Frequent *BRAF*^{V600E} mutation has no effect on tumor invasiveness in patients with Langerhans cell histiocytosis

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Abstract. The oncogenic *BRAF*^{V600E} mutation in patients with Langerhans cell histiocytosis (LCH) has recently been reported. However, the reported frequencies were significantly inconsistent and were based on studies of populations of western ethnic origin. The aim of this study was to identify the presence of *BRAF*^{V600E} mutation in a cohort of Chinese LCH patients and to determine its association with clinicopathological characteristics. Blocks were retrieved from 52 LCH patients and 12 samples were obtained from blood or bone marrow. These were tested for *BRAF*^{V600E} by directly sequencing the entire exon 15 of the *BRAF* gene. To demonstrate the relationship between *BRAF*^{V600E} and invasiveness of LCH, the single or multiple systems classification was used. *BRAF*^{V600E} was the only genetic abnormality within exon 15 of the *BRAF* gene in patients with LCH. Its incidence was 56%, similar to that reported in a United States study, but higher than that reported in German studies. Additionally, the frequencies were similar between patients with single system diseases and those with multiple system diseases. Results of this study showed that *BRAF*^{V600E} was present in a large number of the Chinese LCH patients, confirming the neoplastic nature of LCH. The frequency was similar to that of the USA study but distinctly higher than that from the Europe studies. No additional mutation were identified besides *BRAF*^{V600E}. This mutation did not closely correlate with clinical severity or classification. The finding of *BRAF*^{V600E} in LCH has important implications for both molecular diagnosis and targeted personalized therapy.

Introduction

Langerhans cell histiocytosis (LCH) is a heterogeneous disease, characterized by the accumulation of dendritic cells that have similar features to epidermal Langerhans cells in various organs. LCH is regarded as a rare type of disease with an incidence of 5-6 per million children per year. However, this incidence may be historically underestimated (1). The disease can affect any organ of the human body, with the most frequently involved organs being bones, skin and pituitary (2). Other organs including liver, spleen, lungs, lymph nodes and the central nervous system (CNS), excluding the pituitary, are also involved (3). The clinical course may vary from a self-limiting disease to a rapidly progressive one that might lead to death. Preferentially involving younger people, its significant sequelae usually reduce their quality of lives severely (4).

Although the etiology of LCH was described over a century ago, it remains a controversial issue (5-7). The activating V600E mutation of BRAF, a member of the RAF family of serine threonine kinases, was recently found in 57% of LCH cases (8). As the gene mutation is crucial to pathophysiology and as a therapeutic target, recent studies have attempted to verify the presence of *BRAF*^{V600E} mutation in LCH (9,10). Sahm *et al* (9) reported that the incidence of this mutation was 38% in patients with LCH. In a study with a smaller cohort of patients, Satoh *et al* (10) reported *BRAF*^{V600E} mutation in 56% (9/16) LCH samples but with an extra 13% (2/16) *BRAF*^{600DLAT} insertion. The above results were based on American, German and Australian populations, respectively. The reportedly inconsistent frequencies suggest that geographic distribution would alter the predilection of *BRAF* mutation in LCH patients. This hypothesis was supported by Xu *et al* (3), who showed a higher ratio of adults in Chinese LCH patients. As yet, there is no report concerning the presence of *BRAF*^{V600E} mutation in LCH in an Asian population.

To the best of our knowledge, this is the first study to explore the presence of genetic abnormalities of exon 15 of *BRAF* gene in the Chinese population. To elucidate the relationship between LCH and the *BRAF* signaling pathway, we also investigated whether there are new *BRAF* mutations on exon 15 other than V600E. Twelve blood and marrow samples from 6 patients prior to treatment were tested to verify the hypothesis that the molecular study of monocytes from blood or bone marrow for *BRAF*^{V600E} would contribute to the diagnosis of LCH.

Materials and methods

Patients and tissues. Paraffin blocks from 52 Chinese LCH patients were retrieved from the Department of Pathology at

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the Xiangya Hospital (Changsha, China). The patients included 24 males and 28 females with a median age of 15.4 (range, 1-42) years. Informed consent was obtained from all patients or the patient's parents. Of the 52 samples obtained, 35 were from bone tissue, 7 from brain tissue and 10 from other soft tissue. Twelve blood and bone marrow samples from 6 patients who presented with aggressive multifocal or multisystem diseases were also tested. Institutional Review Board approval from both the Central South University and Xiangya Hospital were obtained for the analysis of the anonymized tissues. The samples were obtained between January, 2008 and December, 2011. Each case was verified by two experienced pathologists and positive results of CD1a or CD207 were routinely recorded by immunohistochemistry. Blocks with numerous lesions were selected to generate the sections. DNA was extracted using a commercial kit (Qiagen, Mainz, Germany) and quantified using a NanoDrop 1000 fluorometer (Thermo, Wilmington, DE, USA). For bone samples, eight 10-μm sections of decalcified tissue were used and 10 min grinding was added prior to DNA extracting.

PCR amplification and direct sequencing. For amplification of the whole sequence of *BRAF* exon 15, primers flanking a 161-bp amplicon of this exon encompassing the V600 codon were designed. The primer sequences used were 5'-TTTTCCTTTACTTACTACACCTCA-3' and 5'-ATAGCCTCAATTCTTACCATCCA-3'. DNA (30 ng) was amplified in a final volume of 20 μl reaction mixture containing 2 μl of PCR buffer (Applied Biosystems, Monza, Italy), 2.5 mmol/l of magnesium chloride (Applied Biosystems), 5 μmol/l of each primer, 1.5 μmol/l of EvaGreen™ Dye (Biotium, Hayward, CA, USA) and 1 unit of Taq polymerase (Roche Diagnostics Ltd., Rotkreuz, Switzerland). PCR was performed in a LightCycler® 480 PCR system (Roche Diagnostics Ltd.) using the following cycling conditions: an initial denaturation at 95˚C for 1 min followed by 50 cycles for 10 sec at 95˚C, annealing at 60˚C for 15 sec and elongation at 72˚C for 25 sec. The samples were then denatured with an initial incubation for 1 min at 95˚C and 1 min at 40˚C. Sequences were determined by bidirectional direct sequencing on a semiautomatic sequencer (ABI 3100 Genetic Analyzer; Applied Biosystem).

Statistical analysis. Prism 6 statistics software was used to analyze the samples. Age was compared by independent sample non-parametric tests. Constituent ratios of gender, organ involvement and clinical classification were compared by Chi-square tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demographic characteristics of patients with LCH. We included 52 LCH cases in this pioneer study. There were 24 male and 28 female individuals with a median age of 15.4 (range, 1-42) years. The distribution of gender and age were similar between the patients with mutated and wild-type *BRAF* (P=1.000 and 0.888, respectively). The frequently involved organs were bones (38/52), CNS (10/52), skin (10/52) and other organs (6/52). Concerning clinical classification, uni-system disease (41/52, 79%) with unifocal involvement or multifocal lesions occurred more frequently than multisystem disease (11/52, 21%) in our study.

Frequency and mutation type of exon 15 of BRAF gene in lesions from patients with LCH. Of the 52 cases of LCH examined, 2 skin samples were not evaluable due to insufficient DNA content. Of the 50 cases tested, 28 (56%) carried a GTG to GAG mutation at codon 600 of *BRAF* (V600E), as identified by sequencing of the PCR product. To demonstrate the potential abnormalities within exon 15 of *BRAF*, the entire exon of the *BRAF* gene was sequenced. In 28 mutated LCH tissues, only *BRAF*^{V600E} was identified (Table I).

Correlation between clinically progressive stages and BRAF^{V600E}. Since *BRAF*^{V600E} is the first genetic abnormality identified in LCH, its impact on clinical characteristics has yet to be determined. Therefore, to determine the relationship between clinical parameters and the mutation, we compared the clinical characteristics between patients with or without *BRAF* mutation. The distributions of clinical classification revealed no difference between patients with wild-type or mutated type of *BRAF* (P=1.000). Furthermore, by analyzing data from our study and those of three previous studies (8-10) (Table II), we failed to identify the correlation of this mutation with progressive stages. In fact, 3 of 4 studies, including the present study, showed that more progressive disease stage is not associated with higher *BRAF*^{V600E} mutation, although no statistical significance was detected.

Discussion

To the best of our knowledge this study has identified, for the first time, the presence of frequent *BRAF*^{V600E} mutation in LCH patients in an Asian population. The incidence of *BRAF*^{V600E} in this cohort of LCH patients was 56%, which was similar to the ratio reported by Badalian-Very *et al* (8) in a USA population (57%), but much higher than that from the Europe study (38%) (9). The exact reason for the inconsistency remains to be clarified, but it may be partly due to the small number of cases in each study or different ethnic origin.

BRAF is the most frequently mutated protein kinase gene in human tumors and exon 15 is the hot region for genetic aberrations (11). Besides *BRAF*^{V600E}, Satoh *et al* (10) reported one somatic *BRAF*^{600DLAT} insertion in one case. In order to obtain the signature of abnormalities within this special functional region, direct sequencing was used to analyze the entire *BRAF* exon 15 instead of site-specific PCR. However, no sequence abnormalities of exon 15 other than V600E were identified in this cohort of patients. The consistent association between *BRAF*^{V600E} and LCH in this report indicated that it should be helpful to specifically detect the mutation of *BRAF*^{V600E} when making a diagnosis of LCH.

Findings of the present and previous studeis failed to associate this mutation with invasiveness of clinical characteristics (8-10). However, the prognostic value of this mutation cannot be evaluated as these studies are not comparable with each other with regards to clinical parameters such as classification, age and original lesions. Additionally, no long-term follow-up data were included in any of these studies. Therefore, multicenter

Table I. Sequencing results of exon 15 of *BRAF*.

Pt	Age (years)	Gender	Organ involved	Sequencing	Pt	Age (years)	Gender	Organ	Sequencing
1	11	F	Bone	*BRAF*^{V600E}	27	14	F	Skin	*BRAF*^{V600E}
2	4	F	Bone	*BRAF*^{V600E}	28	24	F	Spleen	*BRAF*^{V600E}
3	7	M	Bone	*BRAF*^{V600E}	29	18	F	Bone	WT
4	33	M	Bone	*BRAF*^{V600E}	30	9	M	Bone	WT
5	2	M	Bone	*BRAF*^{V600E}	31	2	M	Bone, CNS, skin	WT
6[a]	40	F	Bone	*BRAF*^{V600E}	32	13	M	Bone	WT
7	8	M	Bone	*BRAF*^{V600E}	33	8	F	Bone	WT
8	17	F	Bone	*BRAF*^{V600E}	34	30	F	Bone	WT
9	1.5	M	Bone	*BRAF*^{V600E}	35	15	F	Bone, CNS	WT
10	9	F	Bone	*BRAF*^{V600E}	36	21	M	Bone, CNS	WT
11	19	M	Bone	*BRAF*^{V600E}	37	19	F	Bone, skin	WT
12	17	F	Bone	*BRAF*^{V600E}	38	2	M	Bone	WT
13	3	M	Bone, skin	*BRAF*^{V600E}	39	5	M	Bone	WT
14[a]	22	M	Bone, gum, CNS	*BRAF*^{V600E}	40	3	F	Bone	WT
15[a]	42	M	Bone, skin, liver and pericardium	*BRAF*^{V600E}	41	17	M	Bone	WT
16	30	F	Bone	*BRAF*^{V600E}	42	16	F	Bone	WT
17	5	M	Bone	*BRAF*^{V600E}	43	31	F	Bone	WT
18	18	F	Bone	*BRAF*^{V600E}	44	24	M	Bone	WT
19[a]	39	F	CNS, bone	*BRAF*^{V600E}	45	11	M	Bone	WT
20[a]	16	M	CNS, bone	*BRAF*^{V600E}	46	21	F	CNS	WT
21	7	M	CNS	*BRAF*^{V600E}	47	6	M	CNS	WT
22	7	F	CNS	*BRAF*^{V600E}	48	17	F	Skin, bone	WT
23	13	M	CNS	*BRAF*^{V600E}	49	25	F	LN	WT
24	40	F	Lung	*BRAF*^{V600E}	50	4	M	Skin	WT
25[a]	1	F	LN, skin	*BRAF*^{V600E}	51	17	F	Skin	Insufficient
26	13	M	Skin	*BRAF*^{V600E}	52	4	M	Skin	Insufficient

[a]Both blood and bone marrow samples from these 6 patients were tested for *BRAF*^{V600E} and all were negative. Pt, patient; M, male; F, female; CNS, central nervous system; LN, lymph node; WT, wild-type.

Table II. Clinical classification between patients with MT or WT *BRAF*.

Studies (Refs.)	Origin	Classification	Total	MT, n (%)	WT, n (%)	P-value
Present	Chinese	Single system	43	25 (58.1)	18 (41.9)	0.684
		Multiple systems	7	3 (42.9)	4 (57.1)	
Badalian-Very (8)	United States	Unifocal	44	27 (61.4)	17 (38.6)	0.700
		Multifocal	8	4 (50.0)	4 (50.0)	
Sahm (9)	Germany and Austria	Unifocal	85	31 (36.5)	54 (63.5)	0.154
		Multifocal	4	3 (75.0)	1 (25.0)	
Satoh (10)[a]	UK and France	Single system	9	6 (66.7)	3 (33.3)	0.615
		Multiple systems	7	3 (42.9)	4 (57.1)	

[a]Classification was made based on the data shown in the original paper. MT, mutated type; WT, wild-type.

studies should be conducted to determine the exact incidence of this mutation in Chinese LCH patients and to examine the relationship between *BRAF*^{V600E} and clinical implications.

The pathological features of lesions in LCH include various cell types, such as lymphocytes, macrophages, eosinophilic granulocytes and histiocytes. LCH has been characterized as

either a benign reactive disorder or a neoplastic disease. Recent studies have shown that LCH might be malignant in certain cases. Firstly, it was observed that the original cells affected by LCH might arise from early myeloid precursors rather than mature Langerhans cells (9,12). Secondly and of note, our study (China) along with studies by Badalian-Very *et al* (8) (USA), Satoh *et al* (10) (France) and Sahm *et al* (9) (Germany and Austria) identified the *BRAF*^{V600E} mutation in 31-57% of LCH cases. This finding provided the first molecular insight into the pathogenesis of LCH, likely rendering LCH a neoplastic disease harbouring the *BRAF* mutation. However, whether *BRAF*^{V600E} is a driver- or passenger-mutation, as well as the origin of LCH cells (9,10) and mechanisms activating the MAPK/ERK/AKT cascade other than *BRAF* mutations (8,9) remain to be elucidated.

The finding of *BRAF*^{V600E} has important implications for the diagnosis of LCH. Currently, the diagnostic standards for LCH require positive CD1a and/or CD207 in Langerhans cells from LCH lesions (2). Novel insights into the mechanisms of this mutation may lead the diagnosis of LCH into the molecular era. There are two ways to make the diagnosis of LCH in a large cohort of patients. The first involves testing the mutation directly either by regular mutation analysis or by a new high-resolution amplicon melting analysis method, effective for bone tissue, which is the most involved organ (unpublished data). The second involves immunohistochemically detecting the mutation in LCH with monoclonal antibody VE1, which has high specificity for the mutant BRAFV600E protein (9). Of note, the latter immunohistochemical system has already been applied to the diagnosis of hair cell leukemia, which also harbors a high ratio of *BRAF*^{V600E} mutation (13).

In a real clinical environment, patients with multi-system lesions may not tolerate the invasive procedure required for diagnosis. Since LCH cells originate from myeloid precursors, it is a reasonable hypothesis that the molecular test of *BRAF*^{V600E} may aid in the diagnosis of LCH in these patients. However, in this study, neither patients with single system disease nor patients with multisystem disease had positive *BRAF*^{V600E} mutation in their mononuclear cells (MNCs) from blood and bone marrow. The fact that *BRAF*^{V600E} yielded negative results in both blood and bone marrow, even in the multisystem LCH patients, suggests the presence of few continuous circulating precursor cells with *BRAF*^{V600E}. Thus, the MNC test for *BRAF*^{V600E} mutation in patients is not useful for making the diagnosis of LCH with regard to sensitivity by direct sequencing.

The test for this mutation may be significant for individualized treatment. It is not easy to treat refractory or relapsed LCH patients (14). The BRAF protein inhibitors, such as vemurafenib and dabrafenib, have shown therapeutic activity in metastatic melanoma and other types of cancer harboring the activation mutation of BRAF (15,16). Additionally, a recent report on the successful application of BRAF protein inhibitor to refractory hairy cell leukemia (17) emphasized that LCH can potentially be treated with targeted monotherapy.

In conclusion, we have demonstrated the presence of *BRAF*^{V600E} in a large number of Chinese LCH patients. The frequency was similar to that identified in a USA study, but distinctly higher than that identified in European studies. No additional mutations were identified, with the exception of V600E, in the *BRAF* exon 15. This mutation did not closely correlate with clinical severity or classification. The finding of *BRAF*^{V600E} in LCH has important implications for both molecular diagnosis and targeted personalized therapy.

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