

Higher levels of minimal residual disease in peripheral blood than bone marrow before 1st and 2nd relapse/regrowth in a patient with high-risk neuroblastoma: A case report

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Abstract. More than half of patients with high-risk neuroblastoma (HR-NB) experience relapse/regrowth due to the activation of chemoresistant minimal residual disease (MRD). MRD in patients with HR-NB can be evaluated by quantitating neuroblastoma-associated mRNAs (NB-mRNAs) in bone marrow (BM) and peripheral blood (PB) samples. Although several sets of NB-mRNAs have been shown to possess a prognostic value for MRD in BM samples (BM-MRD), MRD in PB samples (PB-MRD) is considered to be low and difficult to evaluate. The present report describes an HR-NB case presenting higher PB-MRD than BM-MRD before 1st and 2nd relapse/regrowth. A 3-year-old female presented with an abdominal mass, was diagnosed with HR-NB, and treated according to the nationwide standard protocol for HR-NB. Following systemic induction and consolidation therapy with local therapy, the patient achieved complete remission but experienced a 1st relapse/regrowth 6 months after maintenance therapy. The patient partially responded to salvage chemotherapy and anti-GD2 immunotherapy but had a 2nd relapse/regrowth 14 months after the 1st relapse/regrowth. Consecutive PB-MRD and BM-MRD monitoring revealed that PB-MRD was lower than BM-MRD at diagnosis (100 times) and 1st and 2nd relapse/regrowth (1,000 and 3 times) but became higher than BM-MRD before 1st and 2nd relapse/regrowth. The present case highlights that PB-MRD

can become higher than BM-MRD before relapse/regrowth of patients with HR-NB.

Introduction

Neuroblastoma (NB) is the most common solid extracranial tumor in children and accounts for approximately 15% of pediatric cancer-associated deaths. Treatments are tailored for NB patients based on the risk of relapse and death. The International Neuroblastoma Risk Group (INRG) Classification System (1) was developed for pretreatment risk stratification. It used seven risk criteria including the image-based stage (INRG Staging System) (2), age, histologic category, grade of tumor differentiation, MYCN status, presence/absence of 11q aberrations, and tumor cell ploidy, and stratified NB patients into very low-risk, low-risk, intermediate-risk, and high-risk (HR) groups. Approximately half of the NB patients were classified into HR group, whose long-term survival remains no more than 50% despite aggressive multimodal therapy. This is because more than half of HR-NB patients experience relapse/regrowth and relapsed/regrown patients were rarely rescued with less than 10% probability (3-5). Relapse/regrowth is thought to occur due to the activation of chemoresistant minimal residual disease (MRD) remaining in the body following systemic and local cancer therapy. To improve outcomes for HR-NB patients, a more accurate MRD evaluation is required to monitor the disease burden and treatment response (6,7).

MRD in HR-NB patients was commonly evaluated by detecting neuroblastoma-associated mRNAs (NB-mRNAs) with quantitative PCR (qPCR) because no common genetic aberration is identified in NB cells (8,9). Currently, several sets of NB-mRNAs were shown to possess a significant prognostic value for MRD in bone marrow (BM) samples (BM-MRD) (10-13). In addition, we also reported that 7NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) quantitated by droplet digital PCR (ddPCR) had significant and better prognostic information (14).

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Although MRD evaluation was clinically performed with peripheral blood (PB) samples in other cancer types, MRD in PB samples (PB-MRD) in HR-NB patients was believed to show a far lower level than BM-MRD and its clinical significance was not clear (7). Here, we report an HR-NB case presenting higher levels of PB-MRD than BM-MRD before 1st and 2nd relapse/regrowth.

Case report

Patient and samples. The patient was a 3-year-old female diagnosed with INRG stage M (2), HR-NB (1). Disease evaluation was conducted based on the International Neuroblastoma Response Criteria (15). All PB and BM samples were obtained with written informed consent. The use of human samples for the present study was approved by the Ethics Committee at Kobe University Graduate School of Medicine and the study was conducted in accordance with the Guidelines for the Clinical Research of Kobe University Graduate School of Medicine.

Pathological examination. Tumor tissue samples were fixed with 10% buffered formalin for 24 h at room temperature, paraffin-infiltrated overnight, and embedded in the paraffin by auto-processor (Tissue-Tek, Sakura Finetek Japan, Tokyo, Japan) according to manufacturer's instructions. For Hematoxylin and Eosin (HE) staining, tissue blocks were sectioned at 3 μm , dewaxed, and stained by HE at room temperature by auto-stainer (Tissue-Tek, Sakura Finetek Japan) according to manufacturer's instructions. For immunostaining, tissue blocks were sectioned at 3 μm , dewaxed, and heated for 20 min in Bond Epitope Retrieval Solution 2 (Leica Biosystems, Nussloch, Germany). The sectioned slides were incubated with an anti-Synaptophysin primary antibody (Clone 27G12, Leica Biosystems) without dilution, stained with Bond Polymer Refine Detection kit (Leica Biosystems), and counterstained with hematoxylin using Bond-Max automation system (Leica Biosystems) according to manufacturer's instructions. The stained slides were evaluated by pathologist using BX53 microscope (Evident, Tokyo, Japan) at x200 or x400 magnification.

7NB-mRNAs ddPCR assay. 7NB-mRNA ddPCR assay was performed using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μl consisting of 10 μl 2X ddPCR Supermix for probes (Bio-Rad Laboratories), 3.3 μl each of 3 $\mu\text{mol/l}$ sense and antisense primers, 0.5 μl of 10 $\mu\text{mol/l}$ Universal Probe Library probe (Roche, Mannheim, Germany), and 1 μl sample cDNA (corresponding to 12.5 ng total RNA) according to the manufacturer's instructions. The expressions of 7 NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH) and a reference gene mRNA (HPRT1) were determined based on the absolute quantification method according to the Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE) guideline (16,17), and the level of 7NB-mRNAs (combined signature) was calculated as the weighted sum of 7 relative copy numbers (level of each NB-mRNA), in which the reciprocal of 90 percentile in non-NB control PB and BM sample was used for the weighting for each NB-mRNA, as described

previously (14). The following primer and probe sets used in the present case: CRMP1 (accession number NM_001014809) 5'-CCAATCCCTTTATGCTGACG-3' (forward), 5'-GGAACG ATTAAGTTCTCTCCTATTTG-3' (reverse), and Universal Probe Library number 65 probe (Roche), DBH (accession number NM_000787) 5'-TGGGGACTGCTTATTTTG-3' (forward), 5'-TTCTGGGGTCTCTGCAC-3' (reverse), and Universal Probe Library number 3 probe (Roche), DDC (accession number NM_000790) 5'-CTGGAGAAGGGGGAGGAG T-3' (forward), 5'-GCCGATGGATCACTTTGGT-3' (reverse), and Universal Probe Library number 49 probe (Roche), GAP43 (accession number NM_002045) 5'-GAGGATGCT GCTGCCAAG-3' (forward), 5'-GGCACTTTCCTTAGG TTTGGT-3' (reverse), and Universal Probe Library number 26 probe (Roche), ISL1 (accession number NM_002202) 5'-AAGGACAAGAAGCGAAGCAT-3' (forward), 5'-TTC CTGTCATCCCCTGGATA-3' (reverse), and Universal Probe Library number 66 probe (Roche), PHOX2B (accession number NM_003924) 5'-CTACCCCGACATCTACTACTCG-3' (forward), 5'-CTCCTGCTTGCGAACTTG-3' (reverse), and Universal Probe Library number 17 probe (Roche), TH (accession number NM_199292) 5'-TCAGTGACGCCAAGGACA-3' (forward), 5'-GTACGGGTGCAACTTCACG-3' (reverse), and Universal Probe Library number 42 probe (Roche), HPRT1 (accession number NM_000194) 5'-TGACCTTGATTTATT TTGCATACC-3' (forward), 5'-CGAGCAAGACGTTTCAGTC CT-3' (reverse), and Universal Probe Library number 73 probe (Roche).

Clinical course. The patient was admitted to Kobe children's hospital because of fever and leg pain. She had no remarkable medical history and did not show any remarkable abnormalities. Initial laboratory test detected the elevated levels of urine VMA 500 $\mu\text{g/mg}$ Cre, urine HVA 331 $\mu\text{g/mg}$ Cre and serum NSE 553 $\mu\text{g/l}$. Computed tomography (CT) images demonstrated a left adrenal gland mass (Fig. 1A). ^{123}I -metaiodobenzylguanidine (^{123}I -MIBG) scintigraphy revealed multiple bone metastases in the humerus, femur, pelvis, skull, and vertebrae (Fig. 1B). She underwent an open biopsy of the adrenal tumor (Fig. 1C). A pathological examination revealed a poorly differentiated NB with intermediate mitosis-karyorrhexis index (MKI) and non-amplified MYCN status. BM examination showed metastatic NB cells. Based on these findings, she was diagnosed as HR-NB according to the INRG Classification System (1).

Following the diagnosis of HR-NB, she was treated according to the nationwide standard protocol. Induction chemotherapy with one cycle of 05A1 regimen (1,200 mg/m^2 cyclophosphamide (CPA), 1.5 mg/m^2 vincristine (VCR), 40 mg/m^2 pirarubicin (THP), 100 mg/m^2 cisplatin (CDDP)) (18), two cycles of 05A3 regimen (2,400 mg/m^2 CPA, 1.5 mg/m^2 VCR, 40 mg/m^2 THP, 100 mg/m^2 CDDP) (18), and two cycles of ICE regimen (800 mg/m^2 carboplatin (CBDCA), 9,000 mg/m^2 ifosfamide (IFO), 500 mg/m^2 etoposide (VP16)) (19) was completed, and MIBG-avid lesions were disappeared except for the primary lesion. High-dose chemotherapy (12.4 mg/kg busulfan (BU), 180 mg/m^2 melphalan (L-PAM)) with autologous peripheral blood stem cell transplantation (PBSCT) followed by gross total resection of the primary tumor and radiation therapy with proton beam (30.6 Gy) was

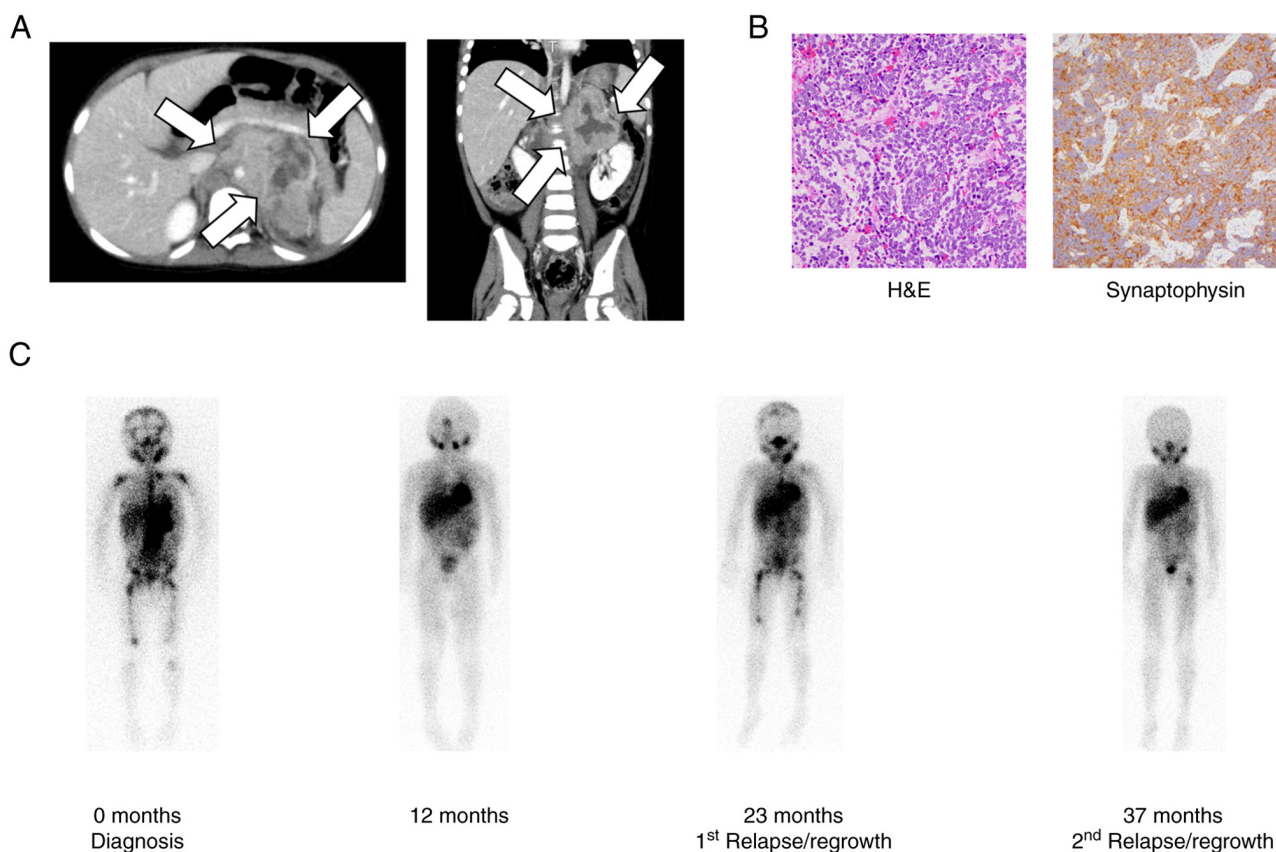


Figure 1. (A) CT images of an abdominal tumor at diagnosis. A tumor with a diameter of ~6 cm (white arrows) was detected in the left adrenal gland. (B) Pathological examination of the adrenal tumor. Small round tumor cells were arranged in nests separated by slender fibers (original magnification, x200). Immunostaining was positive for synaptophysin (original magnification, x400). (C) Representative MIBG images during the entire course of treatment. MIBG-avid lesions were detected in left adrenal gland, right and left upper carpal bones, spine, pelvis, and right and left thigh bones at 0 months, disappeared at 12 months, and reappeared in the left upper carpal bones, spine, pelvis, and right and left thigh bones at 23 months and in the left thigh bone at 37 months. H&E, hematoxylin and eosin staining; MIBG, metaiodobenzylguanidine.

performed, and she achieved complete remission. Maintenance therapy with 13-cis-retinoic acid (13CRA: 6 cycles of a 28-day cycle of 14 consecutive 160 mg/m²/day administration) followed this induction and consolidation therapy.

She developed lower limb pain 6 months after the completion of maintenance therapy. NB cells and MIBG-avid lesions were detected in BM and multiple bones, respectively, and she was diagnosed with 1st relapse/regrowth. Salvage chemotherapy with 4 cycles of TI regimen (500 mg/m² temozolomide (TMZ), 200 mg/m² irinotecan (CPT-11)) and 5 cycles of TC regimen (3.75 mg/m² topotecan, 1,250 mg/m² CPA) was undertaken, and she achieved partial remission. Anti-GD2 immunotherapy (6 cycles of a 28-day cycle of alternating 300 mg/m² dinutuximab with 70 µg/kg filgrastim or 300 mg/m² dinutuximab with 7,000,000 IU/m² teceleukin) was initiated, but she developed lower limb pain again during 4 cycles of the regimen. BM examination did not reveal NB cells, but MIBG-avid lesions appeared in a thigh bone. She was diagnosed with 2nd relapse/regrowth.

MRD monitoring. PB-MRD and BM-MRD were consecutively monitored during the entire course of treatment until 2nd relapse/regrowth (Fig. 2). Both PB-MRD and BM-MRD were highly elevated at diagnosis and decreased with induction chemotherapy. PB-MRD was approximately 100 times lower

than BM-MRD at diagnosis (PB-MRD/BM-MRD designated as MRD ratio 0.011) and after 1 cycle of induction chemotherapy (MRD ratio 0.008) and decreased to the bottom after 3 cycles of induction chemotherapy. PB-MRD was reelected and became higher than BM-MRD after 5 cycles of induction chemotherapy and remained higher than BM-MRD during consolidation and maintenance therapy and follow-up (MRD ratio 2.690, 2.256, 5.091, and 1.596). At 1st relapse/regrowth (MRD ratio 0.001), PB-MRD became almost 1,000 times lower than BM-MRD. With salvage chemotherapy, both PB-MRD and BM-MRD decreased and were reelected. PB-MRD became higher than BM-MRD during salvage chemotherapy and anti-GD2 immunotherapy (MRD ratio 1.500 and 3.477), but lower than BM-MRD at 2nd relapse/regrowth (MRD ratio 0.309).

Discussion

MRD is defined as residual cancer cells that remain in patients following local and systemic therapies. These cancer cells exist as cancer stem cells (CSCs) in primary and metastatic lesions, circulating tumor cells (CTCs) in PB, and disseminated tumor cells (DTCs) in BM (6,7). Since consecutive sampling of primary and metastatic lesions is very difficult, PB and BM samples are commonly used to evaluate MRD in many cancer types. Although a prognostic value of PB-MRD has

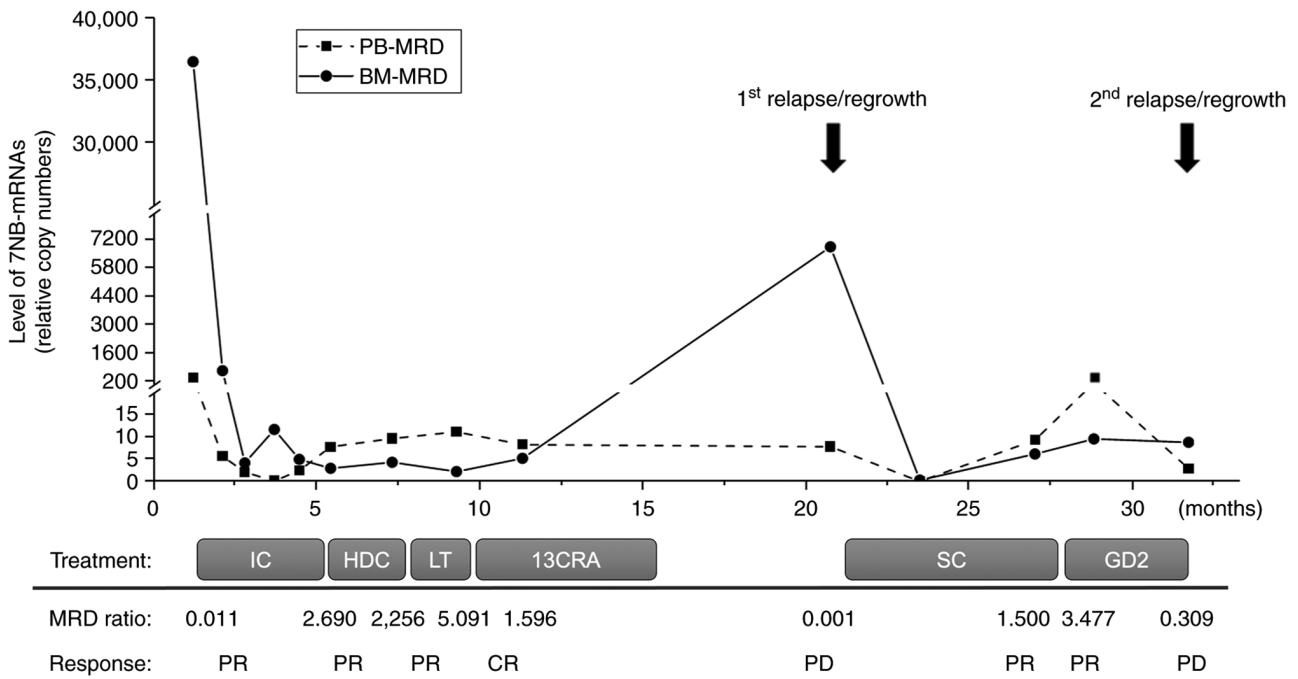


Figure 2. Consecutive PB-MRD and BM-MRD monitoring. Month 0 was defined as the time of diagnosis. 7NB-mRNAs, 7 neuroblastoma-associated mRNAs; 13CRA, 13-cis-retinoic acid; BM-MRD, MRD in bone marrow samples; CR, complete response; GD2, anti-GD2 immunotherapy; HDC, high-dose chemotherapy; IC, induction chemotherapy; LT, local therapy; MRD, minimal residual disease; MRD ratio, PB-MRD/BM-MRD; PB-MRD, MRD in peripheral blood samples; PD, progressive disease; PR, partial response; SC, salvage chemotherapy.

been established for acute lymphoblastic leukemia (ALL) (20), acute myeloid leukemia (AML) (21), and chronic myelogenous leukemia (CML) (22), it depends on cancer types in nonhematopoietic solid tumors.

In HR-NB patients, NB cells were pathologically detected in PB at diagnosis and during chemotherapy (23,24). Expression of two sets of NB-mRNAs in PB samples collected at diagnosis was shown to predict patient outcome (11,25). Although a significant correlation between PB-MRD and BM-MRD was reported by quantitating 5NB-mRNAs (CHGA, DCX, DDC, PHOX2B, TH mRNAs) with qPCR (13) and 7NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) with ddPCR (14,26), respectively, the level of PM-MRD was approximately 10-100 times lower than BM-MRD, raising concern about the sensitivity of PB-MRD. Accordingly, the clinical significance of PB-MRD is not clear for HR-NB patients.

In the present case, PB-MRD was almost 100-1,000 times lower than BM-MRD at diagnosis and 1st relapse/regrowth (MRD ratio 0.011 and 0.001) and after 1 cycle of induction chemotherapy (MRD ratio 0.008). It became higher than BM-MRD before 1st and 2nd relapse/regrowth (MRD ratio 5.091, and 3.477). Although PB-MRD was generally believed to be lower than BM-MRD, it was not always true. PB-MRD (median 5.2) was reported to be higher than BM-MRD (median 3.8) during induction and consolidation therapy in 27 PB and 89 BM samples from 14 and 19 HR-NB patients (14). The amount of DTCs in BM aspirates (=BM-MRD) is almost proportional to the amount of NB cells pathologically detected in BM, whereas CTCs are derived from both primary and metastatic NB tumors residing anywhere in the patient's body including BM. It is generally recognized that the amount of CTCs (=PB-MRDs) is very limited and difficult to predict

by pathological BM examination, MIGB scintigraphy, and CT/magnetic resonance imaging (MRI) imaging. Based on the present case's observation, we suggest the following scenario: PB-MRD will be ~1,000 times lower than BM-MRD when NB cells macroscopically metastasize/invade into BM, whereas PB-MRD can become higher than BM-MRD in at least some patients when NB cells residing in BM are macroscopically eradicated by induction, consolidation, and maintenance therapy. What determines the balance between CTCs and NB tumors (i.e., how many NB cells are released from tumor mass as CTCs) is still an open question and may depend on each patient's specific condition, such as the BM microenvironment after high-dose chemotherapy with autologous PBSCT. Although only a single case's observation, it is tempting to speculate that the elevated PB-MRD before relapse/regrowth implicates the emergence of relapse/regrowth-causing CTCs.

In conclusion, the present case highlights the fact that PB-MRD can become higher than BM-MRD before relapse/regrowth of HR-NB patients. Consecutive PB-MRD and BM-MRD monitoring during the entire course of treatment will be warranted to clarify the clinical significance of PB-MRD for HR-NB patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SI treated the patient, obtained clinical samples, analyzed data and wrote the manuscript. KHNW and CYM acquired and analyzed data. TF, SH, SU, TI, TM, AN, NNak, NNin, AT and NY treated the patient and acquired data. DH, YK and KN designed the study and interpreted data. NNis conceived and designed the study, analyzed and interpreted data, and wrote the manuscript. SI and NNis confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The parents of the patient provided written informed consent for participation in the study.

Patient consent for publication

The parents of the patient provided written informed consent for the publication of any data and/or accompanying images.

Competing interests

The authors declare that they have no competing interests.

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