Abstract. Neuroblastoma is an aggressive pediatric tumor accounting for ~15% of cancer-associated mortalities in children. Despite the current intensive therapy, >50% of high-risk patients experience tumor relapse or regrowth caused by the activation of minimal residual disease (MRD). Although several MRD detection protocols using various reverse transcription-quantitative polymerase chain reaction (RT-qPCR) markers have been reported to evaluate the therapeutic response and disease status of neuroblastoma patients, their clinical significance remains elusive. The present study reports two high-risk neuroblastoma patients, whose MRD was consecutively monitored using 11 RT-qPCR markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) during their course of treatment. The two patients initially responded to the induction therapy and reached MRD-negative status. The patients’ MRD subsequently became positive with no elevation of their urinary homovanillic acid, urinary vanillylmandelic acid and serum neuron-specific enolase levels at 13 or 19 weeks prior to the clinical diagnosis of tumor relapse or regrowth. The present cases highlight the possibility of consecutive MRD monitoring using 11 markers to enable an early detection of tumor relapse or regrowth in high-risk neuroblastoma patients.

Introduction

Neuroblastoma is a common pediatric tumor that accounts for ~10% of pediatric tumors and ~15% of pediatric cancer-associated mortalities (1). It originates from the sympathetic ganglia and/or adrenal medulla, and is characterized by extreme heterogeneity ranging from spontaneous regression to malignant progression (2). Although neuroblastoma patients stratified into the low and intermediate risk groups exhibit an excellent prognosis, the long-term survival rate of high-risk patients remains <40% (3). This is mainly due to tumor relapse or regrowth caused by the activation of chemoresistant minimal residual disease (MRD) (1-3).

To evaluate the therapeutic response and disease status of neuroblastoma patients, several MRD detection protocols based on the expression of multiple reverse transcription-quantitative polymerase chain reaction (RT-qPCR) markers have been reported. These protocols used various sets and quantities of MRD markers, including 3 markers (DCX, PHOX2B and TH) (4), 5 markers (CHGA, DCX, DDC, PHOX2B and TH) (5), 6 markers (CHRNA3, DBH, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) (5), 7 markers (B4GALNT1, DCX, DDC, ELAV4, PHOX2B, STX and TH) (7), 8 markers (CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B and TACC2) (8), and 11 markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) (9). The clinical significance of MRD monitoring in neuroblastoma patients remains to be established (5,7,10,11). The present study reports two high-risk neuroblastoma patients whose MRD was consecutively monitored using 11 markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) during their course of treatment (9).

Patients and methods

Patients. Patients 1 and 2 were four- and two-year old boys, respectively. The patients were diagnosed with stage 4, MYCN-amplified, high-risk neuroblastoma (12) and were

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treated according to the Japan Neuroblastoma Study Group (JNBSG) protocol at Kobe Children's Hospital, Japan [University Hospital Medical Information Network (UMIN) clinical trial registry no. UMIN000005045].

**Imaging diagnostics.** Computed tomography (CT) was performed using an Aquilion PRIME 80 (Toshiba Medical Systems Corp., Ohtahara, Japan). Metaiodobenzylguanidine (MIBG) scanning was performed using an Infinia Hawkeye GP3 (GE Healthcare Life Sciences, Chalfont, UK). Magnetic resonance imaging (MRI) was performed using a Philips Achieva 1.5T (Philips Healthcare, Andover, MA, USA).

**Treatment strategies.** Radiation therapy (RT) was administered as follows: i) Patient 1, 19.8 Gy (as initial local therapy for the tumor bed) plus 12 Gy (as a total body irradiation for relapse); ii) patient 2, 19.8 Gy (as initial local radiotherapy for the tumor bed) plus 30 Gy (as a salvage local therapy).

The drug 13-cis-retinoic acid (13CRA) was administered orally (160 mg/m²/day) over 14 consecutive days in a 28-day cycle, with a total of 6 cycles. Salavage chemotherapy (SC) was administered as follows: i) Patient 1, 1 cycle of irinotecan (300 mg/m²), etoposide (300 mg/m²) and carboplatin (240 mg/m²) (IREC), 1 cycle of ifosfamide (4,800 mg/m²), etoposide (500 mg/m²) and carboplatin (800 mg/m²) (ICE), 1 cycle of IREC, 1 cycle of ICE, 10 cycles of topotecan (3.75 mg/m²) and ifosfamide (6,000 mg/m²) (Topo+IFO), followed by an allogenic bone marrow transplant and best supportive care; ii) patient 2, 1 cycle of IREC, 1 cycle of ICE, 1 cycle of IREC, 1 cycle of ICE, 1 cycle of IREC, 1 cycle of ICE, 1 cycle of Topo+IFO, 2 cycles of temozolomide (1,250 mg/m²) and irinotecan (250 mg/m²), followed by palliative care.

**Samples.** All peripheral blood (PB) and bone marrow (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.

**RNA extraction and cDNA synthesis.** All PB 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 protocol. Total RNA was extracted with a TRIzol Plus RNA purification kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. After evaluating RNA integrity using 1.0% agarose gel electrophoresis (Wako Pure Chemical Industries, Ltd., Osaka, Japan), cDNA was synthesized from 1 or 0.5 ug total RNA using a Quantitect reverse transcription kit (Qiagen, Inc., Valencia, CA, USA) and diluted to a total volume of 80 (or 40) µl. DNase was included in the Quantitect reverse transcription kit.

**RT-qPCR.** RT-qPCR was performed using an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.) in a total volume of 15 µl consisting of 7.5 µl 2X FastStart Universal SYBR-Green master mix (Roche Diagnostics GmbH, Mannheim, Germany), 1.5 µl each of 3 µM sense and anti-sense primers, and 1 µl sample cDNA (corresponding to 12.5 ng total RNA). Each cDNA was amplified with a precycling 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. The primer sequences were as follows: CHRNA3 sense, 5'-TGA AATGGAACCCCTCTGCAC-3' and anti-sense, 5'-GGAAAT CCCCCACAGCATT-3'; CRMP1 sense, 5'-GAGTGCAGCG CATCATC-3' and anti-sense, 5'-GGCCTCTCACA AACTAGGG-3'; DBH sense, 5'-GACCCCAAGGATTAC CTCAAT-3' and anti-sense, 5'-GTTGATGGCATCCAGTTGA C-3'; DCX sense, 5'-CATCCCCAAACACCTCAGAGG-3' and anti-sense, 5'-GGAGGCCAGGTTGCTTGTA-3'; DDC sense, 5'-GGAGAAAGGGGAGGATG-3' and anti-sense, 5'-CAG...
CCGATGGATCCTTTGGAATAA-3'; GABRB3 sense, 5'-GGGTGT CCTTCTGGATCAATT-3' and anti-sense, 5'-TTGTCAAGCA CATGTTGATCC-3'; GAP43 sense, 5'-GGAGATTGCTCT GCCAG-3' and anti-sense, 5'-GGCACTTTTCTTAGGTTT GGT-3'; ISL1 sense 5'-AAGGACAAGAGCCAAGCAT-3' and anti-sense, 5'-TTCTGTGATCCTTCCGGATA-3'; KIF1A sense, 5'-TCTGGCCGACATCACTGACAT-3' and anti-sense, 5'-GGTGAGGCAGGACTGAGG-3'; PHOX2B sense, 5'-CTA CCCCAGCATCTACACTCG-3' and anti-sense, 5'-CTTGCT TGCAGGACTTTG-3'; TACC2 sense, 5'-CCCCACTATTTCG CTCAGAAA-3' and anti-sense, 5'-GGGCTTCTATCCGCA TGAT-3'; TH sense, 5'-GCCAAGGACAAGCTAGG-3' and anti-sense, 5'-AGCGTGATCGGGTCTGAAC-3'; and β2-microglobulin (B2M) sense, 5'-TTCTGCGCCGAGGCGC TATC-3' and anti-sense 5'-TCAGGAAATTTGACTTCCAT TC-3'. Primers were designed using Universal Probe Library Assay Design Center (https://lifescience.roche.com/shop/Categ oryDisplay?catalogId=10001&tab=&identifier=Universal+P
robe+Library&langId=-1) and synthesized by Thermo Fisher Scientific, Inc. The expression of 11 MRD markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) was calculated based on the relative standard curve method (13) using B2M as an endogenous reference for normalization (9), and the expression was scored as positive if it exceeded the normal range. qPCR was repeated 3 times (each sample was analyzed in triplicate).

Results

Clinical courses. Patient 1 presented with a fever and abdominal pain. CT revealed a left adrenal tumor. A biopsy of the tumor demonstrated poorly differentiated neuroblastoma with MYCN-amplified. A MIBG scan revealed abnormal uptake in the abdominal mass, cranial bone, spine, humerus and thigh bone (Fig. 1). BM cytology detected the tumor cells. Urinary homovanillic acid (HVA), urinary vanillylmandelic acid (VMA), and serum neuron-specific enolase (NSE) levels were elevated (Fig. 2). Following two cycles of induction chemotherapy (IC; 1 cycle of 1,200 mg/m^2 cyclophosphamide, 1.5 mg/m^2 vincristine, 40 mg/m^2 pirarubicin and 100 mg/m^2 cisplatin followed by 4 cycles of 2,400 mg/m^2 cyclophosphamide, 1.5 mg/m^2 vincristine, 40 mg/m^2 pirarubicin and 100 mg/m^2 cisplatin), HVA, VMA and NSE levels normalized. No MIBG-avid lesions were detected following an additional three cycles of IC, autologous peripheral blood stem cell transplantation (PBSC), delayed surgical therapy (ST) and RT. During 13CRA treatment, new MIBG-avid lesions emerged in the humerus and thigh bone. At 61 weeks following diagnosis when the NSE level became re-elevated, BM cytology revealed the tumor cells, and tumor relapse was clinically diagnosed. Despite the SC, RT, BM transplantation and high-dose MIBG therapy, the patient succumbed 42 months following diagnosis due to the progression of the tumor.

Patient 2 presented with pallor and an abdominal mass. A CT scan revealed a right adrenal tumor with nodules in the right lung. A biopsy of the tumor revealed poorly differentiated neuroblastoma with MYCN-amplified. An MIBG scan demonstrated abnormal uptake in the right abdominal mass and left thigh bone (Fig. 3). BM cytology revealed the tumor cells. VMA, HVA and NSE levels were elevated (Fig. 4). Following five cycles of IC, PBSCT, ST and RT, the persistent MIBG-avid lesion remained in the liver. Although VMA, HVA and NSE levels normalized during 13CRA treatment, magnetic resonance imaging with contrast media revealed a new liver lesion with marked enhancement on the T1-weighted images. At 67 weeks following diagnosis when the NSE level became re-elevated, the enhanced mass was biopsied and revealed to be a liver neuroblastoma. The patient was subsequently subjected to SC and RT. In total, 33 months following diagnosis, the regrown tumor was classified as progressive disease.

Consecutive MRD monitoring. The expression of 11 markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) was determined in BM and PB samples by RT-qPCR using B2M as an endogenous reference. In patient 1, MRD was consecutively monitored in the BM sample at 4, 8, 28, 48 and 61 weeks following diagnosis (Fig. 2). Following the initial cycle of IC, 7 markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B and TH) were positive. In response to the subsequent cycle of IC, the number of positive markers decreased and became zero following PBSCT.
At 48 weeks following diagnosis, when HVA, VMA and NSE levels remained normal, the single marker PHOX2B became positive. At 61 weeks following diagnosis, when tumor relapse was clinically diagnosed, all 11 markers became positive.

In patient 2, consecutive MRD monitoring was performed in BM and PB samples at 0, 4, 8, 12, 16, 20, 28, 38, 48, 52 and 67 weeks following diagnosis (Fig. 4). At diagnosis, 10 markers (CHRNA3, CRMP1, DBH, DCX, DDC, GAP43, ISL1, KIF1A, PHOX2B and TH) and 2 markers (CRMP1 and GABBR3) were positive in BM and PB samples, respectively. During 5 cycles of IC, the number of positive markers decreased and became zero in the BM and PB samples. At 48 weeks following diagnosis when HVA, VMA and NSE levels normalized, the single markers PHOX2B and CRMP1 became positive in the BM and PB samples, respectively. At 67 weeks following diagnosis, when tumor regrowth was clinically diagnosed, the single markers DBH and CRMP1 were positive in the BM and PB samples, respectively.

Discussion

Due to the fact that more than half of high-risk neuroblastoma patients have experienced tumor relapse or regrowth caused by the activation of MRD (1-3), a precise monitoring of MRD is essential to improve patient prognosis. As accumulating evidence suggest the genetic and phenotypic heterogeneity of MRD (14,15), the expression of MRD markers in BM and PB samples are likely to be dynamic and variable. The inherent nature of neuroblastoma, with extreme heterogeneity in the clinical and biological aspects, may additionally exaggerate the dynamism and variability of MRD marker expression (1-3). In addition, the various reagents used in each treatment protocol potentially modify the level of MRD marker expression, as exemplified by anti-GD2 antibody and MIBG therapies (8). Therefore, it is of critical significance to determine the more effective set of MRD markers for neuroblastoma patients.

The present study consecutively monitored MRD using 11 markers in BM and PB samples of two high-risk neuroblastoma patients during their course of treatment (9). The two patients initially responded to the induction therapy and attained MRD-negative status. However, the patients subsequently had MRD-positive status at 13 and 19 weeks prior to the clinical diagnosis of tumor relapse or regrowth. Although HVA, VMA and NSE levels are known to be falsely elevated in certain neuroblastoma patients (16), they remained normal when MRD was re-detected in the present patients. Although there are limitations to prevent drawing any conclusion from the present study, it warrants the additional evaluation of consecutive MRD monitoring using 11 markers in a larger cohort of neuroblastoma patients in order to clarify the clinical value of MRD.

In conclusion, the findings from the present patients highlight the possibility of consecutive MRD monitoring using 11 markers to enable early detection of tumor relapse or regrowth in high-risk neuroblastoma patients.

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