

Methylated RASSF1a Is the First Specific DNA Marker for Minimal Residual Disease Testing in Neuroblastoma

Janine Stutterheim^{1,3,4}, Fatima Ait Ichou⁴, Emmy den Ouden², Rogier Versteeg³, Huib N. Caron¹, Godelieve A.M. Tytgat¹, and C. Ellen van der Schoot^{2,4}

Abstract

Purpose: PCR-based detection of minimal residual disease (MRD) in neuroblastoma (NB) is presently based on NB-specific transcripts. However, the expression of these targets varies between patients and upon treatment, and only PHOX2B is truly specific. RASSF1a is methylated (RASSF1a_M) in NB, and we investigated whether it can serve as a specific and stable DNA MRD marker.

Patients and Methods: The RASSF1a_M-specific quantitative real-time PCR was tested on control bone marrow (BM; $n = 50$), on 71 NB tumors, and on 159 clinical BM samples at diagnosis and at follow-up of 77 patients. Results were compared with a panel of RNA markers and correlated with prognosis.

Results: RASSF1a_M was present in all stage 4 and 4s tumors ($n = 50$) and in 86% stages 1 to 3 tumors ($n = 21$). The level of methylation in stage 4 NB was correlated with overall survival ($P = 0.02$). RASSF1a_M-PCR was highly specific (only 1 amplification in 50 control samples tested in triplicate) and had a similar sensitivity as the RNA-based PCRs, as shown on clinical samples. Moreover, RASSF1a_M enabled accurate quantification without need for the original tumor.

Conclusions: RASSF1a_M is a novel, highly specific DNA marker for MRD detection in NB, equal to PHOX2B in specificity and sensitivity, and better suitable for MRD quantification. We propose to include RASSF1a_M in further prospective MRD studies in NB alongside RNA MRD markers. In addition, this assay might also be applicable for detection of circulating tumor cells in patients with other cancers with RASSF1a_M such as breast or lung cancer. *Clin Cancer Res*; 18(3); 808–14. ©2011 AACR.

Introduction

In the search for specific methods to assess bone marrow (BM) in patients with neuroblastoma (NB), quantitative real-time PCR (qRT-PCR) using RNA markers (1–3) and GD2 immunocytology (4) are now being standardized for monitoring minimal residual disease (MRD; ref. 5). So far, *PHOX2B* is the only NB-specific marker for MRD detection in NB using qRT-PCR (6). To overcome heterogeneity in expression between tumors, detection of tumor cells by qRT-PCR is preferably done using a panel of NB-specific RNA markers which detects tumor cells with a sensitivity up to 1 in 10^6 normal nucleated BM cells (7).

The expression of the RNA markers in primary tumors varies with a factor 1,000 (7). So for accurate quantification of MRD levels the primary tumor is needed, and it is furthermore not known whether these markers are stably expressed during treatment. DNA is more stable than RNA and is not dependent on level of gene expression. Thus, a DNA marker will enable more accurate quantification of MRD in BM of NB patients. However, a general NB-specific DNA aberration has not been found yet. Common epigenetic changes, such as aberrant DNA methylation, have been described in NB (8–10). A gene, which is often silenced by promoter region methylation (11) in NB and not in normal tissues, is *RASSF1a* (8–10, 12, 13).

The purpose of our study was to determine whether methylated RASSF1a (RASSF1a_M) can serve as a DNA qRT-PCR marker for MRD detection in NB patients with similar sensitivity and specificity as RNA markers and to determine whether MRD levels in the BM can be more accurately quantified using this marker.

Materials and Methods

Patients and samples

BM samples of NB patients, treated at the Emma Children's Hospital/AMC, Amsterdam, the Netherlands, have been collected between 1995 and 2010. We selected 167 samples of which results were already available with a panel

Authors' Affiliations: ¹Department of Pediatric Oncology, Emma Children's Hospital; Departments of ²Hematology and ³Human Genetics, Academic Medical Center and ⁴Sanquin Research at CLB and Landsteiner Laboratory of the AMC, Amsterdam, the Netherlands

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Corresponding Author: C. Ellen van der Schoot, Sanquin Research at CLB and Landsteiner Laboratory of the AMC, Plesmanlaan 125, 1066 CX Amsterdam. Phone: 31-20-5123377; Fax: 31-20-5123474; E-mail: e.vanderschoot@sanquin.nl

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Translational Relevance

Quantitative real-time PCR (qRT-PCR) for detection of minimal residual disease (MRD) in children with neuroblastoma (NB) can be used for evaluation of molecular bone marrow (BM) response and for staging at diagnosis. It is to be expected that future therapies will be guided by measuring MRD levels. Tumor-selective mRNAs markers are sensitive and specific for MRD detection; however, their expression is dependent on the level of gene expression, which is different between patients and can change during treatment. DNA is more stable than RNA and is not dependent on level of gene expression. We describe the first DNA marker for MRD detection in NB. The qRT-PCR detecting methylated RASSF1a (RASSF1a_M) was found to be highly specific for cancer cells, and virtually no amplification was observed in normal BM cells, making this assay also applicable for detection of circulating tumor cells in patients with other cancers with RASSF1a_M such as breast or lung cancer.

of qRT-PCR makers (7) and tested an additional 31 samples which had not been tested with qRT-PCR before. In total, we tested 77 BM samples obtained at diagnosis from 45 patients with INSS (14) stage 4, 26 stages 1 to 3 and 6 stage 4s, and 50 BM samples during treatment from 28 patients with stage 4. From 64 of these patients primary tumor was available. From an additional 7 patients, the primary tumor was tested without corresponding BM samples. The characteristics of patients are shown in Supplementary Table S1. High-risk patients [patients with INSS stage 4 disease ($n = 48$), and patients with low-stage and MYCN amplification ($n = 5$)] were treated according to consecutive protocols (e. g., VECI; ref. 15; $n = 20$), rapid-COJEC (ref. 16; $n = 9$) or GPOH-NB97 (ref. 17; $n = 24$), see Supplementary Table S2 for individual patient data). Written informed consent was obtained from parents/guardians. The Medical Research Ethics Committee of the AMC approved the study.

Control samples

To determine background of the RASSF1a_M qRT-PCR in normal BM, BM samples obtained from 50 children with acute lymphoblastic leukemia in molecular remission, as determined by antigen-receptor qRT-PCR, were tested (18).

RNA extraction, reverse transcription, and quantitative real-time PCR

Total cellular RNA from tumor and BM samples was extracted with TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was synthesized as described (6). All samples were tested with a panel of PCR markers: *PHOX2B*, tyrosine hydroxylase (*TH*), dopamine decarboxylase (*DDC*), cholinergic receptor3 (*CHRNA3*), and growth-associated protein 43 (*GAP43*)

using β -glucuronidase (*GUS*) for normalization, as described previously (7).

DNA extraction and methylation-specific quantitative real-time PCR

DNA was extracted using the QiAmp DNA blood mini kit. Bisulfite conversion was with an EpiTect Bisulfite kit (both Qiagen). Input for bisulfite treatment was 100 ng for tumor samples and 2 μ g for BM samples and eluted in 40 μ L. To control for DNA input, β -actin qRT-PCR was carried out. Primers and probes for β -actin and RASSF1a_M were modified from Lehmann and colleagues (19). Primers and probe for unmethylated RASSF1a (RASSF1a_U) were designed in the same region (spanning exon 1) as RASSF1a_M; primer-probe combinations are listed Supplementary Table S3. Oligonucleotides were synthesized by Eurogentec. qRT-PCR was carried out in an ABI 7500 Fast Step-one-Plus (PE Biosystems). Reactions were carried out in 20 μ L (10 μ L TaqMan Fast Universal PCR MasterMix (Applied Biosystems), 2.6 μ L H₂O, 300 nmol/L forward and reverse primer, 200 nmol/L TaqMan probe, 5 μ L (c)DNA starting with 20 seconds 95°C followed by 50 cycles (1 sec 95°C, 20 seconds 60°C). qRT-PCR assays for RASSF1a_M were carried out in triplicate, RASSF1a_U and β -actin in duplicate.

Assay sensitivity by *in vitro* serial dilutions

Sensitivity of the qRT-PCR assay was assessed by 10-fold diluting DNA isolated from IMR32 NB cells into mononuclear cells (MNC) DNA. Sensitivity was defined as the lowest dilution with at least one positive replicate $\geq 1.0 C_t$ lower than the lowest C_t in MNCs. The quantitative range was defined as the lowest dilution giving a reproducible amplification (C_t of all replicates ≤ 1.5) and with a mean C_t value within 2.6 to 4.0 C_t values from the previous dilution point, with all C_t values ≥ 3.0 lower than the lowest C_t of MNC (20).

Data and statistical analysis

Clinical samples were scored positive if at least 1 of 3 replicates was $\geq 1.0 C_t$ lower than the lowest C_t in control BM.

The percentage of RASSF1a methylation was defined as $[\text{RASSF1a}_M]/([\text{RASSF1a}_M] + [\text{RASSF1a}_U])$. In tumors we corrected for the percentage of tumor cells quantified by morphology.

For quantification with RNA markers, relative values were calculated using the formula: $2^{-\Delta\Delta C_t}$ (ΔC_t primary tumor – ΔC_t BM diagnosis) * 100%. The median relative expression was determined by taking the median of the relative values of all PCR targets.

To compare levels of methylation, the Mann-Whitney test was used, correlation analyses were done using Spearman's test. Survival analyses were done using Kaplan-Meier and log rank statistics, all in SPSS 15.0.

Results

DNA methylation of RASSF1a in NB tumors

We first tested RASSF1a methylation status in 71 NB tumors. In the NB cell line IMR32 only methylated RASSF1a

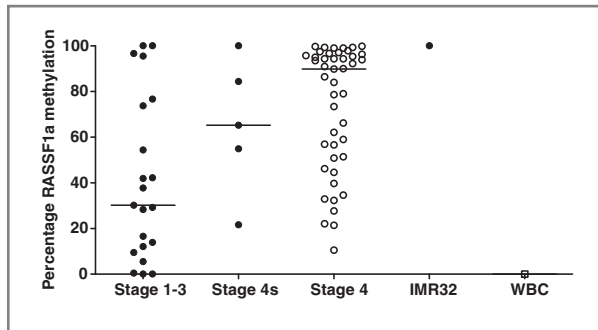


Figure 1. Percentage of RASSF1a methylation determined by qRT-PCR in NB tumors in patients with different disease stages. RASSF1a_M, RASSF1a_U, and β -actin were tested on white blood cells (WBC) from a healthy control on IMR32 cell line cells and on NB tumors of different disease stages: stage 1–3 ($n = 21$), stage 4s ($n = 5$), and stage 4 ($n = 45$).

sequence and in normal white blood cells only unmethylated RASSF1a sequence was detected (Fig. 1). In all stage 4 ($n = 45$) and 4s ($n = 5$) tumors RASSF1a was methylated, with a median percentage of 88% and 65%, respectively. In nonmetastatic NB (stages 1–3) RASSF1a_M was detected in 18 of 21 (86%) of tumors with a median percentage of 30%.

Background of methylated RASSF1a in control BM samples

To define threshold for positivity in BM, we determined the background of RASSF1a_M in control BM (Supplementary Table S4). In only 1 of 50 BM samples amplification in one of the triplicates was observed (C_t 40.9). The threshold for positivity for RASSF1a_M in BM was arbitrarily set on 39.9. Sensitivity of the RASSF1a_M qRT-PCR assay

The sensitivity of RASSF1a_M was tested by assessing dilutions of IMR32 DNA into normal MNC DNA. The percentage tumor cells measured correlated with the percentage tumor cells expected (quantitative range of 10^{-4} ; Fig. 2). The assay reached a sensitivity of detection

of 1 tumor cell in 10^5 MNCs (10^{-5}), equivalent to detection of 2.5 pg (~ 1 allele).

Comparison of RASSF1a_M qRT-PCR results with BM cytology

The qRT-PCR results for RASSF1a_M of all available BM samples ($n = 127$; 77 at diagnosis (13 stage 1 or 2, 13 stage 3, 6 stage 4s and 45 stage 4) and 50 during treatment from 28 stage 4 patients) were compared with BM cytology.

As expected, all BM samples from stage 1 and 2 patients were negative for RASSF1a_M, showing again the specificity of the assay (Table 1). In contrast, in 7 of 13 BM samples from stage 3 patients RASSF1a_M was detected, but in none of these samples all triplicates were positive (Table 1), indicating a low level of infiltration (Supplementary Table S3). In stage 4 and 4s patients, RASSF1a_M was positive in all cytology positive samples ($n = 39$). Furthermore, in 9 cytology-negative samples, RASSF1a_M was positive. In samples taken at follow-up from stage 4 patients many cytology-negative samples were positive for RASSF1a_M ($n = 18$; Table 2).

Comparison of RASSF1a_M qRT-PCR results with qRT-PCR results of a panel of RNA markers in BM samples

To investigate the performance of RASSF1a_M for MRD testing, the assay was tested on clinical samples ($n = 127$) and compared with the PCR results obtained with a panel of RNA markers. As shown in Tables 1 and 2, in 79% of the samples both assays gave similar results (71 positive and 37 negative samples). In 10 samples the RASSF1a_M was negative, whereas the RNA panel gave a positive result, and vice versa in 9 cases the RASSF1a_M was positive and the RNA panel negative. Most discrepant samples were seen in patients with low tumor load (BM obtained from patients at diagnosis with stages 1 to 3 and BM obtained during or after treatment from stage 4 patients). RASSF1a_M detected one extra positive sample compared with the RNA panel in a stage 4s patient at diagnosis, and one panel-positive sample

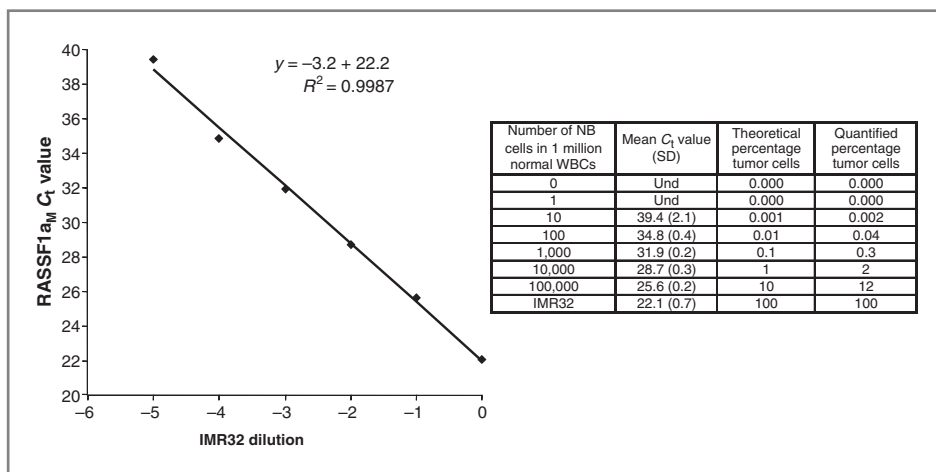


Figure 2. Sensitivity of RASSF1a_M qRT-PCR assay. Sensitivity of the qRT-PCR assay was assessed by diluting DNA isolated from IMR32 NB cells into MNC DNA to obtain a dilution range from 10^{-1} to 10^{-6} . Each dilution was tested in triplicate for RASSF1a_M. In the table the average C_t values of RASSF1a_M are depicted with its SD. The percentage of tumor cells was quantified using the formula: $[\text{RASSF1a}_M] / ([\text{RASSF1a}_M] + [\text{RASSF1a}_U])$.

Table 1. Comparison of RASSF1a_M qRT-PCR with cytology and with qRT-PCR using a panel of RNA targets in BM samples at initial diagnosis from 77 patients

Diagnosis samples	Stage 1,2		Stage 3		Stage 4s		Stage 4	
	Bone marrow cytology							
RASSF1a _M	+	-	+	-	+	-	+	-
+	0	0	0	7	4	1	35	8
-	0	13	0	6	0	1	0	2
	Panel of RNA markers							
RASSF1a _M	+	-	+	-	+	-	+	-
+	0	0	3	4	4	1	43	0
-	3	10	1	5	0	1	1	1

NOTE: +, amplification of marker; -, no amplification of marker.

of a stage 4 patient (only positive for *PHOX2B*) at diagnosis was negative for RASSF1a_M (Table 1).

Comparison of quantification of MRD levels in BM applying RASSF1a_M and a panel of RNA markers

For quantification of MRD levels based on RNA qRT-PCRs, the expression level of the PCR targets in the BM has to be related to their expression in the primary tumor. From 21 patients with stage 4, primary tumor RNA was available. Because the estimated MRD level in the BM varied for the different RNA targets, the median MRD level of the panel of 5 RNA targets was determined for each BM sample as the best estimate for MRD level. In Fig. 3 the correlation between MRD levels (all within the quantitative range) in BM samples determined by RASSF1a_M and by the panel of RNA markers is shown. In 23 of 30 BM samples, both methods quantified more or less (within 1 log) the same MRD level ($R^2 = 0.76$; Spearman's test, $P < 0.001$). In 3 of 30 samples, RASSF1a_M showed MRD levels higher than 1 log than the panel of RNA markers. Because we assumed that all

NB cells were methylated, an overestimation of RASSF1a_M is excluded. Moreover, 2 of these samples showed more than 1% BM infiltration with morphology, confirming that the MRD level is underestimated by the RNA panel. Hence, there are no indications that chemotherapy induces demethylation of *RASSF1a* promoter. In conclusion, MRD quantification in BM is possible using RASSF1a_M with the advantage that the primary tumor is not needed for MRD quantification.

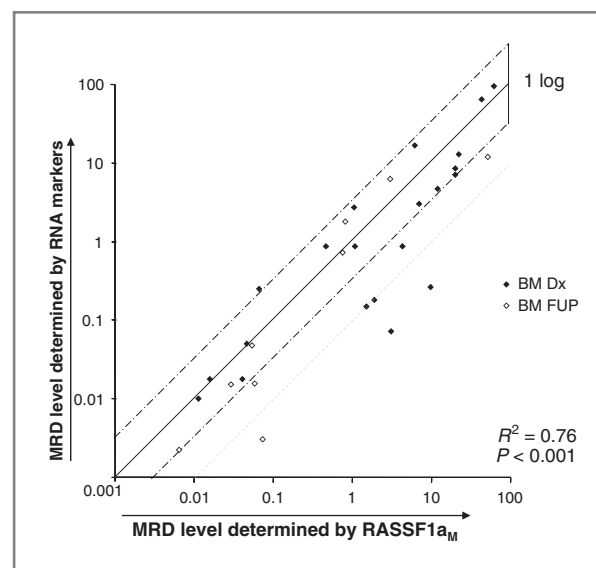
Now we have shown that accurate quantification is possible using RASSF1a_M, we investigated whether the level of BM infiltration at diagnosis is prognostic for survival. As shown in Fig. 4, stage 4 patients with more than 20% of NB cells in the BM had a worse outcome compared with

Table 2. Comparison of RASSF1a_M qRT-PCR with cytology and with qRT-PCR using a panel of RNA targets in BM samples at initial diagnosis during follow up from 50 patients

FU samples	Bone marrow cytology		Panel of RNA markers	
RASSF1a _M	+	-	+	-
+	7	18	21	4
-	0	25	5	20

NOTE: +, amplification of marker; -, no amplification of marker.

Abbreviations: FU: follow-up.

**Figure 3.** Logarithmic correlation between results of RASSF1a_M qRT-PCR and a panel of qRT-PCR targets. Results of PCR-based quantification by either RASSF1a_M or a panel of PCR targets in BM samples at diagnosis ($n = 21$) and BM samples at follow-up ($n = 9$). Quantification by PCR targets was done relative to the primary tumor and the median relative expression of 5 RNA PCR targets is depicted. Spearman's tests were carried out to assess the significance of the relationship between the 2 variables.

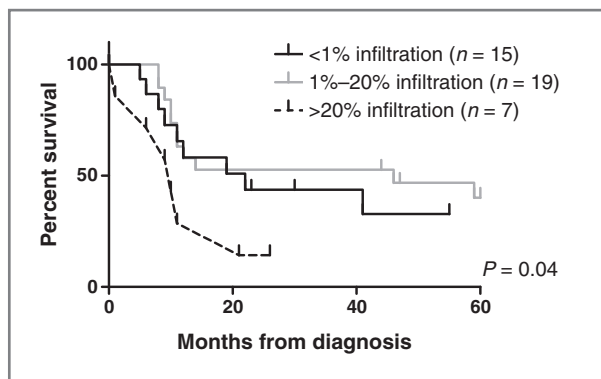


Figure 4. Correlation between tumor cell infiltration in the BM at diagnosis and survival in patients with stage 4 disease older than 1 year of age. Survival curve according to percentage of RASSF1a methylation in BM at diagnosis: solid line = low infiltration (<1%; $n = 15$), gray line = medium infiltration (1%–20%; $n = 19$), dashed line = high infiltration (>20%; $n = 7$); log rank test, 0.04.

patients with lower levels of BM infiltration ($P = 0.04$). Remarkably, there was no difference between patients with low (<1%) or intermediate (1%–20%) MRD levels.

Extent of RASSF1a methylation in NB tumors and survival

Patients with stage 4 NB older than 1 year having tumors with a high percentage RASSF1a methylation (>70%) had a significantly worse outcome than patients with a low percentage of RASSF1a methylation (5 years OS $19\% \pm 9$ vs. $56\% \pm 12$, respectively, $P = 0.02$; Fig. 5). The extent of RASSF1a methylation was higher in MYCN-amplified tumors compared with MYCN-nonamplified tumors (Supplementary Fig. S1A and S1B), in both patients without metastasis (stages 1–3) and with metastasis (stage 4). Thus the extent of RASSF1a methylation seems to correspond to more aggressive NB.

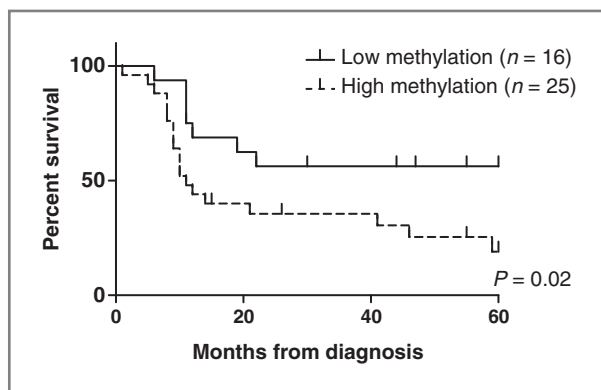


Figure 5. High levels of RASSF1a methylation in NB tumors is correlated with poor outcome in patients with stage 4 disease older than 1 year of age. Survival curve according to percentage of RASSF1a methylation in NB tumors of patients with stage 4 disease older than 1 year of age, dashed line = high methylation level (>70%; $n = 25$), solid line = low methylation level (<70%; $n = 16$); log rank test, 0.02.

Discussion

In this study, we describe methylated RASSF1a as the first DNA marker, which can be used for MRD detection in NB patients. In contrast to most RNA markers, RASSF1a_M was shown to be almost totally specific for NB tumor cells. Furthermore, the DNA-PCR specific for RASSF1a_M was found to reach similar sensitivity as the panel of RNA-PCRs when tested on a large panel of clinical samples of NB patients. In samples with tumor load around the detection limits of both assays, the 2 PCR approaches were complementary, thereby increasing the sensitivity of PCR-based MRD detection. Another advantage is that using RASSF1a_M as target, MRD levels in BM can be more accurately quantified than by using an RNA panel, as the DNA-PCR is unlike the RNA targets not dependent on variable expression levels, and the primary tumor is not needed anymore.

We chose RASSF1a_M as a potential DNA MRD marker, as RASSF1a is one of the most frequently methylated genes in NB and is not methylated in hematologic cells (21). In previous studies, it was shown that RASSF1 was methylated in only a subset of NB, which varied from 70% to 94% of all NB (8–10, 12, 13). However, we now showed that RASSF1a was methylated in all metastatic (stage 4 and 4s) tumors and only in some stage 1 to 3 tumors (3 of 21 tested) no methylation of RASSF1a was observed. None of the 3 patients with an unmethylated RASSF1a_M tumor had BM involvement at diagnosis, determined by qRT-PCR. Yang and colleagues (13) also found that the frequency of RASSF1a methylation was higher in stage 4. In conclusion, although RASSF1a is not methylated in all NB tumors, RASSF1a methylation seemed to be present in all tumors that are able to metastasize to the BM. Furthermore, it remains methylated in tumor cells metastasized to the BM, so it is applicable in most, if not all, patients for MRD detection in the BM.

The applicability of a PCR target for MRD detection is also determined by its background expression in hematologic cells. All RNA targets except *PHOX2B* used for MRD detection in NB are hampered by this background. In contrast, RASSF1a_M was only amplified in one of a triplicate (> C_t 40) in 50 control BM samples. Furthermore, the RASSF1a_M qRT-PCR reached the maximal theoretical sensitivity of 2.5 pg DNA (~1 allele of DNA; ref. 20).

In qualitative comparison between a panel of RNA markers PCR results for both assays were almost similar, only in BM samples with MRD levels around the detection limit for the positive PCR assay there were some discrepancies.

In addition, we showed that RASSF1a_M is a better marker for quantitative analysis of MRD levels than RNA PCR marker. For the CpG-rich region of RASSF1a analyzed in this study, a close correlation between methylation and gene silencing has been shown (11, 22). RASSF1a gene inactivation by allelic loss of chromosome 3p21.3 is a rare event in NB (10, 23, 24). Therefore, the percentage methylation corresponds to the percentage of tumor cell infiltration. Theoretically, a DNA marker is more suitable for MRD detection than an RNA marker. Although the percentage of

methylation varies between tumors and might be changed during therapy, this has less impact (within 1-log) on the estimated MRD level compared with the 3-log variation in expression level seen for RNA markers (7). Indeed, we show that in some cases RNA markers were downregulated in BM metastasis, whereas RASSF1a_M showed comparable quantification as BM morphology. Using RASSF1a_M for quantification of BM infiltration, we now observed that in patients with BM involvement, there was no difference in survival between patients with low (<1%) or intermediate (1%–20%) BM infiltration. Only the few patients with more than 20% NB cells had a significant worse prognosis. This is in line with results of Trager and colleagues (25) who reported that high expression of *TH* at diagnosis corresponds to poor outcome; our finding indicates that the mere presence of BM infiltration is the major prognostic factor, rather than the estimated level of infiltration. This can be explained by the patchy growth pattern of NB, in which the place of the biopsy might determine the level of infiltration.

As suggested by others, RASSF1a_M is prognostic for survival (13). Here we show that a high level of RASSF1a methylation in the primary tumor also seems to be correlated to survival. In both nonmetastatic and metastatic NB the extent of RASSF1a methylation was higher in MYCN-amplified tumors. This can be explained by the model proposed by Palakurthy and colleagues in which MYC facilitates recruitment of the DNA methyltransferase

DNMT3B to the *RASSF1a* promoter, which causes methylation of RASSF1a (26).

RASSF1a methylation is not specific for NB, as it occurs in a broad spectrum of tumors (27). The PCR on bisulfite-converted genomic DNA described in this article might therefore also be used to detect circulating tumor cells in, for example, breast or lung cancer in which now tumor-associated transcripts, such as CK19 (28) and CEA (29), are used.

In conclusion, in this article we present a novel sensitive and specific DNA qRT-PCR marker for MRD detection in BM cells of patients with NB; methylated RASSF1a. The data presented suggest that methylated RASSF1a should be included as marker in prospective MRD studies of NB, not only for detection but also for accurate quantification of MRD levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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