

Minimal residual disease monitoring in neuroblastoma patients based on the expression of a set of real-time RT-PCR markers in tumor-initiating cells

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Abstract. Minimal residual disease (MRD) is derived from tumor-initiating cells (TICs) and is responsible for tumor relapse. Neuroblastoma is characterized by extreme tumor heterogeneity, and more than half of high-risk patients experience tumor relapse. To overcome tumor heterogeneity and achieve more sensitive detection of MRD, several sets of real-time RT-PCR markers have been reported for MRD monitoring in neuroblastoma patients from different centers. However, these markers vary across centers and are still being validated. In the present study, we validated the ability of 14 commonly used real-time RT-PCR markers to detect MRD based on their expression in neuroblastoma TICs, and we developed a novel MRD detection protocol, which scored the samples as MRD-positive when the expression of one of the 11 real-time RT-PCR markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) exceeded the normal range. By using this protocol, we prospectively monitored MRD in 73 bone marrow (BM), 12 peripheral blood stem cell and 8 peripheral blood samples from 14 neuroblastoma patients treated at a single center. We scored 100, 56, 56 and 57% BM cytology-positive, elevated vanillylmandelic acid (VMA), elevated homovanillic acid (HVA) and elevated neuron-specific enolase (NSE) samples as

MRD-positive, respectively. MRD was also positive in 48, 45, 46 and 43% of the BM cytology-negative and normal VMA, normal HVA and normal NSE samples, respectively. These results suggest that the present MRD detection protocol based on the expression of a set of 11 real-time RT-PCR markers in neuroblastoma TICs achieves sensitive MRD monitoring in neuroblastoma patients.

Introduction

Neuroblastoma is one of the most common solid tumors in children and accounts for ~15% of pediatric cancer-related deaths (1,2). Neuroblastoma cells originate from neural crest cells that are committed to the development of the sympathetic nervous system, and display extreme heterogeneity ranging from spontaneous regression to malignant progression. Although the prognosis for neuroblastoma patients has improved considerably, <40% of high-risk patients can expect long-term survival (3). The majority of cancer-related deaths in neuroblastoma are due to tumor relapse caused by minimal residual disease (MRD).

Tumor-initiating cells (TICs) account for only a small population of tumor cells that generate the bulk of the tumor through continuous self-renewal and differentiation, and are found in a variety of cancers (4-6). TICs can persist as MRD locally in the primary tumor or distantly in the blood, bone marrow and metastatic organs (7,8). Neuroblastoma TICs were recently identified as spheres grown in serum-free non-adherent culture and were shown to express stem cell markers, exhibit self-renewal, and form metastatic tumors in immunodeficient mice (9,10).

Sensitive detection of MRD is fundamental when monitoring the disease status and evaluating the treatment response (7,11). In acute lymphoblastic leukemia (ALL), MRD detection with real-time PCR is directed against tumor-specific junctions using

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genomic DNA as a template. This sensitive MRD detection in ALL patients has revealed the most important prognostic factors and is used to stratify treatments in the clinic (12-14). In neuroblastoma, the rationale for introducing the current MRD detection protocols to stratify treatments in the clinic remains unclear (15,16). Since no recurrent fusion genes have been found in neuroblastoma cells, MRD detection in neuroblastoma patients requires well-defined real-time RT-PCR markers that can be assessed using cDNA as a template. Although ideal MRD markers should be exclusively expressed in neuroblastoma cells and not in normal cells, commonly used markers are selected by their ability to experimentally define a cut-off value that distinguishes neuroblastoma cells from normal cells.

Tyrosine hydroxylase (TH) was initially evaluated as a single marker for neuroblastoma MRD detection, but was hampered by its expression in normal cells (17-20). Paired-like homeobox 2b (PHOX2B) was subsequently identified as a more specific single marker, but was not highly expressed in all neuroblastoma tumors (21). To overcome tumor heterogeneity and achieve more sensitive detection of MRD in neuroblastoma patients, several sets of multiple markers have been reported at different centers. Based on gene expression analyses in neuroblastoma tumors, Viprey *et al* (22) identified the following three markers: doublecortin (DCX), PHOX2B and TH, while Cheung *et al* (23) independently identified the following 8 markers: cyclin D1 (CCND1), collapsin response mediator protein 1 (CRMP1), dopa decarboxylase (DDC), GABA A receptor β 3 (GABRB3), ISL LIM homeobox 1 (ISL1), kinesin family member 1A (KIF1A), PHOX2B, and transforming acidic coiled-coil containing protein 2 (TACC2). Furthermore, Stutterheim *et al* (24) selected the following 6 markers: cholinergic receptor, nicotinic, α 3 (CHRNA3), dopamine β -hydroxylase (DBH), DDC, growth-associated protein 43 (GAP43), PHOX2B and TH. These MRD markers are currently being validated.

In the present study, we validated 14 commonly used real-time RT-PCR markers based on their expression in neuroblastoma TICs, and developed a novel MRD detection protocol. By using this protocol, we prospectively monitored MRD in 73 bone marrow (BM), 12 peripheral blood stem cell (PBSC), and 8 peripheral blood (PB) samples from 14 neuroblastoma patients treated at Kobe Children's Hospital, Japan, between November, 2010 and August, 2012.

Materials and methods

Patients and samples. Normal PB samples were obtained from three healthy volunteers. PB, PBSC and BM samples were obtained from 14 neuroblastoma patients who were treated at Kobe Children's Hospital between November, 2010 and August, 2012. Written informed consent was obtained from the patients, and the study was approved by Kobe Children's Hospital Ethics Board. The samples were handled in accordance with the Guidelines for the Clinical Research of Kobe University Graduate School of Medicine. All PB, PBSC and BM samples were separated using Mono-Poly Resolving Medium (DS Pharma Biomedical, Osaka, Japan), and nucleated cells were collected according to the manufacturer's instructions.

Neuroblastoma TICs. Human neuroblastoma BE(2)-C cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and neuroblastoma TICs were isolated as spheres of BE(2)-C cells as described previously (25). Briefly, BE(2)-C cells were cultured at 37°C (5% CO₂ and 95% air) in sphere medium consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (3:1) (Wako Pure Chemical-Industries, Osaka, Japan), 100 U/ml penicillin/streptomycin (PC/SM) (Invitrogen), 2% B27 supplement (Life Technologies, Carlsbad, CA, USA), 40 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA) and 20 ng/ml epidermal growth factor (EGF) (R&D Systems). The cells were subcultured with non-enzymatic cell dissociation solution (Sigma, St. Louis, MO, USA) and maintained for more than 4 weeks in Ultra-Low Attachment culture dishes (Corning, Inc., Corning, NY, USA).

RNA extraction and cDNA synthesis. Total RNA from normal BM was obtained from Clontech (Palo Alto, CA, USA). Total RNA from neuroblastoma TICs as well as PB, PBSC and BM samples was extracted with a TRIzol Plus RNA Purification kit (Life Technologies) according to the manufacturer's instructions. RNA integrity was evaluated by agarose gel electrophoresis. cDNA was synthesized from 1 (or 0.5) μ g of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, USA) and diluted to a total volume of 80 (or 40) μ l.

Real-time RT-PCR. Real-time RT-PCR was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) in a total volume of 15 μ l consisting of 7.5 μ l of 2X FastStart Universal SYBR-Green Master (Roche, Mannheim, Germany), 1.5 μ l each of 3 μ M 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 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec, and one cycle at 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec and 60°C for 15 sec. Each sample was analyzed in triplicate. To correct for differences in the amount of total RNA and in the efficiency of cDNA synthesis, the expression of each marker was calculated based on the relative standard curve method using β 2-microglobulin (B2M) as an endogenous reference for normalization.

Primers. The following primers were used: GD2 synthase (B4GALNT1, NM_001478) 5'-CCAACTCAACAGGCAA CTACAA-3' (sense) and 5'-ATGTCCCTCGGTGGAG AAC-3' (antisense); CCND1 (NM_053056) 5'-GAAGATCGTCGC CACCTG-3' (sense) and 5'-GACCTCCTCCTCGCACTTC T-3' (antisense); CHRNA3 (NM_000743) 5'-TGAAATGGAACC CTCTGAC-3' (sense) and 5'-GGAAATCCCCAACAGC ATT-3' (antisense); CRMP1 (NM_001014809) 5'-GAGTGC AGCCGACATCATC-3' (sense) and 5'-GGGCTCTCCA AAAACTAGGG-3' (antisense); DBH (NM_000787) 5'-GAC CCAAGGATTACCTCATT-3' (sense) and 5'-GTTGATGGC CTCCAGTGAC-3' (antisense); DCX (NM_000555) 5'-CAT CCCCACACCTCAGAAG-3' (sense) and 5'-GGAGGTTCC GTTTGCTGA-3' (antisense); DDC (NM_000790) 5'-GGA GAAGGGGGAGGAGTG-3' (sense) and 5'-CAGCCGATG GATCACTTTG-3' (antisense); GABRB3 (NM_000814)

Table I. Expression profile of real-time RT-PCR markers in neuroblastoma TICs.

Gene name	Normal PB (mean C _T ± SD)	Normal BM (mean C _T ± SD)	Neuroblastoma TICs (mean C _T ± SD)
B4GALNT1	>40	31.593±0.382	25.535±0.122
CCND1	34.051±0.329	28.725±0.096	22.243±0.056
CHRNA3	>40	31.028±0.709	21.538±0.102
CRMP1	>40	34.863±0.957	21.779±0.103
DBH	>40	>40	23.133±0.059
DCX	>40	31.683±0.727	22.037±0.544
DDC	>40	36.150±0.329	21.538±0.102
GABRB3	35.996±0.130	31.952±0.865	22.340±0.102
GAP43	35.639±0.922	31.828±0.920	19.777±0.010
ISL1	>40	>40	24.929±0.044
KIF1A	>40	32.601±0.728	19.901±0.044
PHOX2B	>40	>40	20.351±0.140
TACC2	>40	28.007±1.272	34.782±0.001
TH	>40	32.741±1.224	18.666±0.034

PB, peripheral blood; BM, bone marrow; TICs, tumor-initiating cells. B4GALNT1, β-1,4 N-acetylgalactosaminyltransferase 1; CCND1, cyclin D1; CHRNA3, cholinergic receptor, nicotinic, α3; CRMP1, collapsin response mediator protein 1; DBH, dopamine β-hydroxylase; DCX, doublecortin; DDC, dopa decarboxylase; GABRB3, GABA A receptor β3; GAP43, growth-associated protein 43; ISL1, ISL LIM homeobox 1; KIF1A, kinesin family member 1A; PHOX2B, paired-like homeobox 2b; TACC2, transforming acidic coiled-coil containing protein 2; TH, tyrosine hydroxylase.

5'-GGGTGTCCTTCTGGATCAATTA-3' (sense) and 5'-TTGTCAGCACAGTTGTGATCC-3' (antisense); GAP43 (NM_001130064) 5'-GAGGATGCTGCTGCCAAG-3' (sense) and 5'-GGCACTTTCCTTAGGTTTGGT-3' (antisense); ISL1 (NM_002202) 5'-AAGGACAAGAAGCGAAGCAT-3' (sense) and 5'-TTCCTGTCATCCCCTGGATA-3' (antisense); KIF1A (NM_004321) 5'-CTTGCGACATCACTGACAT-3' (sense) and 5'-GCTGGACAGGGCTGAGAG-3' (antisense); PHOX2B (NM_003924) 5'-CTACCCCGACATCTACACTCG-3' (sense) and 5'-CCTGCTTGCGAACTTGG-3' (antisense); TACC2 (NM_006997) 5'-CCCCACTATTCGCTCAGAAA-3' (sense) and 5'-GGGCTTCTATCCGCATGAT-3' (antisense); TH (NM_199292) 5'-GCCAAGGACAAGCTCAGG-3' (sense) and 5'-AGCGTGACGGGTCGAACT-3' (antisense); and β2-microglobulin (B2M, NM_004048) 5'-TTCTGGCCTG GAGGCTATC-3' (sense) and 5'-TCAGGAAATTTGACTT TCCATTC-3' (antisense).

Standard curves. To mimic MRD-positive PB, PBSC and BM samples, total RNA from neuroblastoma TICs was serially diluted into total RNA from normal PB to yield a total amount of 1.0 μg. The resulting six-step 5-fold dilutions were converted to cDNA yielding 100, 20, 4, 0.8, 0.16 and 0.032% dilutions. To obtain a standard curve, the C_T values for each marker were obtained from triplicate real-time RT-PCR and plotted against the logarithm of the % dilution/100. Due to slight expression of each marker in normal PB, we generated standard curves with R²>0.90 for each marker by limiting the plotted points of the % dilution. The quantitative range was defined as the minimum % dilution that plotted to the standard curve or above.

Normal range. Based on the threshold defined by the European Study Group on MRD detection in ALL (ESG-MRD-ALL), the mean C_T value of a normal sample -3 was set as the maximum C_T value for the normal sample (12). The normal range was then defined as any value less than the % dilution of MRD that corresponded to the maximum C_T value of the normal sample. If the mean C_T value of the normal sample was >40, the normal range was defined as less than the % dilution of MRD that corresponded to the minimum % dilution of MRD in the quantitative range.

Results

Expression profile of real-time RT-PCR markers in neuroblastoma TICs. To achieve more sensitive detection of MRD, we first analyzed the expression of commonly used real-time RT-PCR markers in normal PB, normal BM and neuroblastoma TICs that were isolated as spheres of BE(2)-C cells (25). In addition to the 13 MRD markers that were collectively reported by Viprey *et al* (22), Cheung *et al* (23) and Stutterheim *et al* (24), a well-examined MRD marker, B4GALNT1, was also included in the present study (26). When the expression of these 14 MRD markers were analyzed by real-time RT-PCR, CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH were predominantly expressed in neuroblastoma TICs in which the mean C_T value was >9.0 lower when compared with that in normal PB and BM (Table I). Considering the variation of the mean C_T value for normal PB, BM and neuroblastoma TICs (12), B4GALNT1, CCND1 and TACC2 were excluded from the present study.

Table II. Normal range of real-time RT-PCR markers.

Gene name	Standard curve	R ²	Quantitative range (%)	Normal range in PB/PBSC (%)	Normal range in BM (%)
CHRNA3	$C_T = -3.69 \log (\% \text{ dilution}/100) + 22.28$	0.969	≥ 0.032	<0.032	<0.413
CRMP1	$C_T = -3.04 \log (\% \text{ dilution}/100) + 26.21$	0.911	≥ 0.032	<0.032	<0.207
DBH	$C_T = -3.66 \log (\% \text{ dilution}/100) + 26.60$	0.946	≥ 0.032	<0.032	<0.032
DCX	$C_T = -3.02 \log (\% \text{ dilution}/100) + 27.39$	0.912	≥ 0.160	<0.160	<5.598
DDC	$C_T = -3.71 \log (\% \text{ dilution}/100) + 23.99$	0.983	≥ 0.032	<0.032	<0.051
GABRB3	$C_T = -3.43 \log (\% \text{ dilution}/100) + 25.00$	0.948	≥ 0.032	<0.032	<1.059
GAP43	$C_T = -3.34 \log (\% \text{ dilution}/100) + 24.37$	0.966	≥ 0.032	<0.032	<0.694
ISL1	$C_T = -3.71 \log (\% \text{ dilution}/100) + 25.36$	0.985	≥ 0.160	<0.160	<0.160
KIF1A	$C_T = -4.40 \log (\% \text{ dilution}/100) + 22.43$	0.962	≥ 0.032	<0.032	<0.351
PHOX2B	$C_T = -4.19 \log (\% \text{ dilution}/100) + 23.46$	0.944	≥ 0.032	<0.032	<0.032
TH	$C_T = -3.78 \log (\% \text{ dilution}/100) + 22.13$	0.987	≥ 0.160	<0.160	<0.160

CHRNA3, cholinergic receptor, nicotinic, $\alpha 3$; CRMP1, collapsin response mediator protein 1; DBH, dopamine β -hydroxylase; DCX, doublecortin; DDC, dopa decarboxylase; GABRB3, GABA A receptor $\beta 3$; GAP43, growth-associated protein 43; ISL1, ISL LIM homeobox 1; KIF1A, kinesin family member 1A; PHOX2B, paired-like homeobox 2b; TH, tyrosine hydroxylase.

Standard curves for real-time RT-PCR markers. To mimic the actual expression of neuroblastoma MRD markers in clinical samples as precisely as possible, we generated their standard curves by serially diluting neuroblastoma TICs into normal PB. The 11 MRD markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) were amplified by real-time RT-PCR, and triplicate C_T values for each MRD marker were plotted against the logarithm of the % dilution/100. Although the standard curve for the real-time RT-PCR assay should theoretically have $R^2 > 0.99$, the actual R^2 value is lower than expected due to slight expression in normal PB. In the present study, we generated the standard curve with $R^2 > 0.90$ by limiting the plotted points of the % dilution (Table II). Accordingly, 5 points (100, 20, 4, 0.8 and 0.16% dilution) were plotted against triplicate C_T values for DCX, ISL1 and TH (Fig. 1). For CHRNA3, CRMP1, DBH, DDC, GABRB3, GAP43, KIF1A and PHOX2B, 6 points (100, 20, 4, 0.8, 0.16 and 0.032% dilution) were used (Fig. 1).

Normal range of real-time RT-PCR markers. Although the quantitative range frequently reflects the portion of the standard curve in which the MRD levels can be accurately quantitated, we here defined the quantitative range as the minimum % dilution that was plotted to the standard curve or above. While DCX, ISL1 and TH had a quantitative range of $\geq 0.160\%$ dilution, the quantitative range of CHRNA3, CRMP1, DBH, DDC, GABRB3, GAP43, KIF1A and PHOX2B was $\geq 0.032\%$ dilution (Table II). To account for tumor heterogeneity, we set the maximum C_T value for the PB/PBSC and BM samples as the mean C_T value of the normal PB and BM cells -3, respectively, according to the threshold defined by ESG-MRD-ALL (12). The normal range for each MRD marker in the PB/PBSC and BM samples was then defined as less than the % dilution that corresponded to the maximum C_T value for the PB/PBSC and BM samples, respectively. If the mean C_T value in normal PB and BM cells was > 40 , the normal range was defined as less than the % dilution that corresponded to the minimum % dilu-

tion in the quantitative range. For the PB samples, the normal ranges of all MRD markers did not exceed the minimum % dilution in the quantitative range (Table II). For the BM samples, the normal ranges of DBH, ISL1, PHOX2B and TH did not exceed the minimum % dilution in the quantitative range, whereas the normal ranges of CHRNA3, CRMP1, DCX, DDC, GABRB3, GAP43 and KIF1A were determined to be < 0.413 , < 0.207 , < 5.598 , < 0.051 , < 1.059 , < 0.694 and $< 0.351\%$, respectively (Table II).

MRD sample characteristics. All 93 MRD samples were obtained from 14 neuroblastoma patients (#1-14) who were prospectively treated at Kobe Children's Hospital between November, 2010 and August, 2012. Patients #1-10 developed primary tumors and were treated according to the Japan Neuroblastoma Study Group (JNBSG) protocol. Low-risk patient #1 was subjected to surgical therapy followed by watchful waiting. Low-risk patient #2 was treated with one course of the LI-A regimen (1.5 mg/m² VCR and 300 mg/m² CPA). Intermediate-risk patient #3 was treated with one course of the LI-D regimen (1.5 mg/m² VCR, 900 mg/m² CPA, 30 mg/m² THP and 60 mg/m² CDDP). High-risk patient #4 was treated with five courses of the 05A3 regimen (2,400 mg/m² CPA, 1.5 mg/m² VCR, 40 mg/m² THP and 100 mg/m² CDDP) followed by peripheral blood stem cell transplantation (PBSCT), radiation therapy and surgical therapy. High-risk patient #5 was treated with five courses of the 05A3 regimen followed by PBSCT and radiation therapy. High-risk patient #6 was treated with one course of the 05A1 regimen (1,200 mg/m² CPA, 1.5 mg/m² VCR, 40 mg/m² THP and 100 mg/m² CDDP) and four courses of the 05A3 regimen followed by PBSCT, radiation therapy and surgical therapy. High-risk patient #7 was treated with one course of the 05A1 regimen and four courses of the 05A3 regimen followed by PBSCT and radiation therapy. High-risk patient #8 was treated with one course of the 05A1 regimen and four courses of the 05A3 regimen. High-risk patients #9

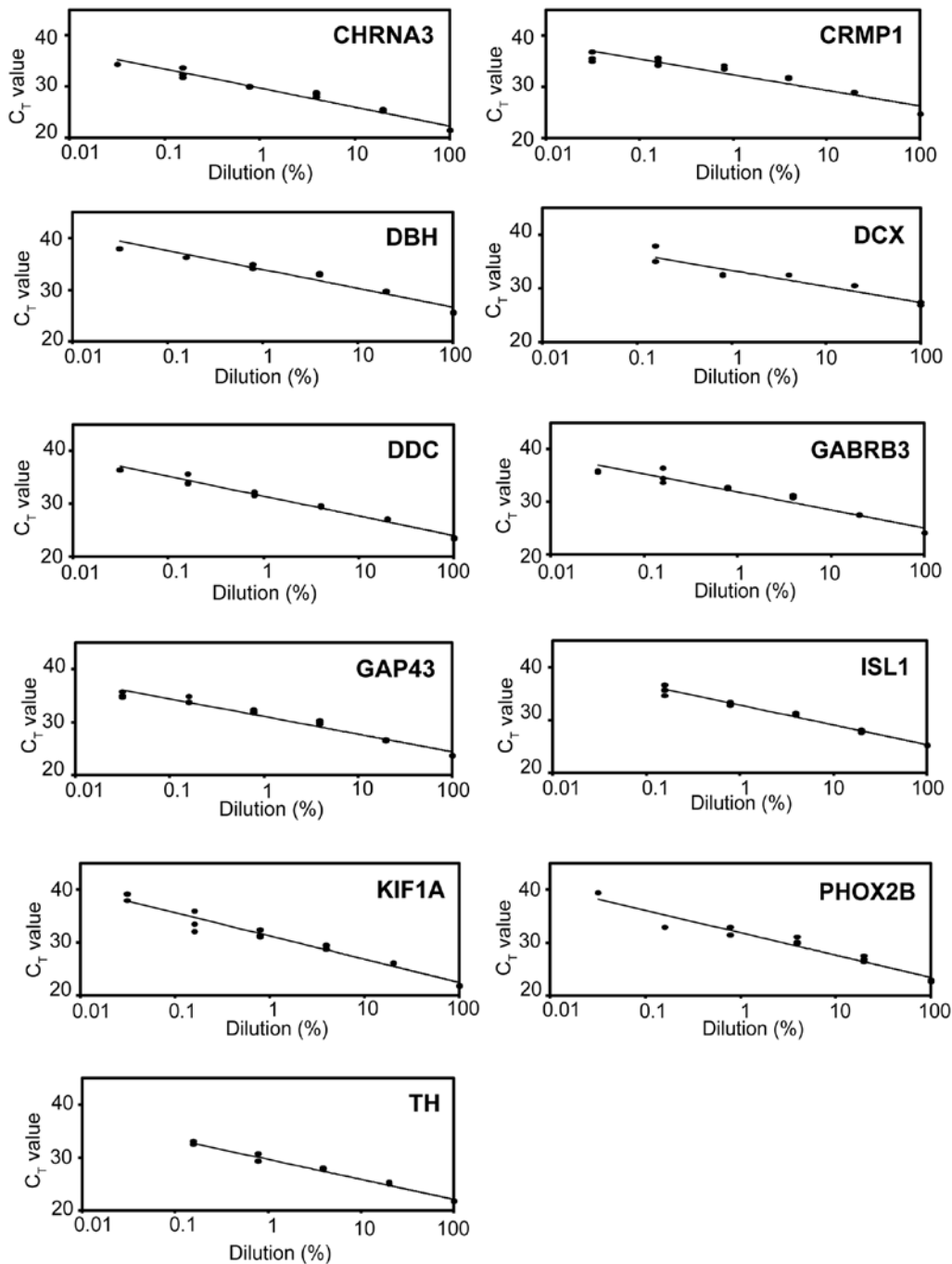


Figure 1. Standard curves for real-time RT-PCR markers. Triplicate C_T values for CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH were plotted against the logarithm of the % dilution/100.

and 10 were treated with one course of the 05A1 regimen and three courses of the 05A3 regimen. Patients #11-14 experienced tumor relapses and were subjected to salvage chemotherapy. Patient #11 had one course of the IREC regimen (300 mg/m² CPT-11, 300 mg/m² VP-16, 240 mg/m² CBDCA) and five courses of the topotecan/CPA regimen (3.75 mg/m² topotecan, 1,250 mg/m² CPA). Patient #12 had three courses of the ICE regimen (6,000 mg/m² IFO, 800 mg/m² CBDCA, 500 mg/m² VP-16) and two courses of the CPT-11 regimen (125 mg/m² CPT-11) followed by bone marrow transplantation (BMT). Patient #13 had two courses of the CPT-11 regimen, six courses of IREC regimen, five courses of the topotecan/CPA regimen,

and three courses of the topotecan/TMZ regimen (3.75 mg/m² topotecan, 750 mg/m² TMZ). Patient #14 had three courses of the ICE regimen and two courses of the IREC regimen. All patients were subjected to MRD sampling as frequently as possible during the course of treatment (Fig. 2).

Expression profile of real-time RT-PCR markers in MRD samples. CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH expression was determined by real-time RT-PCR in the 93 MRD (73 BM, 12 PBSC and 8 PB) samples. Each marker was scored as positive when its expression exceeded the normal range. The number of

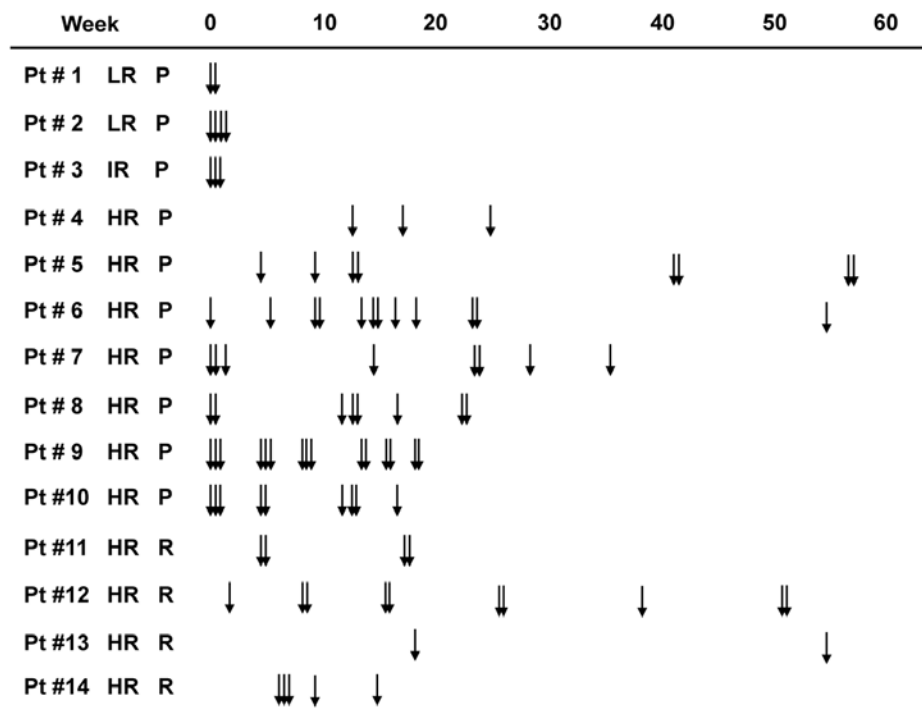


Figure 2. MRD sample characteristics. Downward arrows indicate the time points of MRD sampling. Week 0 was defined as the time when the primary or recurrent tumor was diagnosed. Pt, patient. LR, low-risk; IR, intermediate-risk; HR, high-risk. P, primary tumor; R, recurrent tumor.

positive samples for each marker varied from 4 (GABRB3) to 33 (PHOX2B). PHOX2B and DBH seemed to be sensitive markers in the present BM samples (Table III). Although only a limited number of PBSC and PB samples were analyzed, the sensitivity of each marker also varied between the BM, PBSC and PB samples as described previously (24). Only KIF1A, DCX, CRMP1 and GABRB3 were able to detect MRD in the present PBSC and PB samples (Table III).

Comparison of the present MRD detection protocol with clinical examinations. To validate the applicability of a set of these 11 commonly used real-time RT-PCR markers to monitor MRD, we compared these results with BM cytology, urinary vanillylmandelic acid (VMA), urinary homovanillic acid (HVA) and serum neuron-specific enolase (NSE) data. BM cytology data were obtained from 73 MRD (73 BM) samples, whereas urinary VMA, urinary HVA, and serum NSE data were obtained from 89 MRD (71 BM, 10 PBSC, and 8 PB) samples (Table IV). MRD was scored as positive when the expression of one of the 11 markers exceeded the normal range. Thus, 100, 56, 56 and 57% of BM cytology-positive (n=10), elevated VMA (>15 µg/mg Cr, n=45), elevated HVA (>30 µg/mg Cr, n=43), and elevated NSE (>20 ng/ml, n=49) samples were MRD-positive, respectively (Table IV). MRD was also positive in 48, 45, 46 and 43% of BM cytology-negative (n=63), normal VMA (≤15 µg/mg Cr, n=44), normal HVA (≤30 µg/mg Cr, n=46), and normal NSE (≤20 ng/ml, n=40) samples, respectively (Table IV). As BM samples were obtained from at least two sites (left and right iliac crests), we also compared real-time RT-PCR results from the paired BM samples. Of the 23 pairs of BM cytology-negative samples, 11 pairs were MRD-positive at only one site. The remaining

12 pairs had identical results for both sites; 6 pairs were MRD-negative and 6 pairs MRD-positive.

Discussion

In the present study, we validated the ability of 14 commonly used real-time RT-PCR markers to detect neuroblastoma MRD based on their expression in neuroblastoma TICs. We then developed a novel MRD detection protocol and prospectively monitored MRD in 73 BM, 12 PBSC and 8 PB samples from 14 neuroblastoma patients treated at Kobe Children's Hospital between November, 2010 and August, 2012. Compared to currently used protocols, our protocol had two advantages. First, it determined 11 real-time RT-PCR markers based on their expression in neuroblastoma TICs. Second, it calculated the expression of these markers using the relative standard curve method and scored their expression levels as % dilution.

Several sets of real-time RT-PCR markers have been reported for MRD monitoring in neuroblastoma patients. Neuroblastoma is characterized by extreme heterogeneity that is observed in three levels: i) between different patients, ii) between different tumors within a patient, and iii) between different cells within a tumor. Regarding the heterogeneity between different patients, highly variable expression of CHRNA3, DBH, DDC, GAP43, PHOX2B and TH was detected in tumor samples from 56 patients (24). In terms of the heterogeneity between different tumors within a patient, one of the 5 MRD markers (CHRNA3, DDC, GAP43, PHOX2B and TH) showed a >10-fold difference in relative expression in 6 out of 56 pairs of BM samples obtained from two different sites (27). Regarding heterogeneity between different cells within a tumor, Stutterheim *et al* (27) recently reported that GAP43 and

Table III. Expression profile of real-time RT-PCR markers in MRD samples.

	BM sample	PBSC sample	PB sample
CHRNA3			
Positive	10	0	0
Negative	63	12	8
CRMP1			
Positive	14	0	1
Negative	59	12	7
DBH			
Positive	22	0	0
Negative	51	12	8
DCX			
Positive	9	0	2
Negative	64	12	6
DDC			
Positive	14	0	0
Negative	59	12	8
GABRB3			
Positive	3	0	1
Negative	70	12	7
GAP43			
Positive	9	0	0
Negative	64	12	8
ISL1			
Positive	14	0	0
Negative	55	11	7
KIF1A			
Positive	11	2	1
Negative	62	10	7
PHOX2B			
Positive	33	0	0
Negative	40	12	8
TH			
Positive	12	0	0
Negative	61	12	8

MRD, minimal residual disease; BM, bone marrow; PBSC, peripheral blood stem cell; PB, peripheral blood; CHRNA3, cholinergic receptor, nicotinic, $\alpha 3$; CRMP1, collapsin response mediator protein 1; DBH, dopamine β -hydroxylase; DCX, doublecortin; DDC, dopa decarboxylase; GABRB3, GABA A receptor $\beta 3$; GAP43, growth-associated protein 43; ISL1, ISL LIM homeobox 1; KIF1A, kinesin family member 1A; PHOX2B, paired-like homeobox 2b; TH, tyrosine hydroxylase.

TH expression differed by $>10 C_T$ values across subclones of the same neuroblastoma IMR-32 cells isolated by serial dilution. Due to the differential gene expression between whole tumor cells and TICs (9,25), we validated MRD markers based

Table IV. Comparison of the present MRD detection protocol with clinical examinations.

	MRD Positive	MRD Negative
BM cytology		
Positive	10	0
Negative	30	33
Urinary VMA		
$>15 \mu\text{g/mg Cr}$	25	20
$\leq 15 \mu\text{g/mg Cr}$	20	24
Urinary HVA		
$>30 \mu\text{g/mg Cr}$	24	19
$\leq 30 \mu\text{g/mg Cr}$	21	25
Serum NSE		
$>20 \text{ ng/ml}$	28	21
$\leq 20 \text{ ng/ml}$	17	23

MRD, minimal residual disease; BM, bone marrow; VMA, vanillyl-mandelic acid; HVA, homovanillic acid; NSE, neuron-specific enolase.

on their expression in neuroblastoma TICs instead of whole neuroblastoma cells used by previous studies. Accordingly, B4GALNT1, CCND1 and TACC2 were determined to be inappropriate MRD markers, even though they were selected by their ability to experimentally define a cut-off value with which to distinguish neuroblastoma cells from normal cells in previous studies (23,26).

Although most previous studies determined the expression of MRD markers by real-time RT-PCR using the comparative C_T method, we hypothesized that standard curves generated by serially diluting neuroblastoma TICs into normal PB would reflect more accurate MRD marker expression in clinical samples. Therefore, we calculated MRD marker expression by the relative standard curve method and scored it as the % dilution. While previous studies recommended a set of 3, 6 or 8 markers by which to detect MRD in neuroblastoma patients (22-24), we used a set of 11 MRD markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH). PHOX2B was the most sensitive MRD marker in the present BM samples. Among 40 PHOX2B-negative BM samples, only 7 samples were positive for CRMP1, DBH, DDC, ISL1 and/or TH. Although CRMP1, DCX, GABRB3 and KIF1A were not as sensitive as PHOX2B in the BM samples, they were able to detect MRD in the present PB and PBSC samples. Consequently, a set of these 11 markers was able to detect MRD in more than 40% of the BM cytology-negative, normal urinary VMA, normal urinary HVA and normal serum NSE samples analyzed in the present study.

In summary, the present MRD detection protocol based on the expression of a set of 11 real-time RT-PCR markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) in neuroblastoma TICs achieved sensitive MRD monitoring in neuroblastoma patients who were prospectively treated at a single center.

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