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A practical fluorometric assay method to measure lysosomal acid lipase activity in dried blood spots for the screening of cholesteryl ester storage disease and Wolman disease

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ABSTRACT

Fluorometric measurements of 4-methylumbelliferone (4-MU) are generally used to screen lysosomal storage diseases (LSDs) using dried blood spots (DBSs). However, in DBS, it is difficult to measure lysosomal acid lipase (LAL) activity due to the influence of other lipases in whole blood. Recently, Hamilton used a fluorometric enzyme assay with 4-MU derivatives to measure the LAL activity in DBS. This method requires mercury chloride as stopping reagent, and the fluorescence intensity of 4-MU was measured at an acidic pH. We report a revised method to measure the LAL activity without using toxic mercury chloride and to measure the fluorescence intensity of 4-MU at a basic pH. For this measurement, we established a more practical method that does not require mercury chloride.

The LAL activity in DBS was measured in 51 normal controls, seven obligate carriers and seven patients with CESD. The average LAL activities ± SD in the DBS from the normal, obligate carriers and CESD patients were 0.68 ± 0.2 (range: 0.3–1.08), 0.21 ± 0.1 (range: 0.11–0.41) and 0.02 ± 0.02 (range: 0–0.06) nmol/punch/h, respectively. There was a significant difference between the normal and the CESD. Our method does not require toxic mercury chloride and is an appropriate revised enzyme assay using DBS for screening patients with CESD.

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1. Introduction

Cholesteryl ester storage disease (CESD) and Wolman disease (WD) are recessive autosomal disorders caused by a lysosomal acid lipase (LAL; EC 3.1.1.13) deficiency that hydrolyzes cholesteryl esters and triglycerides [1,2]. Clinically, WD is an infantile form of LAL deficiency characterized by marked hepatosplenomegaly, adrenal calcification and a failure to thrive, while CESD is a late onset form with hepatomegaly, microvesicular steatosis and cirrhosis.

Furthermore, enzyme replacement therapy for patients with LAL deficiency has recently been developed using recombinant enzymes produced by egg whites from transgenic *Gallus* [3]. Therefore, early diagnosis and treatment for WD and CESD require a simple diagnosis method using dried blood spots (DBSs). In particular, WD usually takes a rapid clinical course and requires early treatment via enzyme replacement therapy after screening newborns. Therefore, it is essentially necessary to establish an accurate and appropriate procedure to measure the LAL activity using DBS.

A fluorometric assay using 4-methylumbelliferone (4-MU) derivatives for measuring the LAL activity in fibroblasts was first reported by Guy in 1978 [4]. In this method, 4-methylumbelliferyl-palmitate (4-MUP) was used as an enzyme substrate, and the hydrolysis of this substrate occurred at acidic pH. The fluorescence intensity of the released 4-MU was measured at ~pH 9.5. However, in whole blood, it was difficult to measure the LAL activity due to the influence of other lipases in whole blood. Consequently, DBSs were not used to measure the LAL activity.

Currently, DBS samples are widely used to screen newborns for inborn metabolic errors [5], and fluorometric enzyme assay methods using 4-MU derivatives are commonly used to screen DBS for several lysosomal storage disorders (LSDs) [6]. Hamilton discovered a fluorometric measurement system using 4-MU derivatives to assess the LAL activity in DBS in 2012 [7]. In this method, 4-MUP was used as a substrate, and the enzymatic reaction was performed in the presence and absence of a LAL-specific inhibitor (Lalistat2). The released 4-MU was measured at an "acidic pH (~pH 4)" with mercury chloride.

In diagnostic LSD methods using DBS, the fluorescence intensity of 4-MU has been measured at "basic pHs (greater than pH 10)" [6].

Abbreviations: 4-MU, 4-methylumbelliferone; LAL, lysosomal acid lipase; DBS, dried blood spots; LSD, lysosomal storage disorders; CESD, cholesteryl ester storage disease; WD, Wolman disease; 4-MUP, 4-methylumbelliferyl-palmitate; FWHM, full width at half maximum.

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To date, a LAL enzyme assay method used to measure the released 4-MU under basic conditions has not been reported. At "basic pH", the optimal excitation wavelength required to measure the amount of released 4-MU is usually 365 nm. However, at "acidic pHs (~pH 4)", 355 nm was used for the excitation wavelength [7], and this method could differentiate between normal, carrier and CESD patients. The pH was significantly different between the former and latter cases, but the excitation wavelength was nearly the same, although the excitation wavelength of 4-MU is pH-dependent [8,9].

Regarding the optical filter, the full width at half maximum (FWHM) provides important information that can be used to measure the activity of the released 4-MU correctly. Because the optical filter with a wide FWHM value, it can concentrate light from a larger wavelength and detect additional fluorescence signals. However, because distinguishing between similar fluorescence wavelengths is difficult with these optical filters, it cannot be used normally when the fluorescence spectrum overlaps. These critical points used to measure a more precise wavelength for 4-MU have not been demonstrated in previous studies [6,7]. In the present study, we measured the excitation and mission spectra of 4-MU at several different pH values to observe the relationship between the pH and wavelength and to determine a suitable optical filter for measuring the LAL activity.

Based on these fundamental parameters, we have established a better enzyme assay method for measuring the LAL activity by modifying Hamilton's method [7].

2. Materials and methods

2.1. DBS samples

The 51 normal (adults) DBS samples (EDTA-blood) were obtained with informed consent by Dr. Yoshikatu Eto. The seven CESD and the seven obligate carrier DBS samples (EDTA-blood) were provided by Synageva BioPharma (Lexington, Massachusetts, USA) and Dr. John Hamilton (Department of Biochemistry, Yorkhill Hospital, UK). The samples were stored at 4 °C until analysis. This study was approved by the Ethics Committee of the Southern TOHOKU Research Institute for Neuroscience.

2.2. Reagents and equipment

4-Piperidinyl-1,2,5-thiadiazolyl-3-morpholine carboxylate (Lalistat2) was provided by Chemical Tools. 4-Methylumbelliferyl palmitate(4-MUP) was obtained from Santa Cruz Biotechnology. 4-Methylumbelliferone (4-MU), dimethyl sulfoxide (DMSO) and the cardiolipin solution were purchased from Sigma-Aldrich. Sodium acetate and acetic acid were obtained from Wako Pure Chemical Industries. The filter paper and black 96-well assay plates were purchased from Toyo Roshi Kaisha, Ltd., and Nunc, respectively. The fluorescence intensity was measured with a Corona MTP810 fluorescence microplate reader. A 365-nm filter (full width at half maximum (FWHM): 6 nm) was used for excitation, and a 450-nm filter (FWHM: 12 nm) was used for emission. The excitation and emission spectra were measured with a Shimadzu RF-5300PC spectrofluorometer.

2.3. Excitation and emission spectra

The excitation and emission spectra of 20 nM aqueous 4-MU were measured using a Shimadzu RF-5300PC spectrofluorometer at pH values 4.1, 7.1, 8.4 and 10.1. The excitation spectra were collected at 450 nm, and the emission spectra were collected at 320 nm or 365 nm. A pH titration was performed by adding 0.025–1.0 M NaOH or 0.025–1.0 M HCl to 20 nM aqueous 4-MU (1 nmol/50 mL). A 3-mL aliquot of the 4-MU solution was removed from the beaker at each titration point, and the fluorescence intensity of the sample was measured in a disposable plastic cuvette. The excitation and emission spectra were recorded, and the solution was returned to the beaker. The final volume of the 4-MU solution was 52.6 mL (~20 nM).

2.4. LAL activity measurement

The LAL activity was measured by modifying the method reported by Hamilton [7].

For each sample, a 3-mm disk was punched from a DBS. Each punch was placed into a 1.5 mL tube and eluted in 300 μL of water at room temperature for 1 h.

A 0.345 mM substrate solution was prepared from 1.2 mL of 13.3 mM 4-MUP and 42 mL of 100 mM sodium acetate buffer pH 4.0, 1.0%(v/v) Triton X-100 and 3.0 mL of 0.5% (w/v) cardiolipin. Using a black 96-well plate, the enzymatic reactions were performed in the presence and absence of a LAL inhibitor (Lalistat2). Duplicate enzyme reactions and the single blank reactions used for background subtraction were performed for each sample. For the enzyme reaction with the LAL inhibitor, 50 μL of the substrate in buffer solution, 40 μL of the DBS eluate and 10 μL of 30 μM Lalistat2 were combined in a black 96-well plate. For the enzyme reaction without the LAL inhibitor, 50 μL of the substrate in buffer solution, 40 μL of the DBS eluate and 10 μL of water were combined in a black 96-well plate. The plates were sealed with an adhesive aluminum film and incubated in a 37 °C water bath for 24 h. The LAL reactions were terminated using 200 μL of 150 mM EDTA at pH 11.5 (At this point, the solution pH was ~10.9). A standard curve of 0–33.3 μM 4-MU was prepared via serial dilution of a 100 μM aqueous solution of 4-MU (100 μL/well), and 200 μL of 150 mM EDTA at pH 11.5 was added to each well.

The fluorescence of the plates were read immediately with a Corona MTP810 fluorescence microplate reader using a 365-nm excitation filter (FWHM: 6 nm) and a 450-nm emission filter (FWHM: 12 nm). The LAL activity (nmol/punch/h) was calculated by subtracting the enzymatic activity of the inhibited (with Lalistat2) reaction from that of the uninhibited (without Lalistat2) reaction.

The statistical analysis was performed using parametric approaches; comparisons were made using non-repeated measures ANOVA with Student–Newman–Keuls's post-test.

3. Results

3.1. Excitation and emission spectra of 4-MU

Fig. 1 shows the excitation spectra (emission wavelength = 450 nm) of 4-MU at pH values 4.1, 7.1, 8.4 and 10.1. As shown in Fig. 1, the excitation peak of 4-MU was drastically shifted by the solution pH. At pH 4.1, we observed the maximum excitation peak of 4-MU at 320 nm; this peak shifted from 320 nm to 365 nm when the solution pH was increased. In addition, at "acidic pHs (pH 4.1)", there was no peak near

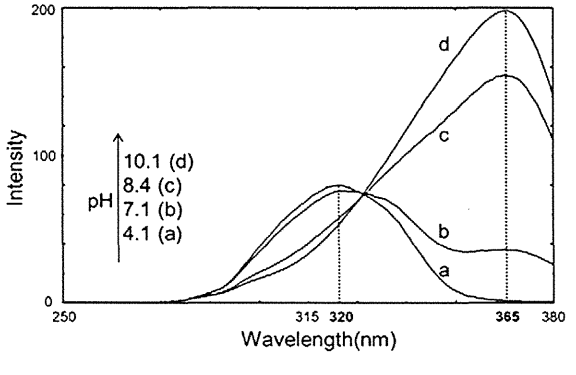

Fig. 1. Excitation spectra (emission wavelength = 450 nm) of 20 nM 4-methylumbelliferone at pH values 4.1 (a), 7.1 (b), 8.4 (c) and 10.1 (d).

365 nm. Therefore, even with a 365-nm excitation at "acidic pH (pH 4.1)", 4-MU does not excite.

Fig. 2A shows the emission spectra (excitation wavelength = 365 nm) of 4-MU at pH values 4.1, 7.1, 8.4 and 10.1. As shown in Fig. 2A, the fluorescence intensity slightly increased as the pH increased, drastically changing from pH 7.1 to pH 8.4. At pH 10.1, we observed the maximum emission at 450 nm.

Similar to the well-known chemical properties of 4-MU, at "basic pH (pH 10.1)", the optimal wavelengths used to measure the amount of released 4-MU are 365 nm (excitation) and 450 nm (emission). However, with excitation at 320 nm, the fluorescence intensity slightly decreased as the pH increased and drastically changed from pH 7.1 to pH 8.4 (Fig. 2B). At pH 4.1, we observed the maximum emission peak at 450 nm (Fig. 2B). Fig. 2B revealed that at "acidic pH (pH 4.1)", the optimal wavelengths required to measure the amount of 4-MU released are 320 nm (excitation) and 450 nm (emission).

3.2. LAL activity measurement

The LAL activity in the DBS was measured in 51 normal, 7 obligate carrier and 7 CESD (Fig. 3). The average LAL activities in the DBS from the normal, the obligate carrier and the CESD were 0.68 ± 0.2 (range: 0.3–1.08), 0.21 ± 0.1 (range: 0.11–0.41) and 0.02 ± 0.02 (range: 0–0.06) nmol/punch/h, respectively. There was a significant difference in the LAL activities between the normal, the obligate carrier and the CESD ($P < 0.05$). We tested 8 normal samples for 20 days. The coefficient of variation (CV) value for the normal subjects was 11.9% (within-run) and 19% (day-to-day). The limit of detection was 0.06 nmol/punch/h, and the limit of quantification was 0.2 nmol/punch/h.

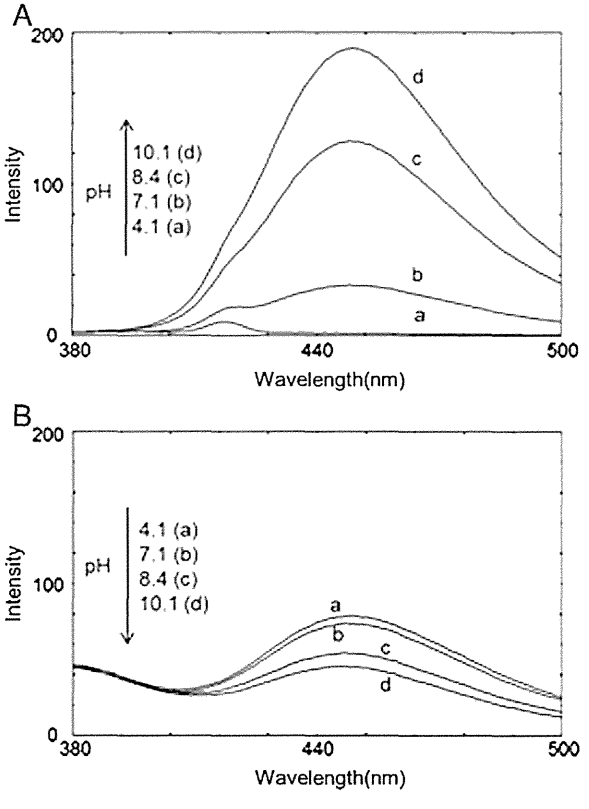

Fig. 2. Emission spectra at excitation wavelength = 365 nm (A) and emission spectra at excitation wavelength = 320 nm (B) of 20 nM 4-methylumbelliferone at pH values 4.1 (a), 7.1 (b), 8.4 (c) and 10.1 (d).

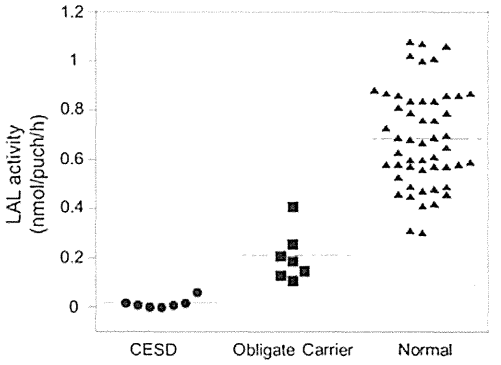

Fig. 3. Comparison of the LAL activity of 51 normal, 7 obligate carriers and 7 CESD samples.

4. Discussion

In this study, we demonstrated that the maximum excitation wavelength required to measure the amount of released 4-MU is different between "basic pH" and "acidic pH" conditions. Based on this fundamental information, we have also developed a revised method for measuring LAL by modifying Hamilton's method [7].

Because the strongest fluorescence intensity of 4-MU was obtained at pH 10 or greater, we used an EDTA solution (pH 11.5) to quench the LAL reaction. Due to the "basic pH" conditions, the fluorescence intensity of the released 4-MU was measured with a fluorescence microplate reader using a 365 nm (FWHM: 6 nm) excitation filter.

Our method has two advantages for screening CESD and WD. First, this technique does not use mercury chloride to quench the LAL reaction. Because mercury chloride is not needed, the screening test for the DBS samples can be carried out safely. Second, we used an excitation filter (365 nm: FWHM; 6 nm) that is commonly used for measuring other lysosome enzymatic activities in DBS. Therefore, screening for CESD and WD could occur at many laboratories using our method. In Hamilton's method, 4-MU was measured under "acidic pH" conditions (~pH 4). To measure the LAL activity under these conditions, we used an excitation filter (e.g., 355 nm: FWHM; ~70 nm) with a large FWHM value, or a band pass excitation filter (e.g., 320 nm: FWHM; ~6 nm).

According to our statistical analysis, the CESD patient group, the obligate carrier group and the normal group could be distinguished, but part of the obligate carrier group and part of the normal group had overlapped. Using DBS, distinguishing the obligate carriers from the normal samples may be difficult, but the DBS of all CESD patients could be detected using our method. Our method can be used to screen newborns for WD and to screen patients with fatty liver/cirrhosis for a high risk of CESD. Furthermore, we can enact early diagnosis and treatment using enzyme replacement therapy in LAL-deficient patients because recombinant enzymes from the egg whites of transgenic *Gallus* are now available [3].

5. Conclusions

Our study demonstrates that a suitable optical filter must be selected for the LAL activity measurement. Based on this finding, we revised a method for measuring the LAL activity by the method published by Hamilton [7]. Our newly developed method for measuring the LAL activity based on the fluorometric analysis of 4-MU will enable safer, more appropriate screening methods for LAL deficiencies (CESD and WD) in high-risk populations or during newborn screening for patients with WD.

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Pediatr Blood Cancer 2014;61:2316–2318

BRIEF REPORT

Proposed Strategy for the Use of High-Dose Chemotherapy With Stem Cell Rescue and Intrathecal Topotecan Without Whole-Brain Irradiation for Infantile Classic Medulloblastoma

Ai Yamada, MD,[1] Hiroshi Moritake, MD, PhD,[1]* Sachiyo Kamimura, MD,[1] Shinji Yamashita, MD,[2] Hideo Takeshima, MD, PhD,[2] and Hiroyuki Nunoi, MD, PhD[1]

We describe a 6-month-old infant with classic medulloblastoma. Gross total resection of the left cerebellar tumor was performed; however, relapse occurred during the administration of intrathecal and intravenous methotrexate-based chemotherapy. After undergoing resection, high-dose chemotherapy was administered consisting of topotecan, melphalan, and cyclophosphamide with autologous peripheral stem cell rescue followed by local irradiation and intrathecal topotecan, which resulted in a complete response for more than two years. The administration of high-dose chemotherapy followed by intrathecal topotecan as maintenance therapy is an effective strategy, without losses in the cognitive function, for avoiding the use of whole-brain irradiation for infantile classic medulloblastoma. Pediatr Blood Cancer 2014;61:2316–2318.
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Key words: histology; high-dose chemotherapy; infantile medulloblastoma; intrathecal topotecan; stem cell transplantation;

INTRODUCTION

Malignant brain tumors are the leading cause of cancer death in children, with medulloblastoma (MB) being the most common lesion [1]. The standard treatment of non-metastatic with near total resection in older children consists of surgery followed by craniospinal irradiation (CSI) and chemotherapy, with an 85% overall survival (OS) at five years [2,3]. On the other hand, children with MB under the 3 years of age have inferior outcomes due to the lack of use of CSI. The application of cranial irradiation (CI) in younger children has been implicated in deficits in learning, attention, processing speed and the endocrine function [4]. The five-year OS for children under the 3 years of age ranges from 13% to 60%, with a poor prognosis [5].

We herein describe a case of infantile recurrent MB that was successfully treated with topotecan (TOPO) using an Ommaya reservoir as maintenance therapy following high-dose chemotherapy (HDCT) with autologous stem cell rescue (ASCT) and local irradiation.

CASE REPORT

A 6-month-old Japanese infant presented with persistent vomiting and lethargy (Fig. 1). Intracranial computed tomography revealed a tumor in the left cerebellum with obstructive hydrocephalus. Surgical gross total resection of the tumor was performed. The tumor consisted of primitive cells with hyperchromatic nuclei and scanty cytoplasm (Fig. 2A). These cells were positive for synaptophysin, INI1, and MIB-1 (Fig. 2B–D). The tumor was diagnosed to be a classic MB. One month later, follow-up intracranial magnetic resonance imaging (MRI) performed prior to starting chemotherapy revealed a tumor in the left cerebellum. Local relapse of MB was pathologically confirmed after tumor resection. Simultaneously, an intraventricular subcutaneous reservoir (Ommaya reservoir) was implanted in the anterior horn of the lateral ventricle. The patient then received chemotherapy consisting of intra-Ommaya methotrexate (MTX) in addition to intravenous cyclophosphamide, MTX, vincristine, carboplatin, and etoposide according to the regimen proposed by Rutkowski et al. [6]. We measured the lumbar cerebrospinal fluid (CSF) level of MTX 24 hours after administration, and the trough level was maintained between 0.17 and 1.77 μM/L. After one cycle of chemotherapy, cranial MRI demonstrated a tumor in the left cerebellum without leptomeningeal spread or leukoencephalopathy. The patient received the second cycle of therapy, and the intra-Ommaya MTX dose was increased from 2 to 6 mg. The concentration of MTX in the CSF ranged between 0.65 and 2.16 μM/L, which is below the toxic level of 5 μM/L. The size and nature of the tumor remained stable. Therefore, myelosuppressive chemotherapy consisting of ifosfamide, carboplatin, and etoposide was administered in order to mobilize peripheral stem cells and perform ASCT. The patient's peripheral stem cells were collected and maintained in liquid nitrogen (CD34-positive cells: 1.79×10^6 cells/kg). Following resection of the residual tumor, the patient received intra-Ommaya TOPO twice weekly for four weeks (eight doses) starting at 0.2 to 0.3 mg [7]. He experienced no symptoms of severe arachnoiditis other than fever. Subsequently, ASCT after HDCT consisting of TOPO, melphalan, and cyclophosphamide (TMC) was performed [8,9]. Posterior fossa radiotherapy at a dose of 36 Gy with an additional boost of 14.4 Gy to the tumor bed was also performed after engraftment. Concurrently, the patient received four doses of weekly intra-Ommaya TOPO (0.4 mg), followed by twice-monthly treatments for four months and then monthly for

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Conflicts of interest: Nothing to declare.

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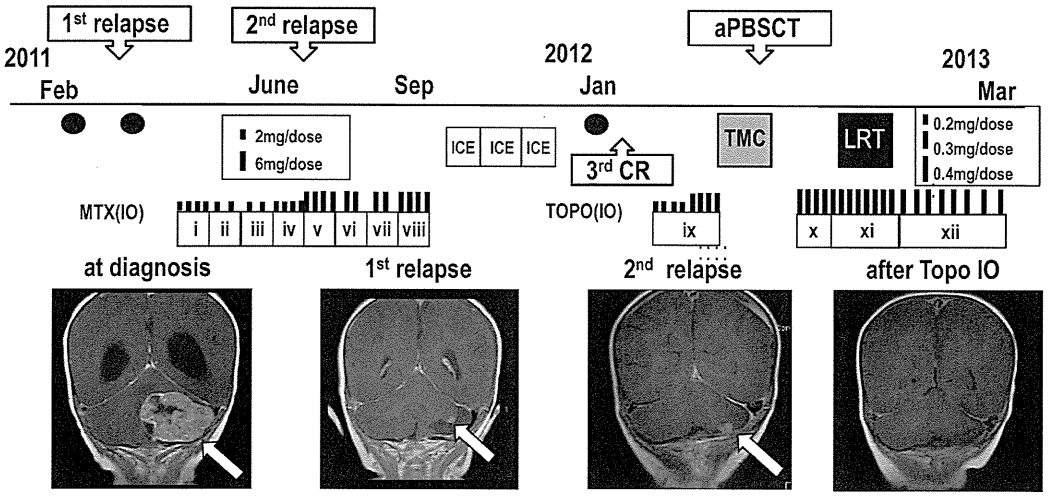

Fig. 1. Clinical course during chemotherapy. ● Excision of brain tumor. (i and v) CY + VCR; (ii, iii, vi, and vii) MTX + VCR; and (iv and viii) CBDCA + Etp (Reference: New Engl J Med 2005; 352:978–986). [ICE] CBDCA: 400 mg/m^2/day, days 1–2; IFO: 1.8 g/m^2/day, days 1–5; Etp: 100 mg/m^2/day, days 1–5. [TMC] TOPO: 4 mg/m^2/day, days −6 to −2; Mel: 70 mg/m^2/day, days −3 to −2; CY: 1 g/m^2/day, days −6 to −4. [TOPO(IO)]: (ix) twice weekly, 4 weeks; (x) weekly, 4 weeks; (xi) twice-monthly, 4 months; (xii) monthly, 8 months. [LRT] posterior fossa = 36Gy in 20 fractions, tumor bed = additional 14.4Gy in 8 fractions, total: 50.4Gy. aPBSCT: autologous peripheral blood stem cell transplantation; CR: complete response; LRT: local radiotherapy; MTX: methotrexate; IO: intra-Ommaya; TOPO: topotecan; CY: cyclophosphamide; VCR: vincristine, CBDCA: carboplatin; Etp: etposide; IFO: ifosfamide; and Mel: melphalan.

eight months [7]. The patient has maintained a status of complete response (CR) for more than two years after achieving his third CR. He has experienced no symptoms of neurotoxicity or leukoencephalopathy, and his intellectual quotient (IQ) was 98 at 3 years and 4 months of age.

DISCUSSION

Children with MB less than 3 years of age are often severely affected by CI. The St. Jude Children's Research Hospital has treated 29 MB children less than 3 years of age with CI [4]. All

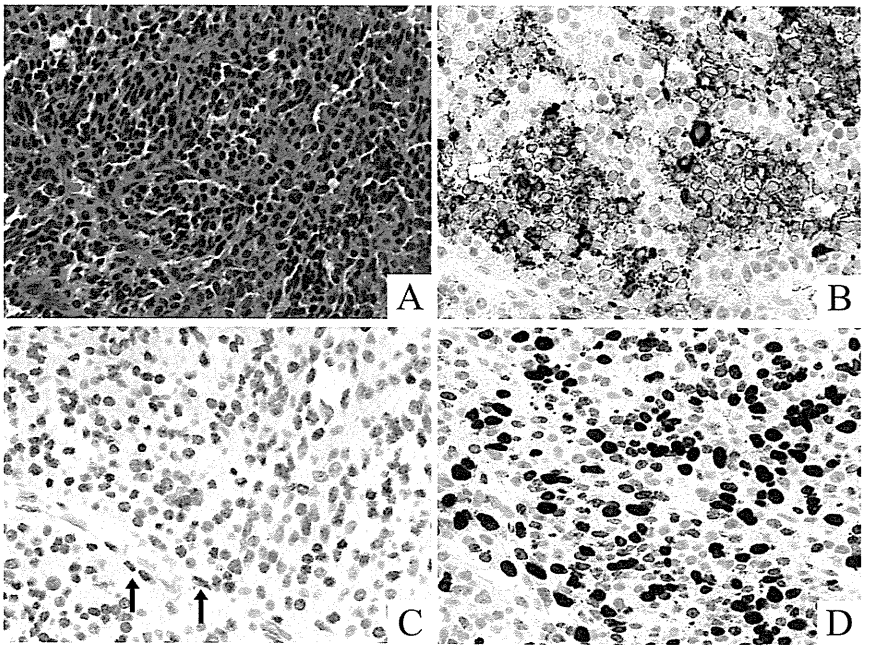

Fig. 2. Microscopic findings of the tumor: (**a**) Hematoxylin and eosin staining. The tumor consists of primitive cells with hyperchromatic nuclei and scanty cytoplasm (x20); (**b**) Synaptophysin immunostaining. The tumor cells are partly positive (x40); (**c**) INI1 immunostaining. The nuclei of the tumor cells and vascular endothelial cells (arrow) are stained (x40); and (**d**) MIB-1 immunostaining. High density of MIB-1 labelled tumor cells indicates brisk mitotic activity (x40).

patients unfortunately lost their cognitive function at a mean of −3.9 IQ points every year after therapy and experienced significant declines in the sensory function. Recently, the German Pediatric Brain Tumor Study Group reported that intrathecal and intravenous MTX-based chemotherapy without CI achieved better EFS and OS than conventional chemotherapy [6]. These findings have helped to make physicians aware of the potential efficacy of intrathecal chemotherapy while avoiding CI in young children. However, classic MB is known to have a strong independent poor prognostic histology. Given the low survival rates of children with classic MB, treatment intensification should be considered in this subgroup [10]. In the HIT-SKK 2000 trial, many relapsed patients with classic MB <3 years of age were treated with intrathecal MTX combined chemotherapy, most of whom received CSI for a cure [11]. The regional relapsed tumor observed in our case has not changed in size, although the dose of intra-Ommaya MTX was increased from 2 to 6 mg. These results suggest limitations in this strategy for treating infantile classic MB.

TOPO, a topoisomerase I inhibitor, is well known to be safe when administered into the CSF. Our patient suffered from the first relapse one month after undergoing tumor resection, suggesting the rapid growth of the tumor. In order to prevent further relapse after resection prior to HDCT with ASCT, we decided to introduce intra-Ommaya TOPO instead of MTX. In a phase 1 trial, the maximum tolerated dose associated with chemical arachnoiditis was 0.4 mg in children [7]. Our patient exhibited few adverse events other than fever at a dose of 0.4 mg. The safety and efficacy of intrathecal TOPO for relapsed various tumors, including MB, after radiotherapy has been published [7,12].

Recently, HDCT with ASCT was introduced for use in patients with pediatric brain tumors in order to avoid radiotherapy in young children [13]. However, the optimal conditioning regimen has not yet been established. Thiotepa and melphalan-based regimens have been applied to treat various brain tumors [13–15]. For example, Pérez-Martínez et al. [13,16] reported that the administration of busulfan and melphalan with an alkylating agent or topoisomerase inhibitor was effective in high-risk pediatric cases of recurrent MB; however, these combinations are associated with considerable toxicity. Furthermore, Kadota et al. [17] found that the application of cyclophosphamide and melphalan for relapsed or progressive MB was well tolerated, although the efficacy was not clearly superior. In the present study, we used TMC as a conditioning regimen, which is safe and active in patients with multiple myeloma or ovarian cancer [8,9]. Importantly, our patient also suffered no severe transplantation-related toxicities. Therefore, TMC may be a safe and effective combination for use in ASCT in patients with MB.

MB has four subgroups (the sonic hedgehog [SHH] group, the WNT group, group 3 and group 4) based on the results of gene expression profiling, with differences in prognosis between the groups [18]. Northcott et al. [19] reported that the results of immunohistochemistry for DKK1 (WNT), SFRP1 (SHH), NPR2 (group 3), and KCNA1 (group 4) can be used to easily classify these subgroups with high reliability. In the near future, it will be necessary to establish an appropriate chemotherapy regimen without the use of CI for subgroups with a good prognosis in order to prevent severe neurocognitive sequelae.

In conclusion, HDCT consisting of TMC and ASCT rescue followed by intrathecal TOPO therapy is an effective strategy for treating relapsed or resistant infantile MB.

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