peripheral blood stem cells of patients with adverse outcomes.

Relevance

Neuroblastoma cells that undergo epithelial to MES transition are believed to become more chemoresistant and give rise to relapse. These MES neuroblastoma cells can escape detection by real-time quantitative polymerase chain reaction with the currently used (ADRN) markers. By using our MES mRNA panel, we may improve the detection of minimal residual disease in neuroblastoma and identify patients at risk for recurrent disease.

identified neuroblastoma-specific MES mRNA markers for detecting MES neuroblastoma cells and then examined the dynamics of these MES markers in samples obtained from patients with high-risk neuroblastoma.

PATIENTS AND METHODS

Cell Lines and Cell Culture

SH-EP2, SH-SY5Y, IMR-32, 691-MES, and 691-ADRN cells were cultured as previously described.[30,31]

Microarray Analysis

Gene expression analysis to detect MES-specific and mesenchymal stromal cell (MSC)–discriminating candidate markers can be found in the Appendix.

Patients and Samples

We analyzed samples (stored remains) obtained from 38 patients with high-risk neuroblastoma treated in accordance with the German NB2004 or Dutch NBL2009 trial[32,33] (Data Supplement). Written informed consent was provided by the patients' parents or guardians. The study was approved by the medical ethics committees (Academic Medical Center, Amsterdam, the Netherlands; MEC07/219#08.17.0836) and the University of Cologne. Clinical samples were collected in EDTA tubes, processed within 24 hours, transferred to PAXgene Blood RNA Tubes (QIAGEN, Venlo, the Netherlands), and stored at −20 °C. DNA was isolated from mononuclear cells stored in 10% dimethylsulfoxide at −180 °C, and hypermethylated *RASSF1A* RT-qPCR was performed as described previously.[34]

Control Samples and Isolation of Nonpathologic Cell Subsets

To assess expression patterns of candidate markers in control tissue, pediatric BM samples of patients with leukemia in molecular remission (at least 3 months after therapy; n = 48), PB samples from healthy volunteers (n = 104), and PBSC samples from children treated for a disease other than neuroblastoma (n = 29) were tested as described previously.[28] Different PB populations were isolated as described previously.[10] Cultured MSCs[35] were provided by Carlijn Voermans, PhD, at the Department of Hematopoiesis, Sanquin Blood Supply Foundation (Amsterdam, the Netherlands). All samples were obtained with informed consent.

RNA Extraction and RT-qPCR

RNA was isolated from clinical and control samples using the PAXgene Blood RNA Kit (QIAGEN). RNA was isolated from cell lines using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Methods for cDNA synthesis and RT-qPCR can be found in the Appendix. Expression was normalized to *GUSB* expression using the following equation: normalized threshold cycle (dCt) = (Ct_{GUSB} − Ct_{marker}).

Data Analysis

For the newly identified MES markers, a threshold for positivity in control BM, PB, and PBSC samples was determined on the basis of their expression in each sample (see Results). In the PBSC cohort, survival was analyzed using the Kaplan-Meier method, and significant differences between the estimated survival curves were analyzed using the log-rank test. Fisher's exact test was used to analyze the differences in MES marker positivity between the relapse and survivor groups. All statistical analyses were performed using SPSS version 23 software (IBM Corporation, Chicago, IL).

RESULTS

Expression of Commonly Used MRD Markers in a Panel of Neuroblastoma Cell Lines

The expression of commonly used MRD markers (*PHOX2B*, *TH*, *DDC*, *DBH*, *GAP43*, *CHRNA3*, and

GD2S)[10,36,37] was measured in two isogenic neuroblastoma cell line pairs (691-MES/691-ADRN and SH-EP2/SH-SY5Y) and in one ADRN cell line (IMR-32). Compared with the corresponding ADRN cell lines, the MES cell lines had significantly lower, albeit variable, expression of six markers; in contrast, *GD2S* was expressed at high levels in both the MES and the ADRN cell lines (Fig 1).

## Identification of Candidate MES Markers for MRD Testing

To detect MES cells in the context of MRD, we compared the gene expression data between pairs of isogenic cell lines and then selected the genes that were significantly upregulated in the MES cell lines compared with the corresponding ADRN cell line and expressed at high levels in MES cells (defined as > 400 units). Subsequently, we filtered for genes with a minimal expression level in PB data sets, which resulted in an initial list of 14 candidate genes (Fig 2A). All 14 of these genes were included in the MES gene signature reported recently by van Groningen et al[24] (Fig 2B). Although *PRRX1* was not included in this initial list because its expression in 700-MES cells was less than 400, we included this gene in our additional analyses because PRRX1 was reported as an immunohistochemistry marker for MES neuroblastoma.[24]

Validation of Candidate Markers

Using RT-qPCR with SYBR Green I dye (Applied Biosystems, Foster City, CA), we examined the expression of all 15 candidate markers in MES cells (691-MES and SH-EP2), ADRN cells (691-ADRN, SH-SY5Y, and IMR-32), and a control PB sample (Data Supplement). As a result of culture problems, 700-ADRN/700-MES was not used for RT-qPCR testing. Our analysis revealed three genes with high expression in MES cells, a significant difference in expression between the MES and ADRN cell lines, and either low or no measurable expression in the control PB sample; these three genes were *POSTN*, *PRRX1*, and *COL3A1*, which encode the proteins periostin, paired related homeobox 1, and collagen type III α1, respectively.

RT-qPCR using TaqMan probes validated expression of these markers, which demonstrated high *POSTN*, *PRRX1*, and *COL3A1* expression in 691-MES and SH-EP2 and low expression in 691-ADRN, SH-SY5Y, and IMR-32 (Fig 3A). *COL3A1* was then excluded because it did not adequately discriminate between ADRN and MES cell lines and was detected in the control PB samples.

Next, to set thresholds for defining positivity, we measured the expression of *POSTN* and *PRRX1* in normal hematologic cells: control BM samples (n = 48), PB samples (n = 104), and PBSCs (n = 29). We measured extremely low expression (or no expression) of both genes in control PBSCs. However, because both *POSTN* and *PRRX1* were expressed at variable levels in the control BM and PB samples (Fig 3B), we examined their expression levels in several subsets of hematologic cells and in MSCs (Fig 3C). We found that both *POSTN* and *PRRX1* were
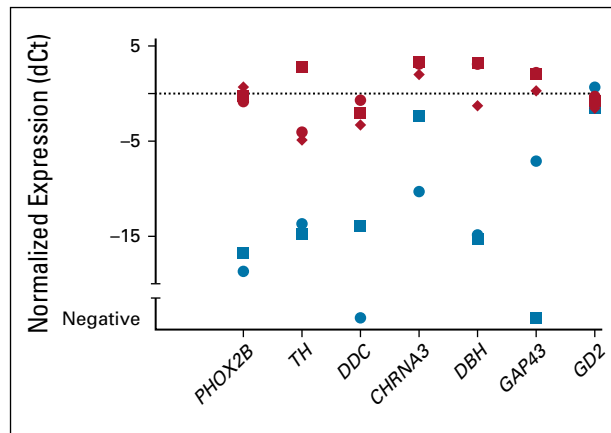
FIG 1. Summary of normalized expression levels of commonly used minimal residual disease markers in a panel of neuroblastoma cell lines. Normalized expression ($dCt = Ct_{GUSB} - Ct_{marker}$) is shown. Mesenchymal (MES) cell lines are indicated in blue. Adrenergic (ADRN) cell lines are indicated in red. Blue circles, SH-EP2; blue squares, 691-MES; red squares, 691-ADRN; red circles, SH-SY5Y; red diamonds, IMR-32.

robustly expressed in MSCs and, to a lesser extent, in T cells; in contrast, *POSTN* was expressed at extremely low levels in B cells. Taken together, these results suggest that the presence of both *POSTN* and *PRRX1* in control BM and PB samples is likely the result of their expression in MSCs.

## Identification of an MSC-Discriminating Marker

Next, we searched for a marker that can be used to discriminate between MSCs and MES mRNA. Microarray analysis identified 14 candidate genes that were highly expressed in MSCs but poorly expressed both in MES cell lines and in hematologic cells (not containing MSCs; Fig 4A; Data Supplement). The specificity of these markers was then tested using MSCs obtained from healthy individuals (n = 2), neuroblastoma cell lines (n = 5), and control PB samples (n = 2) using SYBR Green I dye (Data Supplement). Our analysis revealed that the *FMO3* gene (encoding flavin-containing monooxygenase 3) was the most promising marker for discriminating between MSCs and MES cells. Subsequent testing using an *FMO3*-specific TaqMan probe confirmed high expression in MSCs (comparable to the expression levels of *PRRX1* and *POSTN*), low expression (or no expression) in all neuroblastoma cell lines (Fig 4B), and no expression in hematologic subsets that lacked MSCs.

Threshold for Positivity in BM, PB, and PBSCs

Because MSCs, and to a lesser extent, B cells and T cells, express low levels of *POSTN* and *PRRX1*, we established a threshold for positivity for MES mRNA detection in control BM, PB, and PBSC samples. Forty-eight control BM samples were analyzed for *PRRX1*, *POSTN*, and *FMO3* expression (Data Supplement). In 77% of the control BM samples (37 of 48), *FMO3* expression was similar to or
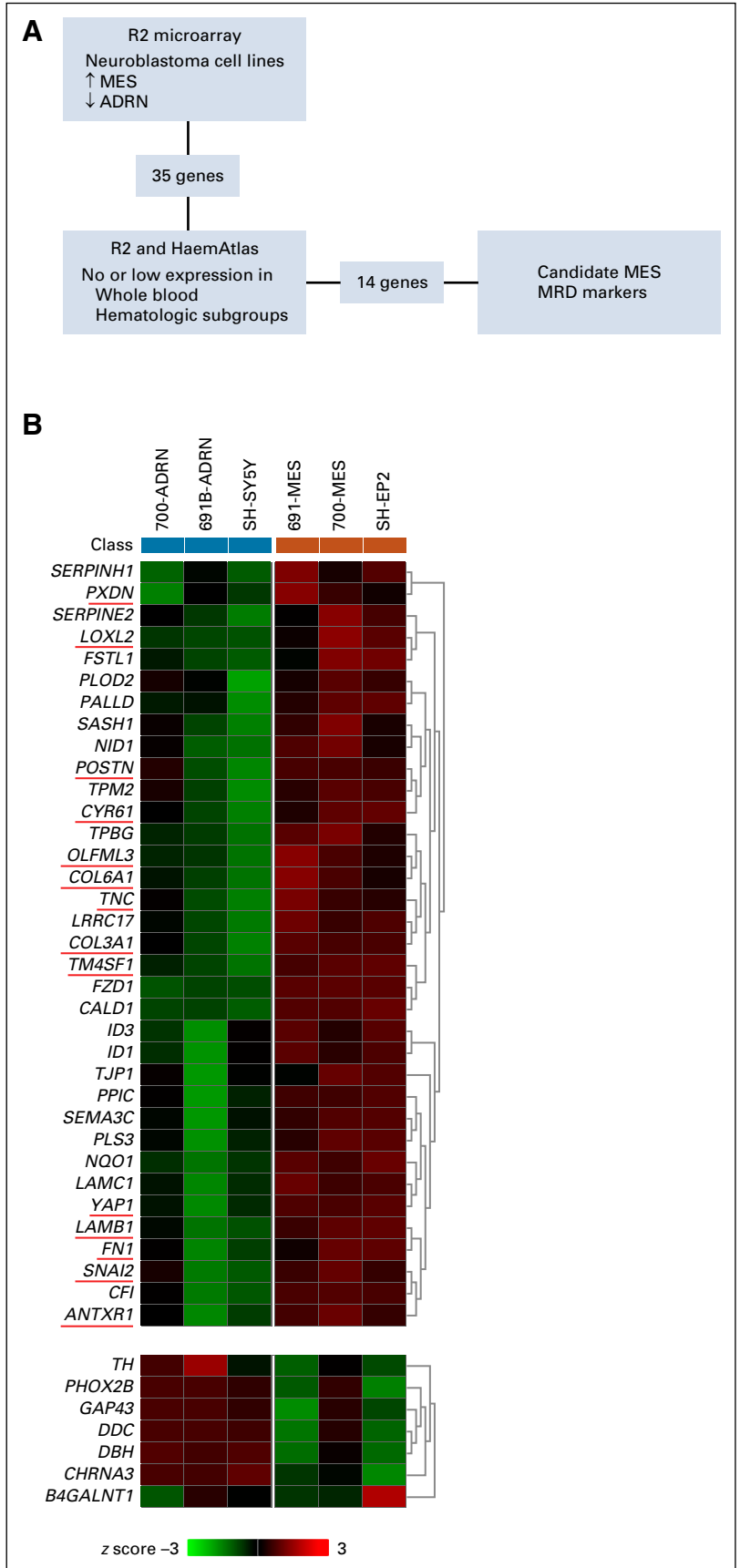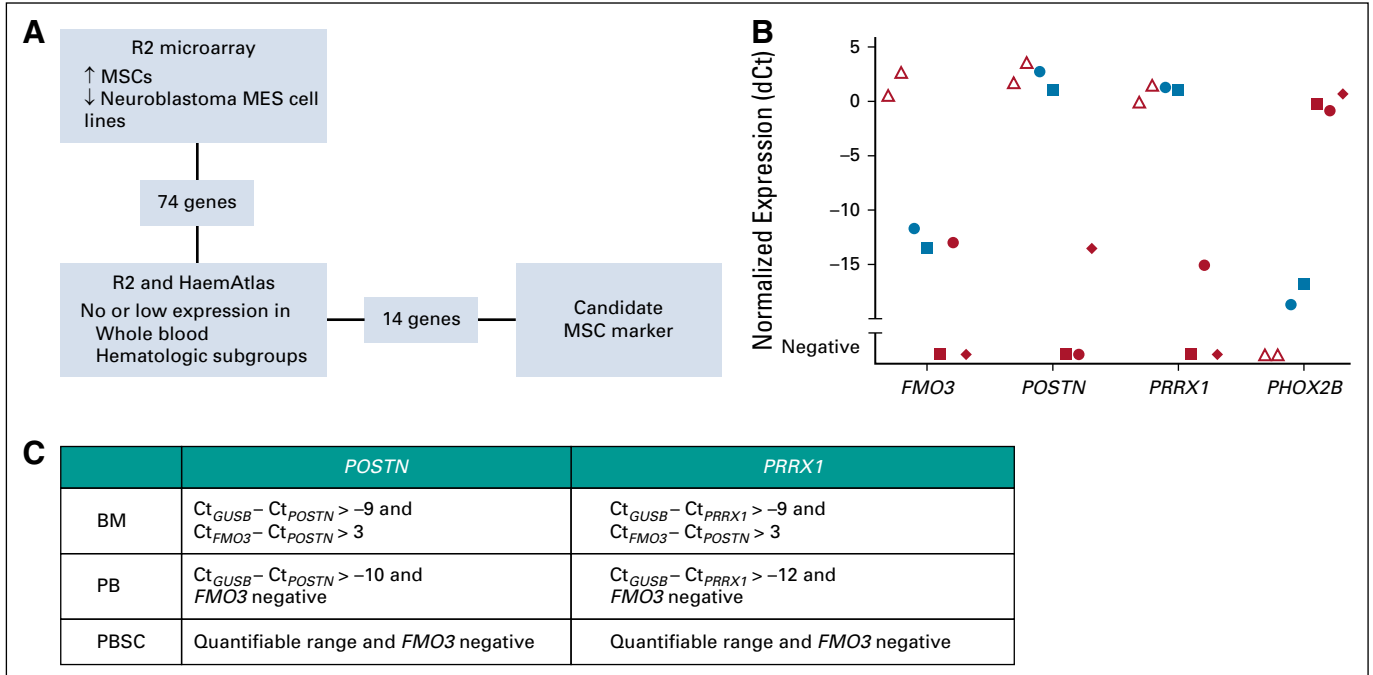
FIG 2. Identification of candidate mesenchymal (MES) markers of minimal residual disease (MRD). (A) Flowchart that shows the strategy used to identify putative MES markers. (B) Heat map that shows the expression profiles of the indicated 35 genes with high expression (red) in MES cell lines (right) and low expression (green) in adrenergic (ADRN) cell lines (left); shown underneath are genes that are commonly used for MRD testing in neuroblastoma. Subsequent filtering for minimal gene expression in normal peripheral blood data sets resulted in 14 candidate MES markers for MRD (underlined in red).
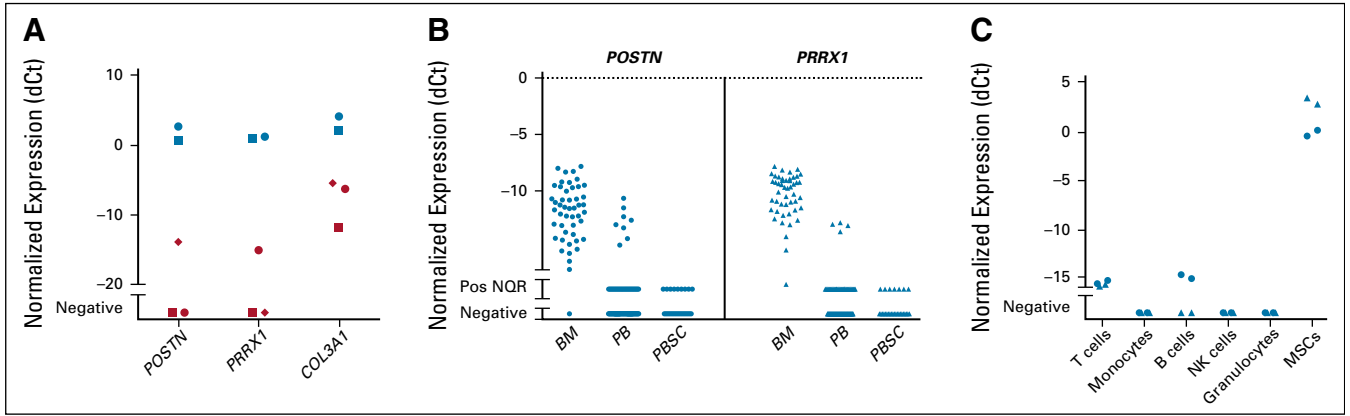
FIG 3. Real-time quantitative polymerase chain reaction analysis of three putative mesenchymal (MES) markers in cell lines and control samples. (A) The indicated genes were measured in MES cell lines (shown in blue) and adrenergic (ADRN) cell lines (shown in red). Blue squares, 691-MES; blue circles, SH-EP2; red circles, SH-SY5Y; red squares, 691-ADRN; red diamonds, IMR-32. Normalized expression (dCt = Ct_{GUSB} − Ct_{marker}) is shown. (B) Normalized expression (dCt = Ct_{GUSB} − Ct_{marker}) of *POSTN* and *PRRX1* measured in control bone marrow (BM), peripheral blood (PB), and peripheral blood stem cell (PBSC) samples. Pos NQR indicates samples that were positive but not within the quantifiable range. (C) Normalized expression of *POSTN* (circles) and *PRRX1* (triangles) measured in the indicated hematologic cell types in control blood. MSC, mesenchymal stromal cell; NK, natural killer.

higher than *POSTN* and *PRRX1*; in contrast, *POSTN* and *PRRX1* expression levels were higher than *FMO3* in the remaining 11 control samples (> 1 dCt between *FMO3* and *POSTN/PRRX1*). On the basis of these results, the positivity threshold for *POSTN* and *PRRX1* was set to a dCt value greater than −9 between *POSTN/PRRX1* and *GUSB* and a dCt value greater than 3 between *POSTN/PRRX1* and

FMO3 (Fig 4C). The threshold for positivity for PB was determined using 104 control PB samples (Data Supplement) and was set to a dCt value greater than −12 for *PRRX1* and a dCt value greater than 10 for *POSTN*, with no measurable expression of *FMO3*. Because the background expression of both *POSTN* and *PRRX1* was extremely low in control PBSC samples, each PBSC sample was scored as

	POSTN	PRRX1
BM	Ct_{GUSB} − Ct_{POSTN} > −9 and Ct_{FMO3} − Ct_{POSTN} > 3	Ct_{GUSB} − Ct_{PRRX1} > −9 and Ct_{FMO3} − Ct_{POSTN} > 3
PB	Ct_{GUSB} − Ct_{POSTN} > −10 and *FMO3* negative	Ct_{GUSB} − Ct_{PRRX1} > −12 and *FMO3* negative
PBSC	Quantifiable range and *FMO3* negative	Quantifiable range and *FMO3* negative

FIG 4. Identification of mesenchymal stromal cell (MSC)–specific markers. (A) Flowchart that shows the strategy used to identify MSC markers. (B) Normalized expression (dCt = Ct_{GUSB} — Ct_{marker}) of FMO3, POSTN, PRRX1, and PHOX2B in MSCs and neuroblastoma cell lines. Mesenchymal (MES) and adrenergic (ADRN) neuroblastoma cell lines are shown in blue and red, respectively. Blue squares, 691-MES; blue circles, SH-EP2; red circles, SH-SY5Y; red squares, 691-ADRN; red diamonds, IMR-32; red triangles, MSC. (C) Thresholds for positivity in bone marrow (BM), peripheral blood (PB), and peripheral blood stem cell (PBSC) samples.

positive for MES mRNA if the Ct value was within the quantitative range,[38] with no measurable expression of *FMO3* (Fig 4C). We therefore tested whether these three molecular markers combined with these established thresholds can be used to detect neuroblastoma-derived MES mRNA in patient samples.

Testing of the MES MRD Panel in Serial PB and PBSC Samples From Patients With High-Risk Neuroblastoma

In 67 serial PB and 15 PBSC samples obtained from 12 patients with high-risk neuroblastoma, we measured *PHOX2B* and MES mRNA (Data Supplement). *PHOX2B* mRNA was detected in all diagnostic samples; this expression decreased to undetectable levels in most patients during induction chemotherapy (IC) and was detected again at relapse. In contrast, MES mRNA was detected in only six samples; five of these samples were PBSCs, and of note, these PBSC samples were negative for *PHOX2B* mRNA.

Next, we examined PBSC samples obtained from 53 patients with high-risk neuroblastoma who were previously studied using ADRN mRNA[39] (Data Supplement). MES mRNA and ADRN mRNA were detected in 15 (28%) and six (11%) of 53 samples, respectively, with little overlap (both MES and ADRN mRNA were detected in only one sample). We previously reported that ADRN mRNA positivity is not correlated with outcome.[39] However, here we found that the presence of *POSTN* and/or *PRRX1* mRNA in PBSC samples was significantly associated with low event-free survival (*P* = .045) and low overall survival (*P* = .047; Fig 5; Data Supplement). Moreover, consistent with our previous findings, we found no correlation between ADRN mRNA and outcome (Data Supplement).

Testing of the MES MRD Panel in BM From Patients With High-Risk Neuroblastoma

We then examined the feasibility of using these RT-qPCR–based markers to study response kinetics in BM samples obtained from patients with high-risk neuroblastoma who experienced recurrent disease (n = 16) and from those with high-risk neuroblastoma who remained in complete remission (n = 13). Specifically, we compared *PHOX2B* mRNA with MES mRNA measured in 95 serial BM samples collected at diagnosis, during therapy, during follow-up, and (where applicable) during relapse (Fig 6; Data Supplement). *PHOX2B* mRNA was detected in all 27 diagnostic BM samples; this expression decreased during IC treatment and was undetectable by the end of IC in most patients (nine of 10 patients with complete remission and 10 of 15 patients who experienced relapse). In the patients with recurrent disease, *PHOX2B* mRNA was detected again in 75% (nine of 12 patients) with systemic relapse (three of the 15 patients with recurrent disease had a local relapse). In contrast, MES mRNA was detected in only 14 of 27 patients at diagnosis, and this number increased to 18 during IC treatment. In analyzing the entire patient group, we found that the prevalence of MES mRNA was significantly higher in the patients who experienced relapse (29 of 54 samples; 53%) compared with the patients with complete remission (13 of 41 samples; 32%; *P* = .03). Finally, we identified two BM samples and one PBSC sample that were negative for all ADRN mRNA markers but were positive for MES mRNA. Moreover, we detected hypermethylated *RASSF1A* DNA, a marker for neuroblastoma,[34] in these samples (Fig 6D). We conclude that the kinetics differ between MES mRNA and ADRN mRNA, and MES positivity is significantly higher in the BM of patients with
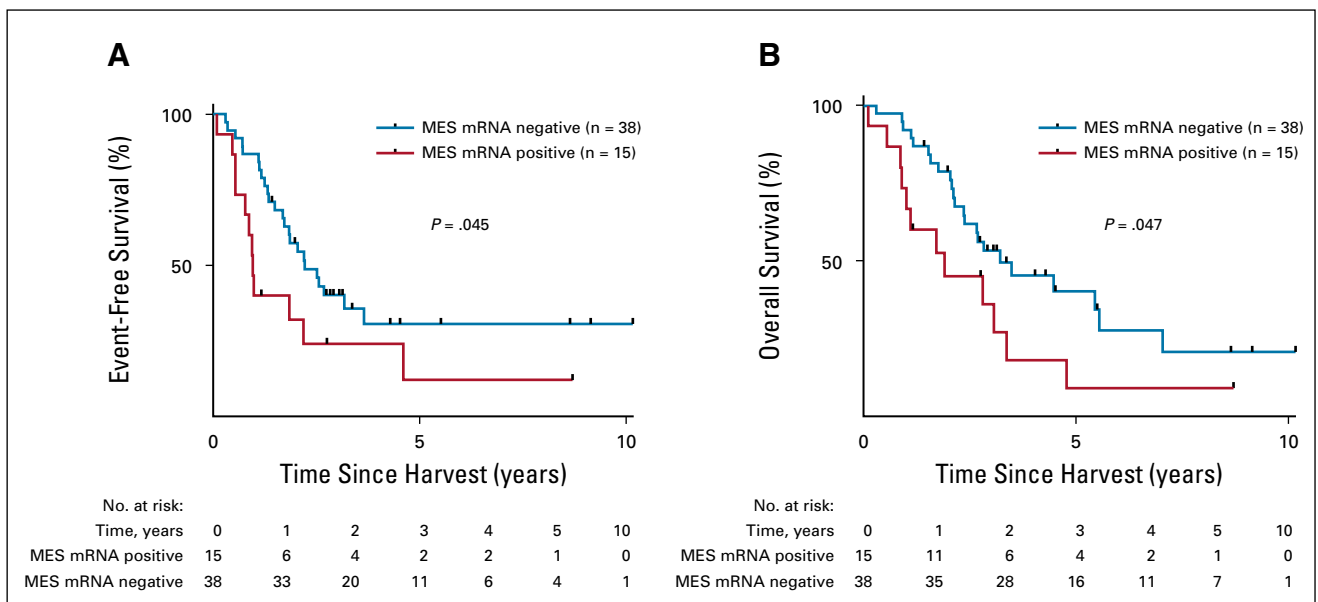
FIG 5. Detection of mesenchymal (MES) markers of minimal residual disease in peripheral blood stem cells. Kaplan-Meier plots of (A) event-free survival and (B) overall survival in patients with peripheral blood stem cell samples that tested negative (blue line) or positive (red line) for MES mRNA.
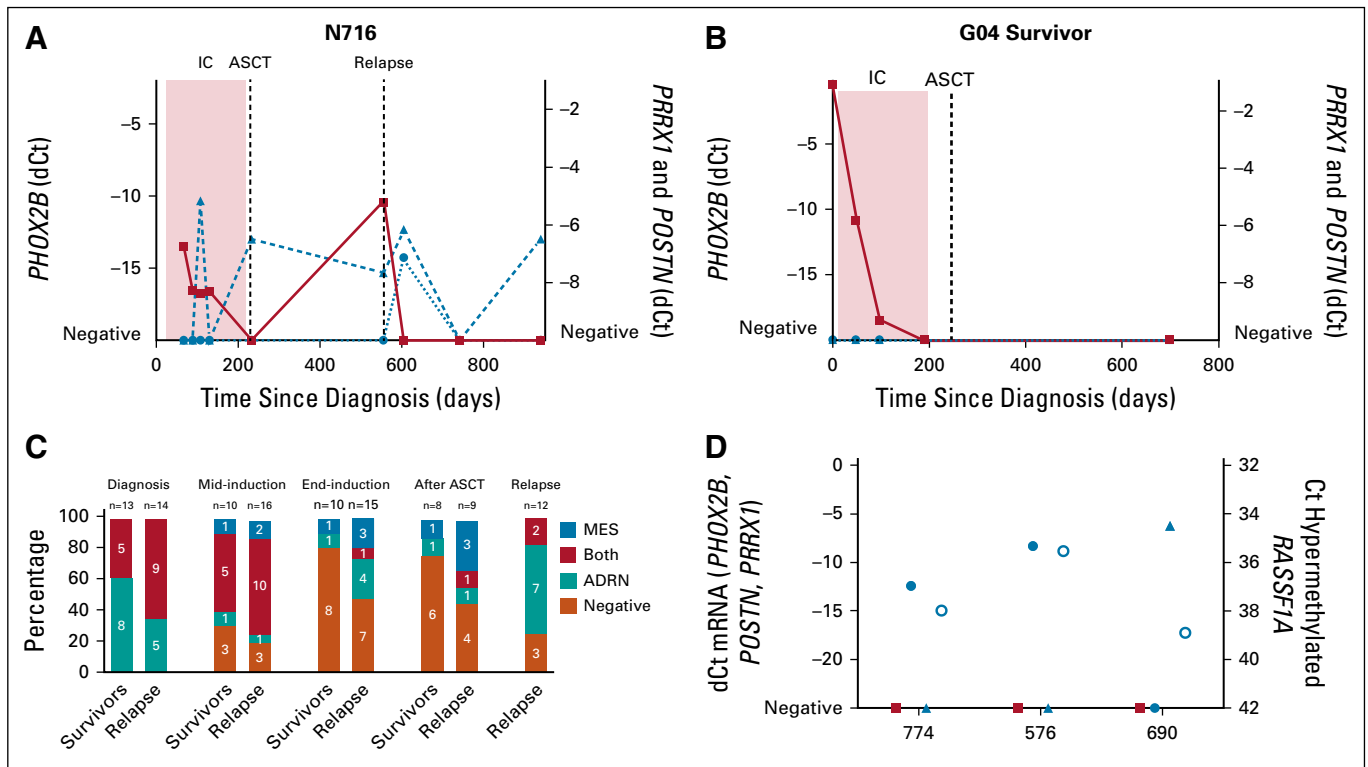
FIG 6. Detection of mesenchymal (MES) minimal residual disease markers in serial bone marrow (BM) samples. (A and B) Time course for patient N716 (a patient with recurrent disease) and patient G04 (a patient in complete remission). Red symbols indicate normalized *PHOX2B* expression measured in BM samples, and blue symbols indicate normalized expression of *POSTN* (circles) and *PRRX1* (triangles). The shaded area indicates treatment with induction chemotherapy (IC). Myeloablative therapy with autologous stem-cell transplantation (ASCT) also is shown. (C) Summary of the results obtained from all serial samples obtained from patients who ultimately experienced relapsed and patients who remained in complete remission (survivors). (D) A peripheral blood stem cell sample obtained from patient 774 and two BM samples obtained from patients 576 and 690 were measured for *PHOX2B* (squares), *POSTN* (filled circles), *PRRX1* (triangles), and hypermethylated *RASSF1A* (open circles). ADRN, adrenergic; Ct, cycle threshold; dCt, normalized cycle threshold.

recurrent disease. Finally, using the tumor-specific DNA marker hypermethylated *RASSF1A*,[34] we found that BM samples that are both ADRN negative and MES positive contain neuroblastoma cells.

DISCUSSION

RT-qPCR–based testing of BM of patients with neuroblastoma is a robust, highly sensitive, and clinically important method for detecting residual disease.[19] However, even high-risk patients who have low or undetectable post-therapy mRNA levels can experience a relapse (48% to 60%).[11,18]

Cellular heterogeneity is a key feature of many cancer types and is caused in part by EMT. We found that the commonly used neuroblastoma MRD markers are expressed predominantly in ADRN cell lines but are rarely expressed in MES cell lines. Thus, neuroblastoma cells that undergo EMT may not be detected using the current MRD marker panel. Ideally, an effective MRD marker should detect the full spectrum of neuroblastoma cell types, including ADRN and MES cells. We found that *GD2S* is expressed at high levels in both MES and ADRN cell lines; however, its

specificity is limited because of its relatively high expression in normal hematologic cells.[10]

To study the expression of MES-specific markers at the time of diagnosis as well as the dynamic expression pattern during follow-up, we identified a panel of markers that includes *POSTN* and *PRRX1*, which have been linked to EMT in several cancer types.[24,40-44] An MRD marker ideally should be expressed at extremely low levels in normal hematologic cells. In our search for MES MRD markers, we performed gene expression analysis. However, a possible limitation of this approach is that dependent on the platform used, the actual gene expression levels can be underestimated, for example, as in the case of *PRRX1* expression in one cell line used in our study. Moreover, genes that have not been reported to be expressed in PB data sets may indeed be expressed on the basis of RT-qPCR analysis. For example, RT-qPCR analysis revealed that both *POSTN* and *PRRX1* are expressed at relatively weak levels in control BM samples, whereas these genes are expressed at barely detectable levels in control PB samples and control PBSCs. A subset analysis revealed that the expression was primarily the result of expression in MSCs; therefore, we added the

discriminative marker *FMO3* to our panel, as this gene is expressed by MSCs but not by neuroblastoma cells. Of note, although we ascribed the expression of *POSTN* and *PRRX1* in control BM samples to MSCs, we cannot rule out the possibility that other cell types, such as osteoblasts, may also contribute to this expression. Nevertheless, because we established strict thresholds for defining positivity, we believe that we avoided detection of normal stromal cells as well as other cell types.

Because MES mRNA was rarely detected in PB samples obtained from patients with high-risk neuroblastoma, its clinical relevance remains unclear, and these results need to be confirmed using a larger cohort. In contrast, MES mRNA was detected in 28% of the PBSC samples obtained from 53 patients and was significantly associated with low event-free survival and overall survival. Of note, we previously reported a relatively low prevalence (9%) of ADRN mRNA in PBSCs obtained from this cohort, and the presence of ADRN mRNA was not associated with either low event-free survival or low overall survival.[39] Therefore, we speculate that the MES cells that reside in the BM circulate during stem-cell mobilization.

By focusing on serial BM samples in high-risk patients, we found that MES mRNA and ADRN mRNA have distinct temporal dynamics. Specifically, ADRN mRNA levels were high at diagnosis and during relapse but decreased during treatment, whereas MES mRNA levels increased during treatment and were associated with patients who ultimately had a relapse. This finding suggests that MES cells may respond differently to therapy compared with ADRN neuroblastoma cells and may play an important role in disease progression and/or recurrence; this notion is consistent with reports that demonstrated the importance of EMT in disease progression and treatment resistance.[24,45] Moreover, our finding that MES mRNA was detected in only 14 of 27 patients at diagnosis (and only rarely at relapse) is consistent with the hypothesis that metastatic cells can undergo an MES-to-epithelial conversion and thus revert to an ADRN phenotype.[46] The relatively small size of our patient cohorts precluded extensive multivariable analyses of survival; however, these exploratory findings suggest that detection of MES markers in the BM and PBSCs during treatment may have prognostic value.

DNA markers for MRD (and recently, circulating cell-free DNA markers) have been shown to provide added value when combined with RNA-based methods for monitoring MRD and for measuring tumor-derived genetic aberrations.[34,47-49] On the other hand, we previously reported discrepancies between RNA-based and DNA-based markers using either methylated *RASSF1A* or patient-specific DNA markers.[34,47] We hypothesize that these discrepancies reflect MES neuroblastoma cells that express reduced levels of ADRN markers but can still be detected using DNA markers.

In conclusion, we report that *POSTN*, *PRRX1*, and *FMO3* mRNA can be used to detect MES neuroblastoma cells in BM and PBSCs in patients with high-risk neuroblastoma. Of note, we also found that MES-based markers have a different expression pattern during treatment than ADRN-based markers. Moreover, although the MES markers are more prevalent in the BM of patients who will ultimately experience relapse, they are rarely present at the actual time of relapse. To study the clinical implications and significance of these finding, this new panel of MES markers should be tested together with currently used MRD markers in a large prospective study.

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EQUAL CONTRIBUTION
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APPENDIX

Microarray Analysis

Mesenchymal (MES)-specific candidate markers were identified by comparing gene expression profiles (Human Genome U133 Plus 2.0 Array; Affymetrix, Santa Clara, CA) among the following isogenic pairs of adrenergic (ADRN) and MES cell lines: 691-MES/691B-ADRN, SH-EP2 (MES)/SH-SY5Y (ADRN), 700-MES/700-ADRN.[24] Specifically, we screened for genes with a 1 log-fold or more difference in expression (within each isogenic pair) and an expression level greater than 400 units, thereby selecting only highly expressed marker genes. To identify discriminating markers for normal mesenchymal stromal cells (MSCs), the top 300 genes with the highest expression were used (Gene Expression Omnibus: GSE68374); the genes with the highest fold difference between MSCs and MES neuroblastoma cell lines were then selected. For each analysis, each gene's expression was compared with its corresponding expression level in reference blood samples obtained from five different data sets (GSE13159, GSE17186, GSE10715, GSE8121, and GSE6575); only genes that are not expressed in hematologic cell types (using HaemAtlas; Watkins et al: Blood 113:e1-e9, 2009) were selected. All gene expression analyses were performed in the genomics analysis and visualization platform R2 (http://r2.amc.nl).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

cDNA was synthesized using 2 to 3 μg RNA, random hexamers (25 μM; Invitrogen, Carlsbad, CA), deoxynucleotide triphosphates (1 mM; Promega, Madison, WI), and Moloney murine leukemia virus reverse transcriptase (100 U; Invitrogen) in a total reaction volume of 40 to 60 μL. The reverse transcription was then heat inactivated, and the reaction volume was increased to 100 to 150 μL.

Primers and probes were designed using Primer Express version 1.5 software (Applied Biosystems, Foster City, CA) or Oligo 6 (Molecular Biology Insights, Colorado Springs, CO) and synthesized by Eurogentec (Liège, Belgium; Data Supplement). The primer/probe combinations for glucuronidase-β (GUSB), β-1,4-N-acetyl-galactosaminyltransferase 1 (B4GALNT1, also known as GD2S), paired-like homeobox 2B (PHOX2B), tyrosine hydroxylase (TH), dopa decarboxylase (DDC), growth-associated protein 43 (GAP43), cholinergic receptor nicotinic α3 (CHRNA3), and dopamine β-hydroxylase (DBH) have been published previously[10,36,37] (Beillard et al: Leukemia 17:2474-2486, 2003). Real-time quantitative polymerase chain reaction (RT-qPCR; maximum, 50 cycles) was performed using StepOnePlus (Applied Biosystems). The initial screening for candidate molecular markers was performed using SYBR Green I dye (Applied Biosystems) combined with a melting curve analysis followed by specific TaqMan probes (Eurogentec). Expression was normalized to GUSB expression using the following equation: normalized threshold cycle (dCt) = $(Ct_{GUSB} - Ct_{marker})$. All RT-qPCR reactions were performed in triplicate (except GUSB, which was performed in duplicate), and mean values were used for analysis. A given sample was scored as follows: positive if all three replicates were positive; positive not quantifiable if amplification was observed in only one or two replicates; and negative if the Ct value was 40 or greater (with the exception of PHOX2B [Ct ≥ 50]).[10,28] Samples with an insufficient Ct_{GUSB} value (Ct > 25, corresponding to < 500 copies) were excluded[28,38] (Beillard et al: Leukemia 17:2474-2486, 2003). The sensitivity and quantitative range of each RT-qPCR assay were assessed using cDNA prepared from 691-MES cells and serially diluted in water.