BRAF^{V600E} Mutation in Cell-Free DNA, Rather than in Lesion Tissues, at Diagnosis Is An Independent Prognostic Factor in Children with Langerhans Cell Histiocytosis

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ABSTRACT

The aim of this study was to investigate the prognostic significance of BRAF^{V600E} in cell-free (cf) DNA (cfBRAF^{V600E}) and lesion tissues (ltBRAF^{V600E}) in pediatric Langerhans cell histiocytosis (LCH). This study included a total of 140 patients with successfully detected cfBRAF^{V600E} and ltBRAF^{V600E} at diagnosis. Treatment response at week 6 was correlated with both cfBRAF^{V600E} and ltBRAF^{V600E}. Moreover, the patients with positive cfBRAF^{V600E} had a much lower 3-year progression-free survival (PFS) rate and a higher progression/reactivation rate than those with negative cfBRAF^{V600E} (47.1% ± 7.6% vs. 78.4% ± 5.1%, $P < 0.0001$; 44.6% vs. 19.0%, $P = 0.001$, respectively). However, no significant difference was found in the 3-year PFS rate or progression/reactivation rate between patients with positive and negative ltBRAF^{V600E} ($P = 0.348$ and 0.596, respectively). In addition, after patients were divided into group A (both cfBRAF^{V600E} and ltBRAF^{V600E} positive, $n = 56$), group B (ltBRAF^{V600E} positive and cfBRAF^{V600E} negative, $n = 28$), and group C (both cfBRAF^{V600E} and ltBRAF^{V600E} negative, $n = 56$), there was a significant difference in the 3-year PFS rate and progression/reactivation rate among the three groups (47.1% ± 7.6%, 92.9% ± 6.1%, and 72.2% ± 6.1%, $P < 0.001$; 44.6%, 3.6%, and 26.8%, $P < 0.001$, respectively). In the multivariate analysis, cfBRAF^{V600E} and age at diagnosis remained independent prognostic factors for 3-year PFS in childhood LCH. Therefore, cfBRAF^{V600E} was more closely associated with important clinical characteristics, treatment response at week 6, and prognosis than ltBRAF^{V600E}.

Introduction

Langerhans cell histiocytosis (LCH), the most common type of histiocytic disease, is characterized by the abnormal proliferation and aggregation of CD1a$^+$/CD207$^+$ cells in almost all the organ systems and dysfunction of the involved organs. The clinical presentations of LCH are very heterogeneous, ranging from a spontaneously regressing single lesion to extensive multiorgan disease with a life-threatening disorder and even to rapid progression or death (1, 2).

Somatic-activating mutations in MAPK pathway genes exist in more than 85% of patients with LCH and are regarded as important causes of the disease (3). Of importance, the recurrent activating *BRAF*^{V600E} mutation has been identified in lesion tissues (lt*BRAF*^{V600E}) in 38% to 64% of all patients with LCH (4–6). Until now, the clinical prognostic value of lt*BRAF*^{V600E} in LCH has remained a controversial issue. Some studies reported that this mutation was correlated with risk organ involvement, increased resistance to first-line therapy, and a poor prognosis (6–8), whereas several other studies did not find an obvious association of this mutation with a poor prognosis (9–10).

Cells release DNA into the circulation; this is referred to as "cell-free DNA" (cfDNA). In cancer, tumor cells release their DNA, which is then present in different proportions as "circulating tumor DNA" within cfDNA (11). cfDNA is becoming a widely used prognostic and predictive biomarker, especially in oncology (11). Several studies, including our previous study, have demonstrated that the *BRAF*^{V600E} mutation in cfDNA (cf*BRAF*^{V600E}) is an attractive target to assess treatment response and prognosis in patients with LCH (12–16).

In this study, we compared the prognostic significance of cf*BRAF*^{V600E} and lt*BRAF*^{V600E} at diagnosis in pediatric LCH and evaluated the correlation of cf*BRAF*^{V600E} with the clinical outcomes of patients. As a consequence, diagnostic cf*BRAF*^{V600E} had more significant prognostic value than lt*BRAF*^{V600E} in childhood LCH.

Materials and Methods

Patients and treatments

Two hundred forty-nine children with newly diagnosed LCH (age <18 years) were enrolled in the Beijing Children's Hospital LCH registry from January 2017 to December 2018. Among them, 140 patients with both biopsy and plasma samples available were included in this study. The clinical characteristics of the children included in this study were compared with those of the children not included in this

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study and two French cohorts (7, 15). This study was approved by the Beijing Children's Hospital Institutional Review Board and was conducted in accordance with the Declaration of Helsinki.

The diagnosis of LCH was based on the morphologic identification of clonal neoplastic proliferation with the expression of CD1a and/or CD207 (Langerin; refs. 17, 18). According to the classification established by the Histology Society, we classified the patients into three categories. In single-system (SS) LCH, only one organ or system was involved and without risk organ (RO; the including liver, spleen, and hematologic system) involvement; In multiple-system (MS) LCH, two or more organs or systems were involved either with (RO^+) or without (RO^-) RO involvement (18).

The patients received a systemic chemotherapy regimen (BCH-LCH 2014 protocol, www.chictr.org.cn, identifier: ChiCTR2000030457) based on LCH-III and LCH-S-2005 protocols (16). Briefly, the first-line therapy was based on a vindesine–steroid combination, consisting of one or two 6-week courses (according to the response to treatment) of intensive initial induction therapy and subsequent maintenance therapy. Disease activity was assessed at treatment initiation, and treatment response was evaluated 6, 12, and 52 weeks thereafter for patients who received the first-line therapy. Patients with a poor response to the first-line therapy were switched to second-line therapy, consisting of cytarabine–vindesine–steroid combined with or without cladribine. Treatment response was evaluated according to the International LCH Study Group Criteria (19). Nonactive disease (NAD) was defined as complete resolution; active disease (AD)/better was defined as continuous regression of disease; AD/intermediate was defined as unchanged disease or regression with some new involvement, and AD/worse was defined as disease progression. Patients who responded to therapy were those who had NAD or AD/better response (19). In addition, the diagnosis of neurodegenerative disease was made according to T2 and FLAIR intense lesions in the cerebellum (peduncles, dentate nuclei), basal ganglia, and/or brainstem and symptoms that included progressive tremors, ataxia, dysarthria, dysmetria, learning disabilities, and behavioral abnormalities (20).

Quantification of cf*BRAF*^{V600E} by ddPCR

Blood samples were collected into EDTA-containing vacutainer tubes from patients with LCH at diagnosis. Plasma cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions and stored at −80°C until quantification. A QX200TM Droplet Digital PCR System (Bio-Rad) was used to determine the presence and level of cf*BRAF*^{V600E}.

We used Tru-Q7 (1.3% Tier) Reference Standard DNA (Horizon Discovery) as a positive control and gDNA from white blood cells of healthy donors as a negative control. For a given patient sample, the assay reported *BRAF*^{V600E} mutation fragments detected as a percentage of detected wild-type *BRAF* fragments. All samples were tested at least in duplicate. To evaluate the limit of detection of the ddPCR assay, Tru-Q7 Reference Standard DNA was serially diluted into gDNA from healthy donors to achieve 8% to 0.01% mutant alleles. The limit of the detection assay was determined at 0.1%. The details of the quantification have been described previously (16).

For patients with LCH with positive lt*BRAF*^{V600E} with or without cf*BRAF*^{V600E}, blood samples were collected at diagnosis, week 6 (after the first cycle of initial induction therapy), week 12 (after the second cycle of initial induction therapy), week 52 (the end of maintenance first-line therapy), and course 8 (the end of intensified second-line therapy) to detect cf*BRAF*^{V600E}. For patients with negative lt*BRAF*^{V600E}, blood samples at diagnosis and week 6 were collected to detect cf*BRAF*^{V600E}. Blood and lesion tissue samples at progression or reactivation were unavailable due to the parents' refusal to sample.

Targeted sequencing of *BRAF*^{V600E} in LCH lesions

Biopsy samples of skin rashes or bone lesions were obtained from 36 to 84 patients, respectively. The other 20 samples were obtained from the lung, lymph nodes, liver, and so on. Genomic DNA was extracted from 10 × 5 μmol/L unstained sections of paraffin-embedded lesion tissue at diagnosis using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. Then, targeted sequencing of genomic DNA was performed by MyGenostics Inc. The sequencing had a minimum depth of 500×.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 software (SPSS Inc.). Differences among groups were compared with the *t* test and one-way ANOVA or the Mann–Whitney *U* test for quantitative variables obeying normal or skewed distribution, respectively, and with the Pearson χ^2 test or Fisher's exact test for qualitative variables. Progression-free survival (PFS) was estimated from the date of diagnosis to the date of one of the following events: Progression, reactivation, or death, whichever came first or last in contact with the patient. The last follow-up date was June 30, 2020. Survival rates were analyzed by the Kaplan–Meier method and compared among subgroups with the log-rank test. A Cox proportional hazards model was used for multivariate analyses (21). A P value of <0.05 was considered statistically significant.

Results

Patients' clinical characteristics

In this study, there was no significant difference in most clinical characteristics, such as age, sex, clinical classification, organ involvement, and 3-year PFS, or prognoses between included patients (140 patients) and excluded patients (109 patients); however, involvement of the oral cavity was higher in included patients (**Table 1**). Compared with the two French cohorts, there was more lung involvement and less hematological involvement in our cohort (Supplementary **Table S1**). Our cohort had a higher frequency of detectable cf*BRAF*^{V600E} in patients with MS RO-LCH (38.8% vs. 10.5%) and SS-LCH (25% vs. 15.8%) than French cohort 1 but a higher frequency of detectable lt*BRAF*^{V600E} in patients with SS-LCH (56.3% vs. 43.9%) and a lower frequency in MS RO-LCH (51.0% vs. 61.4%) than French cohort 2. The discrepancies among the three cohorts might be due to the clinical heterogeneity of LCH and ethnic differences.

The median age at diagnosis was 2.3 years (range, 0 to 13.3 years) for the study cohort. There were 76 boys (54.3%) and 64 girls (45.7%). The patients were classified into three groups: The SS group (64, 45.7%), MS RO^- group (49, 35.0%), and MS RO^+ group (27, 19.3%). The most commonly affected system was the skeleton, as bone lesions were present in 123 patients (87.9%); furthermore, in 90 patients (64.3%), multifocal lesions in the bone were detected. Other commonly affected organs included the skin, lung, ear, and eye. Importantly, six (4.3%) of 12 patients with involvement of the hematopoietic system developed hemophagocytic lymphohistiocytosis (HLH).

In general, the median follow-up period was 31.7 months (range, 1.2–45.5 months). A total of 42 patients (30.0%) experienced disease progression or reactivation during the follow-up period. Twelve patients suffered at least one of the permanent consequences (PCs), including diabetes insipidus (six patients), liver cirrhosis (four

Table 1. Comparison of clinical characteristics in 249 children included or excluded in this study.

Characteristic	Included ($n = 140$)	Excluded ($n = 109$)	P
Age at diagnosis (median)	2.3 years	2.7 years	0.234
Gender			
Male	54.3%	65.1%	0.085
Female	45.7%	34.9%	
Disease extent categories			
SS LCH	45.7%	56.0%	
MS RO$^-$ LCH	35.0%	27.5%	0.182
MS RO$^+$ LCH	19.3%	16.5%	
Involvement			
Bone	87.8%	85.3%	0.577
Skin	27.8%	33.9%	0.333
Liver	16.4%	16.5%	1.000
Spleen	10.7%	9.2%	0.832
Hematopoietic	8.6%	3.7%	0.191
Lung	22.1%	14.7%	0.145
Pituitary	7.9%	7.3%	1.000
Eye	20.0%	19.3%	1.000
Ear	20%	13.8%	0.238
Oral cavity	6.4%	0.0%	0.005
Lymph nodes	11.4%	10.1%	0.838
CNS	5.7%	2.8%	0.357
3-year PFS (%)	65.9% ± 4.5%	63.8% ± 5.0%	0.096

Abbreviations: CNS, central nervous system; MS, multisystem; SS, single system.

patients), and neurodegenerative disease (two patients). In this cohort, two patients (1.4%) died from liver cirrhosis with multiple organ failure and severe infection with secondary HLH. The 3-year PFS rate was 65.9% ± 4.5%.

Impact of *BRAF*^{V600E} on the treatment response of children with LCH

lt*BRAF*^{V600E} was positive in 84 of 140 (60%) patients. cf*BRAF*^{V600E} was positive in 56 (40%) patients, all of whom were positive for lt*BRAF*^{V600E}. Twenty-eight patients (20%) were lt*BRAF*^{V600E} positive but cf*BRAF*^{V600E} negative; no patient was negative for lt*BRAF*^{V600E} but positive for cf*BRAF*^{V600E} at diagnosis and week 6, indicating the reliability of mutation detection. The other 56 (40%) patients were negative for both lt*BRAF*^{V600E} and cf*BRAF*^{V600E}.

We divided 140 patients into three groups: Group A, 56 patients with positive *BRAF*^{V600E} in both cfDNA and lesion tissues; group B, 28 patients with positive lt*BRAF*^{V600E} but negative cf*BRAF*^{V600E}; and group C, 56 patients with negative *BRAF*^{V600E} in lesion tissues and cfDNA.

We analyzed the relationship of the *BRAF*^{V600E} mutation with treatment response in our patients. In total, 120 patients (50, 21, and 49 patients in groups A, B, and C, respectively) were evaluated for their response to the first-line treatment after 6 weeks. Both cf*BRAF*^{V600E} and lt*BRAF*^{V600E} were correlated with treatment response at week 6 ($P = 0.027$ and 0.013, respectively, **Table 2**). Furthermore, a significant association was also observed when the patients were divided into the three groups mentioned above ($P = 0.037$, **Table 2**).

Impact of *BRAF*^{V600E} on the prognosis of children with LCH

First, we found no significant difference in 3-year PFS between patients with positive (group A and B) and negative (group C) lt*BRAF*^{V600E} (72.2% ± 6.1% vs. 68.2% ± 5.4%, respectively, $P = 0.348$, **Fig. 1A**). No difference in the progression/reactivation rate between the two groups was recognized (31.0% vs. 26.8%, $P = 0.596$, **Table 3**). Furthermore, lt*BRAF*^{V600E} was not related to 3-year PFS in different categories of disease extent (**Fig. 1B–D**).

However, cf*BRAF*^{V600E} showed strong prognostic value: The patients with positive cf*BRAF*^{V600E} (group A) had a much lower 3-year PFS rate than those with negative cf*BRAF*^{V600E} (groups B and C, 78.4% ± 5.1% and 47.1% ± 7.6%, respectively, log-rank test; $P < 0.0001$, **Fig. 2A**). Moreover, the progression/reactivation rate was much higher in cf*BRAF*^{V600E}-positive patients than in cf*BRAF*^{V600E}-negative patients (44.6% vs. 19.0%, $P = 0.001$, **Table 2**). In addition, in patients with SS-LCH and MS RO$^-$, positive cf*BRAF*^{V600E} was obviously correlated with a worse 3-year PFS rate than negative cf*BRAF*^{V600E} (57.1% ± 13.7% vs. 82% ± 6.9%, $P = 0.033$; 48.8% ± 12.1% vs. 72.7% ± 8.3%, $P = 0.045$; **Fig. 2B** and C), although a similar prognosis was observed in MS RO$^+$ patients with or without cf*BRAF*^{V600E} (36.3% ± 12.8% vs. 66.7% ± 27.1%, $P = 0.269$, **Fig. 2D**).

We further compared the 3-year PFS rate and progression/reactivation rate among patients in groups A, B, and C. As expected, group A had the worst prognosis, with a 3-year PFS rate of 47.1% ± 7.6%, whereas group B had the best prognosis, with a 3-year PFS rate of 92.9% ± 6.1%; only one child experienced reactivation 1 year after the end of first-line chemotherapy. The treatment outcome of patients in group C was intermediate, and the 3-year PFS rate was 72.2% ± 6.1% (**Fig. 3A**). There was an obvious difference in the progression/reactivation rate among groups A, B, and C (44.6%, 3.6%, and 26.8%, respectively, $P < 0.001$, **Table 3**). Notably, the progression/reactivation rate of group A was higher than those of groups B and C ($P < 0.001$ and $P = 0.049$) and that of group C was also higher than that of group B ($P = 0.011$). Nevertheless, in the three disease extent categories, only in patients with SS-LCH was there a significant difference in PFS among groups A, B, and C (57.1% ± 13.7%, 90.9% ± 8.7%, 78.6% ± 7.8%, respectively; $P = 0.034$, **Fig. 3B**). Although a trend of a worse prognosis was noted in MS RO$^-$ patients (48.8% ± 12.1%, 100%, and 66.7% ± 9.6%, $P = 0.066$, **Fig. 3C**), there was no significant difference in MS RO$^+$ patients (36.3% ± 12.8%, 100%, and 50.0% ± 35.4%, $P = 0.486$, **Fig. 3D**). However, neither cf*BRAF*^{V600E} nor lt*BRAF*^{V600E} was observed to be associated with PCs ($P > 0.05$, **Table 2**).

In the univariate analysis of common prognostic factors for PFS, cf*BRAF*^{V600E}, age at diagnosis, and involvement of the skin, risk organs, or ear were associated with 3-year PFS (**Table 3**). However, in the multivariate Cox analysis, only cf*BRAF*^{V600E} and age at diagnosis remained independent prognostic factors for PFS in childhood LCH (cf*BRAF*^{V600E}, HR, 2.257, $P = 0.034$; age at diagnosis, HR, 2.773, $P = 0.013$; **Table 3**).

Collectively, these data indicate that cf*BRAF*^{V600E}, rather than lt*BRAF*^{V600E}, has a significant effect on the prognosis of children with LCH, mainly in patients with SS and MS RO$^-$ according to our study.

Correlation of *BRAF*^{V600E} at diagnosis with patients' clinical characteristics

We next analyzed correlations of the common clinical characteristics of our patients with cf/lt*BRAF*^{V600E} (**Table 2**). MS RO$^+$ and involvement of the skin, liver, spleen, and ear were correlated with the existence of both lt*BRAF*^{V600E} and cf*BRAF*^{V600E} (**Table 2**). Of importance, cf*BRAF*^{V600E} was also associated with age <3 years, which was another independent prognostic factor, and involvement of the hematopoietic system and oral cavity (risk organ and CNS risk lesion, respectively; **Table 2**).

Further analysis showed obvious differences in age (<3 years), MS RO$^+$, and involvement of the skin, liver, spleen, ear, hematopoietic

Table 2. Correlation of cf*BRAF*^{V600E} and lt*BRAF*^{V600E} at diagnosis with clinical characteristics, treatment response and outcome.

		cf*BRAF*^{V600E}			lt*BRAF*^{V600E}			Combine cf*BRAF*^{V600E} and ltRAF^{V600E}						
Characteristic	***n***	**Negative *n* (%)**	**Positive *n* (%)**	***P***	**Negative *n* (%)**	**Positive *n* (%)**	***P***	**Group A *n* (%)**	**Group B *n* (%)**	**Group C *n* (%)**	***P***	**Group A vs. B**	**Group A vs. C**	**Group B vs. C**
Total	140	84 (60.0)	56 (40.0)		56 (40.0)	84 (60.0)		56 (40.0)	28 (20.0)	56 (40.0)				
Sex														
Male	76	50 (65.8)	26 (34.2)	0.128	36 (47.4)	40 (52.6)	0.052	26 (34.2)	14 (18.4)	36 (47.4)	0.145	0.757	0.057	0.209
Female	64	34 (53.1)	30 (46.9)		20 (31.3)	44 (68.8)		30 (46.9)	14 (21.9)	20 (31.3)				
Age at diagnosis														
<3 years	78	35 (44.9)	43 (55.1)	<0.001	26 (33.3)	32 (51.6)	0.071	43 (55.1)	9 (11.5)	26 (33.3)	<0.001	<0.001	0.001	0.211
≥3 years	62	49 (79.0)	13 (21.0)		30 (48.4)	52 (66.7)		13 (21.0)	19 (30.6)	30 (48.4)				
Disease extent categories														
SS	64	48 (75.0)	16 (25.0)	<0.001	28 (43.8)	36 (56.3)	0.010	16 (25.0)	20 (31.3)	28 (43.8)	<0.001	<0.001	<0.001	0.116[b]
MS RO$^-$	49	30 (61.2)	19 (38.8)		24 (49.0)	25 (51.0)		19 (38.8)	6 (12.2)	24 (49.0)				
MS RO$^+$	27	6 (22.2)	21 (77.8)		4 (14.8)	23 (85.2)		21 (77.8)	2 (7.4)	4 (14.8)				
Involvement														
Bone	123	76 (61.8)	47 (38.2)	0.245	51 (41.5)	72 (58.5)	0.342	47 (38.2)	25 (20.3)	51 (41.5)	0.563[b]	0.743[b]	0.253	1.000
Skin	39	14 (35.9)	25 (64.1)	<0.001	9 (23.1)	30 (76.9)	0.011	25 (64.1)	5 (12.8)	9 (23.1)	0.001	0.016	0.001	1.000[b]
Liver	23	4 (17.4)	19 (82.6)	<0.001	3 (13.0)	20 (87.0)	0.004	19 (82.6)	1 (4.3)	3 (13.0)	<0.001[b]	0.002	<0.001	1.000[b]
Spleen	15	2 (13.3)	13 (86.7)	<0.001	0 (0.0)	15 (100.0)	0.001	13 (86.7)	2 (13.3)	0 (0.0)	<0.001[b]	0.070	<0.001	0.108[b]
Hematopoietic	12	2 (16.7)	10 (83.3)	0.003[b]	2 (16.7)	10 (83.3)	0.124[b]	10 (83.3)	0 (0.0)	2 (16.7)	0.007[b]	0.027[b]	0.015	0.550[b]
Lung	31	15 (48.4)	16 (51.6)	0.135	13 (41.9)	18 (58.1)	0.803	16 (51.6)	2 (6.5)	13 (41.9)	0.081	0.024	0.518	0.070
Pituitary	11	7 (63.6)	4 (36.4)	1.000[b]	3 (27.3)	8 (72.7)	0.526[b]	4 (36.4)	4 (36.4)	3 (27.3)	0.417[b]	0.431[b]	1.000[b]	0.215[b]
CNS	8	4 (50.0)	4 (50.0)	0.713[b]	3 (37.5)	5 (62.5)	0.745	4 (50.0)	1 (12.5)	3 (37.5)	0.903[b]	0.661[b]	1.000[b]	1.000[b]
Lymph nodes	16	9 (56.2)	7 (43.8)	0.745	7 (43.8)	9 (56.2)	0.790	7 (43.8)	2 (12.4)	7 (43.8)	0.834[b]	0.711[b]	1.000	0.711[b]
Ear	28	9 (32.1)	19 (67.9)	0.001	5 (17.9)	23 (82.1)	0.007	19 (67.9)	4 (14.3)	5 (17.9)	0.003	0.057	0.001	0.473[b]
Eye	28	15 (53.6)	13 (46.4)	0.438	12 (42.9)	16 (57.1)	0.730	13 (46.4)	3 (10.7)	12 (42.9)	0.379	0.169	0.820	0.227
Oral cavity	9	1 (11.1)	8 (88.9)	0.003[b]	1 (11.1)	8 (88.9)	0.086[b]	8 (88.9)	0 (0.0)	1 (11.1)	0.013[b]	0.047[b]	0.032[b]	1.000[b]
Response at week 6[a]														
NAD/AD-B	67	45 (67.2)	22 (32.8)	0.027	34 (50.7)	33 (49.3)	0.013	22 (32.8)	11 (16.4)	34 (50.7)	0.037	0.518	0.011	0.174
AD-I/W/S	53	25 (47.2)	28 (52.8)		15 (28.3)	38 (71.7)		28 (52.8)	10 (18.9)	15 (28.3)				
Progression/reactivation														
No	99	68(68.7)	31 (31.3)	0.001	41 (41.4)	58 (58.6)	0.596	31 (31.3)	27 (27.3)	41 (41.4)	<0.001	<0.001	0.049	0.011
Yes	41	16 (39.0)	25 (61.0)		15 (36.6)	26 (63.4)		25 (61.0)	1 (2.4)	15 (36.6)				
Permanent consequence														
No	128	79 (61.7)	49 (38.3)	0.222[b]	54 (42.2)	74 (57.8)	0.124[b]	49 (38.3)	25 (19.5)	54 (42.2)	0.269[b]	1.000[b]	0.162[b]	0.327[b]
Yes	12	5 (41.7)	7 (58.3)		2 (16.7)	10 (83.3)		7 (58.3)	3 (25.0)	2 (16.7)				

[a]For 120 evaluable patients.
[b]The Fisher's exact test.

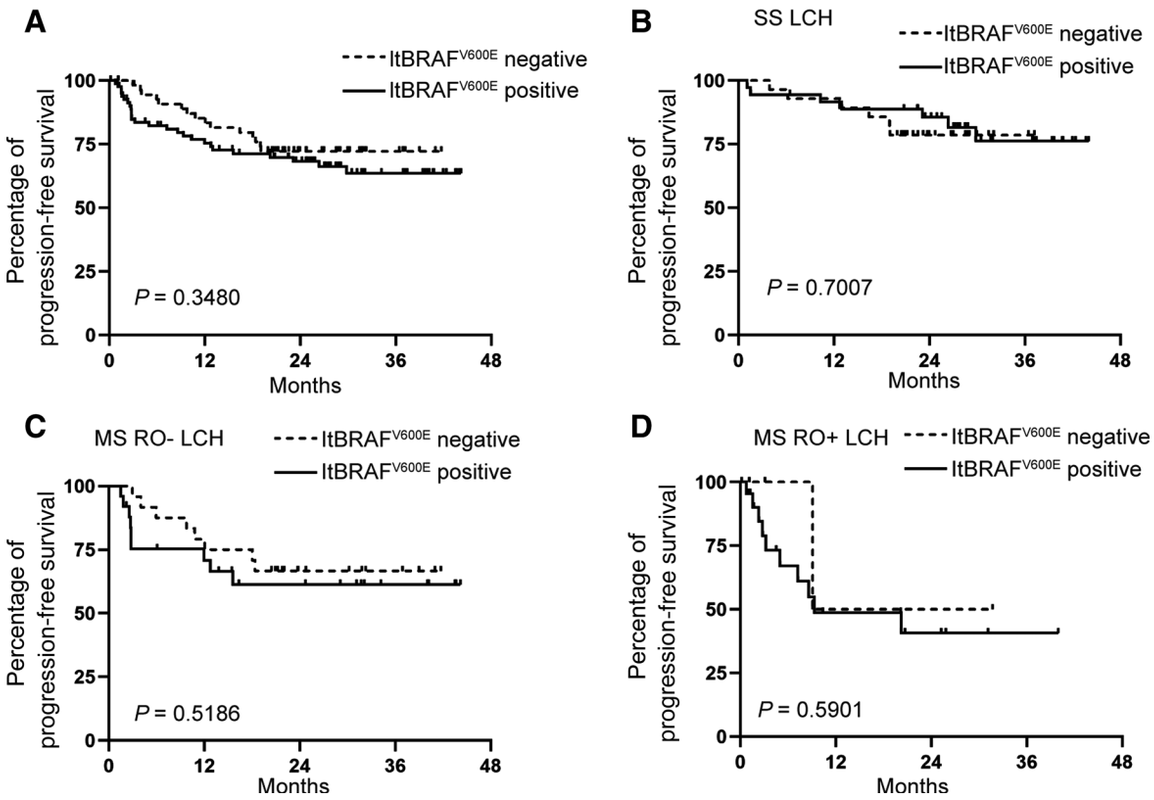

Figure 1.

The prognostic significance of ltBRAF^{V600E} at diagnosis in children with LCH. **A,** 3-year PFS in the whole cohort of 140 patients with LCH; **B,** 3-year PFS in patients with single-system (SS) LCH ($n = 64$); **C,** 3-year PFS in patients with multiple system (MS) risk organ-negative (RO^-) LCH ($n = 49$); **D,** 3-year PFS in patients with MS RO-positive (RO^+) LCH ($n = 27$).

Table 3. Univariate and multivariate analysis of prognostic factors for PFS in children with LCH.

	Univariate	Multivariate[a]		
Prognostic factors	***P***	**HR**	**95% CI**	***P***
lt*BRAF*^{V600E} Positive vs. negative	0.503	0.849	0.408–1.769	0.662
cf*BRAF*^{V600E} Positive vs. negative	0.004	2.257	1.062–4.794	0.034
Gender Male vs. female	0.345	0.965	0.479–1.943	0.920
Age at diagnosis <3 years vs. ≥3 years	<0.0001	2.773	1.242–6.194	0.013
Involvement (yes vs. not)				
Bone	0.565	1.995	0.590–6.744	0.266
Skin	0.033	1.219	0.551–2.694	0.625
Risk organs	0.002	1.594	0.630–4.032	0.325
Pituitary	0.680	0.864	0.211–3.535	0.839
Lung	0.604	0.691	0.300–1.591	0.385
Lymph nodes	0.847	0.568	0.148–2.177	0.409
Ear	0.036	1.149	0.476–2.771	0.757
Eye	0.552	0.827	0.341–2.006	0.675
Oral cavity	0.552	0.823	0.219–3.088	0.772

Abbreviation: HR, hazard ratio.

[a]All factors in univariate analysis were selected in Cox regression of multivariate analysis for PFS.

system, and oral cavity ($P < 0.05$, **Table 2**) among groups A, B, and C. Although there was no difference in the previously mentioned clinical features between groups B and C ($P > 0.05$), more patients in group A presented with these features than patients in group C ($P < 0.05$). Similarly, most of the above clinical features were observed more frequently in group A than in group B ($P < 0.05$), though only a trend in the involvement of the spleen and ear was noted ($P = 0.070$ and 0.057, respectively). Thus, these results suggest that cf*BRAF*^{V600E} has more vital clinical and prognostic value than lt*BRAF*^{V600E}.

Discussion

In this study, our results showed that lt*BRAF*^{V600E} was correlated with the disease clinical classification (SS, MS RO^-, and MS RO^+), involvement of ROs (spleen and liver) and CNS risk lesions (ear), and a poor response to 6-week chemotherapy. Nevertheless, we did not find a relationship of lt*BRAF*^{V600E} with the occurrence of progression/reactivation and a low PFS rate, which has been shown in some recent studies on children and adults with LCH (7, 22). The discrepancy in the prognostic value of lt*BRAF*^{V600E} between other studies and ours may result from the difference in the clinical features of patients and chemotherapy regimens. For example, Zeng and colleagues (22) included 32 adult patients (33%) in their study. Moreover, 91.8%, 62.2%, and 45.7% of the patients in the two studies and ours had SS-LCH, respectively (7, 22). As *BRAF*^{V600E} is underrepresented in patients with SS-LCH, which is associated with a good prognosis, a

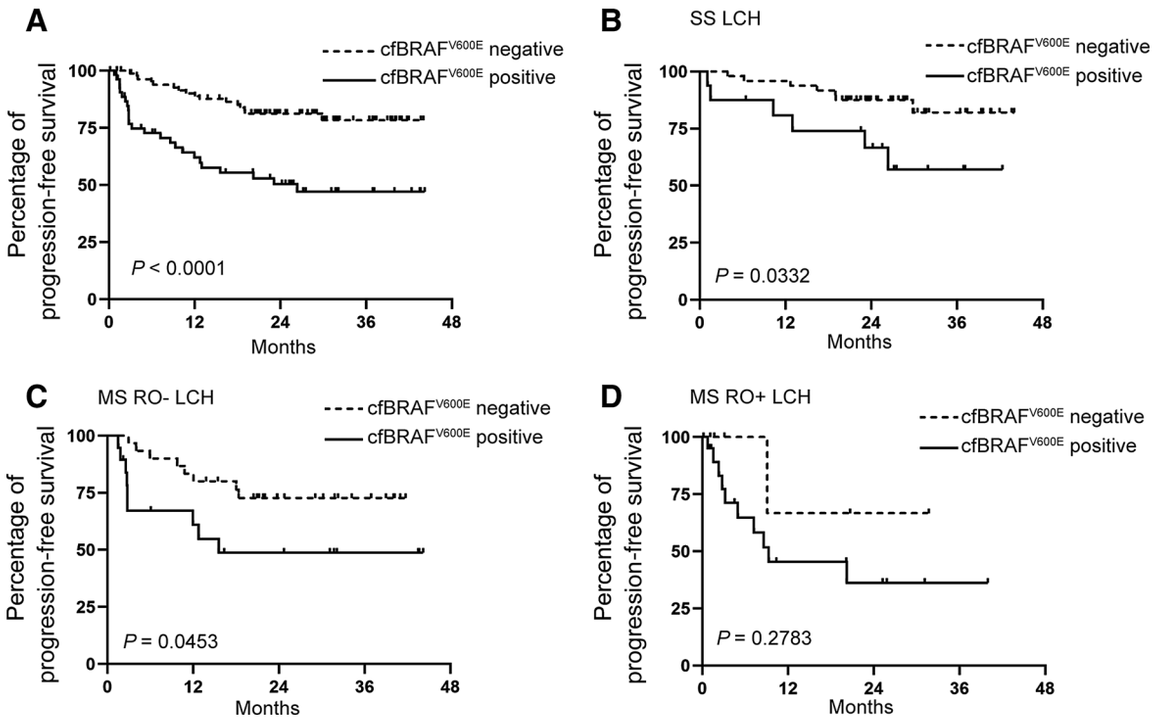

Figure 2.

The prognostic significance of cfBRAF^{V600E} at diagnosis in children with LCH. **A,** 3-year PFS in the whole cohort of 140 patients with LCH; **B,** 3-year PFS in patients with single-system (SS) LCH ($n = 64$); **C,** 3-year PFS in patients with multiple system (MS) risk organ-negative (RO^-) LCH ($n = 49$); **D,** 3-year PFS in patients with MS RO-positive (RO^+) LCH ($n = 27$).

high percentage of patients with SS-LCH would possibly lead to an obvious difference in prognosis between patients with and without *BRAF*^{V600E}. In addition, in view of the fact that lt*BRAF*^{V600E} could be detected in patients with not only progressive disease but also self-limiting Hashimoto–Pritzker disease (23), lt*BRAF*^{V600E}-positive LCH is highly heterogeneous. Other genetic or biological factors, including the types and developmental hierarchy of the cells affected by the *BRAF* mutation, affect the prognostic value of lt*BRAF*^{V600E}. Thus, the prognostic significance and mechanisms of lt*BRAF*^{V600E} need to be clarified in future studies.

Some studies, including ours, have shown that cf*BRAF*^{V600E} detection during the course of chemotherapy is a promising indicator for a prognostic evaluation (12, 14–16), and positive cf*BRAF*^{V600E} at diagnosis predicts a poor prognosis in patients with SS-LCH (16). In this study, we further showed that cf*BRAF*^{V600E} at diagnosis was related to not only some important clinical features and 6-week treatment response but also to the progression/reactivation rate and 3-year PFS rate. Furthermore, the patients with lt*BRAF*^{V600E} could be stratified into cf*BRAF*^{V600E}-positive and -negative groups with obvious differences in 3-year PFS. Patients positive for cf*BRAF*^{V600E} had the worst prognosis. Although both lt*BRAF*^{V600E} positivity and cf*BRAF*^{V600E} positivity were correlated with RO involvement, the latter was an independent risk factor for progression and reactivation even when taking RO^+ as a covariate, highlighting the importance of determining cf*BRAF*^{V600E} at diagnosis. Furthermore, this was also observed in patients with SS-LCH and tended to be maintained in patients with MS RO-LCH. Therefore, cf*BRAF*^{V600E} detection at diagnosis might aid in the risk stratification of children with SS and MS RO-LCH. A new risk stratification system composed of cf*BRAF*^{V600E} at diagnosis and other clinical stratification factors would have more significant prognostic value and identify patients at higher risk of progression/reactivation more accurately. On the other hand, in MS RO^+ patients, the difference in treatment outcome was not observed among groups A, B, and C. This was probably because in the MS RO^+ category, most patients (21/27, 77.8%) were in group A (cf*BRAF*^{V600E} positive), and only two (7.4%) and four (14.8%) patients were in groups B and C, respectively (**Table 2**). Thus, a study with a large sample size would clarify the prognostic significance of cf*BRAF*^{V600E} and its role in risk stratification in the MS RO^+ category.

Until now, the mechanisms of the association of cf*BRAF*^{V600E} with the poor prognosis of children with LCH have not been fully understood. cfDNA is mainly regarded as debris of apoptotic cancer cells (24). Many signals, including cfDNA released by apoptotic cancer cells, may function as mitogens to stimulate adjacent cells to proliferate in a compensatory manner; this is referred to as apoptosis-induced proliferation (25). Alternatively, cfDNA may come from apoptotic cancer cells, which are outcompeted by other cells with stronger proliferative activity; this is referred to as proliferation-induced apoptosis (24). In both cases, cfDNA is always associated with the increased proliferation of some cancer cells that may be resistant to chemotherapy (11). This is possibly true in LCH, which is regarded as a myeloid tumor. Therefore, more attention should be paid to genes or signaling pathways related to the proliferation of LCH cells in future studies. On the other hand, cf*BRAF*^{V600E} could also be simply related to tumor burden, such as the involvement of a large single organ or bulky disease. However, cfDNA is isolated from cell-free plasma and does not

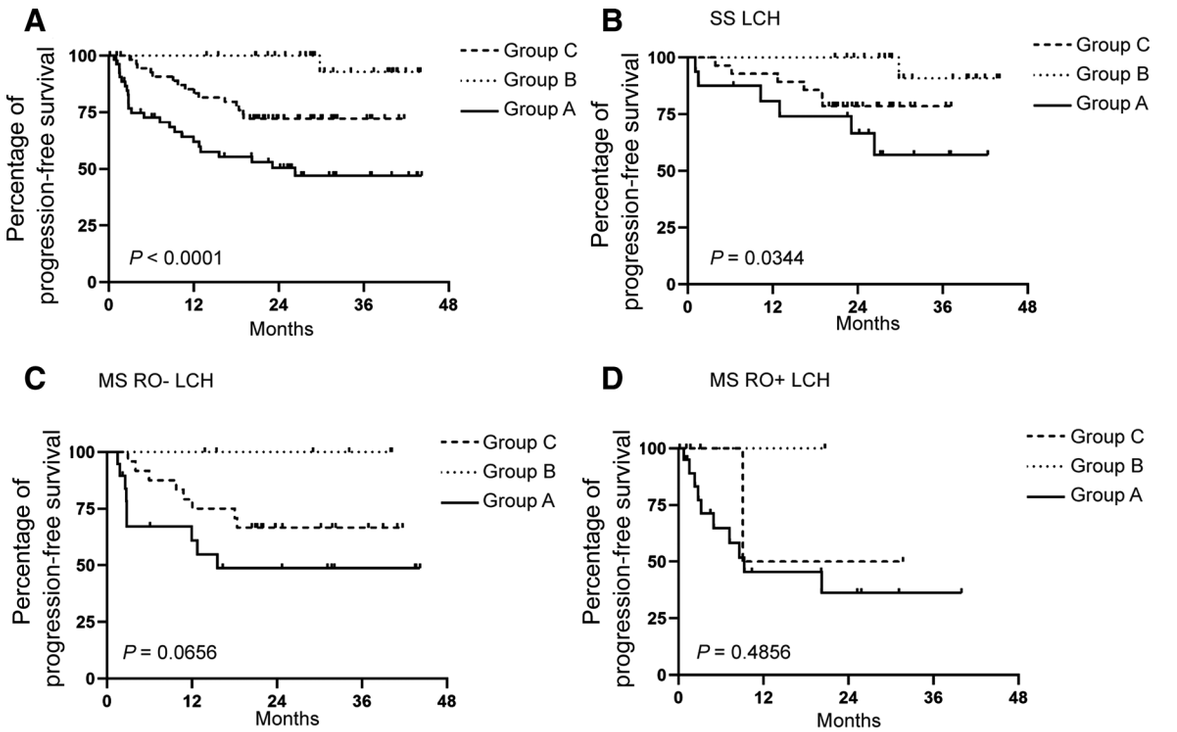

Figure 3.

The prognosis of children with LCH in groups **A, B,** and **C**. **A,** 3-year PFS in the whole cohort of 140 patients with LCH; **B,** 3-year PFS in patients with single-system (SS) LCH ($n = 64$); **C,** 3-year PFS in patients with multiple system (MS) risk organ-negative (RO^-) LCH ($n = 49$); **D,** 3-year PFS in patients with MS RO-positive (RO^+) LCH ($n = 27$).

directly result in the spread or "metastasis" of LCH cells, whereas $CD207^+CD1a^+$ cells present in the circulation of patients with active LCH may directly lead to the spread of LCH cells (26).

In addition to $BRAF^{V600E}$, many mutations in genes of the MAPK signaling pathway have been discovered and reported to play an important role in the pathogenesis of LCH (27). As the prognosis of group C (lt$BRAF^{V600E}$ negative) fell between those of groups A and B (cf$BRAF^{V600E}$ positive and negative, respectively), patients with LCH could be stratified into subgroups with different mutations in the MAPK pathway in addition to $BRAF^{V600E}$. Thus, a study direction is to develop new methods to detect the above mutations in ltDNA and cfDNA at diagnosis and during the course of chemotherapy. This may be helpful for improving patient stratification.

Two mouse models of LCH were established recently. In these LCH models, MAPK inhibitors were capable of restoring migration and apoptosis potential, decreasing disease burden, and reducing the histiocytic infiltration of alveoli and peribronchiolar stroma in the lungs, sinusoidal distension and periportal cellular infiltration in the liver (28, 29). New models with or without cf$BRAF^{V600E}$ could be established on the basis of the above mouse models to test the clinical value of cf$BRAF^{V600E}$ and the efficacy of MAPK inhibitors in cf$BRAF^{V600E}$-positive mice. Furthermore, vemurafenib has shown safety and efficacy in children with refractory MS LCH (30). Thus, the significance of vemurafenib is of great interest and should be elucidated in the treatment of cf$BRAF^{V600E}$-positive SS and MS RO^- patients.

It has been reported that up to 50% of patients with LCH have at least one PC, and PCs are more frequent among patients with multisystem disease and patients who experience reactivation than their counterparts (31). The most common are central nervous system PCs, including central diabetes insipidus and neurodegenerative disease, followed by orthopedic abnormalities and various organ failures. Two studies showed that patients with $BRAF^{V600E}$ had increased risks of frontline treatment failure and neurodegenerative disease development (6, 7). However, in this study, there was no correlation of lt$BRAF^{V600E}$ or cf$BRAF^{V600E}$ with PCs. This may be due to the relatively short follow-up time (median, 31.7 months). A longer follow-up period is needed to clarify the relationship between this mutation and PCs.

In conclusion, our study showed that cf$BRAF^{V600E}$, rather than lt$BRAF^{V600E}$, was an independent prognostic factor and was more closely associated with critical clinical characteristics and treatment outcomes than lt$BRAF^{V600E}$. The detection of gene mutations in cfDNA is of clinical importance in childhood LCH.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

C.-J. Wang: Data curation, writing–original draft. **L. Cui:** Data curation. **H.-H. Ma:** Resources. **D. Wang:** Resources. **L. Zhang:** Resources. **H.-Y. Lian:** Resources. **W.-J. Li:** Investigation. **Q. Zhang:** Investigation. **T.-Y. Wang:** Funding acquisition, project administration. **Z.-G. Li:** Methodology, writing–original draft,

project administration, writing–review and editing. **R. Zhang:** Funding acquisition, methodology, project administration, writing-review and editing.

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