Analysis of the *BRAFV600E* mutation in 19 cases of Langerhans cell histiocytosis in Japan

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Abstract

Langerhans cell histiocytosis (LCH) is a rare disease characterized by clonal proliferation of CD1a- and CD207 (langerin)-positive dendritic cells. Mutated *BRAF* (*p.V600E*) is observed in histiocyte-related diseases and dendritic cell-related diseases, including LCH. *BRAFV600E* is observed in some LCH cases and is thought to be involved in maintaining MAPK activation. We retrospectively analyzed *BRAFV600E* in 19 patients diagnosed with LCH. In our study, direct sequencing for exon 15, a mutation hotspot, demonstrated that 4 out of the 19 patients (21%) harbored a GTG>GAG (valine>glutamic acid) base substitution, which encodes BRAFV600E. The clinical impact of BRAFV600E in such diseases is unclear. The frequency of *BRAFV600E* in our LCH patients from Japan was lower than that reported in the United States and in Germany. However, reports from Asia tend to show a lower rate of the *BRAFV600E* mutation. These results imply the possibility of different genetic backgrounds in the pathogenesis of LCH across various ethnicities. We also performed an immunohistochemical analysis to detect BRAFV600E using the mutation-specific monoclonal antibody. However, immunohistochemical analysis failed to detect any mutated protein in any of the 4 *BRAFV600E*-positive cases. This implies that at present, *BRAFV600E* should be assessed by direct sequencing.

Introduction

Langerhans cell histiocytosis (LCH) is a disease characterized by clonal proliferation of CD1a- and CD207 (langerin)-positive dendritic cells [1, 2]. It is a very rare disease, with a prevalence of 5 to 10 in 1,000,000 people. The male-to-female ratio is 2:1 [3]. LCH affects a wide range of age groups, from newborns to the elderly. The manner of involvement varies, and single-organ local involvement (single-system single-site; SS), single-organ multiple involvement (single-system multi-site; SM), and multi-organ multiple involvement (multi-system multi-site; MM) are recognized. In SS-type LCH, lesion(s) may disappear upon follow-up without treatment, but SM- and MM-type LCH are often treated with chemotherapy [4, 5].

BRAF is a protein belonging to the serine/threonine kinases, and it plays an important role in the mitogen-activated protein kinase (MAPK) pathway. MAPK regulates cell maintenance and proliferation in some solid tumors, such as melanoma and colon cancer. The genetic mutation *BRAFV600E* is observed in histiocyte-related diseases and dendritic cell-related diseases, including LCH [6]. The *BRAFV600E* mutation is also observed in some LCH cases and is thought to be involved in maintaining MAPK activation [7].

Using mass spectrometry analysis, Badalian-Very et al. showed *BRAFV600E* to be present in 35 of 61 cases of LCH (57%) in the United States [8]. Reported frequencies of *BRAFV600E* in LCH patients in other countries are 34 of 89 (38%) in Germany [9], 7 of 15 (47%) in Hungary [10], and 6 of 27 (22%) in South Korea [6]. In contrast with these figures, Tong et al. found no *BRAFV600E* in any of the 18 LCH patients they studied in China recently [11]. This implies that different ethnicities have different rates of *BRAF* mutations

in LCH. We retrospectively analyzed *BRAFV600E* among Japanese people affected with LCH by using direct sequencing and immunohistochemistry (IHC). Here, we present the results of these assays, along with clinical information.

Materials and methods

Patients and data collection

This retrospective analysis was conducted in 19 patients diagnosed with LCH, whose samples were submitted for diagnosis to the Department of Pathology, Kurume University, Kurume, Japan (Table 1). Among the 19 cases, we obtained prognostic information in 14 cases. Patients with histiocytic sarcoma and Erdheim–Chester disease (ECD) were excluded from this study.

Paraffin-embedded tissues were available from all 19 LCH patients. All biopsied samples were obtained prior to any therapy. The study received approval from the Research Ethics Committee of Kurume University and was performed according to the principles of the Helsinki Declaration. The reporting system for materials and clinical information ensured the anonymity of patients.

Morphologic analysis

The diagnosis of LCH was based on clinical and histologic diagnosis. Biopsies were performed in all LCH patients. Histopathological evaluation of initial samples was done using formalin-fixed, paraffin-embedded (FFPE) blocks.

Immunohistochemistry

The original diagnosis was also supported by IHC using CD1a (mouse monoclonal antibody, clone MTB1; Leica Biosystems, Newcastle upon Tyne, UK), CD207 / langerin (mouse monoclonal antibody, clone 12D6;

Leica Biosystems), and S100 (polyclonal rabbit antibody, Dako, Glostrup, Denmark). To evaluate the presence of BRAF protein in LCH, we also performed IHC using a mouse monoclonal BRAFV600E antibody (mouse monoclonal antibody, clone VE1; Spring Bioscience, Pleasanton, CA, USA). The antibody was constructed for detection of the mutant protein BRAFV600E. In particular, the BRAFV600E antibody (dilution: 1:100) was used in IHC with the EnVision G|2 Doublestain System (Dako, Glostrup, Denmark) for detection of the mutant protein BRAFV600E in sections of 2.5-µm thickness cut from FFPE blocks after heat-induced antigen retrieval (boiling for 40 minutes at 95°C, in 10 mM Tris-buffer/1 mM EDTA, pH 9.0).

DNA isolation and *BRAFV600E* mutation analysis in direct sequencing

We performed *BRAF* mutation analysis in 19 LCH patients by direct sequencing. Tumor DNA was isolated from the relevant FFPE tissue blocks by cutting 10-μm-thick sections followed by extraction using KAPA Express Extract Kits (Kapa Biosystems). The median estimate of the tumor percentage in the biopsied sample was 50% (10-90%). *BRAF* exon 15, a mutation hotspot, was amplified by semi-nested polymerase chain reaction (PCR). In the first round, PCR was performed using KAPA2G Robust PCR Kits (forward primer, 5′-TAAACTCTTCATAATGCTTGCTTGCTCTGAT-3′; reverse primer, 5′-AACTCAGCAGCATCTCAGGGCCAA-3′) on a GeneAtlas 482 thermal cycler (ASTEC) with the following cycling conditions: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s; and final extension at 72°C for 10 min. In the second step, PCR was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems) (forward primer, 5′-CATAATGCTTGCTCTGATAGGAAAATGAG-3′; reverse primer, 5′-AACTCAGCAGCATCTCAGGGCCAA-3′) on the GeneAtlas 482 thermal cycler with the following cycling conditions: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s; and final extension at 72°C for 10 min. The positive control was β-actin in each set of amplifications. PCR products were detected by 2% agarose gel electrophoresis and then purified and sequenced on an ABI PRISM 310 Genetic Analyzer (Life Technologies). Analyses of the sequence data were performed by using GENETYX software Ver.10 (GENETYX). The reference sequence was a DNA sequence complementary to the *BRAF* sequence.

Results

Morphologic analysis and Immunohistochemistry

Hematoxylin-eosin (HE) staining of the biopsies showed features of LCH (e.g., abnormal histiocytic cells, eosinophils, small lymphocytes, and macrophages). CD1a, CD207/langerin and S100 were all positive in all cases (Figure 1).

BRAFV600E mutation

BRAFV600E mutation analysis was performed in 19 patients. Direct sequencing for exon 15 demonstrated 4 patients to have a GTG>GAG (valine>glutamic acid) base substitution, which leads to the production of mutant protein BRAFV600E (Figure 2). However no mutated protein was found in patients found to be BRAFV600E-positive by direct sequencing (Figure 1). IHC failed to detect mutated protein in any of the cases, indicating a discrepancy between the direct sequencing results and the IHC results (Table 1).

Clinical course and treatment

Of the 19 patients, 9 were male and 10 were female. The manner of involvement was SS in 9 cases, SM in 1 case, and MM in 9 cases. Table 2 summarizes the sites of involvement at diagnosis. Patient 17 had a complication of central diabetes insipidus. The median observation period was 39 months; 11 patients survived, and 3 died due to either the underlying disease or related complications.

SS-type LCH patients, except patients 4 and 5, underwent follow-up without treatment, while most SM and MM-type LCH patients received chemotherapy (Table 3). Chemotherapy was performed for patient 3 because he experienced disease progression during watchful waiting. Patient 4 received chemotherapy because he experienced gait disturbance and pain due to nerve involvement of the tumor. In 9 patients, the regimen of the JLSG-02 protocol (Ara-C, vincristine, and prednisolone), designed by the Japan LCH Study Group, was used [12]. Patient 11 showed refractory disease and received allogeneic umbilical cord blood transplantation. However, she died due to transplant-related events. In patients 16 and 17, initial treatment was insufficiently effective. They therefore received salvage therapy with cladribine, and remission of the tumor cells was achieved in both patients.

Discussion

Currently, there is agreement that LCH is a clonal neoplasm. In the present report, the fact that the *BRAFV600E* mutation was present in some LCH patients supports the neoplastic nature of LCH. Berres et al. found the *BRAFV600E* mutation in 64% of LCH patients in the United States. Interestingly, the same group found that the presence of *BRAFV600E* in LCH patients was not related to the prognosis [13]. Go et al. reported the *BRAFV600E* mutation to also be present in histiocytic diseases other than LCH [6]. More specifically, they observed the *BRAFV600E* mutation in 1 of 1 (100%) case of Langerhans cell sarcoma, 5 of 8 (62.5%) cases of histiocytic sarcoma, 1 of 10 (10%) cases of giant cell tumor of the bone, and 1 of 20 (5%) cases of tenosynovial giant cell tumor.

In our study, *BRAFV600E* was noted in 4 out of 19 Japanese patients (21%). This proportion is lower than that reported by Badalian-Very et al. in the United States (57%), Sahm et al. in Germany (38%), and Méhes, et al. in Hungary (47%) [8-10]. In contrast, reports from Asia have demonstrated a lower frequency of this mutation. Go et al. showed *BRAFV600E* in 22.2% of LCH cases in South Korea, while Tong et al. did not detect it in any of the 18 cases they analyzed in China [6, 11]. These results imply that the genetic basis underlying the pathogenesis of LCH differs by ethnicity.

Additionally, Badlian-Very et al. previously demonstrated that MEK and ERK, which are downstream effectors of the MAPK pathway, are phosphorylated in LCH irrespective of the existence of the *BRAFV600E* mutation [8]. The fact that MEK and ERK were phosphorylated in LCH irrespective of the BRAFV600E

mutation might explain the finding by Marie et al. that LCH patients with *BRAFV600E* and LCH patients with wild-type *BRAF* had similar prognoses. It remains unknown why LCH patients lacking the *BRAFV600E* mutation have continuous activation of genes downstream of MAPK signaling [8]. A recent report by Noah et al. in the United States showed that 18 out of 40 (45%) LCH patients without the *BRAFV600E* mutation had *MAP2K1* (also known as *MEK1*) mutations. The mutations were found in the exon 2 region or exon 3 region of *MAP2K1* [14]. *MAP2K1* is a gene found downstream of *BRAF*, and this finding suggests that the activation of the MAPK pathway does not require the *BRAFV600E* mutation in some LCH cases. It is therefore speculated that other elements, such as *MAP2K1* mutations, are involved in the pathogenesis of LCH. Further studies are needed to clarify whether *MAP2K1* mutations also occur in Japanese LCH cases and to determine the factors related to disease progression of LCH.

In the present study, a BRAFV600E-specific monoclonal antibody was used to detect the mutant protein of BRAF by IHC. However, no mutated protein was found in any of the 4 *BRAFV600E*-positive patients. Such discordance regarding *BRAF* status between the result of direct sequencing and IHC often occurs [9, 10]. Løes et al. stated that Sanger sequencing yielded superior results to IHC using the same primary antibody that we used, VE1, in colon cancer [15]. This implies that at present, *BRAFV600E* should be assessed by direct sequencing.

The efficacy of vemurafenib, a BRAFV600E inhibitor, has not been definitively assessed in LCH; however, some reports have described it to be effective in histiocytic diseases like hairy cell leukemia and

complicating ECD and LCH [16-18]. Further analyses are needed to evaluate therapeutic effects of vemurafenib against LCH with BRAFV600E. In *BRAFV600E*-negative cases, MEK inhibitors may be candidates for targeted therapy. It should be recognized that many LCH cases are pediatric, and it is therefore necessary to pay close attention to the long-term side effects of cytotoxic chemotherapy.

In the path ahead, molecular-targeted therapies having less serious side-effects need to be developed. Investigating treatment strategies that also take growth and development into account remains an important task. In addition, further studies are warranted to clarify whether *MAP2K1* mutations also occur in Japanese LCH patients and to identify the factors related to disease progression of LCH. Variation in the genetic basis of LCH pathogenesis among different ethnicities is another interesting feature worth exploring and might unravel novel therapeutic targets.

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Conflict of interest

The authors have no potential conflicts to disclose.

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Table 1 Patient profile and the results of BRAFV600E mutation analysis by direct sequencing and IHC and clinical features

Case	Diagnosis	Age (y) at diagnosis	Sex	Extension	Tested tissue	BRAF Status		Extention										Coexistence of DI
						Seq	IHC	Skin	Soft tissue	Oral cavity	Bone	BM	LN	Liver	Spleen	Lung	GI	CNS	
1	LCH	0	F	SS	Skin	Wt	N/E	+	-	-	-	-	-	-	-	-	-	-	-
2	LCH	0	F	SS	Lymph node	Wt	N/E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	LCH	1	F	SS	Lymph node	Wt	N/E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	LCH	1	M	SS	Soft tissue	Wt	N/E	-	+	-	-	-	-	-	-	-	-	-	-
5	LCH	2	M	SS	External ear canal	Wt	N/E	+	-	-	+	-	-	-	-	-	-	-	-
6	LCH	4	F	SS	Bone	Wt	N/E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	LCH	5	M	SS	Bone	V600E	N/E	-	-	-	+	-	-	-	-	-	-	-	-
8	LCH	11	M	SS	Frontal sinus	Wt	N/E	-	-	-	+	-	-	-	-	-	-	-	-
9	LCH	13	F	SS	Thyroid gland	Wt	N/E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	LCH	1	M	SM	Bone	Wt	N/E	-	-	-	+	-	-	-	-	-	-	-	-
11	LCH	0	F	MM	Spleen	Wt	N/E	+	-	-	-	-	-	+	+	+	-	-	-
12	LCH	0	M	MM	Lymph node	Wt	N/E	-	-	-	-	-	+	+	-	-	-	-	-
13	LCH	1	F	MM	Skin	V600E	N/E	+	-	-	+	-	+	-	-	-	-	-	-
14	LCH	1	M	MM	Bone	V600E	N/E	+	-	-	+	-	-	-	-	-	-	-	-
15	LCH	3	F	MM	Bone	V600E	N/E	-	+	-	-	-	+	-	-	-	-	-	-
16	LCH	3	M	MM	Skin	Wt	N/E	+	-	-	-	+	-	-	-	-	-	-	-
17	LCH	27	F	MM	Pituitary gland	Wt	N/E	-	-	+	+	-	-	-	-	-	-	+	+
18	LCH	57	M	MM	Skin	Wt	N/E	+	-	-	-	N/A	+	-	-	-	-	-	-
19	LCH	71	F	MM	Lymph node	Wt	N/E	+	+	-	-	-	+	-	-	-	-	-	-

Seq, result of direct sequence; IHC, result of immunohistochemistry; LCH, Langerhans cell histiocytosis; M, male; F, female; SS, single-system single-site; SM, single-sistem multi-site; MM, multi-system multi-site; N/E, Not estimable; BM, bone marrow; LN, lymph node; GI, stomach and intestine; CNS, central nervous system; DI, diabetes insipidus; N/A, not applicable

Table 2 Treatment and outcome

Case	Initilal therapy	Effect of initial therapy	Salvage therapy	Effect of salvage therapy	Follow-up (months)	Status
1	Watchful waiting	-	-	-	46	Alive
2	L/F	L/F	L/F	L/F	L/F	L/F
3	L/F	L/F	L/F	L/F	L/F	L/F
4	JLSG-02	RD	-	-	23	Alive
5	VCR+PSL	PD	JLSG-02	PD	56	Dead
6	L/F	L/F	L/F	L/F	L/F	L/F
7	Watchful waiting	RD	-	-	7	Alive
8	Watchful waiting	-	-	-	39	Alive
9	L/F	L/F	L/F	L/F	L/F	L/F
10	JLSG-02	RD	-	-	27	Alive
11	JLSG-02	PD	HD Chemo+URCBT	N/A	8	Dead
12	JLSG-02	RD	-	-	84	Alive
13	JLSG-02	RD	L/F	L/F	1	L/F
14	JLSG-02	RD	-	-	40	Alive
15	JLSG-02	RD	-	-	48	Alive
16	JLSG-02	PD	CdA +Ara-C	-	23	Dead
17	JLSG-02	PD	CdA	RD	40	Alive
18	IFRT	RD	-	-	60	Alive
19	MTX	SD	-	-	32	Alive

L/F, loss of follow-up; JLSG-02, Japan Langerhans Study Group Regimen 2002; VCR, vincrisrtine; PSL, prednisolone; IFRT, involved field radiation therapy; MTX, metotrexate; N/A, not applicable; RD, regressive disease; SD, stable disease; PD, progressive disease; HD Chemo, high dose chemotherapy; URCBT, unrelated cord blood transplantation; CdA, cladribine; Ara-C, cytarabine

Figure legends

Figure 1 Morphologic and immunohistochemical findings in patient 15.

A The subcutaneous area is diffusely infiltrated by atypical cells (hematoxylin-eosin; original magnification, ×100).

B The neoplastic cells with pale cytoplasm are medium to large in size (hematoxylin-eosin; original magnification, ×400).

C Atypical histiocytic cells are positive for CD1a (original magnification, ×400).

D Atypical histiocytic cells are positive for CD207 (langerin) (original magnification, ×400).

E Atypical histiocytic cells are positive for S100 (original magnification, ×400).

F Atypical histiocytic cells are negative for BRAFV600E (original magnification, ×400).

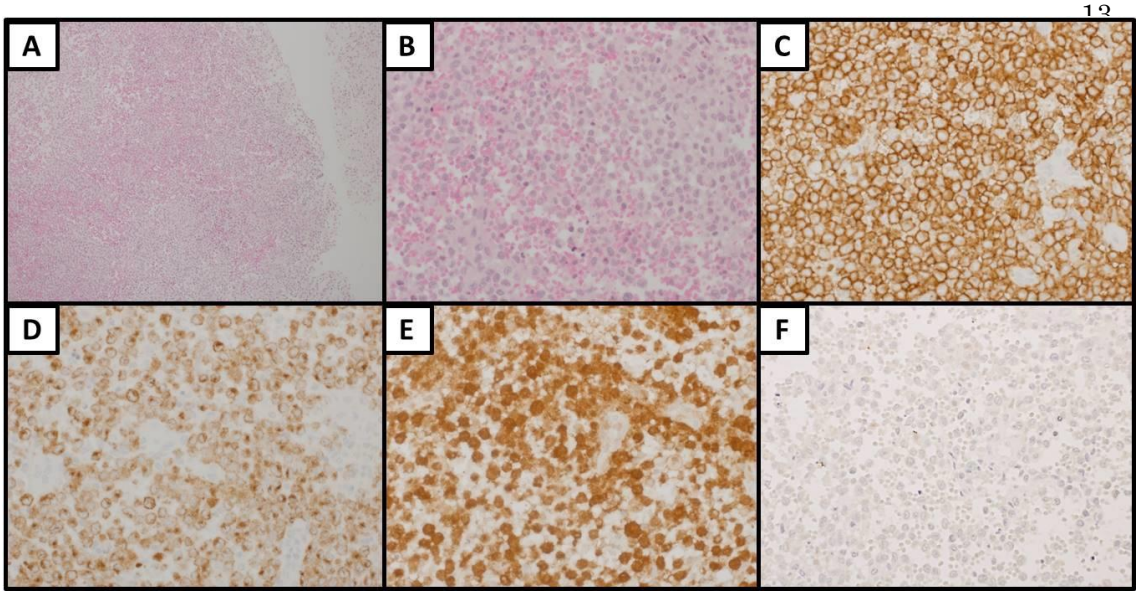

Figure 2 Results of *BRAF* mutation analysis.

Direct sequencing for exon 15 demonstrated 4 cases have GTG>GAG (valine>glutamic acid) base substitution, which encodes BRAFV600E.