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Research Article

BRAF V600E Mutation in Paediatric Langerhans Cell Histiocytosis in Japan: A Single-Centre Retrospective Study

Hidehiro Minegisihi[1], Atsushi Makimoto[1,2]*, Yuichi Yokokawa[1], Motohiro Matsui[1], Yuki Yuza[1] and Kentaro Matsuoka[2]

[1]Department of Hematology and Oncology, Tokyo Metropolitan Children's Medical Center, Japan

[2]Department of Diagnostic Pathology and Laboratory Medicine, Tokyo Metropolitan Children's Medical Center, Japan

***Corresponding Author**: Atsushi Makimoto, Department of Diagnostic Pathology and Laboratory Medicine and Department of Hematology and Oncology, Tokyo Metropolitan Children's Medical Center, Musashidai, Fuchu, Tokyo, Japan.

Received: January 18, 2022; **Published:** February 25, 2022

Abstract

Introduction: Langerhans cell histiocytosis (LCH) is a mitogen-activated protein kinase (MAPK)-driven myeloproliferative neoplastic disorder. Although the BRAF-V600E is one of the major contributors to the MAPK pathway, its association with the clinical features of LCH has not been studied well in Japanese patients.

Methods: Surplus tissue specimens from 21 patients with LCH were received their diagnosis at Tokyo Metropolitan Children's Medical Center were analyzed using a test combining immunohistochemical staining (ICH) with a mutation-specific anti-BRAF -V600E antibody and 3D digital PCR (dPCR) targeting BRAF -V600E in DNA isolated from the tissues. The relationship between the presence of BRAF -V600E and the patients' clinical features was then examined.

Results: BRAF was able to be identified by dPCR in 19 specimens; of these, five had BRAF -V600E either on IHC or by dPCR. While four of the 19 specimens were positive for BRAF -V600E on IHC, one was positive only by dPCR. In the three instances, the mutation was confirmed using both IHC and dPCR. Cohen's Kappa coefficient was 0.68. No significant correlation was found between BRAF -V600E and clinical characteristics or outcomes.

Conclusion: The combination of dPCR and IHC was a practical way of detecting BRAF-V600E in tissue samples. The reliability of dPCR results depended on the quality of the DNA samples. Expansion of the study cohort is warranted especially in Japan, where the incidence of BRAF-mutation positivity in LCH is substantially low.

Keywords: *Langerhans Cell Histiocytosis (LCH); BRAF-V600E; Mitogen-Activated Protein Kinase (MAPK); 3D Digital PCR (dPCR); Immunohistochemical Staining (IHC); Pediatrics*

Introduction

Lesions in Langerhans cell histiocytosis (LCH) are characterized by pathological dendritic cells (DCs) and infiltration of inflammatory cells, which proliferate abnormally in various organs, such as bone, skin and the central nervous system, often causing tissue destruction [1]. The etiology of LCH had been debated (i.e. whether it is a malignancy or inflammation) since the 19th century when Hand described the first case [2]. Because pathological DCs are not morphologically dysplastic and mitosis is rarely observed, researchers were unable to conclude that LCH was true malignancy. The controversy was ended by an epoch-making discovery by Badalian-Very., *et al*. who reported that *BRAF*-V600E, a somatic mutation, occurred in lesional tissue of more than half of the LCH patients they examined [3]. Other activating mutations in BRAF and other genes, such as *MAP2K1* and *ARAF*, were also detected in the cells [1]. These genetic mutations render the mitogen-activated protein kinase (MAPK) pathway constitutively active and are thought to have a major role in LCH etiology.

Clinically, *BRAF*-V600E is associated with high-risk features, irreversible complication, and poor, short-term response to chemotherapy [4]. BRAF inhibitors may benefit a certain subpopulation of these patients [5]. In this regard, subgrouping patients with LCH based on their *BRAF*-V600E status in clinical practice is gaining in importance. Although *BRAF* mutations are typically detected by DNA-based sequencing assays, including next generation sequencing [6], such methods are currently relatively time-consuming or require specific expertise, such as bioinformatics analysis, and are not widely available for routine practice.

The role of immunohistochemistry (IHC) using the mutation-specific *BRAF*-V600E antibody (VE1) has the potential to serve effectively in the place of more costly and technically difficult methods [7]. Ballester., *et al*. reported that the specificity of IHC using VE1 was able to be increased to 100% if optimized, stringent, scoring criteria were applied [8]. The chip-based digital polymerase chain reaction method (dPCR), which was developed in 1999 [9], is an ultrasensitive method combining compartmentalization and PCR amplification of a single DNA fragment and has been optimized for use in simple procedures in the form of a commercially-available kit that is readily usable in clinical practice [10].

Aim of the Study

The present, retrospective study aimed to test the feasibility of a combination of two simple and practical tests, IHC with mutation-specific VE1 and dPCR targeting *BRAF*-V600E mutations, using formalin-fixed, paraffin-embedded (FFPE) LCH samples stored at a single institution in Japan.

Materials and Methods

Patient samples

The present study included consecutive patients who underwent a diagnostic biopsy at Tokyo Metropolitan Children's Medical Center (TMCMC) and received the diagnosis of LCH by histopathological examination between March 1, 2010 and June 30, 2019. FFPE tissue blocks were obtained from these patients and analyzed by ICH and dPCR in accordance with the examination protocol described below. In total, 21 patients (14 males, 7 females) were included. The median age was 3 years (range: 0 - 11 years) at the time of diagnosis.

Examination protocol: dPCR targeting *BRAF*-V600E mutations

dPCR was performed using QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific K.K., Tokyo, Japan) with a final volume of 18 µl on a chip containing 9 µl of 2 x QuantStudio™ 3D Digital PCR Master Mix (Thermo Fisher Scientific K.K., Tokyo, Japan), 0.9 µl of the primer/probe mixture (GeneArt High-Q Strings [Thermo Fisher Scientific K.K., Tokyo, Japan]), and 10 to 50 ng DNA template diluted in 1.8 µl. Thermal cycling was performed according to the manufacturer's instructions as follows: at 96.0°C for 10 minutes; 39 cycles at 60.0°C for 2 minutes; 30 seconds at 98.0°C; and a final elongation step at 60°C for 2 minutes. The primer and probe sequences were designed according to Human BLAT Search [11].

IHC with anti-*BRAF* V600E antibody

IHC was performed using VENTANA BenchMark® XT automated IHC stainer (Roche Diagnostics, Tokyo, Japan) and anti-*BRAF* -V600E antibody (Roche Diagnostics K.K., Tokyo, Japan) at a 1:45 dilution in an antibody diluent. Antigen retrieval was done with ER2 solution for 40 minutes, and diaminobenzidine was used as a chromogen. Two pathologists (KM and AM) microscopically evaluated the positive cells on slides and classified them into four grades according to a previous report [8]. Of these grades, 0+, 1+, 2+ and 3+, and 2+ and 3+ indicated *BRAF* -V600E positivity.

Analyses

The primary endpoint was the proportion of *BRAF* -V600E positivity in the LCH samples. The secondary endpoints included (1) the relationship between the presence of *BRAF* -V600E positivity and time-to-recurrence; (2) differences in clinical characteristics (age of onset, sex, history, disease type, presence/absence of risk organ lesions, white blood cell count and C-reactive protein (CRP) at initial diagnosis, mortality rate, and irreversible complications, such as diabetes insipidus, bone deformity, central nervous system abnormality, and growth disorder) according to BRAF status; and (3) comparison of the reproducibility of the IHC and dPCR results using Cohen's Kappa coefficient. Continuous variables, such as age of onset, were compared using Wilcoxon's test. Qualitative variables, such as the presence of complications, were compared using Fisher's direct test. In each case, the *p*-value was calculated, and the probability was set at 5% on a two-sided test. The Kaplan-Meier method with log-rank test was used to compare recurrence and death.

Ethical Approval and Informed Consent

The study protocol was approved by the institutional review board at TMCMC. In accordance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects issued by the Ministry of Health, Labor and Welfare of Japan, information about this study was made available to the public, and individuals potentially eligible for participation in the study were given an opportunity to refuse via an opt-out clause on the institutional website. Because of the retrospective nature of this research, personal informed consent was waived.

Results

Both procedures were feasible in all 21 samples and were considered applicable to daily clinical practice. Figure 1 summarizes the *BRAF* -V600E status of each sample. Two of the 21 samples showed no significant amplification of *BRAF*, including the wild type, by dPCR probably because of the poor quality of the DNA samples. One of the two samples was *BRAF* -V600E-positive on IHC. Among the 19 samples with both sets of BRAF data, one was positive only on dPCR whereas another was positive only on IHC. Three were positive on both dPCR and IHC, and the remaining 14 were negative on both tests. The proportion of *BRAF* -V600E positivity was 21.0% (four of 19 cases; 95% confidence interval (CI): 6.0 - 45.6%) on dPCR and 23.8% (five of 21 cases; 95% CI: 8.2 - 47.2%) on IHC.

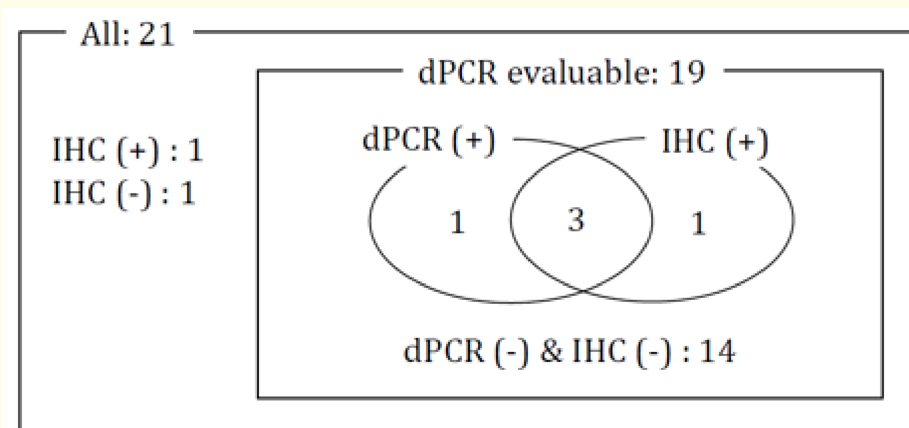

Figure 1: *Pattern of BRAF status per testing method in all 21 patients.*
dPCR: 3D Digital Polymerase Chain Reaction; IHC: Immunohistochemical Staining.

Table 1 summarizes the characteristics of the 21 patients from whom the samples were obtained. Sixteen required chemotherapy consisting of vincristine, methotrexate, and cytarabine, and all the patients showed a good response to the treatment. The remaining five patients initially received surgery or a biopsy. All 21 patients survived, but three experienced a recurrence at the time of the final analysis. The first recurrence was in a 2-month-old male patient who initially presented with swelling of the cervical lymph node; the recurrence was detected four months after completion of the initial treatment. The patient was *BRAF* -V600E-negative on both dPCR and IHC. The second recurrence was in a 2-year-old, female patient who initially presented with a mass in the frontal region. The recurrence was detected 22 months after completion of the initial treatment. This patient was also *BRAF* -V600E-negative on both tests. The third recurrence was in a 2-year-old male patient who initially presented with a mass in the frontal region. The recurrence was detected 17 months after completion of the initial treatment. The patient was *BRAF* -V600E-negative on IHC but not evaluable by dPCR.

No.	Age (years)	Sex	Lesion site	Type	Chemotherapy	Time to relapse (months)	*BRAF* V600E by IHC	*BRAF* V600E by dPCR
1	5	F	Bone	MS	+	-	-	-
2	5	M	Bone	SM	+	-	+	+
3	4	M	Bone, subcutaneous tissue	MS	+	-	+	-
4	5	M	Bone	SS	+	-	-	-
5	3	M	Bone	SM	+	-	-	-
6	1	F	Bone	SS	+	-	-	-
7	6	M	Bone	SS	+	-	-	No amplification
8	2	F	Bone	SS	+	-	-	-
9	1	M	Bone, skin	MS	+	-	+	+
10	0	M	Bone	SS	+	-	-	-
11	8	F	Bone	SS	-	-	+	+
12	0	M	Bone, multiple organs	MS	+	4	-	-
13	0	M	Skin, lung	MS	+	-	-	-
14	2	M	Bone	SS	-	-	-	-
15	2	M	Bone	SS	-	-	-	-
16	11	M	Bone	SM	+	-	-	-
17	2	M	Bone	SM	+	17	+	No amplification
18	3	M	Bone	SS	+	-	-	-
19	8	F	Bone	SS	-	-	-	-
20	2	F	Bone	SM	+	22	-	-
21	5	F	Bone	SS	-	-	-	-

SS: Single System, Single Site; SM: Single System, Multiple Site; MS: Multiple System;
dPCR: 3D Digital Polymerase Chain Reaction; IHC: Immunohistochemical Staining

Table 1: *Patient characteristics (n = 21).*

Since none of the patients among those with a relapse was positive for *BRAF* -V600E on dPCR, evaluation of the secondary endpoints (1) and (2) was done using clinical data on the 21 patients and their *BRAF* -V600E status as evaluated by IHC. Figure 2 shows the recurrence-free survival curve of two groups defined by their *BRAF* -V600E status on IHC (four positive, 17 negative). No significant, intergroup difference was observed either on log-rank test (p = 0.74) or in terms of age at onset, sex, medical history, disease type, and treatment course (Table 1 and 2).

		All patients n = 21	BRAF wild type by IHC n = 5	BRAF V600E by IHC n = 17	p-value
Gender	Male	14	4	10	0.624
	Female	7	1	6	
Age of onset (Median)		3	4	3	0.416
Disease type	SS	11	1	10	0.0286
	SM	5	2	3	
	MS RO -	2	2	0	
	MS RO +	3	0	3	
Lesion	Bone	19	5	14	
	Skin	3	1	2	
	Liver	1	0	1	
	Spleen	1	0	1	
	Lung	1	0	1	
	Lymph node	1	0	1	
Relapse		3	1	2	1.0
Death		0	0	0	

SS: Single System, Single Site; SM: Single System, Multiple Site; MS: Multiple System; RO: Risk Organ

Table 2: *Comparison of clinical data between BRAF status groups.*

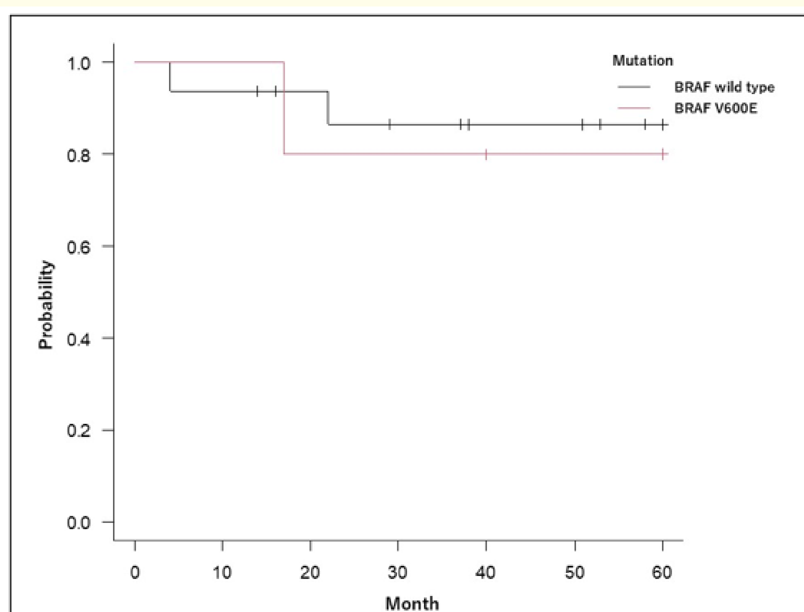

Figure 2: *Recurrence-free survival curves according to BRAF status.*

Reproducibility was calculated based on IHC and dPCR data from the 19 samples. Cohen's Kappa showed substantial agreement at 0.68.

Discussion

LCH is currently thought to be a MAPK-driven myeloproliferative neoplastic disorder based on evidence demonstrating that several mutations, including *BRAF*-V600E, activate the MAPK pathway and strongly contribute its etiology [1]. The proportion of *BRAF*-V600E among patients with LCH is reportedly as high as 64% [1]. Given that this mutation is associated with high-risk features of LCH and a poor response to chemotherapy, it is likely to garner increasing attention both in research and clinical practice [4]. The present study attempted to demonstrate the feasibility and performance of a test combining IHC using a mutation-specific VE1 and dPCR targeting *BRAF*-V600E in FFPE samples of LCH. Both tests proved simple and practicable in the clinical setting.

The present report is the first to examine *BRAF* -V600E in LCH using a dPCR, which can detect low-frequency mosaicism, rendering it unnecessary to use special procedures, such as microdissection, to analyze FFPE specimens in searching for the target mutation [12]. In addition, dPCR uses the end-point PCR reaction, which is less susceptible to inhibitors than real-time PCR or to the instability of DNA sequences resulting from long-term storage [9]. However, the *BRAF* gene, including the wild type, was not able to be amplified in two samples in which a *BRAF*-V600E mutation was not able to be analyzed by dPCR. Therefore, we recommend that in daily clinical practice, DNA be extracted immediately after obtaining a biopsy specimen to ensure higher dPCR accuracy. As with other PCR methods, dPCR has high sensitivity for specific target genes. One sample was negative on IHC but positive on dPCR probably because the proportion of cells with BRAF-V600E was too low to be detected by IHC. IHC has high specificity whereas dPCR has high sensitivity. In fact, in the present study Cohen's Kappa coefficient between PCR and ICH was relatively high at 0.68. Taken together, the findings of the present study indicated that the combination of the two testing methods is simple, practicable, and potential very useful in daily clinical practice.

The proportion of *BRAF*-V600E-positive samples in the present study was unexpectedly low but similar to figures reported by previous studies in Japan [13,14], which were significantly lower than those reported in European [4] or American [3,15] studies, suggesting that ethnic differences may exist in LCH. Although this clinical question is very interesting, the data on this topic are currently insufficient. The development and generalization of cancer-genomic testing may help solve this question eventually and contribute to the development of novel precision treatments for LCH.

The present study had a number of limitations, including its monocentric design and the low proportion of *BRAF*-V600E mutations in Japan. We were unable to demonstrate any relationship between clinical features, including recurrence, and *BRAF*-V600E. The proportion of *BRAF*-V600E positivity, recurrence, and deaths was extremely low compared to figures reported previously by Héritier., *et al* [4]. To clarify the clinical significance of *BRAF*-V600E in Japanese patients with LCH, a multicentric, prospective, clinical study with a fairly large patient cohort is warranted.

Conclusion

The combination of the dPCR and IHC was a practical method of detecting *BRAF*-V600E in FFPE tissue samples. However, the reliability of dPCR results was dependent on the quality of the DNA samples. Although we were unable to demonstrate any relationship between *BRAF*-V600E status and the clinical features of each patient, expanding the study cohort is warranted especially in Japan, where the incidence of *BRAF*-V600E positivity in LCH is very low.

Acknowledgements

The authors thank Mr. James Robert Valera for his assistance with editing this manuscript, their colleagues, Mr. Shinichi Sugisawa, Ms. Maki Fukui, and Ms. Kazue Kinoshita for their technical assistance on the research procedures, and to all the children who battled Langerhans cell histiocytosis for inspiring the authors to write this article.

Conflict of Interest

The authors declared that there are no conflicts of interest.

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Volume 11 Issue 3 March 2021
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