



Level of Seven Neuroblastoma-Associated mRNAs Detected by Droplet Digital PCR Is Associated with Tumor Relapse/Regrowth of High-Risk Neuroblastoma Patients

Thwin, Khin K. M. ; Ishida, Toshiaki ; Uemura, Suguru ; Yamamoto, Nobuyuki ; Lin, Kyaw S. ; Tamura, Akihiro ; Kozaki, Aiko ; Saito, ...

(Citation)

Journal of Molecular Diagnostics, 22(2):236-246

(Issue Date)

2020-02

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

© 2020 American Society for Investigative Pathology and the Association for Molecular Pathology. Published by Elsevier Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0>).

(URL)

<https://hdl.handle.net/20.500.14094/90006833>





Level of Seven Neuroblastoma-Associated mRNAs Detected by Droplet Digital PCR Is Associated with Tumor Relapse/Regrowth of High-Risk Neuroblastoma Patients

Khin K.M. Thwin,^{*} Toshiaki Ishida,[†] Suguru Uemura,^{*} Nobuyuki Yamamoto,^{*} Kyaw S. Lin,^{*} Akihiro Tamura,[†] Aiko Kozaki,[†] Atsuro Saito,[†] Kenji Kishimoto,[†] Takeshi Mori,[†] Daiichiro Hasegawa,[†] Yoshiyuki Kosaka,[†] Nanako Nino,^{*} Satoru Takafuji,^{*} Kazumoto Iijima,^{*} and Noriyuki Nishimura[‡]

From the Department of Pediatrics,^{*} Kobe University Graduate School of Medicine, Kobe; the Department of Hematology and Oncology,[†] Kobe Children's Hospital, Kobe; and the Department of Public Health,[‡] Kobe University Graduate School of Health Science, Kobe, Japan

Accepted for publication
October 17, 2019.

Address correspondence to
Noriyuki Nishimura, M.D.,
Ph.D., Kobe University Grad-
uate School of Health Science,
7-10-14 Tomogaoka, Suma-ku,
Kobe 654-0142, Japan. E-mail:
nnishi@med.kobe-u.ac.jp.

Monitoring of several sets of neuroblastoma-associated mRNAs (NB-mRNAs) by real-time quantitative PCR (qPCR) can be used to evaluate minimal residual disease in NB patients. Droplet digital PCR (ddPCR) is an adaption of qPCR that potentially provides simpler and more reproducible detection of low levels of mRNAs. However, whether minimal residual disease in NB patients can be monitored by ddPCR using a set of NB-mRNAs is not yet tested. In this study, 208 bone marrow (BM) and 67 peripheral blood samples were retrospectively collected from 20 high-risk NB patients with clinical disease evaluation at two Japanese centers between 2011 and 2018, and level of each NB-mRNA (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNAs) was determined by ddPCR. Level of 7NB-mRNAs (defined as the combined signature of each NB-mRNA) was higher in BM than peripheral blood, but correlated significantly with each other. In accordance with disease burden, it varied with disease status (remission, stable, or progression) and collection time point (diagnosis, treatment, post-treatment, or relapse). In 73 post-treatment BM samples, it was significantly higher in 17 relapsed/regrown samples than in 56 non-relapsed/nonregrown samples. Furthermore, ddPCR had a better prognostic value than qPCR in detecting 7NB-mRNAs in the same 73 post-treatment BM samples. This study suggests that ddPCR detection of 7NB-mRNAs is significantly associated with tumor relapse/regrowth in high-risk NB patients. (*J Mol Diagn* 2020, 22: 236–246; <https://doi.org/10.1016/j.jmoldx.2019.10.012>)

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by its extreme heterogeneity, ranging from spontaneous regression to malignant progression.^{1,2} Approximately half of the patients with NB have high-risk disease, whose long-term survival remains as low as 40%. Although intensive multimodal treatment including high-dose chemotherapy with autologous stem cell rescue, surgery, radiotherapy, and immunotherapy have been introduced over the past decades, 50% of high-risk patients still experience tumor relapse/regrowth caused by chemo-resistant minimal residual disease (MRD).^{3–5}

MRD is defined conceptually as residual tumor cells that persistently reside in cancer patients after local and systemic

cancer therapies.⁶ It persists as cancer stem cells in primary tumor, circulating tumor cells in peripheral blood (PB), disseminated tumor cells in bone marrow (BM) and lymph nodes, and micrometastasis in other tissues. These cancer stem cells, circulating tumor cells, and disseminated tumor cells represent the dynamics of MRD in cancer patients.^{7,8} Because of the invasive nature of surgical biopsies, the

Supported in part by Japan Society for the Promotion of Science, Japan KAKENHI Grant-in-Aid for Scientific Research 17K10110.

Disclosures: Part of this study also was funded by institutional research funding to Kobe University by the Sysmex Corporation (N.Nis.). N.Nis. filed an international patent application (PCT/JP2017/045715).

detection and analysis of circulating tumor cells and disseminated tumor cells by less-invasive sampling of BM and PB has been shown to be of clinical relevance in many cancer types.⁹

To improve the outcome of high-risk NB patients, the accurate and sensitive detection of MRD is essential to monitor the disease burden and treatment response. Because no recurrent oncogenic-fusion gene is identified in NB cells, MRD has been identified by detecting NB-associated mRNAs (NB-mRNAs) with real-time quantitative PCR (qPCR), which can define a cut-off value between NB cells and normal BM or PB cells.^{10,11} Although single NB-mRNA such as tyrosine hydroxylase (*TH*) and paired-like homeobox 2b (*PHOX2B*) initially were evaluated in BM and PB samples,^{12,13} multiple NB-mRNAs later were shown to be required to overcome tumor heterogeneity and achieve sensitive MRD detection. Recently, several sets of NB-mRNAs have been reported to have a significant prognostic value for NB patients.^{14–17} However, these NB-mRNAs adopted by different centers vary considerably and remain to be established in the clinics.

We previously validated 11 NB-mRNAs [cholinergic receptor, nicotinic, $\alpha 3$ (*CHRNA3*); collapsin response mediator protein 1 (*CRMP1*); dopamine beta-hydroxylase (*DBH*); doublecortin (*DCX*); dopa decarboxylase (*DDC*); GABA A receptor $\beta 3$ (*GABRB3*); growth-associated protein 43 (*GAP43*); ISL LIM homeobox 1 (*ISL1*); kinesin family member 1A (*KIF1A*); *PHOX2B*; and *TH* mRNAs) based on their expression in NB cancer stem cells by qPCR.¹⁸ This qPCR-based MRD monitoring in BM and PB has enabled the early detection of relapse/regrowth in two high-risk NB cases.¹⁹ In the present study, we tested 7NB-mRNAs (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNAs) for droplet digital PCR (ddPCR)-based MRD monitoring in high-risk NB patients to achieve more robust detection of low-levels of NB-mRNAs than qPCR.

Materials and Methods

NB Patients and Samples

Twenty high-risk NB patients, as defined by the International Neuroblastoma Risk Group,²⁰ who were treated at Kobe Children's Hospital or Kobe University Hospital between June 2011 and January 2018 based on the JN-H-11 (UMIN00005045) or JN-H-15 (UMIN00016848) protocol of the Japanese Children's Cancer Group Neuroblastoma Committee,²¹ were included. All BM and PB samples with written informed consent were collected as frequently as possible during the entire course of high-risk NB treatment. This study was approved by the Ethics Committee at Kobe University Graduate School of Medicine and Kobe Children's Hospital, and was conducted in accordance with the guidelines for Clinical Research of Kobe University Graduate School of Medicine.

Disease Evaluation

Response was graded at every BM and PB sampling time point for computed tomography/magnetic resonance imaging, metaiodobenzylguanidine, and BM assessments based on the available medical records, and these were combined into an overall response.²² Evaluation was conducted in accordance with the International Neuroblastoma Response Criteria,^{23,24} and the response assigned was remission, which corresponded to complete response or very good partial response; stable, which corresponded to partial response, mixed response, or no response; or progression, which corresponded to progressive disease. For patients with multiple collection time points, the most recent disease status was considered a new baseline for subsequent response evaluation. The disease status for all BM and PB samples then were assigned to remission, stable, or progression according to BM and overall response.

Non-NB Control Samples

All BM samples from non-NB controls were purchased from commercial sources; 1 sample from BioChain (Newark, CA), 2 samples from STEMCELL (Vancouver, Canada), 56 samples from AllCells (Alameda, CA), 16 samples from ReachBio (Seattle, WA), and 28 samples from Loza (Walkersville, MD) (Supplemental Table S1). All PB samples from non-NB controls were obtained from adult healthy volunteers; 107 samples were obtained from the Japanese Red Cross Society (Supplemental Table S2). The use of donated blood was approved by the Kobe University Hospital and the Japanese Red Cross Society Review Board.

RNA Isolation and cDNA Synthesis

All fresh BM and PB samples obtained from NB patients and adult healthy volunteers were separated using Mono-Poly resolving medium (DS Pharma Biomedical, Osaka, Japan), and nucleated cells were collected according to the manufacturer's instructions. For all frozen BM cells obtained from commercial sources, nucleated cells were prepared according to the manufacturer's instructions. Total RNA was extracted with a TRIzol Plus RNA purification kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and stored at -80°C until use. After measuring RNA concentration with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and evaluating its integrity using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer instructions, cDNA was synthesized from 1 or 0.5 μg total RNA using a Quantitect reverse transcription kit (Qiagen, Valencia, CA) that included DNase and priming oligo (a mixture of random and oligo-dT sequences), and diluted to a total volume of 80 or 40 μL . All cDNA samples were stored at -80°C (median, 1 year; range, 0–7 years) after processing the BM and PB samples.

ddPCR

ddPCR was performed using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μ L consisting of 10 μ L 2 \times ddPCR Supermix for probes (Bio-Rad Laboratories), 3.3 μ L each of 3 μ mol/L sense and antisense primers, 0.5 μ L of 10 μ mol/L Universal Probe Library probe (Roche, Mannheim, Germany), and 1 μ L sample cDNA (corresponding to 12.5 ng total RNA). After generating droplets with a QX200 Droplet Generator (Bio-Rad Laboratories), each cDNA was amplified with a C1000 Touch Thermal Cycler (Bio-Rad Laboratories). Thermal cycling conditions were a pre-cycling hold at 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 60°C for 1 minute, and a post-cycling hold at 98°C for 10 minutes. The ramp rate was 2°C per second. After ddPCR amplification, droplets were measured with a QX200 Droplet Reader (Bio-Rad Laboratories), and the target copy number was analyzed using QuantaSoft software version 1.6.6 (Bio-Rad Laboratories). By using nontemplate control reactions, thresholds were manually set as positive and negative droplet populations. To correct for differences in the amount of total RNA and the efficiency of cDNA synthesis, the target copy number was normalized using hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) as an endogenous reference. Only cDNA that resulted in a minimum of 0.045 copies per droplet of *HPRT1* mRNA was used to ensure the reproducible ddPCR reaction, and qualified cDNA was diluted further two to eight times to adjust a maximum of 0.45 copies per droplet. Consequently, ddPCR reactions were repeated two to three times for all samples from NB patients and non-NB controls. ddPCR analysis was performed in accordance with the digital Minimum Information for the publication of Quantitative PCR Experiments (MIQE) guidelines.²⁵

qPCR

qPCR was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA) in a total volume of 15 μ L consisting of 7.5 μ L of 2 \times FastStart Universal SYBR-Green Master (Roche), 1.5 μ L each of 3 μ mol/L sense and antisense primers, and 1 μ L of the sample cDNA (corresponding to 12.5 ng of total RNA). Each cDNA was amplified with a pre-cycling hold at 96°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds, and 1 cycle at 95°C for 15 seconds, 60°C for 60 seconds, 95°C for 30 seconds, and 60°C for 15 seconds. Each sample was analyzed in triplicate. To correct for differences in the amount of total RNA and in the efficiency of cDNA synthesis, β 2-microglobulin (*B2M*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and phosphoglycerate kinase 1 (*PGKI*) were used as endogenous references for normalization. Relative mRNA expression was evaluated by the Δ threshold cycle (C_T) method as described previously.¹⁷ Briefly, the C_T value for each gene was set as the cycle number where the amplification signal

reached a threshold of 0.5 over baseline, and 40 was assigned when this threshold was not reached by the 40th cycle. ΔC_T values for the detection genes were calculated as the geometric mean of the C_T values for the detection genes minus the geometric mean of the C_T values of the reference genes. Accordingly, lower ΔC_T values indicate a higher level of NB-mRNA. The qPCR analysis was performed in accordance with the MIQE guidelines.²⁶

Primers and Probes

Primers and probes were designed using ProbeFinder software version 2.50 at the Universal ProbeLibrary Assay Design Center (Roche). The following primers were used for ddPCR and qPCR (available from the Nucleotide Database, <https://www.ncbi.nlm.nih.gov/nucleotide>): *CRMP1* (accession number NM_001014809) 5'-CCAATCCCTTTATGCTGACG-3' (forward) and 5'-GGAACGATTAAGTTCTCTCCTATTTG-3' (reverse), *DBH* (accession number NM_000787) 5'-TGGGGACACTGCCTATTTTG-3' (forward) and 5'-TTCTGGGGTCTCTGCAC-3' (reverse), *DDC* (accession number NM_000790) 5'-CTGGAGAAGGGGGAGGAGT-3' (forward) and 5'-GCCGATGGATCACTTTGGT-3' (reverse), *GAP43* (accession number NM_002045) 5'-GAGGATGCTGCTGCCAAG-3' (forward) and 5'-GGCACTTTCCTTAGGTTTGGT-3' (reverse), *ISL1* (accession number NM_002202) 5'-AAGGACAAGAAGCGAAGCAT-3' (forward) and 5'-TTCCTGTCATCCCCTGGATA-3' (reverse), *PHOX2B* (accession number NM_003924) 5'-CTACCCCGACATCTACTACTCG-3' (forward) and 5'-CTCCTGCTTGCGAAACTTG-3' (reverse), *TH* (accession number NM_199292) 5'-TCAGTGACGCCAAGGACA-3' (forward) and 5'-GTACGGGTCGAACTTCACG-3' (reverse), *HPRT1* (accession number NM_000194) 5'-TGACCTTGATTTATTTTGCATACC-3' (forward) and 5'-CGAGCAAGACGTTTCAGTCCT-3' (reverse), *B2M* (accession number NM_004048) 5'-TTCTGGCCTGGAGGCTATC-3' (forward) and 5'-TCAGGAAATTTGACTTTCCATTC-3' (reverse), *GAPDH* (accession number NM_002046) 5'-AGCCACATCGCTCAGACAC-3' (forward) and 5'-GCCCAATACGACCAAATCC-3' (reverse), and *PGKI* (accession number NM_000291) 5'-GGAGAACCTCCGCTTTCAT-3' (forward) and 5'-GCTGGCTCGGCTTTAACC-3' (reverse). The following Universal ProbeLibrary probes (Roche) were used for ddPCR: *CRMP1* number 65, *DBH* number 3, *DDC* number 49, *GAP43* number 26, *ISL1* number 66, *PHOX2B* number 17, *TH* number 42, and *HPRT1* number 73.

Level of Each NB-mRNA (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA) and 7NB-mRNAs

The level of each NB-mRNA (each signature) was defined as the relative copy number of each NB-mRNA (each NB-mRNA copy number divided by *HPRT1* mRNA copy number and multiplied by 10,000). The level of 7NB-mRNAs

Table 1 Patient Characteristics

Variable	Category	Count
All patients		20
Sex	Male	14
	Female	6
Age at diagnosis	>18 months	3
	<18 months	17
Relapse/regrowth	Positive	12
	Negative	8
MYCN status	Amplified	8
	Nonamplified	12
DNA ploidy	Diploid	13
	Hyperploid	4
	Unknown	3
Histopathology*	Favorable	0
	Unfavorable	20
Bone marrow morphology at diagnosis†	Positive	18
	Negative	2

*Histopathology was either favorable or unfavorable and was evaluated according to the International Neuroblastoma Pathology Classification System.²²

†Bone marrow morphology at diagnosis was either positive or negative and was evaluated according to the International Neuroblastoma Response Criteria Bone Marrow Working Group.^{23,24}

MYCN, MYCN proto-oncogene, bHLH transcription factor.

(combined signature) was defined as the weighted sum of seven relative copy numbers (level of each NB-mRNA). The reciprocal of the 90th percentile in non-NB control samples was used for weighting each NB-mRNA. For the correlation of 7NB-mRNA levels between BM and PB, the level of 7NB-mRNAs in BM was calculated as the mean of right and left samples. For a direct comparison between ddPCR and qPCR, the level of 7NB-mRNAs was defined as the geometric mean of seven relative copy numbers (level of each NB-mRNA).

Statistical Analysis

The differences in the levels of each NB-mRNA and 7NB-mRNAs between two sample populations were evaluated by the *U*-test. Correlation of the level of 7NB-mRNAs between BM and PB samples was assessed by the Spearman rank correlation coefficient. The diagnostic and prognostic values of the levels of each NB-mRNA and 7NB-mRNAs was assessed using receiver operator characteristic analysis.²⁷ Reported *P* values were two sided and *P* < 0.05 was considered statistically significant. Statistical analyses were

performed with KaleidaGraph version 4.5.2 (Synergy Software, Reading, PA) and EZR version 1.38 (Saitama Medical Center, Jichi Medical University, Saitama, Japan, www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html, last accessed March 2019), which is a modified version of R commander designed to add statistical functions frequently used in biostatistics.²⁸

Results

Patient and Sample Characteristics

A total of 20 high-risk NB patients treated at Kobe Children's Hospital or Kobe University Hospital were included in the present study, with a median age of 33 months at diagnosis (range, 11 to 75 months) and a median follow-up time of 32 months (range, 13 to 73 months). Of these 20 patients, 60% (12 of 20) experienced tumor relapse/regrowth, 40% (eight of 20) had MYCN proto-oncogene, bHLH transcription factor–amplified tumors, and 90% (18 of 20) were positive for BM morphology at initial diagnosis (Table 1). During high-risk NB treatment that was widely used in Japan during the study period, BM and PB were sampled as frequently as possible. BM samples usually were collected from the right and left iliac crest.²⁴ Collection time points were classified as follows: diagnosis; treatment including induction chemotherapy, high-dose chemotherapy with autologous peripheral blood stem cell rescue, surgery, and radiotherapy; post-treatment including 13-cis-retinoic acid; and relapse; this resulted in 34 diagnosis samples, 116 treatment samples, 94 post-treatment samples, and 31 relapse samples (Table 2). The disease status at all sampling time points was assigned retrospectively as remission, stable, or progression by evaluating the available medical records, resulting in 72 remission samples, 137 stable samples, and 66 progression samples (Table 2). A total of 103 BM and 107 PB samples were obtained from healthy adults as non-NB controls (Supplemental Tables S1 and S2). In the present study, a total of 275 (208 BM and 67 PB) samples from 20 NB patients and 210 (103 BM and 107 PB) samples from 210 non-NB controls were analyzed by ddPCR.

Level of Each NB-mRNA (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA) in BM and PB

Previously developed qPCR-based MRD monitoring showed early detection of relapse/regrowth by consecutive

Table 2 Sample Characteristics

Sample type	Non-NB control	NB patient	Disease status			Collection time point			
			Remission	Stable	Progression	Diagnosis	Treatment*	Post-treatment†	Relapse
BM, sample/patient	103/103	208/20	58/10	104/18	46/17	24/14	89/16	73/15	22/10
PB, sample/patient	107/107	67/17	14/6	33/11	20/13	10/10	27/9	21/8	9/8

*Treatment comprised all collection time points during induction chemotherapy, high-dose chemotherapy with autologous peripheral blood stem cell transplantation, surgery, and radiation.

†Post-treatment comprised all collection time points from the completion of treatment to the clinical diagnosis of relapse/regrowth.

BM, bone marrow; NB, neuroblastoma; PB, peripheral blood.

Table 3 Detection of NB-mRNAs in Non-NB Control Samples

NB-mRNA	BM non-NB control, 103 samples				PB non-NB control, 107 samples			
	Median*	Interquartile range*	Detectable samples [†]	Positive rate, %	Median*	Interquartile range*	Detectable samples [†]	Positive rate, %
<i>CRMP1</i> mRNA	14.4	7.1–30.9	96	93.2	13.2	7.0–26.2	84	78.5
<i>DBH</i> mRNA	0.0	0.0–3.3	49	47.6	0.0	0.0–0.0	11	10.3
<i>DDC</i> mRNA	0.0	0.0–3.6	47	45.6	0.0	0.0–6.6	37	34.6
<i>GAP43</i> mRNA	2.5	0.0–5.0	65	63.1	7.4	0.0–13.9	72	67.3
<i>ISL1</i> mRNA	2.2	0.0–4.1	63	61.2	0.0	0.0–0.0	26	24.3
<i>PHOX2B</i> mRNA	0.0	0.0–2.9	51	49.5	0.0	0.0–0.0	23	21.5
<i>TH</i> mRNA	0.0	0.0–2.8	51	49.5	0.0	0.0–0.0	19	17.8

*Median and interquartile range are expressed as relative copy number.

[†]Detectable samples comprised the number of samples whose level of each NB-mRNA (relative copy number) was greater than 0 per number of all analyzed samples.

BM, bone marrow; CRMP1, collapsin response mediator protein 1; DBH, dopamine beta-hydroxylase; DDC, dopa decarboxylase; GAP43, growth-associated protein 43; ISL1, ISL LIM homeobox 1; NB-mRNA, neuroblastoma-associated mRNA; PB, peripheral blood; PHOX2B, paired-like homeobox 2b; TH, tyrosine hydroxylase.

MRD monitoring in two high-risk NB cases.^{18,19} To achieve the more robust detection of low levels of NB-mRNA than qPCR-based MRD monitoring, *CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNAs were used for ddPCR-based MRD monitoring in the present study. Although these NB-mRNAs were selected for no or little expression in normal cells by qPCR,

ddPCR detected them in 45.6% to 93.2% and in 10.3% to 78.5% of non-NB control BM and PB samples, respectively (Table 3). The level of each NB-mRNA in BM and PB varied greatly between individuals and collection time points, and was all significantly higher in diagnosis samples (24 BM and 10 PB samples collected from NB patients at diagnosis) than control samples (103 BM and

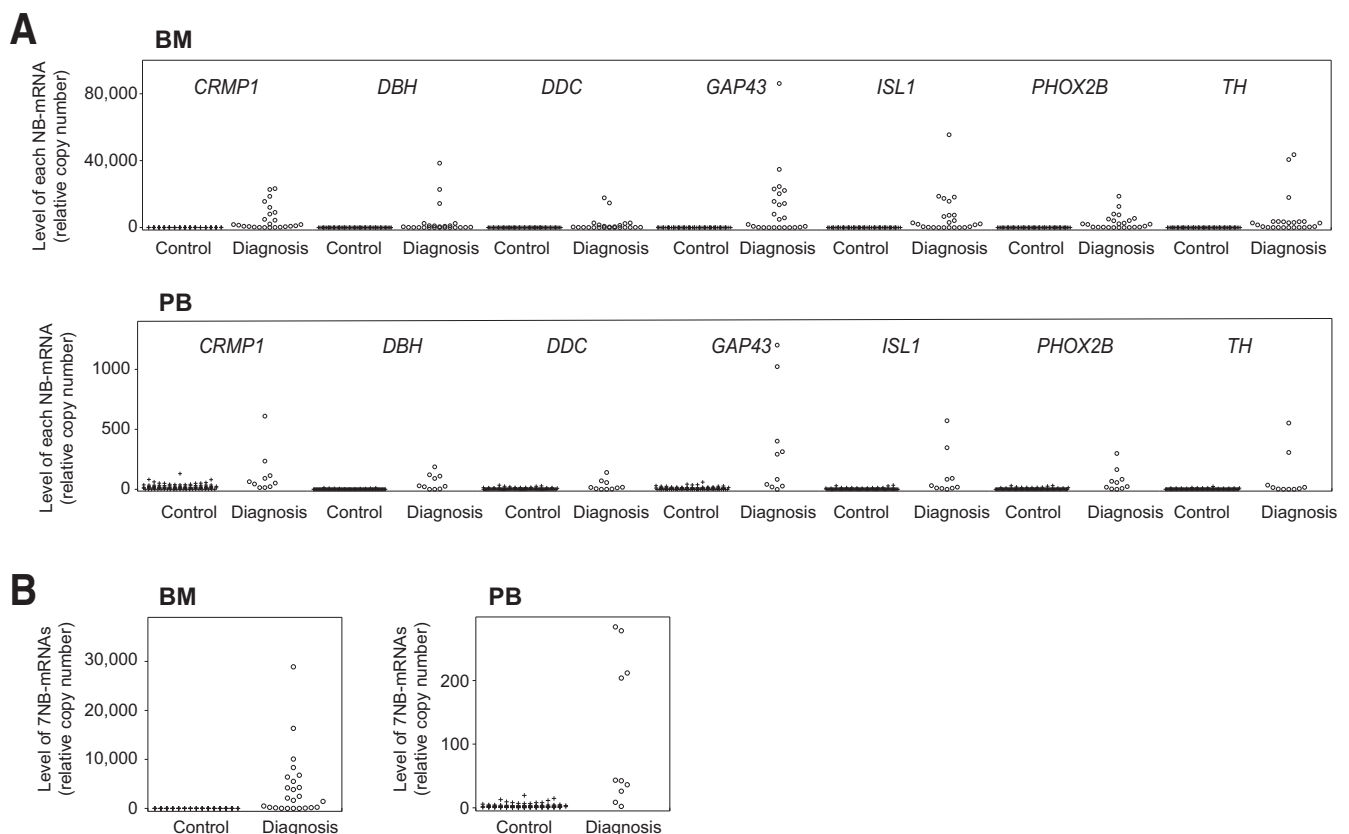


Figure 1 Levels of neuroblastoma-associated mRNAs (NB-mRNAs) [collapsin response mediator protein 1 (*CRMP1*), dopamine beta-hydroxylase (*DBH*), dopa decarboxylase (*DDC*), growth-associated protein 43 (*GAP43*), ISL LIM homeobox 1 (*ISL1*), paired-like homeobox 2b (*PHOX2B*), and tyrosine hydroxylase (*TH*) mRNAs] in bone marrow (BM) and peripheral blood (PB). **A:** Level of each NB-mRNA (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA, relative copy number) in BM and PB samples was determined by droplet digital PCR (ddPCR). **B:** Level of 7NB-mRNAs (relative copy number) in BM and PB samples was determined from the level of each NB-mRNA. $n = 103$ BM and 107 PB control samples obtained from non-NB controls; $n = 24$ BM and 10 PB diagnosis samples collected from high-risk NB patients at diagnosis.

Table 4 ROC Analysis of NB-mRNAs in Diagnostic Samples

NB-mRNA	BM Non-NB control, 103 samples NB diagnosis, 24 samples				PB Non-NB control, 107 samples NB diagnosis, 10 samples			
	TV	Sensitivity, %	Specificity, %	AUC	TV	Sensitivity, %	Specificity, %	AUC
Each signature*								
<i>CRMP1</i> mRNA	85.2	91.7	95.1	0.971	43.9	70.0	91.6	0.859
<i>DBH</i> mRNA	4.9	91.7	83.5	0.911	3.2	80.0	91.6	0.881
<i>DDC</i> mRNA	12.6	75.0	94.2	0.899	11.1	60.0	87.9	0.779
<i>GAP43</i> mRNA	8.0	83.3	87.4	0.905	22.0	90.0	91.6	0.901
<i>ISL1</i> mRNA	13.7	79.2	95.1	0.873	7.3	90.0	84.1	0.900
<i>PHOX2B</i> mRNA	17.9	87.5	98.1	0.974	3.2	90.0	80.4	0.896
<i>TH</i> mRNA	14.5	87.5	97.1	0.904	2.7	70.0	84.1	0.794
Combined signature [†]								
7NB-mRNAs	21.2	87.5	98.1	0.981	8.6	90.0	95.3	0.959

*Each signature is expressed as the relative copy number.

[†]Combined signature is expressed as the weighted sum of seven relative copy numbers.

7NB-mRNAs, *CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA neuroblastoma-associated mRNAs; AUC, area under curve; BM, bone marrow; *CRMP1*, collapsin response mediator protein 1; *DBH*, dopamine beta-hydroxylase; *DDC*, dopa decarboxylase; *GAP43*, growth-associated protein 43; *ISL1*, ISL LIM homeobox 1; PB, peripheral blood; *PHOX2B*, paired-like homeobox 2b; ROC, receiver operator characteristic; *TH*, tyrosine hydroxylase; TV, threshold value.

107 PB samples obtained from non-NB controls) (Figure 1A).

Level of 7NB-mRNAs in BM and PB

MRD previously was scored as positive when the expression of one or two of multiple NB-mRNAs exceeded a cut-off value between NB cells and normal BM or PB cells.^{14–16,18} However, the combined signature of five NB-mRNAs was shown to have a higher diagnostic value than other fewer NB-mRNA signatures in evaluating MRD by qPCR.^{17,29} To test whether the combined signature of our NB-mRNAs also was superior to each NB-mRNA signature in evaluating MRD by ddPCR, receiver operating characteristic analysis was performed for the level of each NB-mRNA and 7NB-mRNAs, defined as the weighting sum of the level of each NB-mRNA, in diagnosis samples (24 BM and 10 PB) and non-NB control samples (103 BM and 107 PB) (Table 4). Consistent with qPCR-based MRD monitoring,¹⁷ the level of 7NB-mRNAs was estimated as a higher area under the curve (AUC) than the level of each NB-mRNA (Table 4). Accordingly, the level of 7NB-mRNAs resulted in a stronger separation between the diagnosis and non-NB control samples (Figure 1B) and was used in the subsequent study.

Correlation of Level of 7NB-mRNAs between BM and PB

Because the correlation of 5NB-mRNA expression between BM and PB was reported in qPCR-based MRD monitoring,¹⁷ the correlation of the level of 7NB-mRNAs between BM and PB was tested in the present ddPCR-based MRD monitoring. Among 208 BM and 67 PB samples, 60 pairs of BM and PB samples were collected concurrently (<3 days apart), at eight diagnosis time points, 24 treatment

time points, 19 post-treatment time points, and nine relapse time points. The level of 7NB-mRNAs in these samples showed a significant positive correlation between BM and PB ($r = 0.613$; $P < 0.0001$), but was approximately 100 times higher in BM than PB (Figure 2).

Level of 7NB-mRNAs in BM and PB at Each Disease Status

To analyze the level of 7NB-mRNAs in relation to disease burden, disease status was evaluated for all BM and PB samples, and was assigned as remission, stable, or progression (see the *Materials and Methods* section). The level

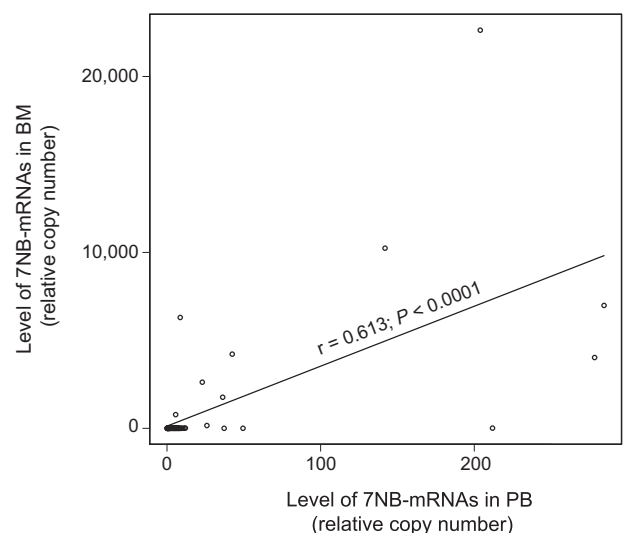


Figure 2 Correlation of the level of 7NB-mRNAs between bone marrow (BM) and peripheral blood (PB). The level of 7NB-mRNAs (relative copy number) in 60 pairs of concurrently collected BM and PB samples was determined by droplet digital PCR (ddPCR).

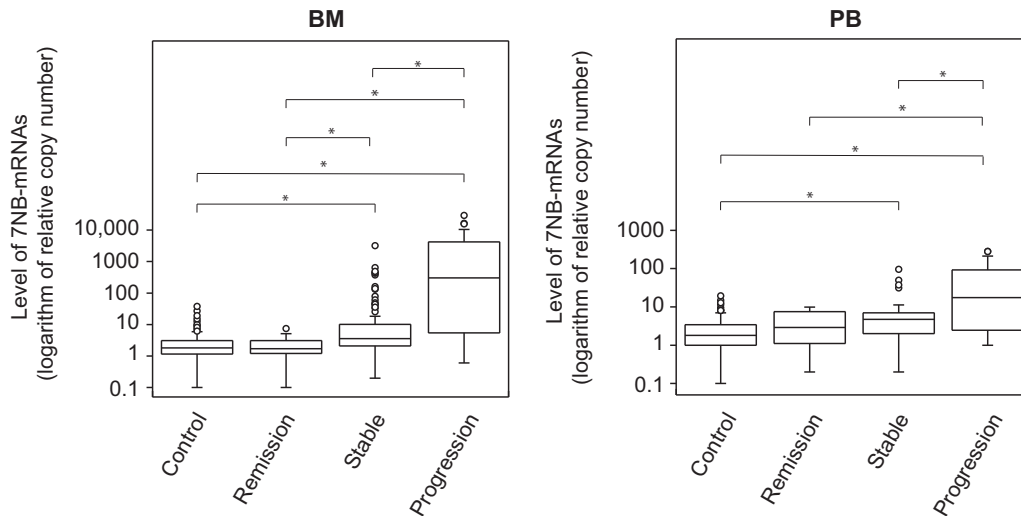


Figure 3 Level of 7NB-mRNAs in bone marrow (BM) and peripheral blood (PB) at each disease status. The level of 7NB-mRNAs (logarithm of relative copy number) in BM and PB at control, remission, stable, and progression was determined by droplet digital PCR (ddPCR). * $P < 0.05$. $n = 103$ BM and 107 PB control samples obtained from non-NB controls; $n = 58$ BM and 14 PB remission samples collected from high-risk NB patients; $n = 104$ BM and 33 PB stable samples collected from high-risk NB patients; $n = 46$ BM and 20 PB progression samples collected from high-risk NB patients.

of 7NB-mRNAs in BM and PB samples changed with disease status. In BM, it differed significantly in every combination of disease status (control, remission, stable, or progression) except for the control-remission combination (Figure 3, Table 5). In PB, it also differed significantly in every combination other than the control-remission and remission-stable combinations (Figure 3, Table 5).

Level of 7NB-mRNAs in BM and PB at Each Collection Time Point

The relationship of the level of 7NB-mRNAs to the collection time point was then analyzed. The level of 7NB-mRNAs in BM and PB samples varied with treatment response and disease burden. In BM, a significant difference was detected in every

Table 5 Detection of 7NB-mRNAs in NB Patient Samples

Sample group	BM, 208 samples			PB, 67 samples		
	Sample number	Median*	Interquartile range*	Sample number	Median*	Interquartile range*
Disease status						
Remission	58	1.6	1.1–3.0	14	2.9	1.1–7.1
Stable	104	3.5	2.0–10.0	33	4.6	2.0–7.0
Progression	46	301.7	5.5–4110.0	20	17.5	2.4–67.9
Collection time point						
Diagnosis	24	1880.8	146.8–5738.1	10	42.8	28.6–209.9
Treatment [†]	89	3.8	2.0–12.4	27	5.2	1.6–7.1
Post-treatment [‡]	73	1.8	1.1–3.1	21	2.8	1.3–6.1
Relapse	22	16.9	2.2–1221.5	9	3.5	1.8–12.0
BM nonrelapse, 97 samples						
BM relapse, 89 samples						
Collection time point						
Diagnosis	7	6395.8	315.5–13214.0	17	1650.9	109.3–4194.7
Treatment [†]	34	10.1	2.5–56.5	55	3.3	2.0–5.5
Post-treatment [‡]	56	1.7	1.1–2.4	17	3.5	1.5–4.5

*Median and interquartile range are expressed as relative copy number.

[†]Treatment comprised all samples collected during induction chemotherapy, high-dose chemotherapy with autologous peripheral blood stem cell transplantation, surgery, and radiation.

[‡]Post-treatment comprised all samples collected from the completion of treatment to the clinical diagnosis of relapse/regrowth.

7NB-mRNAs, *CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA neuroblastoma-associated mRNAs; BM, bone marrow; *CRMP1*, collapsin response mediator protein 1; *DBH*, dopamine beta-hydroxylase; *DDC*, dopa decarboxylase; *GAP43*, growth-associated protein 43; *ISL1*, ISL LIM homeobox 1; PB, peripheral blood; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

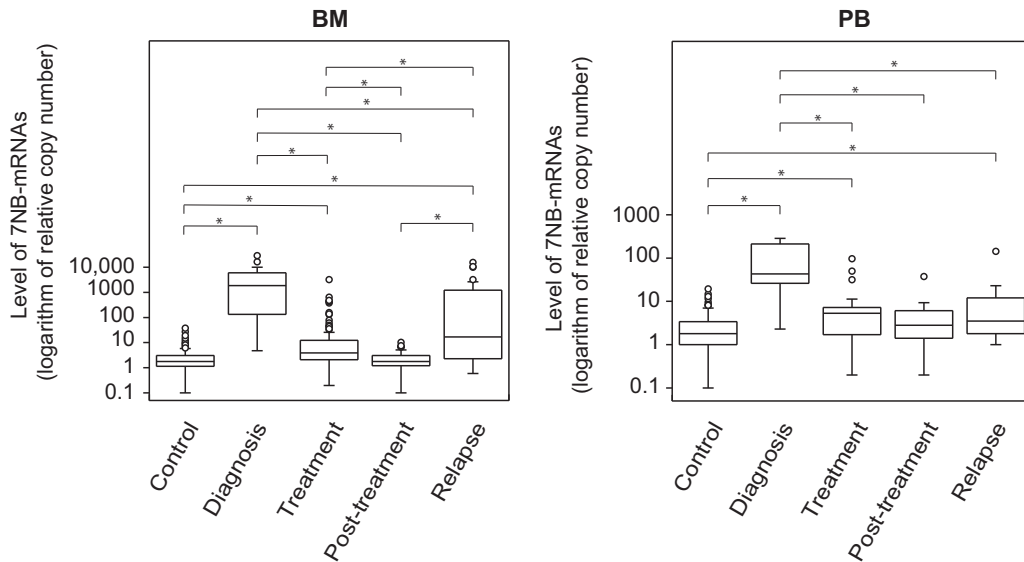


Figure 4 Level of 7NB-mRNAs in bone marrow (BM) and peripheral blood (PB) at each collection time point. The level of 7NB-mRNAs (logarithm of relative copy number) in BM and PB at control, diagnosis, treatment, post-treatment, and relapse was determined by droplet digital PCR (ddPCR). * $P < 0.05$. $n = 103$ BM and 107 PB control samples obtained from non-NB controls; $n = 24$ BM and 10 PB diagnosis samples collected from high-risk NB patients; $n = 89$ BM and 27 PB treatment samples collected from high-risk NB patients; $n = 73$ BM and 21 PB post-treatment samples collected from high-risk NB patients; $n = 22$ BM and 9 PB relapse samples collected from high-risk NB patients.

combination of different collection time points (control, diagnosis, treatment, post-treatment, and relapse) except for the control–post-treatment combination (Figure 4, Table 5). In PB, significant difference was shown only in the combinations containing control or diagnosis other than the control–post-treatment combination (Figure 4, Table 5).

Association between Level of 7NB-mRNAs in BM and Tumor Relapse/Regrowth

Because the level of 7NB-mRNAs in BM differed significantly between post-treatment and relapse samples (Figure 4), its association with patient outcome then was

investigated. Diagnosis and post-treatment BM samples were divided further into nonrelapsed/nonregrown (non-relapse) and relapsed/regrown (relapse) samples because of the considerably different sampling time points (ranging from induction chemotherapy to surgery and radiotherapy) in treatment BM samples (Table 5). In 24 diagnosis samples, the level of 7NB-mRNAs in seven nonrelapse samples did not differ significantly from 17 relapse samples (Figure 5). In 73 post-treatment samples, 17 relapse samples expressed a significantly higher level of 7NB-mRNAs than 56 nonrelapse samples (Figure 5), suggesting a significant association between the level of 7NB-mRNAs in BM and tumor relapse/regrowth.

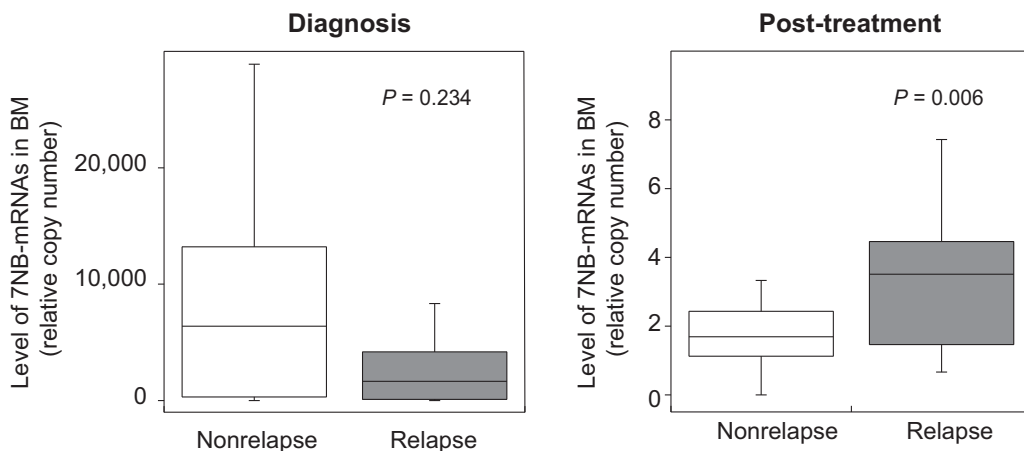


Figure 5 Association between the level of 7NB-mRNAs in bone marrow (BM) with tumor relapse/regrowth. The level of 7NB-mRNAs (relative copy number) in nonrelapse and relapse BM samples at diagnosis and post-treatment was determined by droplet digital PCR (ddPCR). $n = 7$ diagnosis and 56 post-treatment BM samples collected from high-risk NB patients (nonrelapse); $n = 17$ diagnosis and 17 post-treatment BM samples collected from high-risk NB patients (relapse).

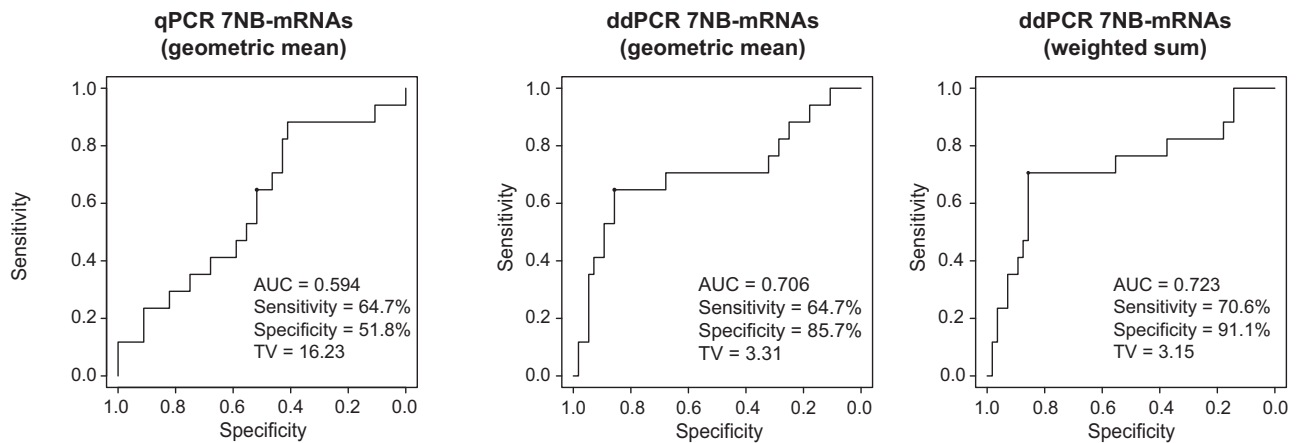


Figure 6 Prognostic values for real-time quantitative PCR (qPCR) and droplet digital PCR (ddPCR) detection of 7NB-mRNAs in post-treatment bone marrow (BM) samples. Receiver operator characteristic curves were plotted for the level of qPCR 7NB-mRNAs (geometric mean) and ddPCR 7NB-mRNAs (geometric mean and weighted sum) in 56 nonrelapse and 17 relapse post-treatment BM samples. AUC, area under curve; TV, threshold value.

Prognostic Values for qPCR and ddPCR Detection of 7NB-mRNAs in Post-Treatment BM Samples

Although ddPCR potentially provides more simple and reproducible detection of low levels of NB-mRNAs than qPCR, their prognostic values have never been compared in the same samples from NB patients. First, we determined the level of each NB-mRNA (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA) in the same 73 (56 nonrelapse and 17 relapse) post-treatment BM samples by qPCR (Supplemental Table S3). The receiver operator characteristic curve then was plotted for level of qPCR 7NB-mRNAs (geometric mean).¹⁷ The level of qPCR 7NB-mRNAs (geometric mean) estimated threshold value was 16.23 and the AUC was 0.594 (Figure 6).

To make a direct comparison between qPCR and ddPCR, the receiver operator characteristic curve was plotted for the level of ddPCR 7NB-mRNAs (geometric mean) in the same 73 (56 nonrelapse and 17 relapse) post-treatment BM samples (Supplemental Table S3). The level of ddPCR 7NB-mRNAs (geometric mean) estimated a threshold value of 3.31 and an AUC of 0.706 with significant accuracy (AUC > 0.7) (Figure 6), showing that ddPCR can provide a better prognostic value than qPCR in detecting MRD in NB patient samples. The level of ddPCR 7NB-mRNAs (weighted sum) resulted in the further improved estimation of threshold value of 3.15 and AUC of 0.723 with significant accuracy (AUC > 0.7) (Figure 6), validating a better prognostic value of ddPCR. These results suggested that a high level of ddPCR 7NB-mRNAs in BM was associated with tumor relapse/regrowth in high-risk NB patients.

Discussion

In the present study, we determined the level of *CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA in BM and PB by ddPCR for evaluating MRD in high-risk NB

patients. To our knowledge, this is the first study of ddPCR-based MRD monitoring in NB patients and shows that ddPCR detection of 7NB-mRNAs in BM has a significant prognostic value in predicting tumor relapse/regrowth of high-risk NB patients (Figure 6).

ddPCR is the most sensitive method to measure the abundance of specific nucleic acids and potentially provides more accuracy and reproducibility in quantitating NB-mRNAs compared with qPCR.^{30,31} To effectively make the most of the advantage of ddPCR over qPCR,^{32,33} *HPRT1* mRNA was carefully validated as an endogenous reference for NB-mRNA and the qualified cDNA template was prepared to yield 0.045 to 0.45 copies per droplet of *HPRT1* mRNA. Accordingly, each NB-mRNA was detected in more than 45% and 10% of non-NB control BM and PB samples, respectively (Table 2). The ddPCR detection of low levels of NB-mRNA in more than 100 non-NB control samples showed for the first time that there was no significant difference in the expression of NB-mRNAs between remission and non-NB control samples (Figures 3 and 4). This finding prompted us to reconsider the cut-off value for MRD evaluation in NB patient samples. Previously, we and others set a cut-off value for each NB-mRNA based solely on its expression in normal cells, and then scored patient samples as MRD positive when the expression of one or two NB-mRNA exceeded the cut-off value.^{18,34,35} However, a recent study reported that the combined signature of multiple NB-mRNAs had a better diagnostic value than other fewer NB-mRNA signatures in evaluating MRD by qPCR.¹⁷ In the present study, we also confirmed that the level of 7NB-mRNAs estimated higher AUCs than the level of each NB-mRNA by ddPCR (Table 4).

ddPCR potentially provides more simple and reproducible detection of low levels of NB-mRNAs than qPCR, although their direct comparison has not been reported. The present study tested whether ddPCR detection of 7NB-mRNAs predicted tumor relapse/regrowth more accurately than qPCR detection of the same 7NB-mRNAs in the same

post-treatment BM samples from high-risk NB patients. The present results have shown clearly for the first time that ddPCR detection of 7NB-mRNAs estimates a higher AUC (>0.7) than qPCR detection of 7NB-mRNAs (Figure 6, Supplemental Table S3). It warrants a further validation of ddPCR detection of 7NB-mRNAs in a prospective clinical studies.

Although the correlation of NB-mRNA expression between BM and PB by qPCR has remained elusive,^{17,35} the present ddPCR-based MRD monitoring showed its positive correlation. Consistent with qPCR detection of 5NB-mRNAs (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH* mRNA) in high-risk NB patients with relapsed/refractory disease,¹⁷ ddPCR detection of 7NB-mRNAs (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA) showed a significant positive correlation between BM and PB, but was approximately 100 times higher in BM than PB in high-risk NB patients (Figure 2). Although PB sampling was less invasive and able to capture the broader landscape of tumors than BM sampling,³⁶ PB was not likely a sensitive surrogate for BM in NB patients.

The most striking result of the present study was the association of ddPCR detection of 7NB-mRNAs with relapse/regrowth in post-treatment BM samples from high-risk NB patients (Figures 5 and 6). This would suggest that ddPCR-based MRD monitoring in BM enabled risk stratification of high-risk NB patients. Several other studies previously have shown that monitoring NB-mRNAs in BM and PB by qPCR provided a prognostic value for high-risk NB patients.^{14–17} The presence of high levels of 5NB-mRNAs (*CHRNA3*, *DDC*, *GAP43*, *PHOX2B*, and *TH* mRNA) during and at the end of induction therapy in BM was associated with a poor outcome,¹⁴ whereas the presence of high levels of 3NB-mRNAs (*DCX*, *PHOX2B*, and *TH* mRNA) was associated with a poor outcome at diagnosis in BM or PB and at the end of induction therapy in BM.¹⁵ For the more specified subgroups of NB patients, progression-free survival of relapsed/refractory NB patients was associated with the level of 5NB-mRNAs (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH* mRNA),¹⁷ and survival of NB patients treated with anti-disialoganglioside antibody also was predicted by the level of 4NB-mRNAs (*B4GALNT1*, *CCND1*, *ISL1*, and *PHOX2B* mRNA) after two cycles of immunotherapy in BM.¹⁶

However, there were several limitations in the present study. Although detection of 7NB-mRNAs (*CRMP1*, *DBH*, *DDC*, *GAP43*, *ISL1*, *PHOX2B*, and *TH* mRNA) in BM was associated strongly with tumor relapse/regrowth and correlated significantly with that in PB, its prognostic value in PB has remained elusive. Similarly, the level of 7NB-mRNAs at diagnosis in BM did not predict tumor relapse/regrowth in contrast to the strong predictive power of 3NB-mRNAs (*DCX*, *PHOX2B*, and *TH* mRNA) at diagnosis for patient outcome.¹⁵ These limitations might be derived from the restricted number of NB patients and hospitals in the present study.

In conclusion, ddPCR is a simple and reproducible method to detect low levels of NB-mRNAs in BM and PB samples collected from high-risk NB patients. High levels of 7NB-mRNAs in BM detected by ddPCR predicts tumor relapse/regrowth in high-risk NB patients. ddPCR-based MRD monitoring would improve detection of MRD, evaluation of disease status and treatment response, and prediction of prognosis in high-risk NB patients. The prospective clinical study testing ddPCR detection of 7NB-mRNAs in the context of uniform therapy in larger numbers of NB patients and hospitals will be warranted to establish its clinical significance.

Acknowledgments

We thank the Pediatric Department staff at Kobe University Hospital and the Hematology and Oncology Department staff at Kobe Children's Hospital for collecting BM and PB samples.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.jmoldx.2019.10.012>.

References

1. Brodeur GM: Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003, 3:203–216
2. Nakagawara A, Li Y, Izumi H, Muramori K, Inada H, Nishi M: Neuroblastoma. *Jpn J Clin Oncol* 2018, 48:214–241
3. Maris JM, Hogarty MD, Bagatell R, Cohn SL: Neuroblastoma. *Lancet* 2007, 369:2106–2120
4. Maris JM: Recent advances in neuroblastoma. *N Engl J Med* 2010, 362:2202–2211
5. Tolbert VP, Matthay KK: Neuroblastoma: clinical and biological approach to risk stratification and treatment. *Cell Tissue Res* 2018, 372:195–209
6. Burchill SA, Selby PJ: Molecular detection of low-level disease in patients with cancer. *J Pathol* 2000, 190:6–14
7. Mordant P, Lorient Y, Lahon B, Castier Y, Lesèche G, Soria J-C, Massard C, Deutsch E: Minimal residual disease in solid neoplasia: new frontier or red-herring? *Cancer Treat Rev* 2012, 38:101–110
8. Uemura S, Ishida T, Thwin KKM, Yamamoto N, Tamura A, Kishimoto K, Hasegawa D, Kosaka Y, Nino N, Lin KS, Takafuji S, Mori T, Iijima K, Nishimura N: Dynamics of minimal residual disease in neuroblastoma patients. *Front Oncol* 2019, 9:455
9. Wang H, Stoecklein NH, Lin PP, Gires O: Circulating and disseminated tumor cells: diagnostic tools and therapeutic targets in motion. *Oncotarget* 2017, 8:1884–1912
10. Beiske K, Ambros PF, Burchill SA, Cheung IY, Swerts K: Detecting minimal residual disease in neuroblastoma patients—the present state of the art. *Cancer Lett* 2005, 228:229–240
11. Brownhill SC, Burchill SA: PCR-based amplification of circulating RNAs as prognostic and predictive biomarkers—focus on neuroblastoma. *Pract Lab Med* 2017, 7:41–44
12. Burchill SA, Bradbury FM, Smith B, Lewis IJ, Selby P: Neuroblastoma cell detection by reverse transcriptase-polymerase chain reaction

- (RT-PCR) for tyrosine hydroxylase mRNA. *Int J Cancer* 1994, 57: 671–675
13. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, Kleijn I, Dee R, Hoofst L, van Noesel MM, Bierings M, Berthold F, Versteeg R, Caron HN, van der Schoot CE, Tytgat GAM: PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. *J Clin Oncol* 2008, 26:5443–5449
 14. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, Caron HN, van der Schoot CE, Tytgat GAM: The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. *Eur J Cancer* 2011, 47:1193–1202
 15. Viprey VF, Gregory WM, Corrias MV, Tchirkov A, Swerts K, Vicha A, Dallorso S, Brock P, Luksch R, Valteau-Couanet D, Papadakis V, Laureys G, Pearson AD, Ladenstein R, Burchill SA: Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: a European HR-NBL1/SIOPEN Study. *J Clin Oncol* 2014, 32:1074–1083
 16. Cheung N-KV, Ostrovskaya I, Kuk D, Cheung IY: Bone marrow minimal residual disease was an early response marker and a consistent independent predictor of survival after anti-GD2 immunotherapy. *J Clin Oncol* 2015, 33:755–763
 17. Marachelian A, Villablanca JG, Liu CW, Liu B, Goodarzi F, Lai HA, Shimada H, Tran HC, Parra JA, Gallego R, Bedrossian N, Young S, Czarniecki S, Kennedy R, Weiss BD, Goldsmith K, Granger M, Matthay KK, Groshen S, Asgharzadeh S, Spoto R, Seeger RC: Expression of five neuroblastoma genes in bone marrow or blood of patients with relapsed/refractory neuroblastoma provides a new biomarker for disease and prognosis. *Clin Cancer Res* 2017, 23:5374–5383
 18. Hartomo TB, Kozaki A, Hasegawa D, Pham TVH, Yamamoto N, Saitoh A, Ishida T, Kawasaki K, Kosaka Y, Ohashi H, Yamamoto T, Morikawa S, Hirase S, Kubokawa I, Mori T, Yanai T, Hayakawa A, Takeshima Y, Iijima K, Matsuo M, Nishio H, Nishimura N: Minimal residual disease monitoring in neuroblastoma patients based on the expression of a set of real-time RT-PCR markers in tumor-initiating cells. *Oncol Rep* 2013, 29:1629–1636
 19. Hirase S, Saitoh A, Hartomo TB, Kozaki A, Yanai T, Hasegawa D, Kawasaki K, Kosaka Y, Matsuo M, Yamamoto N, Mori T, Hayakawa A, Iijima K, Nishio H, Nishimura N: Early detection of tumor relapse/regrowth by consecutive minimal residual disease monitoring in high-risk neuroblastoma patients. *Oncol Lett* 2016, 12:1119–1123
 20. Cohn SL, Pearson ADJ, London WB, Monclair T, Ambros PF, Brodeur GM, Faldum A, Hero B, Iehara T, Machin D, Mosseri V, Simon T, Garaventa A, Castel V, Matthay KK, Force IT: The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report. *J Clin Oncol* 2009, 27:289–297
 21. Hishiki T, Matsumoto K, Ohira M, Kamijo T, Shichino H, Kuroda T, Yoneda A, Soejima T, Nakazawa A, Takimoto T, Yokota I, Teramukai S, Takahashi H, Fukushima T, Kaneko T, Hara J, Kaneko M, Ikeda H, Tajiri T, Nakagawara A: Results of a phase II trial for high-risk neuroblastoma treatment protocol JN-H-07: a report from the Japan Childhood Cancer Group Neuroblastoma Committee (JNBSG). *Int J Clin Oncol* 2018, 23:965–973
 22. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B, Stram DO, Gerbing RB, Lukens JN, Matthay KK, Castleberry RP: The international neuroblastoma pathology classification (the Shimada system). *Cancer* 1999, 86:364–372
 23. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castleberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993, 11: 1466–1477
 24. Burchill SA, Beiske K, Shimada H, Ambros PF, Seeger R, Tytgat GAM, Brock PR, Haber M, Park JR, Berthold F: 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* 2017, 123:1095–1105
 25. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT, Bustin SA: The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. *Clin Chem* 2013, 59:892–902
 26. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009, 55:611–622
 27. Zweig MH, Campbell G: Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 1993, 39:561–577
 28. Kanda Y: Investigation of the freely available easy-to-use software 'EZ' for medical statistics. *Bone Marrow Transplant* 2013, 48: 452–458
 29. Kreissman SG, Seeger RC, Matthay KK, London WB, Spoto R, Grupp SA, Haas-Kogan DA, Laquaglia MP, Yu AL, Diller L, Buxton A, Park JR, Cohn SL, Maris JM, Reynolds CP, Villablanca JG: Purged versus non-purged peripheral blood stem-cell transplantation for high-risk neuroblastoma (COG A3973): a randomised phase 3 trial. *Lancet Oncol* 2013, 14:999–1008
 30. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA: Quantitation of targets for PCR by use of limiting dilution. *Bio-techniques* 1992, 13:444–449
 31. Hindson CM, Chevillet JR, Briggs HA, Galichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M: Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* 2013, 10:1003–1005
 32. Jennings LJ, George D, Czech J, Yu M, Joseph L: Detection and quantification of BCR-ABL1 fusion transcripts by droplet digital PCR. *J Mol Diagn* 2014, 16:174–179
 33. Alikian M, Whale AS, Akiki S, Piechocki K, Torrado C, Myint T, Cowen S, Griffiths M, Reid AG, Apperley J, White H, Huggett JF, Foroni L: RT-qPCR and RT-digital PCR: a comparison of different platforms for the evaluation of residual disease in chronic myeloid leukemia. *Clin Chem* 2017, 63:525–531
 34. Cheung IY, Feng Y, Gerald W, Cheung N-KV: Exploiting gene expression profiling to identify novel minimal residual disease markers of neuroblastoma. *Clin Cancer Res* 2008, 14: 7020–7027
 35. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, Yalcin B, Dee R, van Noesel MM, Berthold F, Versteeg R, Caron HN, van der Schoot CE, Tytgat GAM: Detecting minimal residual disease in neuroblastoma: the superiority of a panel of real-time quantitative PCR markers. *Clin Chem* 2009, 55:1316–1326
 36. Heitzer E, Ulz P, Geigl JB: Circulating tumor DNA as a liquid biopsy for cancer. *Clin Chem* 2015, 61:112–123