original reports

Hypermethylated *RASSF1A* as Circulating Tumor DNA Marker for Disease Monitoring in Neuroblastoma

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abstract

PURPOSE Circulating tumor DNA (ctDNA) has been used for disease monitoring in several types of cancer. The aim of our study was to investigate whether ctDNA can be used for response monitoring in neuroblastoma.

METHODS One hundred forty-nine plasma samples from 56 patients were analyzed by quantitative polymerase chain reaction (qPCR) for total cell free DNA (cfDNA; albumin and β-actin) and ctDNA (hypermethylated *RASSF1A*). ctDNA results were compared with mRNA-based minimal residual disease (qPCR) in bone marrow (BM) and blood and clinical patient characteristics.

RESULTS ctDNA was detected at diagnosis in all patients with high-risk and stage M neuroblastoma and in 3 of 7 patients with localized disease. The levels of ctDNA were highest at diagnosis, decreased during induction therapy, and not detected before or after autologous stem-cell transplantation. At relapse, the amount of ctDNA was comparable to levels at diagnosis. There was an association between ctDNA and blood or BM mRNA, with concordant results when tumor burden was high or no tumor was detected. The discrepancies indicated either low-level BM infiltration (ctDNA negative/mRNA positive) or primary tumor/soft tissue lesions with no BM involvement (ctDNA positive/mRNA negative).

CONCLUSION ctDNA can be used for monitoring disease in patients with neuroblastoma. In high-risk patients and all patients with stage M at diagnosis, ctDNA is present. Our data indicate that at low tumor load, testing of both ctDNA and mRNA increases the sensitivity of molecular disease monitoring. It is likely that ctDNA can originate from both primary tumor and metastases and may be of special interest for disease monitoring in patients who experience relapse in other organs than BM.

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INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor of childhood. In approximately 50%, patients present with high-risk (HR) disease and are treated with intensive multimodality treatment protocols that encompass induction therapy, primary tumor surgery, myeloablative chemotherapy with autologous stem-cell rescue, local irradiation, and anti-GD2–based immunotherapy[1] (Appendix Fig A1). Despite this intensive therapy, in approximately one half of HR patients, the tumor will relapse and result in a fatal outcome.[2] Assessment of treatment response is based on the International Neuroblastoma Response Criteria. Meta-iodobenzylguanidine (MIBG) scintigraphy, imaging (magnetic resonance imaging/positron emission tomography scans), and bone marrow (BM) examinations by histology or (immuno)cytology are combined to assess the extent of disease.[3] Because the median age at diagnosis is 18.8 months,[4] response evaluation that is based on imaging and BM testing often must be performed under general anesthesia. Therefore, alternative methods for monitoring response would potentially result in fewer risks to these patients. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a more sensitive technique for detection and monitoring of minimal residual disease (MRD) in neuroblastoma. Several prospective studies investigating the clinical significance of this technique for mRNA-based MRD detection in HR neuroblastoma are ongoing or have been published.[5-8]

However, even with mRNA-based RT-qPCR, many patients with low or negative MRD results during treatment will experience recurrent disease.[5,9] As an alternative to mRNA, circulating tumor DNA (ctDNA) might be a valuable source of tumor-derived material. ctDNA comprises circulating DNA fragments (cell free DNA [cfDNA]) that carry tumor-specific alterations, which can be found in the plasma of patients with cancer.[10-12] Because of the invasiveness of tumor biopsy and the lack of repeated biopsies during follow-up, the use of liquid biopsies is being investigated.

ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Can hypermethylated *RASSF1A* be used as a circulating tumor DNA (ctDNA) marker for minimal residual disease detection in neuroblastoma?

Knowledge Generated

When testing cell free DNA, we were able to detect tumor-derived hypermethylated *RASSF1A* in all patients with stage M disease at diagnosis. ctDNA levels decreased during treatment and were high again at relapse. Comparison between ctDNA and blood- or bone marrow (BM)–derived mRNA revealed that discrepancies were found when BM infiltration was low or when there were primary tumor lesions without BM involvement.

Relevance

ctDNA is an interesting source for monitoring disease in patients with neuroblastoma. Our data indicate that testing of both ctDNA and mRNA increases the sensitivity of molecular disease monitoring.

Several studies have shown the feasibility of detecting mutations or tumor-specific translocations in the ctDNA by high-depth targeted sequencing or mutation-specific PCR to monitor disease in various types of adult cancer.[13-19] However, neuroblastoma tumors, like many pediatric tumors, lack recurrent mutations and translocations. Broader analysis, such as whole-exome sequencing (WES) and shallow whole-genome sequencing (sWGS), to detect tumor-specific mutations or copy number alterations have been performed successfully using cfDNA of patients with neuroblastoma at diagnosis and relapse.[20-26] Nevertheless, these techniques are only informative when the ctDNA content is approximately ≥ 10%[27] and are, therefore, less suited for the detection of MRD, when the tumor burden is low.

In contrast to the copy number alterations and tumor-specific mutations, a methylation-specific qPCR assay could potentially be a more general and sensitive ctDNA marker. Previously, our group demonstrated that the *RASSF1A* gene is inactivated by hypermethylation in all stage M and MS and in 86% of localized neuroblastoma tumors. Hypermethylated *RASSF1A* (*RASSF1Am)* can be detected in BM with a similar sensitivity as mRNA and has shown added value in mRNA-negative BM.[28] In addition, *RASSF1Am* already has been described as a prognostic ctDNA marker at diagnosis.[29] The aim of this study was to investigate the feasibility of using ctDNA (*RASSF1Am* in plasma) to monitor treatment response in patients with HR/stage M neuroblastoma. We retrospectively performed qPCR for *RASSF1Am* on cfDNA from stored remains of previously collected plasma samples of patients with localized or metastatic neuroblastoma at diagnosis and for patients with HR/stage M neuroblastoma during treatment and at relapse. To test the additional value of ctDNA monitoring, we compared it with other techniques for disease monitoring: MIBG scans, urinary catecholamines, immunocytology, and RT-qPCR RNA-based MRD detection in BM and peripheral blood (PB).

METHODS

Between 2013 and 2016, from all consecutively diagnosed patients who were included in this study (N = 56), 149 PB samples for mRNA and cfDNA and 105 BM samples for mRNA were tested. Because stored remains were used, not all patients had samples for all time points. In this feasibility study, both HR and non-HR patients were included. Patients were treated at the Amsterdam University Medical Center (UMC), Erasmus Medical Center, or the Princess Máxima Center for Pediatric Oncology. Written informed consent from parents or guardians was obtained for all patients. The study was approved by the medical research ethics committee of Amsterdam UMC (MEC07/219#08.17.0836). Clinical data (including urinary catecholamines (homovanillic acid and vanillylmandelic acid) and imaging data (primary tumor [longest diameter], MIBG Curie score[30]) were collected from electronic patient files. Seventy-three PB samples from healthy adult male volunteers were collected as controls from Sanquin Blood Supply (Amsterdam, the Netherlands). Because *RASSF1Am* is also present in plasma of pregnant women, women were excluded as control donors. Pediatric PB control samples were collected from the Amsterdam UMC (Appendix Table A1).

Sample Collection, DNA Isolation, Bisulfite Conversion, and Real-Time qPCR

Methods for sample collection, DNA isolation, bisulfite conversion, and real-time qPCR for *RASSF1Am*[28] and mRNA markers[31] can be found in the Appendix and Appendix Table A2.

Data Analysis

Total cfDNA was quantified by qPCR for albumin (*ALB*) or β-actin (*ACTB*). A maximum of a 3.3-Ct difference between preconverted *ALB* and postconverted *ACTB* was accepted to ensure decent conversion. Samples with a ΔCT between *ALB* and *ACTB* > 3.3 are not included in the analysis for *RASSF1Am*. *RASSF1Am* was scored positive

not quantifiable (PNQ) if not all wells of the triplicate were positive or one of the replicates had a Ct value > 1.5 than the mean Ct of the replicates. Quantification of *RASSF1Am* was performed relative to the neuroblastoma cell line IMR-32. For quantification with mRNA markers, relative values were calculated using the equation $2^{\Delta\Delta}$CT (ΔCT sample – ΔCT IMR-32) × 100%. The median relative expression of 5 markers was used for the analysis. cfDNA and ctDNA levels were not normally distributed and are presented as median (interquartile range). Kruskal-Wallis tests were used for comparison of cfDNA or ctDNA levels. McNemar's test was used for concordance between ctDNA and PB and BM mRNA MRD levels. All statistical analyses were performed with SPSS version 23 (IBM Corporation, Chicago, IL) or GraphPad Prism 8 (GraphPad Software, La Jolla, CA) software.

RESULTS

Patients and Samples

From 48 patients with HR and/or stage M and 8 patients with non-HR neuroblastoma, 149 samples were tested in this study (Fig 1). From the 8 patients with non-HR neuroblastoma, only diagnostic samples were tested. Patient characteristics are listed in Table 1. Six of the 149 patient samples and 12 of 73 healthy control samples were not included for *RASSF1Am* qPCR because too much DNA had been lost during bisulfite conversion (Fig 1). In 2 of 61 adult control samples, *RASSF1Am* amplification was observed (Ct value, 40.1 and 37.1), but this occurred in only 1 of 3 replicates. In the 20 pediatric control samples, no amplification of *RASSF1Am* was found.

Amount of cfDNA

The amount of cfDNA per milliliter of plasma was determined by *ALB* or *ACTB* qPCR in 73 adult and 20 pediatric control samples and compared with 31 samples from patients with neuroblastoma (diagnosis or relapse). Compared with adult or pediatric control samples, samples from patients with neuroblastoma at diagnosis or relapse (all stages) had significantly more cfDNA (median, 1.5 ng/mL [interquartile range, 0.4-4.2 ng/mL], 3.1 ng/mL [interquartile range, 1.4-6.7 ng/mL], and 22.07 ng/mL [interquartile range, 5.7-98.90 ng/mL]; $P < .0001$ and $P = .0045$, respectively). Patients with stage M disease at diagnosis had the highest cfDNA levels (median, 73.1 ng/mL; interquartile range, 5.2-285.5 ng/mL; Fig 2A). There was no significant difference in total cfDNA levels during treatment and follow-up (Fig 2B). In the 28 samples where ctDNA was detected and quantified, the cfDNA levels were higher compared with the 86 patient samples where no ctDNA was detected (median, 34.2 ng/mL [range, 9.2-98.7 ng/mL] *v* 7.9 ng/mL [range, 3.5-25.8 ng/mL]; $P = .044$). Twenty-nine samples with detectable but not quantifiable ctDNA had significantly higher cfDNA levels (median, 12.9 ng/mL; range, 4.4-39.7 ng/mL) compared with adult control samples (median, 1.5 ng/mL; range, 0.4-4.2 ng/mL; $P < .001$). When no ctDNA was detected, cfDNA levels were still higher compared with adult control samples (median,

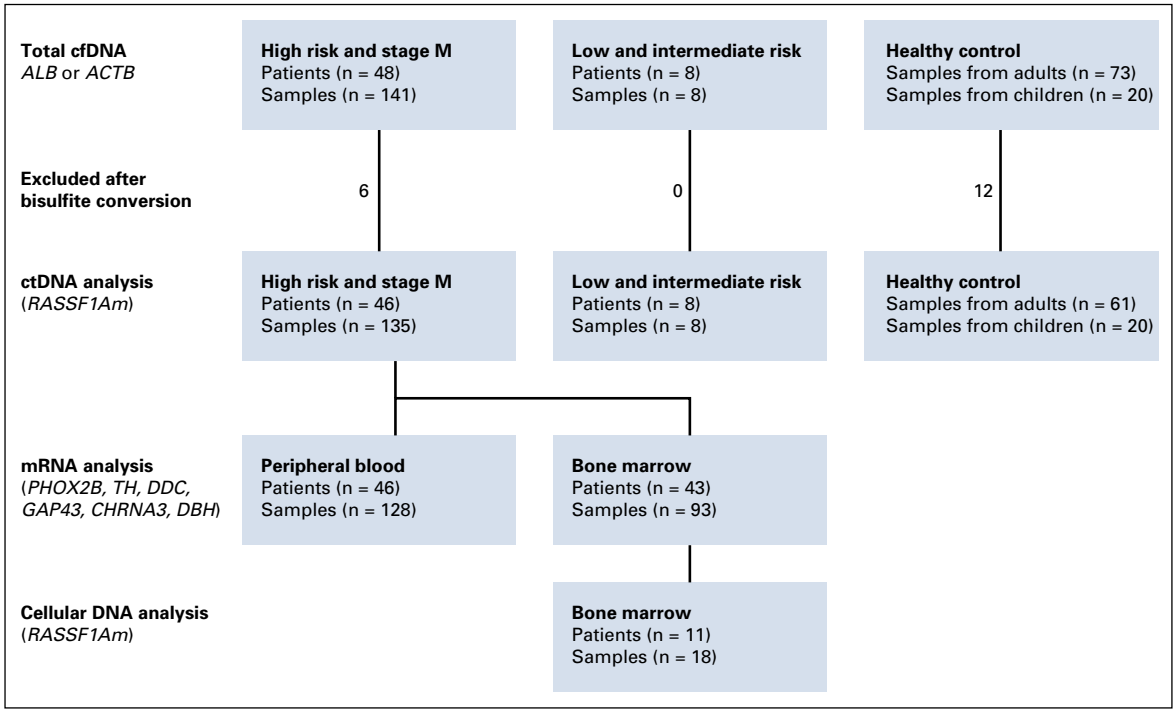

FIG 1. Flowchart of samples tested for total cell free DNA (cfDNA), number of samples excluded after too much DNA had been lost after bisulfite conversion, and number of samples tested for circulating tumor DNA (ctDNA) by hypermethylated *RASSF1A* (*RASSF1Am*). *ACTB*, β-actin; *ALB*, albumin.

TABLE 1. Patient Characteristics

Characteristic	No.
No. of patients	
Total	56
Female	26
Male	30
Age at diagnosis, months	
Median	33.6
Minimum	1.4
Maximum	394.9
Age > 18 months	45
MYCN status	
Amplified	16
Not amplified	37
Unknown	3
Chromosome 1p status	
Deletion	18
Normal	26
Unknown	12
Stage[a]	
L1, L2	10
M	45
MS	1
Risk group	
Low	5
Medium	5
High	46

[a]Stage according to the International Neuroblastoma Staging System.

7.9 ng/mL; range, 3.5-25.8 ng/mL; $P < .001$; Fig 2C). Compared with pediatric control donors, only patients with quantifiable ctDNA levels had significantly higher levels of cfDNA ($P = .0007$).

Level of ctDNA

In all diagnostic samples from patients with stage M neuroblastoma, *RASSF1Am* was detected. In 3 of 7 diagnostic samples from patients with localized disease and in 1 sample from a patient with stage MS disease, ctDNA was detected, although not in the quantitative range. During induction chemotherapy (patients with HR/stage M disease only), in 14 (38%) of 37 patients, ctDNA was detected (median Ct value, 30.6; min-max range, 24.7-33.8). At surveillance, 3 samples were positive, and these patients eventually experienced recurrent disease. In 8 of 9 samples from patients with relapse at the time of sampling, ctDNA was detected. Results are listed in Table 2 and in more detail in Appendix Table A3. The levels of ctDNA were highest at diagnosis, decreased during induction therapy, and undetectable at the end of induction chemotherapy. At relapse, ctDNA levels were comparable to levels at diagnosis (Fig 2D). The percentage of ctDNA of total cfDNA, calculated with the equation [*RASSF1Am* / (*RASSF1Am* + unmethylated *RASSF1A*) × 100], was 94% (range, 82%-98%) in the 14 diagnostic samples from patients with stage M disease. In the 28 samples where *RASSF1Am* could be quantified, the median percentage of ctDNA was 87% (range, 0.7%-99.9%); 29 additional samples were positive for *RASSF1Am* but could not be quantified.

Comparison of ctDNA and the Detection of Neuroblastoma mRNA in PB and BM

To study whether ctDNA, measured as *RASSF1Am*, can be used as an MRD marker in patients with HR/stage M disease, we compared it with our panel of mRNA markers.[31] In 128 matched PB samples, ctDNA could be compared with neuroblastoma mRNA, which demonstrated 79% concordant results (Fig 3A). Compared with the individual mRNA markers, *RASSF1Am* was more often positive, but the combined mRNA markers identified the same positive samples as *RASSF1Am* (Appendix Table A4).

In 93 matched BM mRNA and ctDNA (PB) samples, double-negative or double-positive results were found in 77% (Fig 3B). In contrast to PB, the BM mRNA panel identified more positive samples than ctDNA, and the individual markers *PHOX2B* and *TH* correlated best with *RASSF1Am* (Appendix Table A4).

Discrepant Findings Between ctDNA and PB or BM mRNA MRD

Discrepant results between ctDNA and mRNA were detected in 27 PB and 21 BM samples, respectively, and listed in Table 3 and Appendix Table A4. Total cfDNA levels in the ctDNA-positive/mRNA-negative samples were relatively high, with a median of 38.92 and 11.09 ng/mL for the BM mRNA-negative and PB mRNA-negative samples, respectively. From 3 of 5 BM mRNA-negative/ctDNA-positive samples, cryopreserved BM cells were available and tested all negative for *RASSF1Am*. In some patients (N850, N865, N732), the high levels of ctDNA probably correlated with the large primary or local relapse tumors, and these patients had no or very little BM infiltration.

In the ctDNA-negative samples, in general, the cfDNA levels were lower, with a median of 6.1 ng/mL for the BM mRNA-positive/ctDNA-negative and 1.52 ng/mL for the PB mRNA-positive/ctDNA-negative samples. In this group, the mRNA levels (in both BM and PB) were very low, mostly < 0.1%. From 15 of the 16 BM mRNA-positive/ctDNA-negative samples, cryopreserved cells were available and tested for *RASSF1Am*; of the 5 positive samples, 4 were not in the quantitative range, which indicated low levels of BM infiltration. In the samples from patients N777, N798, N2011, N2014, and N802, no ctDNA was detected. Apart from the very-low mRNA levels (only used for research purposes), N777 and N798 were considered to be in

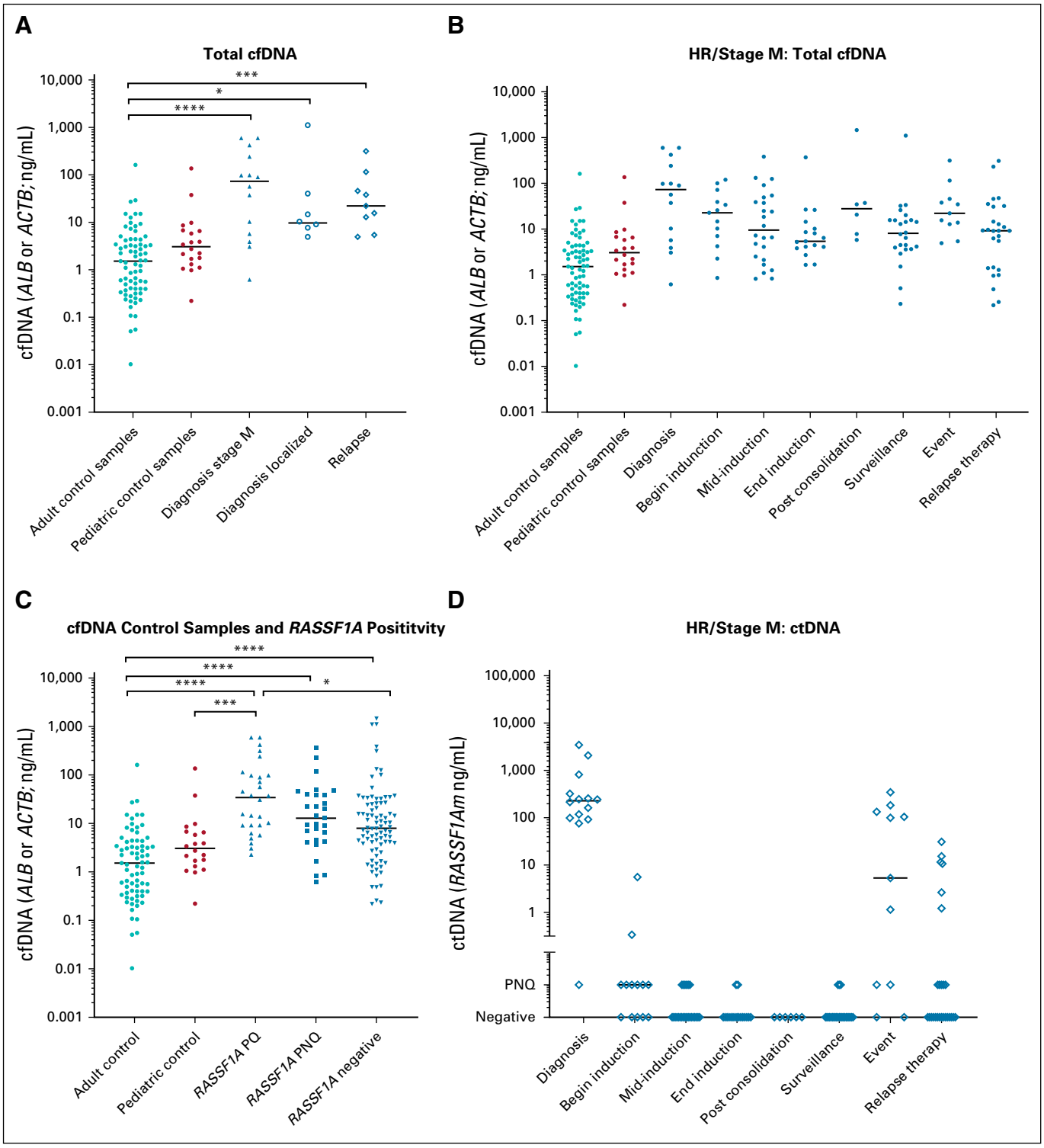

FIG 2. Amount of cell free DNA (cfDNA) and circulating tumor DNA (ctDNA). (A) Comparison of amount cfDNA (measured by albumin [*ALB*] or β-actin [*ACTB*]) between patients with neuroblastoma and healthy control donors. (B) Amount of cfDNA (measured by *ALB* or *ACTB*) at different time points during treatment. (C) Amount of cfDNA in samples with ctDNA compared with samples from healthy control donors and samples without ctDNA detected. (D) Amount of ctDNA (measured by hypermethylated *RASSF1A* [*RASSF1Am*]) at different time points during treatment. Begin induction indicates until 2 courses of induction therapy; mid-induction indicates after 3-5 courses of induction chemotherapy, unless additional courses were given after 6 courses, and samples before last course were also included at this time point; end induction indicates at the end of induction therapy; surveillance indicates during follow-up or at relapse suspicion; and event indicates relapse or progression. HR, high risk; PNQ, positive not quantifiable; PQ, positive and quantified. (*) $P < .05$, (**) $P < .01$, (***) $P < .001$, (****) $P < .0001$.

TABLE 2. Sample Characteristics: Detection of *RASSF1Am*

Characteristic	Total, No.	*RASSF1Am*, No.		
		Positive	PNQ	Negative
No. of samples	143			
Diagnosis samples				
Localized and stage MS	8	0	4	4
Stage M	14	13	1	0
Follow-up samples				
Beginning of induction therapy	13	2	6	5
Mid-induction therapy	24	0	6	18
End of induction therapy	17	0	2	15
Postconsolidation	6	0	0	6
Surveillance	24	0	3	21
Progression	2	1	0	1
Relapse/refractory disease	9	6	2	1
Relapse therapy	26	6	5	15

NOTE. Positive indicates that *RASSF1Am* circulating tumor DNA was detected and quantified, and negative indicates that no *RASSF1Am* circulating tumor DNA was detected.

Abbreviations: PNQ, positive not quantifiable; *RAFSSF1Am*, hypermethylated *RAFSSF1A*.

complete remission at that time. Subsequent samples (if available) showed negative MRD results. At the time of sampling for patients N2011 and N2014, the MIBG score was very low. Therefore, it is likely that the (biologically active) tumor load in these patients was very low. Patient N802 was treated for an isolated CNS relapse.

In the samples from patients N2012, N2013, N2016, N2024, N2029, and N2031, no ctDNA was detected, while low amounts of mRNA were detected in the BM. In the case of restricted, minimal BM disease, mRNA detection was more sensitive than ctDNA (Appendix Table A4). However, in some patients, a primary tumor was still present (median, 50 mm) while ctDNA was negative (Table 3).

DISCUSSION

ctDNA in plasma is a powerful source for the detection of tumor-derived aberrations in a minimally invasive setting. Many ctDNA studies in adults for the detection of MRD are based on detection of tumor-specific mutations by targeted sequencing or digital droplet PCR (ddPCR).[12,16,17] Because recurrent mutations are not common in neuroblastoma,[32] tumor-specific aberrations need to be characterized before they can be used as an MRD marker. However, temporo-spatial heterogeneity has been reported in neuroblastoma by several studies,[20,26] which raises the question of whether we should only use the small part of the tumor that is derived from the biopsy to design tumor-specific MRD markers. In the current study, we show that *RASSF1Am* is a universal marker for detecting ctDNA in patients with neuroblastoma. The use of *RASSF1Am* as an MRD marker has several potential benefits. First, it is a sensitive marker, with a sensitivity of 1 tumor cell in 10^5 mononuclear cells.[28] Second, *RASSF1Am* qPCR can be used in all patients with stage M neuroblastoma because it has been shown that *RASSF1A* is hypermethylated in all previously tested stage M neuroblastoma tumors.[28] Third, detection of *RASSF1Am* is less costly compared with WES and even sWGS (approximately 40- and 10-fold less expensive, respectively). Finally, we show in this report that *RASSF1Am* in plasma is tumor specific. Hypermethylation of *RASSF1A* has been described in several types of cancer and in physiologic circumstances in placental cells.[33] *RASSF1A* is not methylated in normal hematologic cells.[28,33,34] However, in 2 of 61 samples from healthy individuals, we detected very low, nonquantifiable levels of *RASSF1Am*. In

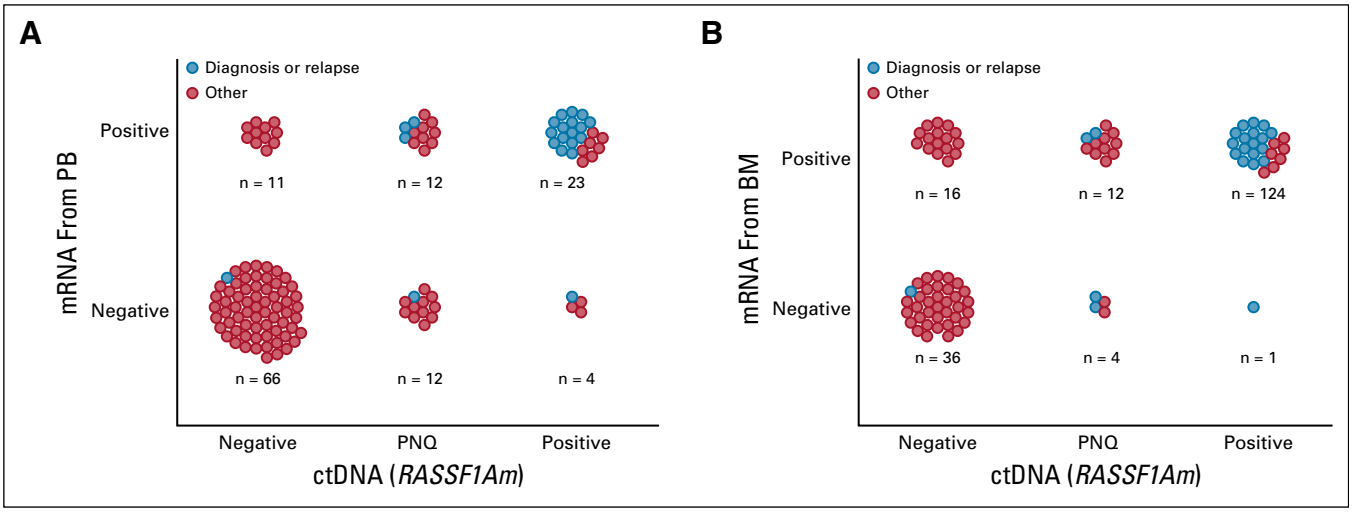

FIG 3. (A) Association between mRNA in peripheral blood (PB) samples and circulating tumor (ctDNA). (B) Association between mRNA in bone marrow (BM) samples and ctDNA. PNQ, positive not quantifiable; *RASSF1Am*, hypermethylated *RASSF1A*.

TABLE 3. Assessment of Discrepant Samples

		Discrepancy, Value																			
Sample	Time Point	ctDNA (ng/mL)		cfDNA (ng/mL)		PB mRNA (No.)		BM mRNA (No.)		BM % *RASSF1A*		BM IC (No.)		MIBG Curie Score		Primary Tumor		Catecholamines (HVA, VMA; No.)	
ctDNA pos, BM neg (n = 5)	Midinduction (n =1)	PNQ (n = 4)		Med	38.92	Neg	(1)	Neg	(5)	Neg	3	Neg	(4)	Pos	4	Neg	1	Not elevated	(3)
	End induction (n = 1)	PQ (n = 1; 105)		Min	12.87	< 0.1%	(4)					Quest	(1)	Med	2	Pos	1; 95	Elevated	(1)
	Relapse (n = 3)			Max	368.62									Min	1				
														Max	9				
ctDNA pos, PB neg (n = 16)	Diagnosis (n = 1)	PNQ (n = 12)		Med	11.02	Neg	(16)	Neg	(1)	Neg	1	Neg	(6)	Neg	2	Neg	0	Not elevated	(11)
	Begin induction (n = 6)	PQ (n = 4)		Min	0.86			< 0.1%	(8)			Quest	(3)	Pos	4	Pos	9	Elevated	(5)
	Midinduction (n = 3)	Med	13.0	Max	119.52			< 1%	(2)			< 1%	(2)	Med	12	Med	51		
	End induction (n = 1)	Min	5.6											Min	2	Min	30		
	Surveillance (n = 1)	Max	76.7											Max	24	Max	111		
	Relapse (n = 1)																		
	Relapse therapy (n = 3)																		
BM pos, ctDNA neg (n = 16)	Midinduction (n = 8)	Neg (n = 16)		Med	6.11	Neg	(14)	< 0.1%	(11)	Neg	10	Neg	(9)	Neg	2	Neg	4	Not elevated	(7)
	End induction (n = 4)			Min	0.51	< 0.1%	(2)	< 1%	(5)	PNQ	4	Quest	(4)	Pos	11	Pos	6	Elevated	(8)
	Postconsolidation (n = 1)			Max	309.73					0.062	1	< 1%	(1)	Med	4	Med	50		
	Progression (n = 1)													Min	1	Min	26		
	Surveillance (n = 1)													Max	19	Max	73		
	Relapse therapy (n = 1)																		
PB pos, ctDNA neg (n = 11)	Progression (n = 1)	Neg (n = 9)		Med	1.52	< 0.1%	(8)	Neg	(3)	PNQ	1	Neg	(4)	Neg	2	Neg	2	Not elevated	(10)
	Postconsolidation (n = 1)			Min	0.25	0.18	(1)	< 0.1%	(1)	0.06	1			Pos	3			Elevated	(1)
	Surveillance (n = 5)			Max	1,099.24									Med	1				
	Relapse therapy (n = 4)													Min	1				
														Max	5				

NOTE. Time point refers to the time point of the sample. ctDNA refers to *RASSF1Am* level relative to IMR-32. PB and BM mRNA levels are relative to IMR-32. BM % *RASSF1A* is *RASSF1Am* / (total *RASSF1A*). BM IC indicates anti-GD-2 IC on BM cytospins. MIBG Curie score[30] refers to segments 1-9. Primary tumor indicates largest diameter of primary tumor measured by magnetic resonance imaging in millimeters. Catecholamines are HVA and/or VMA elevation.

Abbreviations: BM, bone marrow; ctDNA, circulating tumor DNA; HVA, homovanillic acid; IC, immunocytology; Max, maximum; Med, median; MIBG, meta-iodobenzylguanidine; Min, minimum; Neg, negative; PB, peripheral blood; PNQ, positive not quantifiable; Pos, positive; PQ, positive and quantifiable; Quest, questionable; *RASSF1Am*, hypermethylated *RASSF1A*; VMA, vanillylmandelic acid.

addition, in other studies, infrequent detection of *RASSF1Am* has been observed in plasma samples from healthy control participants.[35,36] Therefore, when detecting very-low levels of *RASSF1Am* in patients with neuroblastoma (indicated as PNQ range), results should be analyzed with caution.

It has been shown that neuroblastoma tumors shed high amounts of ctDNA in the plasma.[25,26,37] In the current study, we found a median cfDNA concentration of 73.1 ng/mL at diagnosis for patients with stage M disease. This study confirms that cfDNA levels of patients with neuroblastoma are significantly higher than that of healthy donors, with patients with stage M disease having the highest levels. However, the levels we found are lower compared with previously published studies.[25,26,37] This inconsistency may be due to differences in isolation of cfDNA because we did not use a circulating nucleic acid kit or to differences in quantification methods. We used qPCR, whereas Chicard and colleagues[25,26] used the Qubit fluorometric assay (Thermo Fisher Scientific, Waltham, MA). We found the majority of the cfDNA (94% at diagnosis) to be tumor derived in patients with stage M or HR disease, which is also supported by previous research.[26,37]

We tested 143 samples from 54 patients with neuroblastoma and detected ctDNA in 57 samples. ctDNA was detected at diagnosis in all 14 patients with stage M and 4 of 8 patients with localized and stage MS neuroblastoma. Misawa et al[29] described detection of *RASSF1Am* at diagnosis in the serum of 17 of 68 patients (all stages) and in 11 of 18 patients with stage M disease. There are two likely causes for the increased ctDNA detection in our study. First, we used plasma, whereas Misawa et al tested serum, which is known to be more contaminated by genomic DNA originated from leukocytes during ex vivo clotting.[38] Second, Misawa et al used conventional PCR, which is less sensitive than qPCR. In the current study, ctDNA levels decreased during induction chemotherapy and were high again at relapse. This suggests that with increasing tumor burden, ctDNA levels also increase. Our group has previously described that hypermethylation of *RASSF1A* is variable in tumors of patients with stage MS (median, 65%) and localized (median, 30%) disease[28]; therefore, the level of ctDNA can be slightly underestimated in these patients when using *RASSF1Am* as marker.

We compared the performance of ctDNA with PB and BM mRNA in 128 and 93 samples, respectively. There was a strong correlation between ctDNA and BM mRNA when tumor burden was high or no tumor was detected. However, in some samples, discrepancies were observed for which additional clinical data about tumor response status were retrieved. Most patients in whom we detected relatively high levels of ctDNA compared with PB or BM mRNAs still had considerable tumor volumes or negative or low MIBG scores (data not shown); therefore, it is likely that the ctDNA in these patients originated from the primary tumor. No ctDNA was detected in 17 samples with very low PB or BM mRNA levels ($< 1\%$). Two of these patients were in complete remission but in the other 15 patients, considerable tumor volumes were detected on imaging or urine catecholamines were still positive, which indicate the need to optimize pre-analytic sample handling and prospective study of cfDNA kinetics in well-characterized patient cohorts with available paired (nuclear) imaging and BM assessment.

While the detection of ctDNA is very promising for future MRD studies, the current study has some limitations. Stored remains were used, which resulted in missing samples and paired clinical data. Prospective collaborative studies on the use of ctDNA in the new SIOPEN HR-2 (ClinicalTrials.gov identifier: NCT04221035) patient cohort are being initiated within the SIOPEN liquid biopsy group. For detection and quantification of low levels of ctDNA in the plasma, DNA extraction methods can be optimized with an isolation method specific for cfDNA, and ddPCR[39] may be a more suited technique compared with qPCR.[40] Moreover, large amounts of cfDNA (up to 96%) could be destroyed during bisulfite conversion[41]; therefore, we are investigating alternative methylation-specific ddPCR methods. Finally, Stutterheim et al[28] showed that the percentage of *RASSF1Am* can be variable in neuroblastoma tumors, especially in tumors of patients with localized disease. Previous studies showed that *RASSF1A* was the most frequent hypermethylated tumor suppressor gene in neuroblastoma as well as identified other hypermethylated tumor suppressor genes, and inclusion of these genes as MRD markers might increase the sensitivity.[42,43]

In this study, we used *RASSF1Am* as a ctDNA marker. We analyzed 135 sequential samples at diagnosis, during treatment, and at follow-up for 46 patients with HR/stage M neuroblastoma. In conclusion, ctDNA can be used for monitoring disease in patients with neuroblastoma. In HR patients and all patients with stage M at diagnosis, ctDNA is present. Our data indicate that at low tumor load, the testing of both ctDNA and mRNA increases the sensitivity of molecular disease monitoring. It is likely that ctDNA can originate from both primary tumor and metastases and may be of special interest for disease monitoring in patients who experience relapse in other organs than the BM.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Pinto NR, Applebaum MA, Volchenboum SL, et al: Advances in risk classification and treatment strategies for neuroblastoma. J Clin Oncol 33:3008-3017, 2015
2. Park JR, Kreissman SG, London WB, et al: Effect of tandem autologous stem cell transplant vs single transplant on event-free survival in patients with high-risk neuroblastoma: A randomized clinical trial. JAMA 322:746-755, 2019
3. Park JR, Bagatell R, Cohn SL, et al: Revisions to the International Neuroblastoma Response Criteria: A consensus statement from the National Cancer Institute Clinical Trials Planning Meeting. J Clin Oncol 35:2580-2587, 2017
4. London WB, Castleberry RP, Matthay KK, et al: Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol 23:6459-6465, 2005
5. Viprey VF, Gregory WM, Corrias MV, et al: Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: A European HR-NBL1/SIOPEN study. J Clin Oncol 32:1074-1083, 2014
6. Cheung NK, Ostrovnaya I, Kuk D, et al: Bone marrow minimal residual disease was an early response marker and a consistent independent predictor of survival after anti-GD2 immunotherapy. J Clin Oncol 33:755-763, 2015
7. Kreissman SG, Seeger RC, Matthay KK, et al: Purged versus non-purged peripheral blood stem-cell transplantation for high-risk neuroblastoma (COG A3973): A randomised phase 3 trial. Lancet Oncol 14:999-1008, 2013
8. Burchill SA, Beiske K, Shimada H, et al: Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma on behalf of the International Neuroblastoma Response Criteria Bone Marrow Working Group. Cancer 123:1095-1105, 2017
9. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, et al: The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. Eur J Cancer 47:1193-1202, 2011
10. Schwarzenbach H, Hoon DS, Pantel K: Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 11:426-437, 2011
11. Gormally E, Caboux E, Vineis P, et al: Circulating free DNA in plasma or serum as biomarker of carcinogenesis: Practical aspects and biological significance. Mutat Res 635:105-117, 2007
12. Wan JCM, Massie C, Garcia-Corbacho J, et al: Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer 17:223-238, 2017
13. Leary RJ, Kinde I, Diehl F, et al: Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2:20ra14, 2010
14. Diehl F, Schmidt K, Choti MA, et al: Circulating mutant DNA to assess tumor dynamics. Nat Med 14:985-990, 2008
15. McBride DJ, Orpana AK, Sotiriou C, et al: Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Genes Chromosomes Cancer 49:1062-1069, 2010
16. Dawson SJ, Tsui DW, Murtaza M, et al: Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 368:1199-1209, 2013
17. Tsao SC, Weiss J, Hudson C, et al: Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. Sci Rep 5:11198, 2015
18. Tie J, Kinde I, Wang Y, et al: Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol 26:1715-1722, 2015
19. Buono G, Gerratana L, Bulfoni M, et al: Circulating tumor DNA analysis in breast cancer: Is it ready for prime-time? Cancer Treat Rev 73:73-83, 2019

20. Van Roy N, Van Der Linden M, Menten B, et al: Shallow whole genome sequencing on circulating cell-free DNA allows reliable noninvasive copy-number profiling in neuroblastoma patients. Clin Cancer Res 23:6305-6314, 2017
21. Combaret V, Audoynaud C, Iacono I, et al: Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients. Cancer Res 62:3646-3648, 2002
22. Combaret V, Bergeron C, Noguera R, et al: Circulating MYCN DNA predicts MYCN-amplification in neuroblastoma. J Clin Oncol 23:8919-8920, 2005; author reply 8920
23. Combaret V, Bréjon S, Iacono I, et al: Determination of 17q gain in patients with neuroblastoma by analysis of circulating DNA. Pediatr Blood Cancer 56:757-761, 2011
24. Combaret V, Iacono I, Bellini A, et al: Detection of tumor ALK status in neuroblastoma patients using peripheral blood. Cancer Med 4:540-550, 2015
25. Chicard M, Boyault S, Colmet Daage L, et al: Genomic copy number profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma. Clin Cancer Res 22:5564-5573, 2016
26. Chicard M, Colmet-Daage L, Clement N, et al: Whole-exome sequencing of cell-free DNA reveals temporo-spatial heterogeneity and identifies treatment-resistant clones in neuroblastoma. Clin Cancer Res 24:939-949, 2018
27. Mouliere F, Chandrananda D, Piskorz AM, et al: Enhanced detection of circulating tumor DNA by fragment size analysis. Sci Transl Med 10:eaat4921, 2018
28. Stutterheim J, Ichou FA, den Ouden E, et al: Methylated RASSF1a is the first specific DNA marker for minimal residual disease testing in neuroblastoma. Clin Cancer Res 18:808-814, 2012
29. Misawa A, Tanaka S, Yagyu S, et al: RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: A prognostic marker. Br J Cancer 100:399-404, 2009
30. Yanik GA, Parisi MT, Shulkin BL, et al: Semiquantitative mIBG scoring as a prognostic indicator in patients with stage 4 neuroblastoma: A report from the Children's Oncology Group. J Nucl Med 54:541-548, 2013
31. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al: Detecting minimal residual disease in neuroblastoma: The superiority of a panel of real-time quantitative PCR markers. Clin Chem 55:1316-1326, 2009
32. Pugh TJ, Morozova O, Attiyeh EF, et al: The genetic landscape of high-risk neuroblastoma. Nat Genet 45:279-284, 2013
33. Chan KC, Ding C, Gerovassili A, et al: Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem 52:2211-2218, 2006
34. Hesson LB, Cooper WN, Latif F: The role of RASSF1A methylation in cancer. Dis Markers 23:73-87, 2007
35. Scheffer PG, de Haas M, van der Schoot CE: The controversy about controls for fetal blood group genotyping by cell-free fetal DNA in maternal plasma. Curr Opin Hematol 18:467-473, 2011
36. White HE, Dent CL, Hall VJ, et al: Evaluation of a novel assay for detection of the fetal marker RASSF1A: Facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. PLoS One 7:e45073, 2012
37. Bettegowda C, Sausen M, Leary RJ, et al: Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6:224ra24, 2014
38. Wong FC, Sun K, Jiang P, et al: Cell-free DNA in maternal plasma and serum: A comparison of quantity, quality and tissue origin using genomic and epigenomic approaches. Clin Biochem 49:1379-1386, 2016
39. Hindson BJ, Ness KD, Masquelier DA, et al: High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83:8604-8610, 2011
40. Brunetti C, Anelli L, Zagaria A, et al: Droplet digital PCR is a reliable tool for monitoring minimal residual disease in acute promyelocytic leukemia. J Mol Diagn 19:437-444, 2017
41. Grunau C, Clark SJ, Rosenthal A: Bisulfite genomic sequencing: Systematic investigation of critical experimental parameters. Nucleic Acids Res 29:e65, 2001
42. Hoebeeck J, Michels E, Pattyn F, et al: Aberrant methylation of candidate tumor suppressor genes in neuroblastoma. Cancer Lett 273:336-346, 2009
43. Kiss NB, Kogner P, Johnsen JI, et al: Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. BMC Med Genet 13:83, 2012

APPENDIX

Sample Collection

Clinical samples were collected in EDTA tubes and processed within 24 hours, and 2 mL peripheral blood (PB) or 0.5 mL bone marrow (BM) was transferred to PAXgene Blood RNA Tubes (QIAGEN, Venlo, the Netherlands) and stored at –20°C. The remainder of the blood samples were centrifuged to separate plasma from the PB cells (1,375 × *g* for 10 minutes without brake). Subsequently, the plasma was stored at –20°C. Mononuclear cells were isolated from the remaining BM sample by Ficoll density centrifugation and cryopreserved in 10% dimethyl sulfoxide.

DNA Isolation and Bisulfite Conversion

Dependent on the available plasma volume, DNA was extracted by using the QIAamp DNA Mini Blood Kit (QIAGEN) for 200 μL plasma, or the MagNA Pure 96 isolation robot (Roche, Basel, Switzerland) for 500-1,000 μL plasma and eluted in H_2O. After DNA isolation, bisulfite conversion was performed using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. Converted DNA samples were used directly or stored at –20°C.

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (qPCR) was performed as previously described (van Wezel EM, et al: J Mol Diagn 17: 43-52, 2015). Primers and probes were obtained from Eurogentec (Liege, Belgium). Primer and probe sequences have been described previously[28] (Scheffer PG, et al: BJOG 118:1340-1348, 2011) and are listed in Appendix Table A2. To control for DNA input, Albumin (*ALB*) (before bisulfite conversion) and β-actin (*ACTB*; after bisulfite conversion) qPCRs were carried out. Subsequently, qPCRs for unmethylated and hypermethylated *RASSF1A* were performed. qPCR for *ALB*, *ACTB*, and unmethylated *RASSF1A* was performed in duplicate; hypermethylated *RASSF1A* was tested in triplicate.

PB and BM mRNA Reverse Transcriptase qPCR

Total RNA from whole blood and BM samples was extracted using the PAXgene Blood RNA kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized and reverse transcriptase (RT) qPCR, with a maximum of 50 cycles was performed for β-glucuronidase (*GUSB*), paired ctDNA in neuroblastoma like homeobox 2B (*PHOX2B*), tyrosine hydroxylase (*TH*), dopa decarboxylase (*DDC*), growth-associated protein 43 (*GAP43*), cholinergic receptor nicotinic α-3 (*CHRNA3*), and dopamine β-hydroxylase (*DBH*), as has been described previously.[31] Expression was normalized to *GUSB* expression using the following equation: [normalized threshold cycle (ΔCT) = (Ct*GUSB* – Ctmarker)]. All RT-qPCR reactions were performed in triplicate (except *GUSB*, which was performed in duplicate), and mean values were used for analysis. Samples were scored for positivity according to previously published thresholds[9,31] (Stutterheim J, et al: J Clin Oncol 26:5443-5449, 2008). Samples with an insufficient Ct*GUSB* value (Ct value > 25, corresponding to < 5,000 copies) were excluded (Stutterheim J, et al: J Clin Oncol 26:5442-5449, 2008; Beillard E, et al: Leukemia 17:2474-2486, 2003; Gabert J, et al: Leukemia 17:2318-2357, 2003).

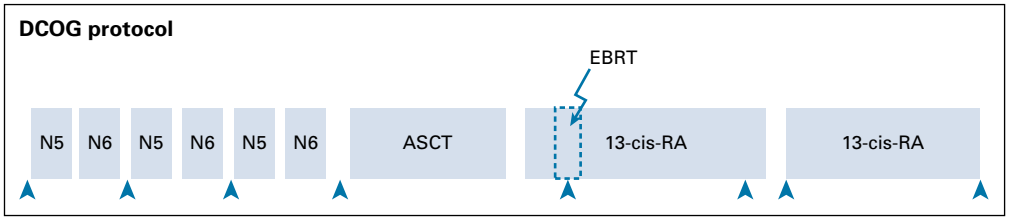

FIG A1. Overview of the DCOG NBL2009 treatment protocol. ASCT, autologous stem-cell transplantation and myeloablative therapy (carboplatin, etoposide, and melphalan); cis-RA, *cis*-retinoic acid; EBRT, external beam radiation therapy; N5, vindesine, etoposide, and cisplatin; N6, vincristine, dacarbazine, ifosfamide, and doxorubicin; S, surgery (was performed after vindesine, etoposide, and cisplatin and vincristine, dacarbazine, ifosfamide, and doxorubicin courses; optimal timing of surgery was discussed).

TABLE A1. Pediatric Control Group Data

Donor	Age, Years[a]	Sex
1	0.0	F
2	1.1	M
3	5.2	M
4	4.2	M
5	6.0	M
6	3.9	M
7	5.3	M
8	0.0	M
9	3.0	F
10	7.2	M
11	4.5	F
12	6.3	M
13	6.2	F
14	0.8	F
15	3.7	F
16	0.5	F
17	3.7	F
18	10.2	M
19	0.5	F
20	5.9	M

[a]Median age, 4.05 years; range, 0-10.2 years.

TABLE A2. Primer and Probe Sequences

Target	Forward Primer	Reverse Primer	Probe
Methylated *RASSF1A*	5′-GCG TTG AAG TCG GGG TTC-3′	5′-CCC GTA CTT CGC TAA CTT TAA ACG-3′	5′FAM-ACA AAC GCG AAC CGA ACG AAA CCA-TAMRA
Unmethylated *RASSF1A*	5′-TGT GTT TGT TAG TGT TTA AAG TTA GTG AAG TAT G-3′	5′-ACA CTC CAA CCA AAT ACA ACC CTT- 3′	5′FAM-CAC ACC CAA CAA ATA CCA ACT CCC ACA ACT-TAMRA
β-Actin	5′-TGG TGA TGG AGG AGG TTT AGT AAG T-3′	5′-AAC CAA TAA AAC CTA CTC CTC CCT TAA-3′	5′FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-TAMRA
Albumin	5′-TGA AAC ATA CGT TCC CAA AGA GTT T-3′	5′-CTC TCC TTC TCA GAA AGT GTG CAT AT-3′	5′FAM-TGC TGA AAC ATT CAC CTT CCA TGC AGA-TAMRA

TABLE A3. Clinical and Sample Data

	Clinical Data							Sample Results								
Patient No.	Age at Diagn, Months	Stage	*MYCN* Status	MIBG Segm 1-9	BM	Event	Death	Diagn	Progress 4S>4	Begin Induct	Mid-Induct	End Induct	PC	Surveill	Relapse/ Refract Disease	Relapse Therapy
N2001	74.4	M	0	0	Unk	1	1	1								
N2010	30.7	M	0	21	Pos	1	1				9					
N2011	43.7	M	0	22	Pos	0	0	1			0	0				
N2012	56.6	M	1	23	Pos	1	1				0 of 2	0	0			
N2013	52.5	M	0	23	Pos	0	0					0	0			
N2014	27.3	M	0	26	Pos	0	0				0	0				
N2015	6.8	M	0	0	Pos	1	0		1		9	0				
N2016	98.7	M	0	15	Pos	1	1								9 + 0	3 of 3
N2018	64.3	M	1	2	Neg	0	0	1		0	0	0				
N2019	20.2	L2	0	0	Neg	1	0	0								
N2020	22.5	M	1	4	Neg	1	1	1		0		0	0			
N2021	44.2	M	1	0	Neg	1	1	1								
N2022	26.3	M	1	20	Pos	1	1	1		0	0		0			
N2023	23.0	L2	0	0	Neg	1	0	0								
N2024	227.7	M	0	17	Pos	1	1	1		1	0 of 3					
N2026	28.0	L2	1	0	Neg	0	0			0						
N2028	76.0	L2	0	0	Neg	1	1	1								
N2029	78.6	M	1	18	Pos	1	1	1			1 of 2					
N2031	19.4	M	0	26	Pos	0	0			1	0	0				
N2032	13.0	M	0	5	Neg	1	0				0					0
N2033	5.4	M	0	7	Pos	0	0			1	0 of 2					
N2034	59.5	M	1	0	Neg	0	0					1				
N2035	1.4	L1	0	0	Neg	0	0	0								
N2037	9.7	L1	0	0	Neg	1	0	1								
N2042	19.2	L1	0	0	Neg	0	0	0								
N2043	76.4	M	0	Unk	Neg	1	1								1	
N2045	39.2	L1	0	0	Neg	0	0	1								
N2046	79.5	M	0	17	Pos	1	1	1								
N621	52.2	M	0	19	Pos	1	1								1	5 of 9
N649	23.6	M	0	14	Pos	1	1						2 of 3			1 of 4
N712	11.5	M	0	21	Pos	1	0							0		

(Continued on following page)

TABLE A3. Clinical and Sample Data (Continued)

	Clinical Data							Sample Results								
Patient No.	Age at Diagn, Months	Stage	*MYCN* Status	MIBG Segm 1-9	BM	Event	Death	Diagn	Progress 4S>4	Begin Induct	Mid-Induct	End Induct	PC	Surveill	Relapse/ Refract Disease	Relapse Therapy
N731	6.5	M	0	19	Unk	0	0							9		
N732	18.7	M	1	0	Pos	1	0							0	1	1 of 2
N733	70.7	M	0	22	Pos	1	0							0		
N764	197.4	M	0	15	Pos	1	1								1	
N769	87.1	M	0	16	Pos	0	0							0 of 2		
N770	84.0	M	0	0	Neg	1	1							1 of 3	2 of 2	0 of 3
N772	36.5	M	0	20	Pos	1	1							0 of 3	1 of 2	0
N777	113.1	M	9	22	Pos	0	0							0 of 2		
N785	5.9	M	1	2	Pos	0	0							0		
N798	79.0	M	0	5	Pos	0	0					0		0 of 2		
N800	65.3	M	0	15	Pos	0	0							0		
N802	56.4	M	0	27	Pos	1	1									9 + 0
N808	23.9	M	1	4	Neg	0	0					0				
N809	23.2	L2	1	0	Neg	0	0				1	0	0	0		
N810	39.1	M	1	25	Pos	1	1						0			
N819	7.2	L2	1	0	Neg	0	0			0						
N834	21.4	M	1	3	Pos	1	0	1		1	1			9	1	
N837	28.0	M	0	1	Pos	0	0	1		1	0 of 3	1		9		
N841	8.4	M	9	7	Neg	1	0	1			1					
N847	24.7	M	0	Unk	Unk	1	1									1 of 2
N848	2.8	MS	0	0	Unk	0	0	1								
N850	49.8	M	0	0	Neg	1	0	1		1	0 of 2	0				
N851	394.9	M	0	1	Pos	Unk	Unk	9		1		0		0 of 2		
N864	36.7	M	9	25	Pos	0	0					0		0		
N865	20.0	M	1	1	Pos	0	0	1		1	0	0				

NOTE. *MYCN* status: 0 = not amplified, 1 = amplification. MIBG segm 1-9: Curie scoring.[30] Event/death: 1 = yes, 0 = no. Sample characteristics: 1 = pos, 0 = neg, 9 = failure during bisulfite treatment; if multiple samples during period, results are depicted as number pos of total samples during period.

Abbreviations: BM, bone marrow; Diagn, diagnosis; Induct, induction; MIBG, meta-iodobenzylguanidine; Neg, negative; Pos, positive; Progress, progression; Refract, refractory; Segm, segment; Surveill, surveillance; Unk, unknown.

TABLE A4. *RASSF1Am* Results Versus mRNA Panel and Individual mRNA Markers

	RASSF1A_P		
Marker	**Negative**	**Positive**	**McNemar's Test *P***
PB_MRD_total			.442
Negative	66	16	
Positive	11	35	
PB_PHOX2B			> .001
Negative	74	23	
Positive	3	28	
PB_DDC			> .001
Negative	42	28	
Positive	0	4	
PB_TH			> .001
Negative	75	33	
Positive	2	18	
PB_CHRNA3			> .001
Negative	70	32	
Positive	7	19	
PB_DBH			.004
Negative	71	22	
Positive	6	29	
BM_MRD_total			.027
Negative	36	5	
Positive	16	36	
BM_PHOX2B			.078
Negative	37	6	
Positive	15	35	
BM_DDC			.012
Negative	14	10	
Positive	1	7	
BM_TH			.087
Negative	43	19	
Positive	9	22	
BM_CHRNA3			.001
Negative	48	22	
Positive	4	19	
BM_GAP43			> .001
Negative	49	23	
Positive	3	18	

Abbreviations: BM, bone marrow; MRD, minimal residual disease; PB, peripheral blood; *RASSF1Am*, hypermethylated *RASSF1A*.

