Circulating cell-free *BRAF* V600E during chemotherapy is associated with prognosis of children with Langerhans cell histiocytosis

Langerhans cell histiocytosis (LCH), a rare neoplasm predominantly affecting young children, is characterized by accumulation of abundant CD1a$^+$ CD207$^+$ histiocytes with inflammatory lesions.[1] Recurrent *BRAF* V600E mutations have been identified in approximately 50% of LCH patients, which are correlated with high-risk features of LCH and increased resistance to the first-line therapy.[2,3] Several recent studies have indicated cell-free (cf) *BRAF* V600E analysis in plasma can serve as a promising biomarker in LCH.[4,5] However, the prognostic significance of cf*BRAF* V600E during chemotherapy remains to be proven. In this study, we monitored cf*BRAF* V600E at different time points during chemotherapy in children with LCH, and evaluated the correlation of the dynamics of cf*BRAF* V600E with patients' clinical outcomes.

Of the 151 consecutive patients with newly diagnosed LCH (age <18 years) who were included in the Beijing Children's Hospital LCH registry from February 2017 to June 2018, 102 patients with available biopsy and plasma samples were enrolled in this study (*Online Supplementary Figure S1*). The patients were treated with a systemic chemotherapy regimen based on LCH-III and LCH-S-2005 protocols (*Online Supplementary Table S1*).[6-8] Genomic DNA (gDNA) was extracted from unstained sections of paraffin-embedded tissue at diagnosis. Serial blood samples were collected from the patients at five time points: the time of diagnosis, week 6 (after the first

Table 1. Clinical characteristics of 81 patients with *BRAF* V600E positive Langerhans cell histiocytosis (LCH) according to cell-free *BRAF* V600E detection at diagnosis.

Variables	Total n	Cell-free *BRAF V*600E at diagnosis		*P*
		Negative n (%)	Positive n (%)	
Total	81	26 (32.1)	55 (67.9)	
Sex				
Male	42	12 (28.6)	30 (71.4)	0.634
Female	39	14 (35.9)	25 (64.1)	
Age(years) at diagnosis,				
< 3 years	56	13 (23.2)	43 (76.8)	0.019
≥ 3 years	25	13 (52.0)	12 (48.0)	
Median (range)	1.5 (0.2 - 11.6)	3.3 (0.3 - 11.6)	1.3 (0.2 - 11.1)	0.038
Clinical classification				
SS LCH	33	20 (60.6)	13 (39.4)	
MS RO$^-$ LCH	23	4 (17.4)	19 (82.6)	< 0.001
MS RO$^+$ LCH	25	2 (8.0)	23 (92.0)	
Involvement				
Bone	71	23 (32.4)	48 (67.6)	1.000
Unifocal bone	15	6 (40.0)	9 (60.0)	0.541
Multifocal bone	56	17 (30.4)	39 (69.6)	
Skin	35	5 (14.3)	30 (85.7)	0.004
Skin SS LCH	6	3 (50.0)	3 (50.0)	0.026
Skin MS LCH	29	2 (6.9)	27 (93.1)	
Liver	21	2 (9.5)	19 (90.5)	0.013
Spleen	13	1 (7.7)	12 (92.3)	0.052
Hematologic	8	0	8 (100.0)	0.050
Pituitary	9	1 (11.1)	8 (88.9)	0.259
Central nervous system	3	0 (0)	3 (100.0)	0.547
Lung	17	3 (17.6)	14 (82.4)	0.242
Lymph nodes	10	3 (30.0)	7 (70.0)	1.000
Ear	27	2 (7.4)	25 (92.6)	0.001
Eye	16	4 (25.0)	12 (75.0)	0.565
Oral	18	4 (22.2)	14 (77.8)	0.397
Thyroid	4	1 (25.0)	3 (75.0)	1.000
Thymus	2	1 (50.0)	1 (50.0)	0.542
Mediastinum	3	1 (33.3)	2 (66.7)	1.000
Response at week 6*				
NAD / AD better	31 (43.1)	13 (65.0)	18 (34.6)	
AD intermediate	25 (34.7)	6 (30.0)	19 (36.5)	0.030
AD worse	16 (22.2)	1 (5.0)	15 (28.8)	
2-year progression-free survival (%)	52.2 ± 6.3	92.3 ± 5.2	34.7 ± 7.3	< 0.001
2-year overall survival (%)	97.0 ± 2.1	100	95.6 ± 3.0	0.343

*For 71 evaluable patients. SS: single-system; MS: multiple system; RO: risk organ; NAD: non-active disease; AD: active disease.

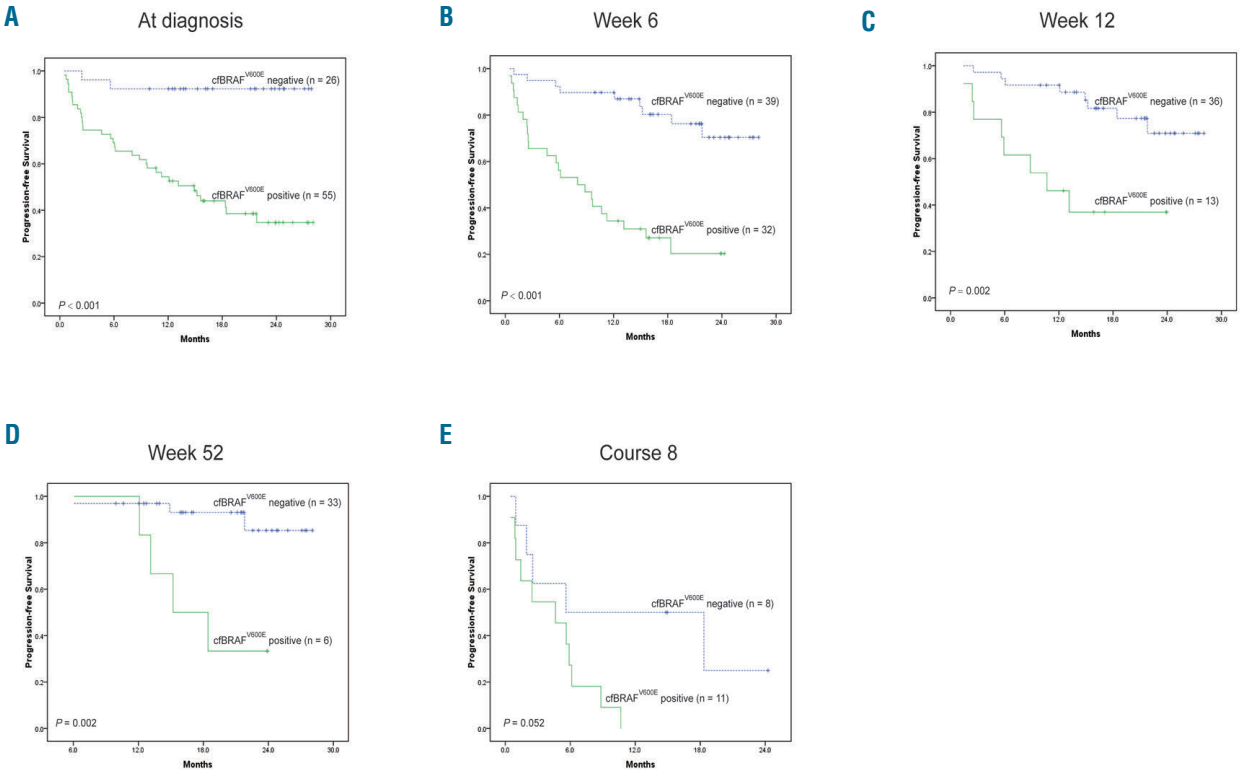

Figure 1. The prognostic significance of cf*BRAF* V600E at five time points during chemotherapy in children with Langerhans cell histiocytosis (LCH). (A) At diagnosis; (B) at week 6; (C) at week 12; (D) at week 52 of the first-line therapy; (E) at the end of course 8 of the second-line therapy.

initial induction therapy), week 12 (after the second initial induction therapy), week 52 (at the end of the maintenance treatment of the first-line therapy), and course 8 (at the end of the intensification treatment of the second-line therapy). Plasma cfDNA were isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen). *BRAF* V600E mutation in tissue gDNA or cfDNA was determined using QX200™ Droplet Digital polymerase chain reaction system (Bio-Rad, Hercules, CA, USA) (*Online Supplementary Table S2*). The limit of the detection assay was determined at 0.1%.

BRAF V600E status in tissue samples was successfully determined in 117 LCH patients. Among these, 88 (75.2%) patients carried the *BRAF* V600E mutation in the tissue samples. There was no significant difference in survival of patients with or without the mutation (*Online Supplementary Table S3*). cf *BRAF* V600E in plasma was detected in 102 of the 117 patients at diagnosis, and was further monitored at four time points during chemotherapy in most of them (*Online Supplementary Figure S1*). There was no difference in the patients' characteristics, suggesting no substantial selection bias (*Online Supplementary Table S4*).

cf*BRAF* V600E at diagnosis was positive in 55 (67.9%) of 81 patients with *BRAF* V600E mutation in tissue samples. cf*BRAF* V600E was not identified in any of the 21 patients without the mutation in tissue samples. There was a relationship between cf*BRAF* V600E and the disease features (Table 1). cf*BRAF* V600E was identified in 92.0% (23 of 25) of patients with multiple system (MS) RO^+ LCH, 82.6% (19 of 23) of patients with MS RO^- LCH, and 39.4% (13 of 33) of patients with single system (SS) LCH (P<0.001). The presence of cf*BRAF* V600E was correlated with age, liver involvement, hematologic system, ear or skin. Moreover, the 6-week response rate was much lower in children with positive cf*BRAF* V600E than in those with negative detection (34.6% *vs.* 65.0%, P=0.032). Two-year progression-free survival (PFS) was much lower in the cf*BRAF* V600E positive group (34.7%±7.3% *vs.* 92.3%±5.2%, P<0.001) (Figure 1A). Furthermore, the 13 SS LCH patients with positive cf*BRAF* V600E obviously had worse PFS than the 20 SS patients with negative detection (44.9%±20.4% *vs.* 100%, P=0.003) (*Online Supplementary Figure S2A*). Positive cf*BRAF* V600E had no significant impact on the PFS of the two groups in MS RO^- or RO^+ patients (*Online Supplementary Figure S2B and C*). In a multivariate analysis, cf*BRAF* V600E at diagnosis remained an independent prognostic factor for PFS in childhood LCH (HR: 5.263, 95%CI: 1.134-24.425, P=0.034) (*Online Supplementary Table S5*).

Thirty-two out of 71 (45.1%) patients with available plasma samples at week 6 had detectable cf*BRAF* V600E. cf*BRAF* V600E at week 6 was also closely related to the treatment response (6-week response rate: 28.1% *vs.* 56.4%, P=0.005) and PFS (20.3%±8.4% *vs.* 70.4%±9.0%, P<0.001) (Figure 1B). cf*BRAF* V600E was quantified in 49 patients at week 12. Thirteen patients (26.5%) had positive detection. Similarly, The PFS of patients with positive cf*BRAF* V600E at week 12 was lower than that of negative group (36.9%±13.8% *vs.* 70.9%±9.4%, P=0.002) (Figure 1C).

cf*BRAF* V600E was sequentially monitored at four time points in 39 patients who were treated by the first-line therapy and determined at three time points in 19 patients treated with the second-line therapy (Figure 2A

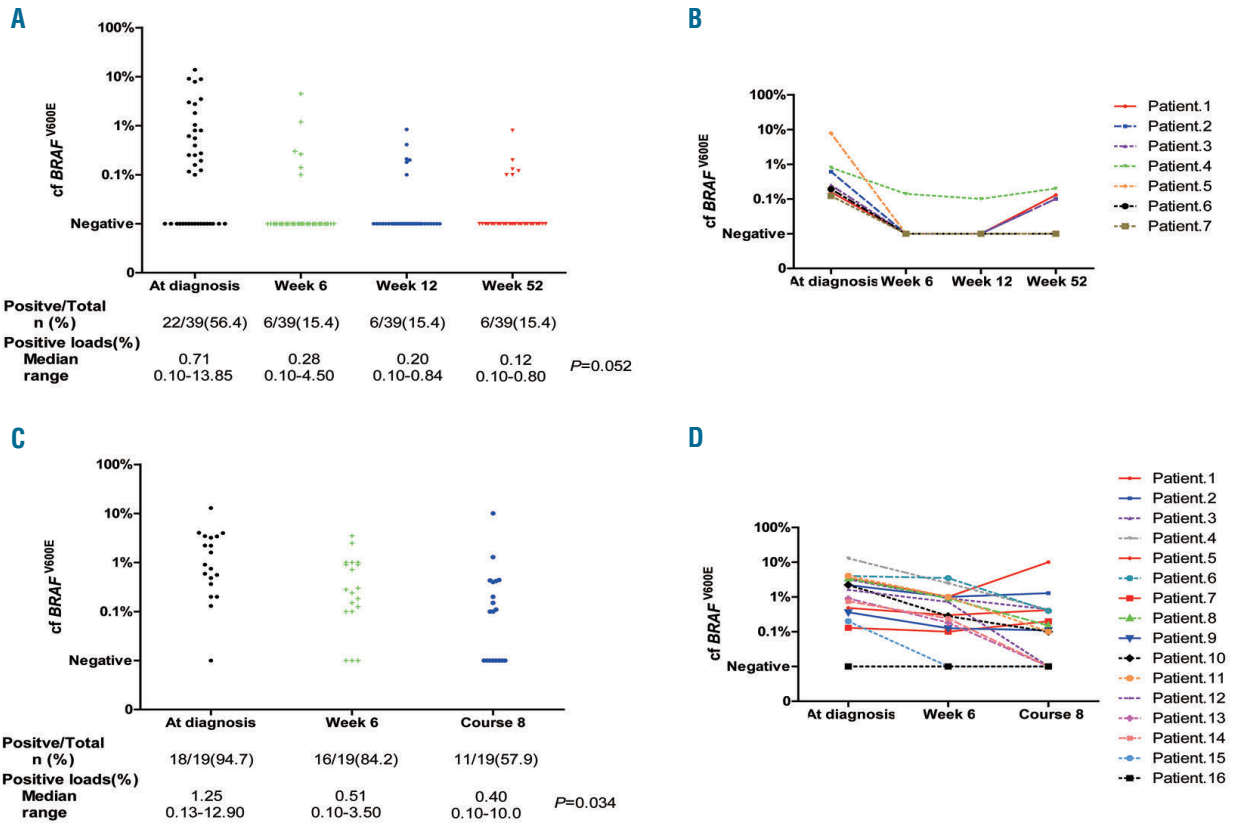

Figure 2. Sequential detection of cf*BRAF* V600E during follow up in children with Langerhans cell histiocytosis (LCH). (A) The levels of cf*BRAF* V600E at four time points in 39 patients treated by the first-line therapy. (B) Dynamics of cf*BRAF* V600E levels in seven relapsed patients treated by the first-line therapy. *BRAF* V600E reappeared at week 52 in Patients 1-3, while the mutation remained persistently detectable at all the four time points in Patient 4. The cfDNA turned negative from week 6 to week 52 in Patients 5-7. (C) The levels of cf*BRAF* V600E at three time-points in 19 patients treated by the second-line therapy. (D) Dynamics of cf*BRAF* V600E in 16 relapsed patients treated by second-line therapy. Patients 1-11 had persistently positive detection from diagnosis to course 8. In particular, the levels of mutation obviously increased at course 8 in Patients 1, 5 and 7. Patients 12-15 had negative detection at course 8. Patient 16 had been negative for cf*BRAF* V600E since diagnosis.

and C). The PFS of the six patients with positive detection at week 52 was much worse than that of 33 patients with negative detection (33.3%±19.2% *vs.* 85.3%±8.6%, *P*=0.002) (Figure 1D). Notably, cf*BRAF* V600E detection turned from negative to positive at week 52 in three patients who relapsed 0-6 months later (Figure 2B, Patients 1-3; *Online Supplementary Table S6*). Eleven out of 19 (57.9%) patients treated by the second-line therapy had persistently positive cf*BRAF* V600E detection from diagnosis to course 8 (Figure 2D, Patients 1-11) and relapsed, showing a tendency for worse PFS compared with other patients with negative detection (0 *vs.* 25.0%±19.8%, *P*=0.052) (Figure 1E and *Online Supplementary Table S6*). In addition, cf*BRAF* V600E was positive at diagnosis in two out of 9 (22.2%) SS patients who were not given any chemotherapy, and turned to negative detection 3-12 months later. The other seven children kept negative detection until one year. None of these patients had progression or relapse.

Detection of gene mutation in cfDNA is becoming a convenient and reliable tool for molecular testing, for new insights into tumor heterogeneity, and for monitoring residual disease.[9] LCH has been recently redefined and treated as a myeloid neoplastic disorder, and progression or relapse has been the main cause of treatment failure.[10] This study demonstrated that positive detection of cf*BRAF* V600E at diagnosis and at the four subsequent time points during chemotherapy had a relevant impact on PFS of children with LCH.

In this study, cf*BRAF* V600E mutations at diagnosis were more frequently found in patients with MS LCH than in patients with SS disease. The presence of cf*BRAF* V600E was correlated with decreased PFS in SS LCH patients, but not in MS patients. The latter might be due to the fact that MS LCH patients were cf*BRAF* V600E positive in the majority of patients. A previous study showed that a French cohort had lower frequency of detectable cf*BRAF* V600E in patients with MS RO- LCH (41.7% *vs.* 82.6%) and SS LCH (14.3% vs. 39.4%) compared to this study (*Online Supplementary Table S7*).[4] It should be noted that the patients in this study were younger at diagnosis and had more lung involvement or less hematologic involvement compared with those patients in the French study. The difference in the positive rate of cf*BRAF* V600E might be due to the high heterogeneity of LCH and racial diversity.

Our findings showed that patients with LCH who remained or became cf*BRAF* V600E positive during chemotherapy were more likely to relapse. Thus, the dynamics of cf*BRAF* V600E level during treatment should be closely followed. Moreover, it has been recently demonstrated that BRAF inhibitors have a dramatic effectiveness in treating patients with *BRAF* V600E positive and refractory LCH, and persistently positive cf*BRAF*

V600E was associated with a higher reactivation risk after drug discontinuation.[11] Future prospective studies with large sample size and long-term follow up were needed to confirm the prognostic significance of cf*BRAF* V600E detection.

It was reported that measurement of *BRAF* V600E from whole blood was more accurate than cfDNA detection in several MS-LCH patients to measure disease activity.[12] In addition, the levels of the *BRAF* V600E alleles in both cfDNA and whole blood were not totally consistent with the clinical condition of several LCH patients treated with the BRAF inhibitor.[12,13] The most likely reason was that the *BRAF* V600E mutation was found in different cells, such as monocytes, dendritic cells, lymphocytes, or $CD34^+$ hematopoietic cell progenitors.[14] Precise monitoring of *BRAF* V600E mutations in the specific cell subsets might be necessary in some patients in order to obtain accurate results.

In summary, this study showed that quantitative analysis of cf*BRAF* V600E during chemotherapy had an impact on PFS of children with LCH. The challenge is to carefully integrate cf*BRAF* V600E detection into clinical practice with the aim of improving outcome for these patients.

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