# *BRAF* V600E-positive cells as molecular markers of bone marrow disease in pediatric Langerhans cell histiocytosis

Langerhans cell histiocytosis (LCH) is an inflammatory myeloid hematologic malignancy in which genetic alterations in the MAPK pathway promote the differentiation of hematopoietic stem cells (HSC) into mononuclear phagocytic lineages such as monocytes and dendritic cells, resulting in apoptosis resistance, local inflammation, and impaired lymph node migration.[1-9] The *BRAF* V600E mutation is detected in various differentiation stages of myeloid cells, including HSC or nearby progenitors and mononuclear phagocytic cells, especially in high-risk systemic LCH.[1-9] Bone marrow (BM) involvement in LCH, which causes cytopenia, is characterized by CD1a-positive immunohistochemical staining.[10-15] However, the *BRAF* mutation status and clinical impact of BM disease (BMD) at the molecular level are not fully understood.

In order to clarify the clinical impact of measurable BMD in LCH, we investigated somatic mutations in paired tumor and BM samples using a sensitive detection system. We retrospectively performed mutational analyses of 59 LCH tumors by targeted amplicon sequencing using custom-designed primers and subsequently analyzed somatic mutations in 41 paired BM samples using allele-specific droplet digital polymerase chain reaction (ddPCR). *BRAF* V600E was identified in 25 of 41 (61%) tumor samples. Measurable BMD was detected in 21 of the 25 (84%) cases positive for *BRAF* V600E, but was not detected in cases positive for other mutations. High mutational loads in BM were significantly associated with multisystem LCH with risk organ involvement, younger age, and disease progression. *BRAF* V600E in BM was detectable in patients who were refractory to treatment, and the mutational load in BM cells was higher than those in peripheral blood mononuclear cells. A high mutational burden in the BM defines a distinct clinical phenotype of high-risk, young-age patients with multisystem LCH and potential alternative risk factors.

Between 1996 and 2020, 65 Japanese pediatric patients diagnosed with LCH by a central or local pathology review were enrolled. The patients were treated with various protocols, including those of the Japan LCH Study Group -96 and -02 and the Japanese Pediatric Leukemia/Lymphoma Study Group LCH-12 in the following 13 domestic centers: Hirosaki University Hospital, Miyagi Children's Hospital, Nihon University Hospital, Chiba Children's Hospital, Tokyo Medical and Dental University Hospital, Tohoku University Hospital, Niigata University Hospital, Kyoto University Hospital, Fujita Medical University Hospital, National Center for Child Health Hospital, Hyogo Children's Hospital, Shizuoka Children's Hospital, and Yamaguchi University Hospital.[16] The parents/guardians of the study participants, who were minors, provided informed consent. The study was conducted according to the principles of the Declaration of Helsinki and approved by the ethics committee of Hirosaki University Graduate School of Medicine. We collected 65 biopsied LCH tumor specimens and 41 paired BM samples. We extracted genomic DNA from formalin-fixed, paraffin-embedded (FFPE) tumor specimens (50–70-μm thick) using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In the BM samples, mononuclear cells were separated by erythrocyte hemolysis, and DNA was extracted. In the peripheral blood samples, plasma and peripheral blood mononuclear cells (PBMC) were separated by centrifugation, and erythrocyte hemolysis was performed before DNA extraction. DNA from frozen or fresh BM and PBMC was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). We evaluated 59 LCH cases using PCR-based targeted next-generation sequencing (NGS), excluding six samples with poor PCR amplification. The amplicon libraries were analyzed for mutations in exons 12 and 15 of *BRAF* and exons 2 and 3 of *MAP2K1* (*Online Supplementary Table S1*). For original data, please contact kkudo@hirosaki-u.ac.jp.

Of the 59 LCH tumor specimens analyzed, we detected somatic mutations in 54 (92%) at various allele frequencies (2.3–35%, median 10%) by targeted NGS. *BRAF* and *MAP2K1* were mutated in 46 (78%) and eight (14%) samples, respectively. *BRAF* V600E was identified in 33 (56%) samples. In-frame deletions in exon 12 of *BRAF* were found in ten (17%) samples (*Online Supplementary Table S2*). We also identified a novel mutation, *BRAF* V600delinsDL (c.1798_1799insATT, p.Val600delinsAspLeu), which was confirmed to be an activating mutation (data not shown).

Next, we conducted mutational analysis of the 41 paired BM samples using allele-specific ddPCR to detect the following mutations: *BRAF* V600E, *BRAF* exon 12 deletion, *MAP2K1* exon 2/3 deletion, *BRAF* V600D, and *BRAF* V600delinsDL (*Online Supplementary Table S1*; *Online Supplementary Figure S1*). In this study, only patients who underwent BM examination at the physician's decision because of fever, inflammatory reaction, mild cytopenia, or staging were included. Most patients underwent staging such as computed tomography (CT), magnetic resonance imaging (MRI), X-ray, bone scintigraphy, and BM examination instead of positron emission tomography

(PET)-CT, and none of the patients changed their disease type during the course.

Measurable BMD was defined as molecularly detectable BM cells with an allele frequency of 0.01% or higher based on the ddPCR analysis of somatic mutations in BM samples. The clinical significance of BMD was examined in 38 patients, excluding three patients for whom quantitative mutational analysis was not applicable. These 38 patients consisted of 12 patients with multisystem LCH with risk organ involvement (MSRO+), 13 with multisystem LCH without risk organ involvement (MSRO-), eight with multifocal bone disease (MFB), and five with single-system LCH with a single lesion (SS) (Table 1). We detected BMD before treatment at various allele frequencies (0.03–7.0%, median 0.83%) in 21 of the 38 (55%) patients, consisting of 12 MSRO+, seven MSRO-, two MFB, and no SS patients. Contrarily, the remaining 17 BMD-negative patients comprised six MSRO-, six MFB, and five SS patients, and no patient with MSRO+. Among them, there were 11 patients with mutations other than *BRAF* V600E, including four MSRO-, three MFB, and four SS patients (Table 1). The *BRAF* V600E mutation was detected in 21 of 25 (84%) BM samples from *BRAF* V600E-positive LCH, whereas no other variants were identified, indicating notable BMD in *BRAF* V600E-positive LCH cases (Figure 1A). The rate of BM involvement in the MSRO+, MSRO-, MFB, and SS cases was 100%, 54%, 25%, and 0%, respectively (Figure 1B). In the 25 cases of *BRAF* V600E-positive LCH, the median variant allele frequency (VAF) of *BRAF* V600E in the BM of the MSRO+, MSRO-, and MFB patients was 1.0% (range, 0.20–7.0%), 0.030% (range, 0–2.2%), and 0% (range, 0–0.51%), respectively (Figure 1B). However, no somatic mutations were detected in the 13 patients with other types of mutations. Mutational burden varied among the four clinical phenotypes (Kruskal–Wallis, $P<0.001$). Notably, MSRO+ disease showed the highest mutational burden (Mann–Whitney test, $P<0.001$) (Figure 1B).

We also investigated the relationship between BMD and age, disease progression, and disease relapse. BMD was detected only in patients younger than 3 years of age, except for one patient aged 4.6 years. BMD-positive patients had a lower median age than BMD-negative patients (0.9 years *vs*. 5.4 years, Mann–Whitney test, $P=0.012$) (Figure 1C). The cumulative incidence of reactivation and relapse in the 21 patients who were positive for BMD at diagnosis was higher than that in the 17 patients who were negative for BMD (79% *vs*. 36%, $P=0.006$ by log-rank test) (Figure 1D). This suggests that BMD positivity is a risk factor for disease progression.

In order to identify the origin of the mutation-positive cells, we measured the somatic mutational load in paired BM and peripheral blood mononuclear cells (PBMC) from 13 patients at 31 time points, before and after initial chemotherapy. There were patients in whom mutated cells were present only in BM cells after chemotherapy or early relapse. The median VAF of *BRAF* V600E was 0.65% (range, 0.016–6.7%) in the BM cells and 0.16% (range, 0–4.0%) in the PBMC, indicating a higher mutational load in the former (paired *t*-test, $P<0.001$) (Figure 1E). These results suggest that the precursors in *BRAF* V600E-positive LCH are BM cells rather than PBMC. In order to assess BMD, we also measured the mutational load of *BRAF* V600E in 12 patients whose samples were available at multiple time points during treatment and disease progression. Refractory patients showed a high mutational load during the treatment (Figure 1F). Furthermore, we examined CD34-positive cells in BM samples of four patients and detected CD34-positive cells harboring *BRAF* V600E in three MSRO+ patients. These results indicate that BM is a source of immature precursor cells harboring *BRAF* V600E, causing residual disease even during chemotherapy, and that BMD is a potential target for the treatment of *BRAF* V600E-positive LCH.

Hematopoietic involvement is defined based on clinical criteria, such as anemia and/or thrombocytopenia. The clinical significance of CD1a positivity remains to be confirmed because of its limited sensitivity.[10-15] Measurable BMD was detected in 84% of *BRAF* V600E-positive cases, whereas cytopenia and CD1a positivity were detected in only 32% and 11% of cases, respectively. A comprehensive pathology review, including immunostaining using with BRAF V600E antibody clone VE1, was performed on patients from whom additional BM pathology specimens were obtained. The results showed no specific findings in the BM clots. Furthermore, BRAF V600E was detected by immunostaining in only one of 25 patients. These results suggest that allele-specific ddPCR is more reliable than CD1a or BRAF V600E immunostaining for BMD identification (Table 2).

The initial treatment comprised prednisolone, cytarabine, and vincristine for 6 weeks for all patients with systemic LCH, and in most progressive patients, the combination of cytarabine and cladribine was administered as second-line therapy.[16] In addition, refractory patients P20, P21, and P41 were treated with vemurafenib, a BRAF inhibitor. Transfusion-dependent cytopenia was improved rapidly in all three patients, showing clinical efficacy. However, only one of the three patients showed a rapid decrease in BMD, whereas the remaining two patients showed an increase in BMD, contrary to the clinical efficacy (Figure 1F). Further studies are needed to elucidate this mechanism.

We acknowledge that this was a retrospective study with different treatments and is biased toward high-risk patients receiving BM aspiration. Interestingly, BMD was identified not only in the MSRO+ patients but also in the MSRO- and MFB patients, suggesting that BMD is potentially a risk factor in these groups. Five patients, P2, P4,

**Table 1.** Characteristics of 38 patients subjected to allele-specific digital droplet polymerase chain reaction of bone marrow samples.

| Case | Sample | Age, Y | Sex | Disease type | Progression/ relapse | Observation period, Y | VAF of BM, % | VAF of PBMC, % | Somatic variants |
|---|---|---|---|---|---|---|---|---|---|
| P1 | BM | 1.8 | M | MSRO- | relapse | 3.7 | 0.25 | NA | *BRAF* V600E |
| P2 | BM | 5.4 | F | MSRO- | relapse | 3.9 | 0.00 | NA | *BRAF* V600E |
| P3 | BM | 16.1 | F | SS | neg | 2.4 | 0.00 | NA | *BRAF* N486_P490del |
| P4 | BM | 0.8 | F | MSRO+ | relapse | 3.2 | 4.46 | NA | *BRAF* V600E |
| P5 | BM | 0.9 | M | MSRO- | relapse | 3.2 | 0.94 | 0.30 | *BRAF* V600E |
| P6 | BM | 1.7 | F | MSRO- | relapse | 3.1 | 0.00 | NA | *BRAF* N486_P490del |
| P7 | BM | 2.3 | M | MFB | relapse | 2.4 | 0.26 | 0.00 | *BRAF* V600E |
| P8 | BM | 1.7 | F | MFB | neg | 2.2 | 0.51 | 0.09 | *BRAF* V600E |
| P9 | FFPE | 0.8 | F | MSRO- | neg | 22.4 | 0.00 | NA | *MAP2K1* Q56_Q61>R |
| P10 | FFPE | 0.4 | M | MSRO- | neg | 14.0 | 0.00 | NA | *BRAF* V600D |
| P11 | FFPE | 1.4 | F | MSRO- | relapse | 12.1 | 2.24 | NA | *BRAF* V600E |
| P12 | FFPE | 0.6 | F | MSRO+ | neg | 10.6 | 0.98 | NA | *BRAF* V600E |
| P13 | FFPE | 5.4 | F | MSRO- | relapse | 9.9 | 0.00 | NA | *BRAF* N486_P490del |
| P14 | FFPE | 1.7 | F | MFB | neg | 4.8 | 0.00 | NA | *BRAF* V600delinsDL |
| P15 | FFPE | 0.4 | F | MSRO- | neg | 3.0 | 0.13 | NA | *BRAF* V600E |
| P16 | FFPE | 1.5 | F | MSRO+ | neg | 2.8 | 6.96 | NA | *BRAF* V600E |
| P17 | FFPE | 0.1 | M | MSRO- | neg | 2.4 | 0.00 | NA | *BRAF* N486_P490del |
| P18 | BM | 7.2 | M | MFB | neg | 1.5 | 0.00 | 0.00 | *BRAF* V600E |
| P19 | BM | 6.9 | F | MFB | neg | 3.8 | 0.00 | 0.00 | *BRAF* V600D |
| P20 | BM | 0.1 | M | MSRO+ | progression | 1.5 | 0.42 | 0.20 | *BRAF* V600E |
| P21 | BM | 0.3 | F | MSRO+ | progression | 1.7 | 0.54 | 0.27 | *BRAF* V600E |
| P22 | BM | 8.1 | M | SS | neg | 1.4 | 0.00 | 0.00 | *MAP2K1* Q56_V60del |
| P23 | BM | 2 | F | MSRO+ | relapse | 1.3 | 1.07 | 0.26 | *BRAF* V600E |
| P24 | BM | 0.8 | M | MFB | relapse | 1.3 | 0.00 | 0.00 | *MAP2K1* Q56_Q61>R |
| P25 | BM | 1.4 | F | SS | neg | 1.1 | 0.00 | 0.00 | *MAP2K1* E102_I103del |
| P26 | BM | 14.1 | M | SS | neg | 1.2 | 0.00 | 0.00 | *BRAF* V600E |
| P28 | BM | 2.5 | F | MSRO- | neg | 1.2 | 0.03 | 0.00 | *BRAF* V600E |
| P29 | BM | 5.4 | M | SS | neg | 1.0 | 0.00 | 0.00 | *MAP2K1* Q56_G61>R |
| P30 | BM | 10 | M | MFB | neg | 1.1 | 0.00 | 0.00 | *MAP2K1* E102_I103del |
| P31 | BM | 1.5 | M | MSRO+ | progression | 1.8 | 0.83 | 0.59 | *BRAF* V600E |
| P32 | BM | 0.7 | M | MSRO+ | progression | 1.0 | 0.20 | NA | *BRAF* V600E |
| P35 | FFPE | 4.6 | F | MSRO+ | progression | 0.7 | 5.39 | NA | *BRAF* V600E |
| P36 | FFPE | 0.8 | M | MSRO+ | neg | 0.3 | 0.21 | 0.00 | *BRAF* V600E |
| P37 | BM | 3 | F | MSRO- | neg | 0.6 | 0.03 | 0.02 | *BRAF* V600E |
| P38 | BM | 1.2 | M | MFB | neg | 0.5 | 0.00 | 0.00 | *BRAF* V600E |
| P39 | BM | 0.4 | M | MSRO+ | progression | 0.4 | 4.10 | 3.50 | *BRAF* V600E |
| P40 | FFPE | 0.3 | F | MSRO- | relapse | 5.4 | 0.84 | NA | *BRAF* V600E |
| P41 | BM | 0.5 | M | MSRO+ | progression | 0.3 | 3.40 | 1.20 | *BRAF* V600E |

BM: bone marrow; FFPE: formalin-fixed paraffin-embedded; M: male; F: female; MSRO-: multisystem disease; MSRO+: multisystem disease with risk organ involvement; MFB: multifocal bone disease; VAF: variant allele frequency; PBMC: peripheral mononuclear cell. cells; NA: not applicable.

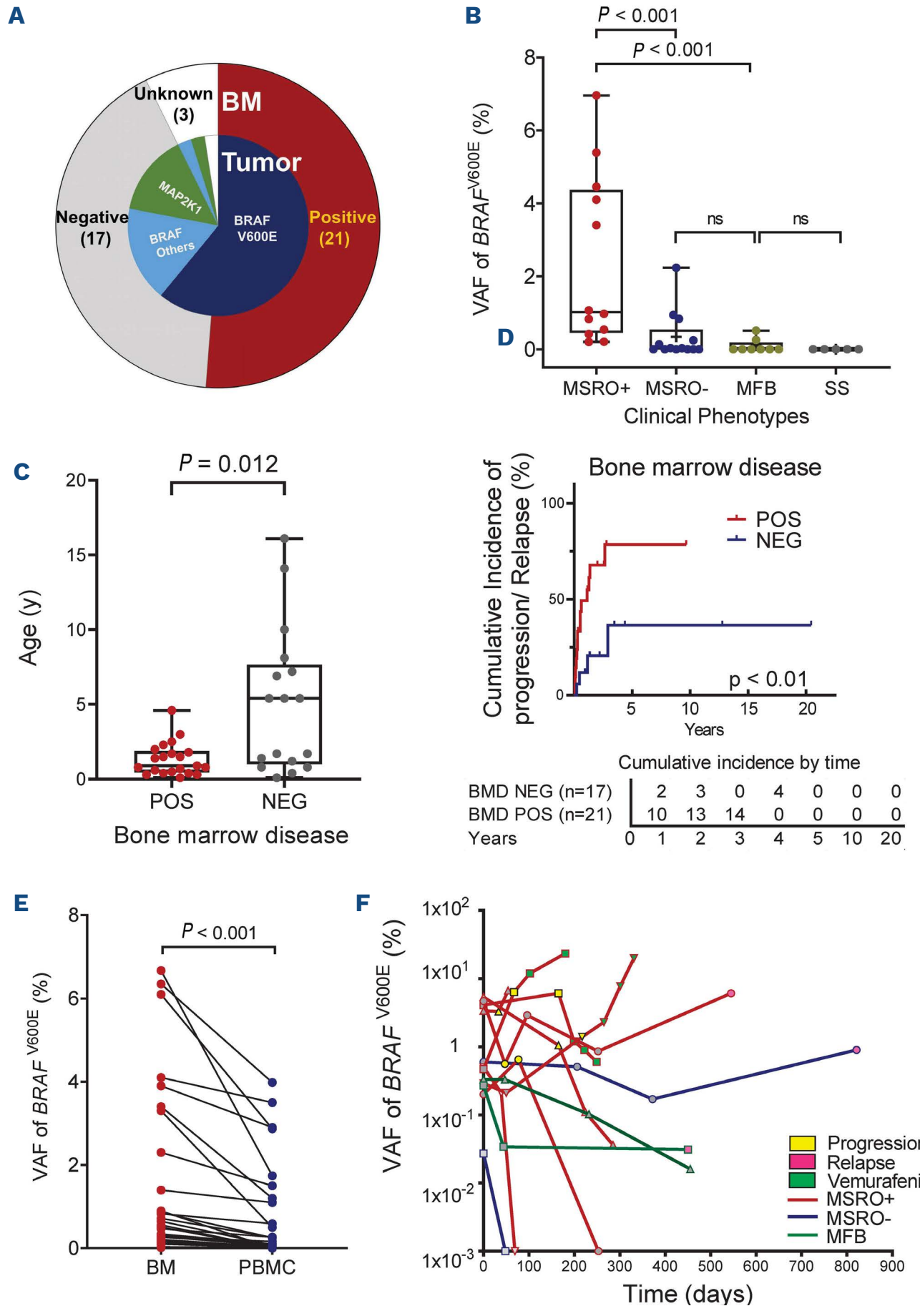


**Figure 1. Bone marrow disease in pediatric Langerhans cell histiocytosis.** (A) Distribution of somatic mutations in 41 paired tumor and bone marrow (BM) samples in patients with Langerhans cell histiocytosis (LCH). We performed polymerase chain reaction (PCR)-based targeted next generation sequencing using custom-designed primers. Genomic DNA (40–100 ng) was amplified using KOD FX Neo DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) under the following conditions: 98 °C for 30 seconds (s), followed by 42 cycles at 95 °C for 5 s, 55–60 °C for 5 s, and 72 °C for 30 s. The amplicon target and primer sequences are shown in the *Online Supplementary Table S1*. All somatic mutations were identified using CLC Genomic Workbench v.4.9 (CLC bio, Aarhus, Denmark) for sequence alignment with the human genome reference genome (hg19). (B) Measurable bone marrow disease (BMD) and clinical phenotypes in 38 cases. We conducted droplet digital PCR assay using PrimePCR ddPCR Mutation Assay (dHsaCP2000027, dHsaCP2000028; Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol for validation of the *BRAF* V600E variant; the sequences, annealing temperature, and two-dimensional plots of each custom primer are shown in the *Online Supplementary Table S1*. A total of 80–200 ng DNA was used for the assay. All samples were analyzed in duplicates, and only those that were positive in both were defined as positive. The outcome data were analyzed using QuantaSoft version 1.7.4.0917 (Bio-Rad). MSRO+; multisystem with risk organs; MSRO-: multisystem without risk organs; MFB: multiple focal bone disease; SS: single system, single

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lesion. (C) BMD and age. (D) The cumulative incidence curves of progression and relapse. BMD-positive patients (red line) have a higher LCH progression risk than BMD-negative patients (blue line). (E) Comparison of the mutational burdens in paired mononuclear cells of the BM and peripheral blood. The variant allele frequency (VAF) of *BRAF* V600E was analyzed in 31 paired cell samples. (F) Persistent detection of BMD in LCH during treatment. Serial quantitative mutation analyses at multiple time points were performed in 12 applicable cases (8 MSRO+, 2 MSRO-, and 2 MFB). Nine patients, including 7 MSRO+, 1 MSRO-, and 1 MFB case, relapsed or had disease progression, representing active diseases that required a change in treatment. In 3 patients, somatic mutations were no longer detectable.

**Table 2.** CD1a positivity and cytopenia in *BRAF* V600E-positive Langerhans cell histiocytosis cases.

| Case | Cytopenia | CD1a positivity of BM | BRAF V600E positivity (VE1) of BM | VAF of *BRAF* V600E in BM, % |
|---|---|---|---|---|
| P1 | No | Negative | Negative | 0.25 |
| P2 | No | Negative | Negative | 0.00 |
| P4 | No | Negative | Negative | 4.46 |
| P5 | No | Negative | Negative | 0.94 |
| P7 | No | Negative | Negative | 0.26 |
| P8 | No | Negative | Negative | 0.51 |
| P11 | No | Negative | Negative | 2.24 |
| P12 | No | Negative | Negative | 0.98 |
| P15 | No | NA | Negative | 0.13 |
| P16 | Yes | NA | Negative | 6.96 |
| P18 | No | NA | NA | 0.00 |
| P20 | Yes | Positive | NA | 0.42 |
| P21 | Yes | Negative | Positive | 0.54 |
| P23 | No | Negative | Negative | 1.07 |
| P26 | No | Negative | NA | 0.00 |
| P28 | No | Negative | Negative | 0.03 |
| P31 | Yes | NA | Negative | 0.83 |
| P32 | No | Negative | NA | 0.20 |
| P35 | Yes | Positive | Negative | 5.39 |
| P36 | Yes | NA | Negative | 0.21 |
| P37 | No | Negative | Negative | 0.03 |
| P38 | No | NA | NA | 0.00 |
| P39 | Yes | Negative | NA | 4.10 |
| P40 | No | Negative | NA | 0.84 |
| P41 | Yes | Negative | NA | 3.40 |
| | 8/25 cases (32%) | 2/19 cases (11%) | 1/17cases (6%) | 21/25 cases (84%) |

BM: bone marrow; NA: not applicable; VAF: variant allele frequency.

P5, P9, and P37, were found to have complications of central diabetes insipidus, two of which were negative for BMD. Two patients, P4 and P31, developed neurodegenerative diseases, and both were positive for BMD. However, the association between BMD and late complications was not clear. The effect of BMD should be evaluated prospectively.

Our results confirm previous mutational analysis results in BM samples in a small number of patients.[6-9] Furthermore, we present novel findings that BMD positivity correlates with prognosis and that quantitative analysis defines the disease type. These results support the current theory of *BRAF* mutation acquisition in HSC or nearby progenitor cells in systemic LCH.[1-4,6]

In conclusion, we found that BMD was frequently detected at the molecular level in pediatric *BRAF* V600E-positive LCH. A high mutational load in the BM was correlated with distinct clinical features of young-age patients with high-risk multisystem LCH, suggesting that measurable BMD is a novel risk factor that could provide an alternative to cytopenia or CD1a positivity. Further prospective studies with larger cohorts are required.

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**Contributions**

KK, TTo and EI designed the study; KK, TT, RK and TT performed the experiments; KK, TT and RK analyzed and interpreted the data; KK and EI wrote the manuscript; KK, TS, TK, SS, MI, CI, KA, HK, TD, HK, MI, YS, AT, DH, YI, KW, KS, YS, MK, KaK, IK, KU, SA, AS, HY, HK, RF and KT evaluated the patients and collected the clinical data; KK, TK, AK and AN collected formalin-fixed paraffin-embedded samples and pathological data. All authors have read and approved the final manuscript.

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**Data-sharing statment**

For original data, please contact kkudo@hirosaki-u.ac.jp

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