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Detecting Minimal Residual Disease in Neuroblastoma: The Superiority of a Panel of Real-Time Quantitative PCR Markers

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BACKGROUND: PCR-based detection of minimal residual disease (MRD) in neuroblastoma (NB) patients can be used for initial staging and monitoring therapy response in bone marrow (BM) and peripheral blood (PB). *PHOX2B* has been identified as a sensitive and specific MRD marker; however, its expression varies between tumors. Therefore, a panel of markers could increase sensitivity.

METHODS: To identify additional MRD markers for NB, we selected genes by comparing SAGE (serial analysis of gene expression) libraries of healthy and NB tissues followed by extensive real-time quantitative PCR (RQ-PCR) testing in samples of tumors (n = 56), control BM (n = 51), PB (n = 37), and cell subsets. The additional value of a panel was determined in 222 NB samples from 82 Dutch stage 4 NB patients (54 diagnosis BM samples, 143 BM samples during/after treatment, and 25 PB samples).

RESULTS: We identified 2 panels of specific RQ-PCR markers for MRD detection in NB patients: 1 for analysis of BM samples (*PHOX2B*, *TH*, *DDC*, *CHRNA3*, and *GAP43*) and 1 for analysis of PB samples (*PHOX2B*, *TH*, *DDC*, *DBH*, and *CHRNA3*). These markers all showed high expression in NB tumors and no or low expression in control BM or PB samples. In patients' samples, the *PHOX2B* marker detected most positive samples. In PB samples, however, 3 of 7 *PHOX2B*-negative samples were positive for 1 or more markers, and in BM examinations during treatment, 7% (6 of 86) of the *PHOX2B*-negative samples were positive for another marker.

CONCLUSIONS: Because of differences in the sensitivities of the markers in BM and PB, we advise the use of 2 different panels to detect MRD in these compartments.

Neuroblastoma (NB)[7] is the most common extracranial solid neoplasm in children. Approximately 40% of NB patients have high-risk disease with dissemination in bone marrow (BM), bone, distant lymph nodes, liver, and other organs. In patients older than 1 year, the presence of marrow disease is a strong indicator of high-risk NB, and this form of NB has a poor prognosis *(1, 2)*. Therefore, detection of BM metastasis is crucial for correction of clinical staging and risk assessment at diagnosis. Furthermore, detection of residual NB cells in BM during therapy can be used to monitor the response to therapy *(3, 4)* and to evaluate stem cell harvests *(5, 6)*.

Cytology testing, the classic method for evaluating BM infiltration, has a sensitivity of 0.1% *(7, 8)*. Real-time quantitative PCR (RQ-PCR) *(9)* and immunocytology *(10)* are much more sensitive for monitoring minimal residual disease (MRD). The protocol for antidisialoganglioside (anti-GD2) immunocytology has been standardized internationally *(11)*, whereas RQ-PCR markers are still being validated.

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[7] Nonstandard abbreviations: NB, neuroblastoma; BM, bone marrow; RQ-PCR, real-time quantitative PCR; MRD, minimal residual disease; PB, peripheral blood; SAGE, serial analysis of gene expression; INSS, International Neuroblastoma Staging System; ALL, acute lymphoblastic leukemia; B-ALL, B-cell ALL; T-ALL, T-cell ALL; Ct, threshold cycle.

Recently, we identified *PHOX2B*[8] (paired-like homeobox 2b) as a specific NB marker, and *PHOX2B* is presently the best single marker for PCR-based MRD detection in NB cases *(12)*. *PHOX2B* is not highly expressed in all NB tumors, however, and its expression varies greatly between tumors. Furthermore, it is not known whether *PHOX2B* is stably expressed during treatment. We therefore assumed that the sensitivity of MRD detection could be increased by adding other MRD markers. Markers commonly used for MRD detection in NB, such as *TH* (tyrosine hydroxylase) and *B4GALNT1* (beta-1,4-N-acetyl-galactosaminyl transferase 1; also known as GD2 synthase), are hampered by their expression in healthy BM, peripheral blood (PB), and/or PB stem cells *(9, 13, 14)*.

We therefore addressed the following questions: Can we identify other, potentially better RQ-PCR markers for MRD detection in NB patients, and is the use of a panel of markers indeed more sensitive in detecting MRD than the use of 1 specific marker, *PHOX2B*?

We first selected candidate marker genes by serial analysis of gene expression (SAGE). We then refined our selection by RQ-PCR testing in NB tumors and established thresholds for positivity by RQ-PCR testing of control tissues, such as BM, PB, and their cell subsets. Finally, we determined the additional value of a panel of markers over that of a single marker.

Materials and Methods

PATIENTS AND SAMPLES

Samples from 82 Dutch patients with stage 4 NB [staged according to the International Neuroblastoma Staging System (INSS)] (54 BM samples at diagnosis, 143 BM samples during treatment, and 25 PB samples) were collected between 1986 and 2007 at Emma Children's Hospital/Academic Medical Center, Amsterdam, or Sophia Children's Hospital/Erasmus Medical Center, Rotterdam. Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue7 summarizes the patients' characteristics; data of individual patients and the origins of the samples are presented in Table 2 in the online Data Supplement. BM and PB sampling was done according to the treatment protocol (at diagnosis, during treatment, and after treatment). Furthermore, 72 NB tumor samples were derived mainly from stage 4 patients (see Table 3 in the online Data Supplement).

To compare RQ-PCR and immunocytology, we tested cDNA prepared from 32 BM samples obtained from 32 German patients with stage 4 disease (see Tables 1 and 4 in the online Data Supplement). Informed consent was given to use stored remains of samples for research purposes. The study was approved by the Medical Research Ethics Committee.

CONTROL SAMPLES AND ISOLATION OF NONPATHOLOGIC CELL SUBSETS

As in our previous study *(12)*, pediatric BM samples (n = 51) of children in molecular remission of acute lymphoblastic leukemia (ALL) *(15)* and PB samples (n = 37) from children and healthy volunteers were used as control tissues for the analysis of expression patterns of candidate MRD markers.

The different cell populations present in BM and PB were selected by magnetic cell sorting with CD3 (n = 4), CD14 (n = 4), CD19 (n = 4), CD56 (n = 4), and CD34 (n = 12) magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Granulocytes and platelets were purified from EDTA-treated blood (n = 4) as previously described *(16)*. For PB cells, we used apheresis buffy coats from healthy volunteers. $CD34^+$ cells were isolated from PB stem cells from granulocyte colony-stimulating factor–mobilized and chemotherapy-mobilized PB from patients treated for a disease other than NB. Immature B cells and T cells were obtained from mononuclear BM cells (>90% blasts) from patients with precursor B-cell ALL (B-ALL) (n = 5) and T-cell ALL (T-ALL) (n = 4). Cultured megakaryocytes (n = 3) *(17)*, cultured immature myeloid cells (n = 2) *(18)*, mesenchymal stromal cells (n = 4) *(19)*, human BM-derived endothelial cells (n = 3) *(20)*, adipocytes (n = 2) *(21)*, smooth muscle cells (n = 2) *(22)*, and fibroblasts (n = 2) *(23)* were kindly provided by several departments at the Sanquin Blood Supply Foundation (Amsterdam, the Netherlands) and the Academic Medical Center (Amsterdam, the Netherlands). All samples were obtained with informed consent.

SAMPLE PREPARATION

Samples were always processed within 24 h after collection into EDTA-containing tubes, and the samples were used directly; alternatively, isolated cells were cryopreserved in 10% DMSO and stored at −180 °C.

[8] Human genes: *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase; *B4GALNT1*, beta-1,4-N-acetyl-galactosaminyl transferase 1 (also known as GD2 synthase); *GUSB*, glucuronidase, beta (also known as GUS); *DBH*, dopamine beta-hydroxylase (dopamine beta-monooxygenase); *DDC*, dopamine decarboxylase (aromatic L-amino acid decarboxylase); *GAGE* family, G antigen family; *UCHL1*, ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase), also known as PGP9.5; *NEFM*, neurofilament, medium polypeptide; *POSTN*, periostin, osteoblast specific factor; *CHRNA3*, cholinergic receptor, nicotinic, alpha 3; *STMN2*, stathmin-like 2; *STMN4*, stathmin-like 4; *CHGB*, chromogranin B (secretogranin 1); *SNAP91*, synaptosomal-associated protein, 91kDa homolog (mouse); *GAP43*, growth associated protein 43.

SAGE ANALYSIS

Candidate MRD markers were selected by comparing SAGE mRNA values for healthy tissues with SAGE mRNA values for NB tissues. Expression databases of 4 NB tumors (stages 4 and 4s; see Table 3 in the online Data Supplement) and 11 cell lines obtained by SAGE technology were analyzed, as has previously been described *(24)*. SAGE libraries of >30 healthy tissues were also available in the Human Transcriptome Map; these libraries originated from the NCBI SAGE Web site (http://www.ncbi.nlm.nih.gov/SAGE/). At the time of analysis, this Web site contained data for tissues and cell lines from brain, kidney, lung, breast, colon, ovary, prostate, pancreas, skin, muscle, vascular tissue, hemangioma, fibroblasts, and leukocytes, but it contained no BM SAGE libraries.

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total cellular RNA was extracted from tumor, BM, and PB samples by the RNA-Bee method (Campro Scientific) according to the manufacturer's instructions. cDNA was synthesized as described previously *(12)*.

PRIMERS/PROBES

Primers and probes were designed with Primer Express software (version 1.5; Applied Biosystems) and Oligo 6 (Molecular Biology Insights) and were based on published gene sequences (see Table 5 in the online Data Supplement). All amplicons spanned an intron of at least 500 bp, and no amplification of genomic DNA was observed. Primers and probes were synthesized by Eurogentec. Primer/probe combinations for the housekeeping gene *GUSB* (glucuronidase, beta; also known as GUS), *B4GALNT1* (i.e., for GD2 synthase), and *TH* have been published previously *(13, 25, 26)*.

REAL-TIME QUANTITATIVE PCR

RQ-PCR was performed in an ABI Prism 7900 Sequence Detection System (Applied Biosystems) as described previously *(12)*. Initial investigation of potential molecular markers was carried out with the SYBR Green I dye used to detect PCR products. The specificity of the PCR was determined by melting curve analysis *(27)*. When genomic DNA was amplified, primer combinations were modified.

Finally, to obtain maximum sensitivity and specificity, we used specific probes (TaqMan; Eurogentec). *GUSB* was used for normalization [normalized threshold cycle (ΔCt) = $Ct_{GUSB} - Ct_{marker}$]. The number of *GUSB* copies was determined with dilutions of *GUSB* plasmid DNA (Ipsogen). Negatively testing samples with <5000 *GUSB* copies were excluded. The mean Ct value for *GUSB* for all BM and PB samples was 22.6 (range, 1000–250 000 *GUSB* copies). All RQ-PCR reactions were carried out at least in duplicate, and mean values were used.

ASSAY SENSITIVITY BY IN VITRO SERIAL DILUTIONS

The sensitivity and quantitative range of RQ-PCR assays were assessed by seeding N206 and NGP NB cells into PB cells at concentrations of 1 tumor cell in 10^2 to 10^7 nucleated healthy cells, as described previously *(12)*.

IMMUNOCYTOLOGY

Cytospin preparations were immunocytologically stained and evaluated by the BM laboratory of the German Society of Pediatric Oncology and Hematology NB group (Cologne) according to the standardized European method *(11)*.

DATA AND STATISTICAL ANALYSIS

When a marker showed amplification in control tissue with a TaqMan RQ-PCR assay, a threshold for positivity was determined. This threshold was defined according to the rules adapted from the European Study Group on MRD detection in ALL *(28)*. Clinical samples were scored as positive if the Ct value was less than 40 and mean ΔCt was ≥3.0 Ct values lower than the mean ΔCt of the normal tissue. Samples with Ct values of ≥40 or of 1 Ct above the background were scored as negative. Samples with a ΔCt value between these were scored as inconclusive and were in the background range. The mean ΔCt in normal tissues was determined by averaging the ΔCt values of control samples that showed amplification. ΔCt values are expressed as the mean (SD).

Results

SELECTION OF CANDIDATE MRD MARKERS BY SAGE ANALYSIS

From SAGE libraries, we selected 28 genes that show high expression in NB tumors, especially in stage 4, and little or no expression in healthy tissues, particularly not in vascular tissue, hemangioma, fibroblasts, and leukocytes (Table 1; see Table 6 in the online Data Supplement). The ratio of expression in NB tissues relative to that in healthy tissues was >100 for 7 markers and 10–100 for 13 of the 28 markers. The 8 other genes showed little or no expression in hematologic or other confounding tissues (data not shown). The 28 selected markers also included previously described markers *TH (29, 30)*, *DBH* [dopamine beta-hydroxylase (dopamine beta-monooxygenase)] *(14)*, and *DDC* [dopamine decarboxylase (aromatic L-amino acid decarboxylase)] *(31)*. To enable comparison of the results of markers selected by SAGE, we also included 5 previously described markers: *B4GALNT1 (32)*, the *GAGE* family *(33)*, *UCHL1* [ubiquitin carboxyl-terminal

Table 1. Candidate genes for MRD detection selected using SAGE expression profile analysis and RQ-PCR on NB tumors and control BM.

Gene	Hs No.[a]	SAGE tag counts, NB[b]	SAGE tag counts, healthy tissue[c]	ΔCt, tumor[d]	ΔCt, BM[e]
PHOX2B	87202	16.0	0.02	3.6 (0.6–6.1)	No amplification
TH	435609	32.1	0.02	3.9 (2.1–6.6)	−12.5
DDC	359698	28.6	0.6	2.0 (−1.5–4.9)	No amplification
DBH	591890	15.2	0.02	4.5 (1.2–6.3)	−13.7
CHRNA3	89605	16.2	1.0	3.5 (2.8–4.1)	−13.3
GAP43	134974	49.6	0.7	5.2 (3.9–5.4)	−9.9
SNAP91	368046	11.2	2.1	3.7 (3.4–4.4)	−11.3
STMN2	521651	79.0	1.2	6.6 (5.4–8.7)	−10.1
STMN4	201058	23.6	1.3	0.0 (−1.6–2.1)	−12.8
CHGB	516874	251.0	0.7	5.6 (2.6–7.8)	−11.0
B4GALNT1	159481	Marker published in literature[f]		0.4 (−0.3–1.1)	−10.6

[a] Hs number as given in UniGene http://www.ncbi.nlm.nih.gov/unigene.
[b] Expression in the SAGE libraries of all cell lines and 4 NB tumors (mean tag count per 100 000).
[c] Tag counts of control tissues (mean tag counts per 100 000).
[d] Mean normalized Ct value ($\Delta Ct = Ct_{GUSB} - Ct_{marker}$) and range for 4 primary NB tumors.
[e] Mean ΔCt value and range for 3 control BM samples.
[f] Cheung et al. *(32)*.

esterase L1 (ubiquitin thiolesterase); also known as PGP9.5] *(34)*, *NEFM* (neurofilament, medium polypeptide) *(35)*, and *POSTN* (periostin, osteoblast specific factor) *(36)*; see flow sheet in Fig. 1.

SCREENING FOR NB SPECIFICITY OF POTENTIAL MRD MARKERS WITH THE SYBR GREEN I DYE

All 33 markers were tested on RNA from 4 NB tumors (including 1 stage 4 tumor used for SAGE expression analysis) and RNA from 3 pediatric control BM samples. On the basis of the high expression in all 4 tested NB tumors and little or no expression in the 3 BM samples, we selected the following 10 genes as potential markers (flow sheet in Fig. 1): *PHOX2B*, *TH*, *DBH*, *DDC*, *CHRNA3* (cholinergic receptor, nicotinic, alpha 3), *STMN2* (stathmin-like 2), *STMN4* (stathmin-like 4), *CHGB* [chromogranin B (secretogranin 1)], *SNAP91* [synaptosomal-associated protein, 91kDa homolog (mouse)], and *GAP43* (growth associated protein 43). *B4GALNT1* was added for comparison with the novel markers.

EXPRESSION OF CANDIDATE MARKERS IN NB TUMORS WITH TaqMan PROBES

To confirm the expression of the potential markers in NB tumors, we tested 56 NB tumor samples by RQ-PCR analysis (Table 2). All NB tumors showed high expression of all 11 markers, although the expression levels varied as much as 1000-fold between tumors. The highest mean expression values were observed for *STMN2*, *CHGB*, *TH*, and *GAP43*, whereas *STMN4* and *B4GALNT1* showed the lowest expression. *PHOX2B* expression was intermediate. Because the marker with the highest expression varied between tumors, each of the markers could theoretically be the best marker for a particular patient.

EXPRESSION OF CANDIDATE MARKERS IN CONTROL TISSUE

To select the most specific markers and to define threshold levels for positivity, we measured the expression of the 11 potential markers in control BM (n = 51) and PB (n = 37) (Table 2). We selected markers with no or a low frequency of positive samples and/or low expression levels in the positive samples (see flow sheet in Fig. 1). On the basis of these results, we selected 6 markers (*PHOX2B*, *TH*, *DBH*, *DDC*, *GAP43*, and *CHRNA3*). The other 5 candidate markers (*B4GALNT1*, *CHGB*, *STMN2*, *SNAP91*, and *STMN4*) showed relatively high expression levels in almost all control hematologic samples.

As described previously *(12)*, *PHOX2B* was not expressed in any of the control samples. Although the expression levels of all other markers were low, they showed some amplification (especially in control BM): *DDC* tested positive only once in BM (1 of 51 samples) and PB (1 of 37 samples). *DBH* tested positive only once in PB (1 of 37 samples) but was relatively frequently expressed in BM (29 of 51 samples). *CHRNA3*

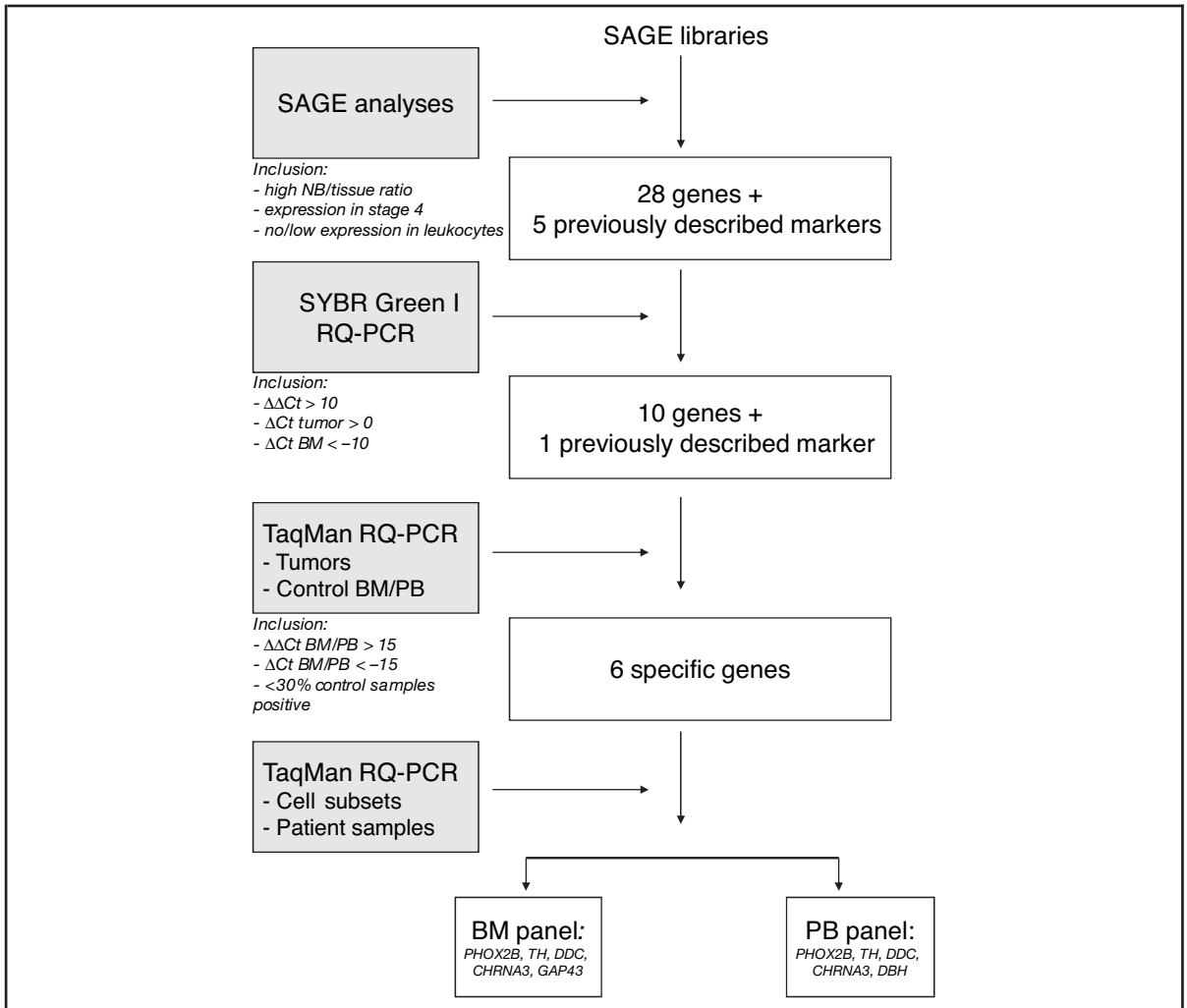

Fig. 1. Flow sheet of study design.

was rather specific in PB (7 of 37) and less so in BM (31 of 51). In contrast, *TH* and *GAP43* showed expression in a considerable number of BM and PB samples, but both genes had a very high tumor/nontumor expression ratio.

Because control PB and BM showed varied expression of the markers, we selected 2 panels of 5 markers each for further testing: a panel for BM [*PHOX2B*, *DDC*, *CHRNA3* (lowest expression in BM), and *TH* and *GAP43* (both with a high tumor/nontumor expression ratio)] and a set for PB [*PHOX2B*, *DDC*, *DBH*, *CHRNA3* (all 4 markers with low expression in PB) and *TH* (high tumor/nontumor expression ratio)].

EXPRESSION OF MARKERS IN CELL SUBSETS

To define which cell subsets contribute to the amplification of the markers in control BM and PB, we measured expression levels in several cell lineages for the 6 selected markers. Fig. 2A shows results for the different subsets of cells present in PB (granulocytes, monocytes, platelets, T cells, B cells, and natural killer cells). Fig. 2B shows results for hematopoietic precursor cells present in BM, and Fig. 2C shows test results for different cell types present in the BM microenvironment. Overall, we found more frequent and higher expression of the markers in BM-derived cells than in PB cells.

PHOX2B and *DBH* were never expressed in PB cell subsets. The other markers were only incidentally positive, mainly in myeloid cells and natural killer cells. Interestingly, *GAP43* was expressed in the B cells of all 4 tested donors.

For BM cell subsets, we tested 12 samples of $CD34^+$ hematopoietic progenitor cell fractions. *PHOX2B* was the

Table 2. Mean normalized expression levels of candidate genes by RQ-PCR with TaqMan probes in NB tumors (n = 56), control BM samples (n = 51), and control PB samples (n = 37).

	Tumor			BM			PB		
Marker	Positive samples[a]	Expression[b]	Range[c]	Positive BM samples[a]	Expression[b]	Threshold[d]	Positive PB samples[a]	Expression[b]	Threshold[d]
PHOX2B	56/56	−1.6 (2.0)	−6.3–4.0	0/51	No amplification	No threshold	0/37	No amplification	No threshold
DDC	56/56	−1.8 (2.4)	−8.0–1.7	1/51	−16.8	−13.8	1/37	−18.0	−15.0
CHRNA3	56/56	−0.9 (2.2)	−6.6–2.8	31/51	−15.6 (1.3)	−12.6	7/37	−14.7 (0.8)	−11.7
TH	56/56	2.2 (2.1)	−3.4–5.0	15/51	−15.3 (1.1)	−12.3	10/37	−15.2 (0.8)	−12.2
GAP43	56/56	1.8 (1.7)	−3.5–6.3	20/51	−14.9 (1.6)	−11.9	29/37	−13.8 (1.5)	−10.8
STMN4	56/56	−3.0 (1.6)	−7.0–0.2	44/51	−14.5 (1.4)	−11.5	37/37	−12.4 (1.0)	−9.4
DBH	56/56	1.1 (2.7)	−5.0–5.2	29/51	−13.5 (1.8)	−10.5	1/37	−18.0	−15.0
STMN2	56/56	3.6 (1.8)	−2.0–6.6	50/51	−11.3 (5.5)	−8.3	37/37	−12.5 (1.6)	−9.5
CHGB	56/56	2.5 (1.9)	−3.0–6.1	49/51	−12.4 (1.3)	−9.4	37/37	−10.7 (2.0)	−7.7
SNAP91	56/56	1.5 (1.3)	−2.9–4.0	43/51	−13.4 (2.5)	−10.4	26/37	−14.6 (2.1)	−11.6
B4GALNT1	56/56	−2.3 (1.7)	−7.2–2.5	49/51	−13.8 (1.5)	−10.8	30/37	−14.4 (1.1)	−11.4

[a] Number of positive tumor, BM, or PB samples of the total number of samples tested.
[b] All samples represent the mean (SD) of normalized Ct values ($\Delta Ct = Ct_{GUSB} - Ct_{marker}$).
[c] ΔCt value of lowest and highest expression of tumor samples.
[d] Threshold for positivity, defined as the mean ΔCt minus 3(Ct).

only marker not expressed in these samples; all other markers showed expression in some of the $CD34^+$ cell samples. Surprisingly, *TH* was expressed in 8 of 12 $CD34^+$ samples. Furthermore, we tested $CD34^+$ cells in vitro that had differentiated toward the granulocytic and megakaryocytic lineage. Leukemic cells of T-ALL and precursor B-ALL patients were tested as representatives of their nonpathologic counterparts. Again, *PHOX2B* was not expressed in any of the other hematopoietic cells, and *DDC* was expressed only in some megakaryocyte samples. The other markers were sporadically positive in all other BM subsets. *DBH* and *CHRNA3* expression was especially high in the T-ALL samples (n = 4) and also positive, albeit at a lower level, in all B-ALL samples (n = 5). *GAP43* was expressed at a low level in virtually all subsets and at a very high level in all stromal cells (mesenchymal stromal cells, fibroblasts, adipocytes, and smooth muscle cells).

SENSITIVITY OF THE RQ-PCR ASSAYS

The sensitivity of the 6 selected markers was assessed by in vitro dilutions of NB cells from 2 cell lines (NGP and N206) into nonpathologic PB cells. With NGP cells, *PHOX2B* and *DBH* were the most sensitive markers (1 tumor cell in 10^7 PB cells) with a quantitative range of 10^{-6} (see Table 7 in the online Data Supplement). In N206 cells, *DDC* and *DBH* were the most sensitive. All 5 PB markers (*PHOX2B*, *TH*, *DDC*, *DBH*, and *CHRNA3*) reached a sensitivity of 10^{-6}.

COMPARISON OF RQ-PCR RESULTS WITH BM ANTI-GD2 IMMUNOCYTOLOGY RESULTS

We also compared the RQ-PCR results for 4 BM MRD markers with anti-GD2 immunocytology results in BM samples from 32 German patients with stage 4 disease (Table 3). All samples positive for anti-GD2 tested positive with the panel. In addition, 5 of 16 immunocytology-negative samples tested positive with the panel, and 3 of these patients died of their disease.

PANEL OF MARKERS COMPARED WITH *PHOX2B* IN DIAGNOSTIC BM SAMPLES AND PB SAMPLES

To determine the added value of a panel of MRD targets relative to the application of a single marker (*PHOX2B*), we tested our selected BM and PB panels with 222 samples from 82 Dutch patients with stage 4 NB (54 BM samples at diagnosis, 143 BM samples during treatment, and 25 PB samples). In the diagnosis BM samples, we also compared the results of the 5 RQ-PCR markers with morphologic findings. As we have previously shown *(12)*, all 42 samples that were morphologically positive were also positive for *PHOX2B*; 6 of the 12 morphologically negative cases were also positive for *PHOX2B* (see Table 8 in the online Data Supplement). The other markers were not always positive in morphologically positive BM samples; sometimes only inconclusive PCR results were obtained. In morpho-

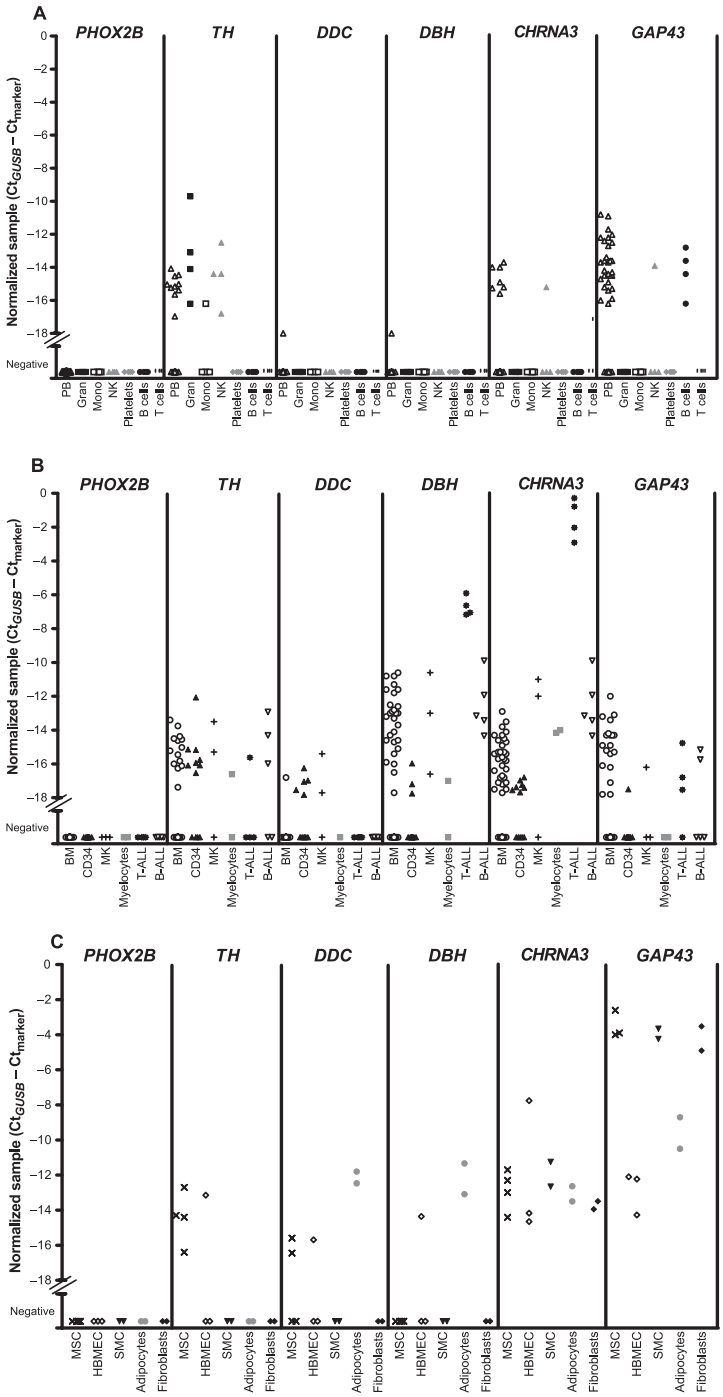

Fig. 2. Marker expression in control PB, BM, and cell subsets.

Markers were tested on control PB and its subsets (A), control BM and hematopoietic precursors (B), and BM environment cells (C). PB (n = 37); Gran, granulocytes (n = 4); Mono, monocytes (n = 4); NK, natural killer cells (n = 4); platelets (n = 4); B cells (n = 4); T cells (n = 4); BM samples (n = 51); CD34, CD34$^+$ cells (n = 12); MK, megakaryocytes (n = 3); myelocytes (n = 2); T-ALL (n = 4); B-ALL (n = 5); MSC, mesenchymal stromal cells (n = 4); HBMEC, human BM-derived endothelial cells (n = 3); SMC, smooth muscle cells (n = 2); adipocytes (n = 2); fibroblasts (n = 2).

Table 3. Comparison of RT-PCR assay results to immunocytology results for BM samples from 32 stage 4 patients of the German Society of Pediatric Oncology and Hematology.[a]

	Immunocytology	
RT-PCR	Positive	Negative
PHOX2B		
Positive	14	4
Negative	2	12
TH		
Positive	16	3
Inconclusive	0	6
Negative	0	7
DDC		
Positive	14	3
Inconclusive	0	2
Negative	2	11
CHRNA3		
Positive	14	2
Inconclusive	0	4
Negative	2	10
Panel		
Positive	16	5
Negative	0	11

[a] Positive, presence of NB cells or mRNA; negative, absence of NB cells or mRNA; inconclusive, result in the range of background of marker expression. Because of a lack of material, GAP43 was not with these samples.

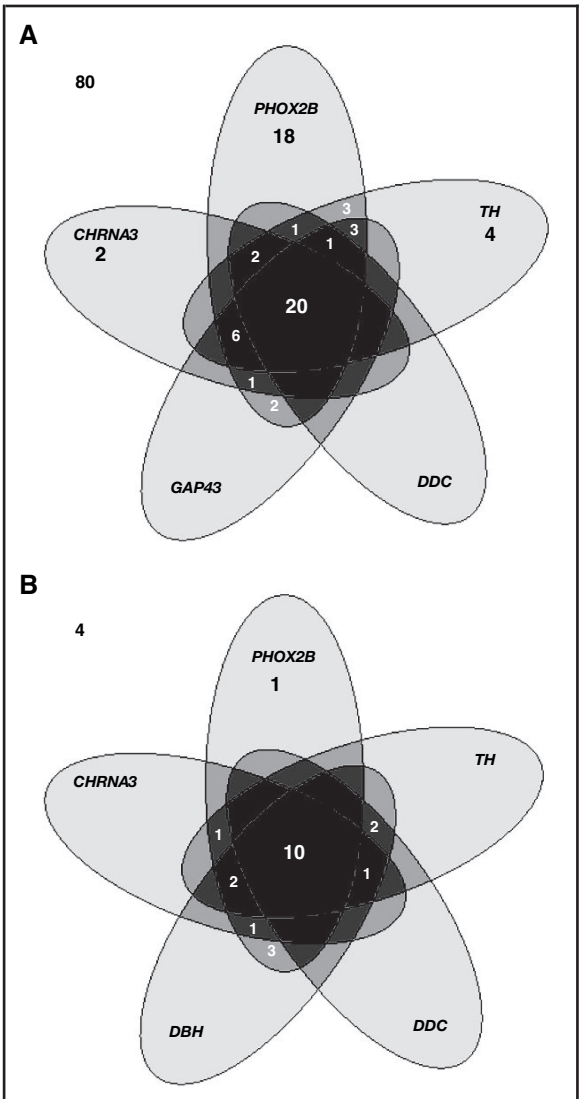

Fig. 3. Value of markers in MRD detection.

BM samples (n = 143) obtained during and/or after treatment of 67 patients (A) and PB samples (n = 25) obtained at diagnosis or during treatment of 25 patients (B) were tested with a panel of RQ-PCR markers. Each ellipse represents positive results for 1 marker. Number outside of ellipses indicates number of samples with no positive result for any marker.

logically negative samples, all of the other markers also detected positive samples; however, no additional positive samples were detected by any 1 of the other markers, in contrast to *PHOX2B*. Only 1 of 6 histologically negative and *PHOX2B*-negative patients died, compared with 5 of 6 histologically negative and *PHOX2B*-positive patients.

Given that lower tumor loads are present during or after treatment, we also investigated the panel of markers with such samples (n = 143). Of these 143 BM samples from 67 patients, 39 samples showed positive results with both *PHOX2B* and the panel of other markers. In 18 *PHOX2B*-positive samples, the other markers tested negative (Fig. 3A). On the other hand, in 6 (7%) of 86 *PHOX2B*-negative samples, 1 of the other markers gave a positive result. As is shown in Fig. 3A, only *TH* (4 additional positive samples) and *CHRNA3* (2 additional positive samples) increased the value of the panel. In PB samples, the added value of the other markers seemed to be higher. As is shown in Fig. 3B, only 1 sample was positive for *PHOX2B* and negative for the other markers. Markers *TH*, *DDC*, and *DBH* were all positive in 3 of 7 *PHOX2B*-negative PB samples.

Discussion

We previously demonstrated that *PHOX2B* is a very good RQ-PCR marker for MRD detection in NB. In the

present study, we searched for other specific RQ-PCR markers for MRD detection in NB patients and found that the sensitivity of MRD detection is increased by the application of a marker panel (*PHOX2B*, *TH*, *DDC*, *DBH*, *CHRNA3*, and *GAP43*). These markers were all highly expressed in NB tumors and were not expressed or expressed at low levels in control BM, PB, and their cell subsets. A seeding experiment demonstrated that each PCR reached a sensitivity of detection of 1 tumor cell in 10^6 nonpathologic cells. Three of the 6 selected markers have previously been applied as MRD markers in NB: *TH (29, 30)*, *DDC (31)*, and *DBH (14)*. In contrast, other commonly used markers [*B4GALNT1 (32)*, *GAGE (33)*, *UCHL1 (34)*, *NEFM (35)*, and *POSTN (36)*] were clearly less specific.

Recently, other groups also have identified novel MRD markers, but with gene expression profiling *(37, 38)*. Although we analyzed only 4 tumors by SAGE analysis, we identified many of the same genes, including *PHOX2B*. As our group has previously described *(12)*, *PHOX2B* is a very good marker for measuring MRD in both BM and PB compartments, owing to its high sensitivity and total specificity. A positive *PHOX2B* result clearly implies tumor infiltration. This features represents a major advantage of *PHOX2B* over other markers, which can have low expression in BM, PB, and/or PB stem cell samples from control individuals; however, because we have now defined threshold levels for these markers by testing large numbers of PB and BM samples, a positive result for 1 of the other markers also can be interpreted as tumor cell infiltration. We used these expression data in control tissues to select 2 panels of MRD markers for testing BM and PB samples. These panels include the markers that are most specific for BM (*PHOX2B*, *TH*, *DDC*, *CHRNA3*, *GAP43*) and PB (*PHOX2B*, *TH*, *DDC*, *DBH*, and *CHRNA3*).

After establishing threshold levels in control tissues, we also determined the origin of illegitimate expression in different cell subsets. These results could be of clinical importance in MRD studies when the *PHOX2B* result is negative and other markers have yielded inconclusive results. Expression levels in nonpathologic cell subsets could then be used as another tool to discriminate between nonpathologic expression and expression derived from tumor cells. Moreover, we are the first to report the expression of RQ-PCR MRD markers in the $CD34^+$ cell subset. Because autologous stem cell harvests are often selected for $CD34^+$ cells, expression levels in these nonpathologic subsets are important to set a threshold level for positivity in this fraction. Surprisingly, we detected some expression of all markers except *PHOX2B* in $CD34^+$ samples. There is some discussion in the literature regarding whether reinfusion of a contaminated harvest is correlated with worse survival prospects *(5, 6, 39, 40)*. To avoid false-positive detection of tumor mRNA in these harvests, we recommend that a cutoff level for the $CD34^+$ fraction be used for markers expressed in this subset. Furthermore, we obtained high expression levels for some markers in lymphoid precursor cells and in different cells in the BM microenvironment (especially for *GAP43*), which might explain the amplifications in control BM.

Initially, we considered testing our samples only with *PHOX2B*, because *PHOX2B* is the most specific and sensitive marker; however, marker genes could be expressed heterogeneously between patients, within a tumor, and between a tumor and its metastasis. In our tumor set of 56 tumors, all marker genes, including *PHOX2B*, were indeed heterogeneously expressed. Such heterogeneity in expression could lead to different sensitivities per marker gene for each patient. A marker gene that shows the highest expression in a tumor would theoretically be the most sensitive marker for MRD detection in that patient. Therefore, the sensitivity of *PHOX2B* used as the sole MRD marker will be lower in patients with relatively low *PHOX2B* expression. In these cases, other markers with higher expression could contribute to more-sensitive detection of MRD.

In BM samples taken at diagnosis, *PHOX2B* can be used as the only marker because all morphologically positive samples were also positive for *PHOX2B*; however, our analysis of 222 samples from the Dutch patients (both BM and PB) showed that 9 samples were *PHOX2B* negative, whereas other markers showed positive results. In PB samples, 3 of 7 *PHOX2B*-negative samples were positive for 1 or more of the other markers, and *TH* or *CHRNA3* were positive in 6 (7%) of 86 *PHOX2B*-negative BM samples obtained during treatment. Presumably, other markers contribute to sensitivity, especially when tumor load is low. Moreover, the cutoff level for most markers was lower in PB than in BM, making these markers more sensitive in PB.

We selected our markers by comparing SAGE libraries of NB tissues to SAGE libraries of nonpathologic tissues. The SAGE libraries contained only 4 NB tumors; therefore, it is possible that we missed some candidate markers. Our use of this method, however, revealed a panel of useful markers, including *PHOX2B*.

In conclusion, the use of different panels of sensitive and specific markers for each compartment is most sensitive for detecting MRD with RQ-PCR. This new point of view offers MRD researchers insight into which panel of markers to use for MRD detection with RQ-PCR. These panels should include *PHOX2B*, because we have shown that *PHOX2B* is not only completely specific but also the most sensitive marker. Because such low levels of MRD can now be detected,

large prospective studies are needed to determine the clinical relevance of MRD monitoring with RQ-PCR analysis.

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