Genetic landscape of adult Langerhans cell histiocytosis with lung involvement

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MAPK alterations are present in most lesions from adult pulmonary LCH patients. In patients with refractory progressive disease, the identification of these alterations, including *BRAF* deletions, is important to guide the choice of targeted treatment. <http://bit.ly/2Qoknsn>

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ABSTRACT The clinical significance of the *BRAF*^{V600E} mutation in adult Langerhans cell histiocytosis (LCH), including pulmonary Langerhans cell histiocytosis (PLCH), is not well understood. Similarly, the spectrum of molecular alterations involved in adult LCH has not been fully delineated. To address these issues, we genotyped a large number of adult LCH biopsies and searched for an association of identified molecular alterations with clinical presentation and disease outcome.

Biopsies from 117 adult LCH patients, 83 with PLCH (median age 36.4 years, 56 females, 38 multisystem disease, 79 single system disease, 65 current smokers) were genotyped for the *BRAF*^{V600E} mutation. In 69 cases, LCH lesions were also genotyped by whole-exome sequencing (WES) or targeted gene panel next-generation sequencing (NGS). Cox models were used to estimate the association of baseline characteristics with the hazard of LCH progression.

MAPK pathway alterations were detected in 59 out of 69 cases (86%) (*BRAF*^{V600E} mutation: 36%, *BRAF*^{N486_P490} deletion: 28%, *MAP2K1* mutations: 15%, isolated *NRAS*^{Q61} mutations: 4%), while *KRAS* mutations were virtually absent in PLCH lesions. The *BRAF*^{V600E} mutation was not associated with LCH presentation at diagnosis, including smoking status and lung function, in PLCH patients. *BRAF*^{V600E} status did not influence the risk of LCH progression over time.

Thus, MAPK alterations are present in most lesions from adult LCH patients, particularly in PLCH. Unlike reports in paediatric LCH, *BRAF*^{V600E} genotyping did not provide additional information on disease outcome. The search for alterations involved in the MAPK pathway, including *BRAF* deletions, is useful for guiding targeted treatment in selected patients with refractory progressive LCH.

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Introduction

Langerhans cell histiocytosis (LCH) is an inflammatory myeloid neoplasia characterised by accumulation in involved tissues of abnormal cells believed to be of the monocyte/macrophage lineage that harbour pathogenic mutations activating the mitogen-activated protein kinase (MAPK) pathway [1]. LCH can affect patients of all ages, from neonates to the elderly, and it has a wide spectrum of clinical presentations and variable outcomes. In adults, lung involvement (pulmonary Langerhans cell histiocytosis (PLCH)) is commonly observed either as part of multisystem disease or as an isolated LCH localisation and occurs almost exclusively in young adult smokers [2]. The prognosis of PLCH may vary from spontaneous resolution to progressive severe disease leading to respiratory failure and, ultimately, to lung transplantation [2]. With the exception of pulmonary hypertension (PH), PLCH outcome is difficult to predict in an individual patient, with no reliable predictive factors having been identified [2].

Although the presence of the *BRAF*^{V600E} mutation in tissue biopsies from adult LCH patients has been widely reported [3–9], the impact of this mutation on clinical presentation and disease outcome, particularly in PLCH patients, remains elusive [7, 10]. Other alterations of the MAPK pathway (*i.e.* *MAP2K1* mutations) have been identified in LCH lesions, including in the lung [6–9, 11–13]. In PLCH, we also identified *NRAS*^{Q61} mutations, either isolated or, more frequently, concurrent with *BRAF*^{V600E} mutations [7]. Consistent with this finding, KAMATA *et al.* [14] reported that the expression of *KRAS*^{G12D} within myeloid lung cells in mice leads to PLCH-like lesions, although the presence of *KRAS* mutations has not been accurately assessed in PLCH lesions. Finally, in a substantial proportion of cases, the genomic alterations that could be involved in MAPK activation in adult LCH lesions remain unknown.

To address these issues, we took advantage of the large cohort of adult patients followed in our national reference centre in order to 1) widely genotype a large number of LCH biopsies and 2) search for an association of identified molecular alterations, particularly the *BRAF*^{V600E} mutation, with clinical presentation and disease outcome.

Material and methods

Patients and Langerhans cell histiocytosis samples

One hundred and seventeen adult LCH patients (median age 36.4 years, 56 females, 79 with single system disease at diagnosis) were included in this study. Of these patients, 23 were involved in a previous study [7] and 83 (71%) had PLCH (isolated lung involvement (n=51); 65 current smokers and 16 ex-smokers).

This study population was part of 194 patients with histologically confirmed LCH seen at our centre during the same period (figure 1) and were representative of the entire cohort (see supplementary tables S1 and S2 for details).

Formalin-fixed paraffin-embedded (FFPE) LCH biopsies were available for all patients and were evaluated by haematoxylin and eosin (H&E) staining and anti-CD1a immunostaining (clone O10, Agilent Technologies, les Ulis, France). CD1a-positive areas were macro-dissected for molecular biology analysis [7].

All tissue specimens were genotyped for the presence of *BRAF*^{V600E} and *NRAS* mutations. In 69 cases, 50 with PLCH, the size of the LCH biopsy allowed for comprehensive molecular analysis. Additionally, for 18 of these patients, frozen LCH tissue was also available for whole-exome sequencing (WES). Peripheral blood mononuclear cells (PBMCs) were available for 14 of these samples, which were used as a paired germline control for WES analysis. The remaining four frozen tissue samples without available PBMCs were analysed only for somatic hotspot alterations.

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FIGURE 1 Flow chart of the 117 Langerhans cell histiocytosis (LCH) patients included in the study. PLCH: pulmonary Langerhans cell histiocytosis.

In 10 cases, CD11c-positive blood cells (purified from thawed DMSO-frozen PBMCs by flow cytometry as previously described [15]) were available for molecular analysis.

LCH presentation at the time of biopsy and the molecular genotyping performed on the tissue specimens are shown in supplementary figure S1 and supplementary table S3.

As PLCH occurs almost exclusively in smokers [2], grossly normal lung tissue from 10 smoker patients, obtained at the time of thoracic surgery for localised lung carcinoma, was also genotyped for *KRAS*.

The study was performed in accordance with the Helsinki Declaration and was approved by the INSERM Institutional Review Board and Ethics Committee in Paris, France (IRB no. 13–130). All patients provided informed consent for the use of their tissue specimens and clinical information for research.

Molecular genotyping techniques

DNA extraction was performed as previously described [7].

Whole-exome sequencing

Whole-exome capture was performed on the genomic platform of the Imagine Institute (Paris, France) using the SureSelect Human All Exon 58 Mb V6 Kit (Agilent Technologies) [16]. Sequencing was conducted on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Variants were annotated and analysed using the Polyweb software interface (www.polyweb.fr/) and Alamut software (Interactive Biosoftware, Rouen, France). The mean depth of coverage obtained was 142X (range: 92.60X–257X, mean: 124X for PBMCs and 155X for tissue biopsies), with >98% and >96% of the targeted exonic bases covered by at least 15 and 30 independent sequencing reads, respectively (see supplementary material for details).

Gene panel next-generation sequencing

A custom-designed next-generation sequencing (NGS) panel of 74 genes involved in MAPK, phosphoinositide 3-kinase, cell cycle and tyrosine kinase receptor signalling pathways [12, 13, 17] was used for the comprehensive genotyping of LCH tissue biopsies (supplementary table S4). In addition, a custom-designed NGS panel of 78 genes involved in myeloid haematological disorders [18] was performed on frozen LCH specimens (supplementary table S5).

Pyrosequencing

BRAF^{V600E} and *NRAS*^{G12/G13/Q61} genotyping was performed as previously described [7]. *BRAF* exon 12 deletions described in paediatric LCH [19] were searched using a specific custom-designed pyrosequencing assay (see supplementary material and supplementary figure S2 for details).

Enhanced *ice*-COLD-PCR

The sensitive detection of mutations in *BRAF* codon 600, *NRAS* codons 12, 13 and 61, and *KRAS* codons 12 and 13 was performed by enhanced *ice*-COLD-PCR (E-*ice*-COLD-PCR; improved and complete enrichment coamplification at lower denaturation temperature PCR) [7].

Data collection

Data on patient demographics, smoking habits, clinical symptoms and signs, LCH localisation and lung function tests at the time of diagnosis and during follow-up, as well as systemic treatments for LCH received by the patients during the study, were retrieved from the database. Patients were categorised as having single system or multisystem disease according to the Histiocyte Society criteria [20].

During follow-up, based on the global outcome of their disease, LCH patients were classified as having nonactive disease if all signs and symptoms had resolved. Otherwise, they were classified as having active disease, which was further subdivided into better, stable or worse [20]. All patients who received systemic treatment for their LCH were categorised as having progressive disease.

The outcome of LCH lung involvement was based on variations over time of lung function tests and/or the occurrence of a new pneumothorax during follow-up [21]. Lung volumes were evaluated by plethysmography, forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) by the flow-volume curve. Diffusing capacity of the lung for carbon monoxide (*D*_{LCO}) was measured using the single-breath method and the predictive values were determined as previously described [21]. A worsening of lung function was defined as a decrease of 15% or more in FEV₁, FVC and/or *D*_{LCO} values compared with baseline values [21]. Patients who developed chronic respiratory failure, who were receiving long-term supplemental oxygen and/or who had developed PH, were classified as having progressive lung disease. It is noteworthy that multisystem LCH patients with lung involvement could have progressed either because of the global outcome of their disease and/or because of lung disease progression. Finally, at the time of last follow-up, patient status (alive or deceased) was recorded.

Statistical analysis

Summary statistics that included means with standard deviation, medians with interquartile range (IQR), or percentages were calculated.

The distribution of right-censored end points (time to progression or death) was estimated by the Kaplan–Meier method. Univariable Cox models were used to estimate the strength of the association of baseline characteristics, including mutations, as measured by the hazard ratio on the hazard of progression. Variables selected with the outcome at the 5% level were introduced into a multivariable Cox model. Model selection was performed using a stepwise procedure based on the Akaike information criterion.

Statistical analyses were performed using SAS (SAS Inc., Cary, NC, USA) and R (www.R-project.org/) software. All tests were two-sided, with p-values of 0.05 denoting statistical significance.

Results

Landscapes of molecular abnormalities detected in LCH lesions

WES analysis, performed on frozen tumour lesions from 14 LCH patients (11 PLCH) with a matched PBMC specimen, revealed an average number of 14 somatic mutations targeting an average of 13 genes (table 1). Four additional frozen lesions were analysed for somatic hotspot alterations.

A *BRAF*^{V600E} mutation or *BRAF*^{N486_P490} deletion was detected in 14 lesions and *MAP2K1* alterations were detected in two lesions. Additionally, *NRAS*^{G12D} and *NRAS*^{Q61K} mutations were identified in one and three lesions, respectively, concurrently with a *BRAF*^{V600E} mutation. Hence, a genomic alteration activating the MAPK pathway was identified in 16 out of 18 LCH cases (89%).

Among the additional mutations identified, two missense mutations predicted to be deleterious (based on the algorithms used) were detected in two distinct lesions (*NT5C1B-RDH14*^{R191H} and *CP*^{G895A}) and three frameshift alterations were detected in three other lesions (a small insertion in *CIC* and *UNC5B*, and a small deletion in *DTHD1*) (table 1).

As there is a known association of myeloid malignancies with a histiocytic disorder related to LCH known as Erdheim–Chester disease (ECD) [22], we investigated 17 of these 18 frozen LCH biopsies using a custom-designed NGS panel for myeloid disorders. In one LCH lesion (skin biopsy), two pathogenic mutations were identified in *TET2* (Q114X, variant allele frequency (VAF) 12% and G1370R, VAF 5%), as well as an in-frame internal tandem duplication (ITD) in *FLT3* (*FLT3*-ITD), which were present concurrently with *BRAF*^{V600E} and *NRAS*^{G12D} mutations.

TABLE 1 Selection of variants detected by whole-exome sequencing (WES) using the Polyweb interface (www.polyweb.fr/) for the 14 lesions analysed with a matched peripheral blood mononuclear cells (PBMC) control sample[#]

Gene	Variation [¶]	Type	Patient number (VAF)	Consequence	Description	Exon	Amino acid variant	Genomic variant	PolyPhen2	SIFT
BRAF	7_140453136_A_T (rs113488022)	SNV	10 (10%) 23 (4%) 27 (1%) 56 (4%) 59 (2%) 103 (11%)	Missense	V-raf murine sarcoma viral oncogene homolog B	15	V600E	c.1799T>A	Probably damaging	Deleterious
BRAF	7_140477837_AATGTGACAGCACCTA_A	Deletion	8 (2.5%) 16 (12%) 30 (4%) 31 (2%)	In-frame deletion	V-raf murine sarcoma viral oncogene homolog B	12	LNVTAPT486LT	c.1457_1471del [§]	–	–
CIC	19_42799295_A_AG	Insertion	67 (34%)	Frameshift	Capicua transcriptional repressor	20	Q1593QA	c.4778_4779ins ^f	–	–
CP	3_148896396_C_G (rs139633388)	SNV	57 (10%)	Missense	Ceruloplasmin (ferroxidase)	16	G895A	c.2684G>C	Probably damaging	Deleterious
DTHD1	4_36285990_TAAAC_T	Deletion	57 (10%)	Frameshift	Death domain containing 1	1	KQ97X	c.289_292del [§]	–	–
HMCN1	1_186086719_C_A (rs376333827)	SNV	57 (21%)	Missense	Hemicentin 1	77	L3938I	c.11812C>A	Possibly damaging	Benign
MAP2K1	15_66729093_TCTGGA_T	Deletion	2 (14%)	Frameshift	MAPK kinase 1	3	L101Dfs*18 ⁺	c.301_305del [§]	–	–
MAP2K1	15_66729093_TGGAGAT_T	Deletion	57 (7%)	In-frame deletion	MAPK kinase 1	3	LEIK 102LK	c.303_308del [§]	–	–
NRAS	1_115256530_G_T (rs121913254)	SNV	10 (14%) 27 (4%) 59 (8%)	Missense	V-ras neuroblastoma RAS viral oncogene homolog	3	Q61K	c.181C>A	Possibly damaging	Deleterious
NT5C1B-RDH14	2_18765937_C_T	SNV	103 (15%)	Missense	NT5C1B-RDH14 readthrough	4	R191H	c.572G>A	Probably damaging	Deleterious
PCLO	7_82584553_C_A (rs202091944)	SNV	57 (17%)	Missense	Piccolo presynaptic cytomatrix protein	5	G1906C	c.5716G>T	Probably damaging	Benign
PPFIA2	12_81657159_T_C (rs368764392)	SNV	57 (15%)	Missense	PTPRF	31	K1189R	c.3566A>G	Benign	Deleterious
UNC5B	10_73056470_G_GATATTCCAGCTGC ATACCACTCTGGCAGAGGTGAGGGAAG TCGGGGCCAC (rs879908823)	Insertion	71 (50%)	Frameshift	Interacting protein (liprin), alpha 2 Unc-5 homolog B (<i>Caenorhabditis elegans</i>)	15	I821IF	c.2460_2461ins ^f	–	–

VAF: variant allele frequency; SIFT: Sorting Intolerant from Tolerant (<https://sift.bii.a-star.edu.sg/>); SNV: single nucleotide variant; MAPK: mitogen-activated protein kinase; PTPRF: protein tyrosine phosphatase, receptor type, f polypeptide. [#]: eleven patients had pulmonary Langerhans cell histiocytosis (PLCH) at diagnosis; [¶]: variation format: chromosome_genomic position_allele reference_allele mutated (rs number); ⁺: fs*18 is a variant that causes a frameshift (the new reading frame ends on a downstream 18-position STOP codon); [§]: del=deletion; ^f: ins=insertion.

Since the identified mutations mainly involved the MAPK pathway, we extended the genomic analysis to a larger series of LCH lesions using a custom-designed NGS panel of 74 genes including MAPK pathway genes. Among the 69 LCH (50 PLCH) lesions analysed, including the 18 lesions sequenced by WES, a *BRAF* alteration was identified in 46 cases (67%) and consisted of 25 *BRAF*^{V600E} mutations (36%), 20 *BRAF*^{N486_P490} deletions (29%, present concurrently with a *BRAF*^{V600E} mutation in one case) and one *BRAF*^{G469A} mutation (lung biopsy). The *BRAF*^{G469A} mutation was previously identified in *BRAF*-mutated lung adenocarcinomas [23]. This mutation belongs to class 2 of the *BRAF* mutants and is insensitive to the *BRAF* inhibitor vemurafenib [24].

MAP2K1 alterations were detected in 10 lesions (14.5%) and consisted of six *MAP2K1* mutations and four *MAP2K1* deletions. All mutations were localised to exons 2 and 3, encoding the autoregulatory domain and the catalytic core of *MAP2K1* (also called MEK1). Five of these mutations are activating: Q56P [25], K57N [26], the E102_I103 deletion [12], G128D and C121S [13]. An R108W mutation not functionally evaluated previously was identified in one case. This mutation, located in the protein kinase catalytic domain, was predicted to be deleterious by 3/3 algorithms and was shown by peptide arrays to have a critical role in the surface of MEK1 for tubulin interaction [27]. Another case harboured an undescribed L101Dfs*18 frameshift deletion, which generates a stop codon 18 positions downstream.

Isolated *NRAS*^{Q61} mutations were observed in three cases (two lung tissue and one bone tissue). In addition, 11 other lesions (lung (n=8) and skin (n=3)) harboured *NRAS* mutations (*NRAS*^{G12D} (n=2) and *NRAS*^{Q61} (n=9)) in association with other alterations (*BRAF*^{V600E} in nine cases). Finally, one isolated *KRAS*^{G12S} mutation was observed in a skin biopsy and only one lung biopsy sample harboured a *KRAS*^{G12D} mutation, which was identified concurrently with a *BRAF*^{V600E} mutation and an *NRAS*^{Q61R} mutation. No *KRAS*^{G12D} mutations were identified by NGS in the control lung tissue obtained from 10 smokers. All these findings were confirmed by at least one additional genotyping technique (pyrosequencing and/or E-Ice-COLD-PCR).

Overall, a pathogenic alteration of the MAPK pathway was observed in 59 of the 69 tested lesions (86%; see table 2 and figures 2 and 3). More precisely, 44 of the 50 PLCH patients' lesions (88%) contained a MAPK alteration. Several other mutations that could potentially be involved in LCH pathogenesis were identified concurrently with MAPK alterations (see table 2 and figure 2, and detailed in supplementary table S6). The prevalence of the identified molecular alterations did not significantly differ according to the site of LCH biopsy (p=0.60).

No MAPK alterations could be identified in purified CD11c⁺ blood cells available from 10 LCH patients (six multisystem), although eight out of 10 paired lesions harboured genomic alterations.

Correlation between MAPK alterations and clinical features

For the 69 LCH patients whose lesions were widely genotyped, no difference in clinical features was identified according to the different MAPK alterations, both at diagnosis and during follow-up. The cumulative incidence of LCH progression during the study period was not different according to the different mutations identified (figure 4; p=0.6, Gray's test).

As the *BRAF*^{V600E} mutation is a driving mutation in LCH and was shown to be correlated with LCH severity in paediatric LCH [15, 28], we specifically evaluated the clinical impact of this mutation. Notably, in other MAPK related diseases and particularly in melanoma, this mutation was shown to be an independent prognostic factor, whereas other MAPK activating mutations did not have the same prognostic impact [29, 30]. For this purpose, 48 additional patients whose LCH tissues were genotyped for *BRAF*^{V600E} were included in this analysis (mutated in 37.5% of cases). The characteristics of LCH at diagnosis were comparable to those of the 69 patients whose lesions were widely genotyped, with the exception that the latter were somewhat younger (p=0.051) and had bone involvement less frequently (p=0.04). Considering the entire study population (n=117), no association was found between *BRAF*^{V600E} status and LCH presentation at diagnosis (table 3).

Among the 83 PLCH patients, no difference in smoking status (p=0.42) or the proportion of pneumothorax (p=0.29) was identified according to the presence or absence of the *BRAF*^{V600E} mutation in tissue lesions. Lung function at diagnosis was available for 73 patients (88%) in comparable proportions for patients with and without the *BRAF*^{V600E} mutation. The proportion of restrictive pattern, airflow obstruction, or impairment of *D*_{LCO} (<80% of predicted values) was similar between the two groups of patients (p=0.088, p=0.44 and p=0.11, respectively).

Patients were followed for a median time of 59.5 months (IQR 32–117.5) and, based on the global outcome of LCH, 70 patients overall (60%) experienced a progression of LCH during the study period. This consisted of progression in 18 LCH patients without pulmonary involvement and in 52 patients with LCH lung localisation at diagnosis. It is noteworthy that only 38 of the latter 52 were related to lung progression in

TABLE 2 Results of genotyping of the 69 patients whose Langerhans cell histiocytosis (LCH) lesions were widely genotyped by next-generation sequencing (NGS) and pyrosequencing for *BRAF* deletions

Patient	Predominant variant	Additional variants
1	<i>BRAF</i> ^{V600E}	–
2	<i>MAP2K1</i> ^{L101Dfs*18}	–
3 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
4 [#]	<i>MAP2K1</i> ^{C121S}	–
5 [#]	<i>MAP2K1</i> ^{R108W}	<i>DUSP</i> ^{D231N} <i>ERBB2</i> ^{T791I}
6 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
7 [#]	<i>MAP2K1</i> ^{G128D}	<i>NRAS</i> ^{Q61K}
8 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
9 [#]	WT	–
10 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61K}
11 [#]	<i>MAP2K1</i> ^{G128D}	–
12	<i>BRAF</i> ^{V600E}	–
13 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
14 [#]	WT	–
15	<i>BRAF</i> ^{N486_P490} deletion	–
16 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
17 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61K}
18 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61R} <i>ERBB4</i> ^{R838X} <i>MET</i> ^{S244F} <i>NOTCH1</i> ^{c.6181-1G>A} <i>PPP6C</i> ^{P296S}
19 [#]	WT	–
20	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61L} <i>EGFR</i> ^{P753S}
21 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
22 [#]	<i>NRAS</i> ^{Q61K}	–
23 [#]	<i>BRAF</i> ^{V600E}	–
24 [#]	WT	–
25 [#]	<i>BRAF</i> ^{V600E}	–
26 [#]	<i>BRAF</i> ^{V600E}	–
27 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61K}
28	<i>BRAF</i> ^{N486_P490} deletion	–
29	WT	–
30 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
31 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
32 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
33 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61R} <i>KRAS</i> ^{G12D}
34 [#]	<i>BRAF</i> ^{nonV600E} (G469A)	–
35 [#]	<i>MAP2K1</i> ^{E102_I103} deletion	–
36 [#]	<i>BRAF</i> ^{V600E}	–
37 [#]	<i>BRAF</i> ^{V600E}	–
38 [#]	<i>MAP2K1</i> ^{E102_I103} deletion	–
39	<i>BRAF</i> ^{V600E}	–
40 [#]	<i>BRAF</i> ^{V600E}	<i>BRAF</i> ^{N486_P490} deletion
41 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
42 [#]	<i>BRAF</i> ^{V600E}	–
43 [#]	<i>BRAF</i> ^{V600E}	–
44 [#]	<i>BRAF</i> ^{V600E}	–
45 [#]	<i>BRAF</i> ^{V600E}	–
46 [#]	<i>BRAF</i> ^{N486_P490} deletion	–
47	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{G12D} <i>TET2</i> ^{Q114X} <i>TET2</i> ^{G1370R} <i>FLT3</i> -ITD
48 [#]	WT	–

Continued

TABLE 2 Continued

Patient	Predominant variant	Additional variants
49 [#]	<i>BRAF</i> ^{N486_P490} deletion	<i>NRAS</i> ^{Q61R}
50	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{G12D}
51 [#]	<i>BRAF</i> ^{V600E}	—
52 [#]	<i>BRAF</i> ^{N486_P490} deletion	—
53	<i>BRAF</i> ^{N486_P490} deletion	—
54	WT	—
55	<i>NRAS</i> ^{Q61R}	—
56 [#]	<i>BRAF</i> ^{V600E}	—
57	<i>MAP2K1</i> ^{E102_I103} deletion	<i>IDH1</i> ^{G105D}
58 [#]	<i>NRAS</i> ^{Q61K}	—
59 [#]	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61K}
60 [#]	<i>MAP2K1</i> ^{Q56P}	—
61	<i>BRAF</i> ^{V600E}	—
62 [#]	<i>BRAF</i> ^{N486_P490} deletion	—
63	<i>KRAS</i> ^{G12S}	—
64	<i>BRAF</i> ^{N486_P490} deletion	—
65	WT	—
66 [#]	<i>BRAF</i> ^{N486_P490} deletion	—
67 [#]	WT	—
68 [#]	<i>MAP2K1</i> ^{K57N}	—
69	WT	—

WT: wild type; ITD: internal tandem duplication. [#]: patients with pulmonary Langerhans cell histiocytosis (PLCH) at diagnosis (n=50).

FIGURE 2 Mutational landscape in 69 Langerhans cell histiocytosis (LCH) patients whose lesions were widely genotyped by next-generation sequencing (NGS) and pyrosequencing for *BRAF* deletions. The basic plot was created using cBioPortal (www.cbioportal.org/) and the altered genes are listed on the left-hand side of the diagram. All *BRAF* mutations are *BRAF*^{V600E} mutations except for one, which is a *BRAF*^{G469A} mutation. The *BRAF*, *NRAS*, *MAP2K1*, *KRAS*, *EGFR*, *DUSP4*, *ERBB2*, *ERBB4* and *MET* genes belong to, or are linked to, the mitogen-activated protein kinase (MAPK) pathway, and *IDH1* and *TET2* belong to the family of epigenetic modifiers. [#]: mutation; [¶]: small deletion.

FIGURE 3 Distribution of mitogen-activated protein kinase (MAPK) alterations among the 69 Langerhans cell histiocytosis (LCH) patients whose lesions were evaluated by next-generation sequencing (NGS) and pyrosequencing for *BRAF* deletions (displayed as percentages of identified MAPK alterations). *MAP2K1* comprises both mutations and deletions. Concurrent genomic alterations were not recorded. WT: wild type.

PLCH patients. Thirty-seven patients needed systemic LCH treatment, consisting mainly of corticosteroids associated with vinblastine and/or cladribine. A comparable proportion of patients with and without *BRAF*^{V600E} mutated lesions received systemic LCH treatment ($p=0.21$, Fisher's exact test). Seven patients (6%) died (five linked to LCH) and two received transplants due to lung involvement. The probability of disease progression over time was similar regardless of the *BRAF*^{V600E} status of the lesions (figure 5a).

Among the PLCH population, 38 patients (46%) experienced lung progression (including 10 patients with respiratory failure, long-term oxygen use, or PH). *BRAF*^{V600E} status was not associated with lung involvement outcome (figure 5b; $p=0.55$, Gray's test).

In the univariable analysis, an older age at diagnosis was associated with an increased risk of disease progression in patients (hazard ratio 1.37 per decade, 95% CI 1.04–1.80; $p=0.027$), including the group of PLCH patients (hazard ratio 1.37, 95% CI 1.02–1.80; $p=0.027$). Additionally, the presence of airflow obstruction was associated with lung progression (supplementary table S7). In the multivariable analysis, only the presence of airflow obstruction remained associated with an increased risk of lung progression (hazard ratio 2.00, 95% CI 1.05–3.84; $p=0.036$) (table 4).

FIGURE 4 Cumulative incidence of disease progression among the 69 Langerhans cell histiocytosis (LCH) patients whose lesions were widely genotyped, according to the different mitogen-activated protein kinase (MAPK) alterations identified [*BRAF*^{V600E} (n=25); other *BRAF* alterations (n=21; *BRAF* deletions (n=19), other (n=2)); non-*BRAF* alterations (n=14; *MAP2K1* (n=10), *NRAS/KRAS* (n=4)); wild type (WT; n=9)].

TABLE 3 Comparison of clinical features at diagnosis of the 117 Langerhans cell histiocytosis (LCH) patients according to the $BRAF^{V600E}$ status of their lesions

Characteristic	$BRAF^{V600E}$ (n=43)	Without $BRAF^{V600E}$ (n=74)	p-value
Female	20 (46.5)	36 (48.6)	0.85
Age years	38.0 (26.6–50.0)	35.2 (25.0–45.8)	0.24
Multisystem	12 (27.9)	26 (35.1)	0.54
Organs involved [#]			0.78
1	31 (72)	48 (64.9)	–
2	7 (16.3)	16 (21.6)	–
3	2 (4.7)	6 (8.1)	–
4	3 (7)	4 (5.4)	–
Lung	31 (72.1)	52 (70.3)	1.00
Bone	10 (23.3)	23 (31.1)	0.40
Pituitary [¶]	6 (13.9)	10 (13.5)	1.00
Skin	7 (16.3)	12 (16.2)	1.00
Lymph node	3 (7)	6 (8.1)	1.00

Data is presented as n (%) or median (interquartile range (IQR)) unless otherwise stated. [#]: risk organ involvement (liver or haematological involvement) and central nervous system localisation were not assessed, as they concerned only three patients each in the entire study population; [¶]: diabetes insipidus (associated (or not) with anterior hypophysis deficiency).

Discussion

In this study, we found the following salient results: 1) MAPK alterations were present in nearly 90% of the LCH lesions; 2) besides $BRAF^{V600E}$ mutations, $BRAF^{N486_P490}$ deletions were identified in a substantial proportion of cases; 3) $KRAS$ mutations were virtually absent from PLCH lesions; and 4) $BRAF^{V600E}$ mutation was not associated with either the initial presentation or the outcome of PLCH.

MAPK alterations were present in 86% of the LCH biopsies evaluated by NGS and only a small minority of cases were found to be wild type (WT). Moreover, because we did not perform RNA sequencing on tissue specimens, we cannot exclude the possibility that additional MAPK alterations, such as $BRAF$ kinase fusion, were missed [19, 31]. Thus, in the vast majority of adult LCH cases, particularly PLCH cases, the disease appears to be driven by genomic alterations involving the MAPK pathway.

The $BRAF^{V600E}$ mutation was identified in 36% of cases and in similar percentages among the subgroups of patients whose lesions were evaluated by NGS or by gene-specific genotyping. This frequency of $BRAF^{V600E}$ mutations was significantly lower than the 64% observed by CHAKRABORTY *et al.* [19] in

FIGURE 5 Cumulative incidence of disease progression among all Langerhans cell histiocytosis (LCH) patients whose lesions were genotyped for the presence of the $BRAF^{V600E}$ mutation. a) Disease progression among the 117 patients tested according to the presence or absence of the $BRAF^{V600E}$ mutation, based on the assessment of the global outcome of their LCH localisations. b) The same comparison for the 83 pulmonary Langerhans cell histiocytosis (PLCH) patients at diagnosis who experienced pulmonary progression during the study period.

TABLE 4 Multivariable analysis of factors associated with pulmonary outcome among the 83 pulmonary Langerhans cell histiocytosis (PLCH) patients at diagnosis[#]

Characteristic	Hazard ratio (95% CI)	p-value
Age at diagnosis	1.03 (1.00–1.05)	0.083
Airflow obstruction	2.00 (1.05–3.84)	0.036

[#]: airflow obstruction (forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) ratio <70%).

paediatric LCH, but close to the 42% reported by HERITIER *et al.* [28] in paediatric LCH with lung involvement. Conversely, we identified *BRAF*^{N486_P490} deletions in up to 30% of our cases, in similar proportions from both WES and a dedicated pyrosequencing assay, which is much higher than the 6% frequency reported in paediatric LCH [19]. In contrast, the *MAP2K1* alterations observed in this series occurred in a similar proportion as that reported in paediatric LCH (14.5% and 12%, respectively) [19]. Finally, no isolated *KRAS* mutations were identified in PLCH and, thus, our results do not support a role for this mutation in the development of PLCH lesions, unlike that recently suggested in a murine model in which a *KRAS* mutation was induced in lung myeloid cells [14].

The identification of these different MAPK alterations has an impact on MAPK-targeted treatment. While the *BRAF*^{V600E} mutation is sensitive to BRAF inhibitors [32, 33], *BRAF*^{N486_P490} deletion was shown to be relatively resistant to “first-generation” *BRAF*^{V600E} inhibitors but sensitive to MEK inhibitors [19]. Similarly, LCH patients whose lesions harbour *MAP2K1* mutations were shown to respond to MEK inhibitors [19, 34–36].

In this series of exclusively LCH cases, only one lesion contained molecular alterations involved in myeloid haematological disorders. This case included a *TET2* mutation as well as in-frame duplication in *FLT3*, concurrently with *BRAF*^{V600E} and *NRAS*^{G12D} mutations. *TET2* is widely involved in haematological malignancies and in clonal haematopoiesis of indeterminate potential [37, 38]. The in-frame duplication in *FLT3* found here corresponds to a recurrent alteration observed in acute myeloid leukaemia [39]. At more than 4 years of follow-up, our patient did not develop any haematological disorders. The rare observation of molecular alterations in myeloid neoplasms is contrary to what has been reported in ECD and may be explained by the younger age of our patients compared with ECD patients [22].

An important result of this study in the clinical context is the absence of an association between the *BRAF*^{V600E} mutation (tested in all cases) and either the initial presentation or the outcome of the patients. This result contrasts with that found in paediatric LCH, in which the *BRAF*^{V600E} mutation was associated with recurrence, severity and resistance to first-line therapy [15, 28]. However, an important difference in adult LCH is that risk organ involvement (*i.e.* liver, spleen, or haematological system), which is associated with disease severity in paediatric LCH [1], is rarely present [20] (as was the case in the present series).

In this large cohort of PLCH patients, we did not find any association between smoking and *BRAF*^{V600E} status, contrary to that previously suggested based on a small series of patients [5]. Importantly, the outcome of PLCH patients based on lung function variations over time was not influenced by the *BRAF*^{V600E} status of their lesions. The nearly 50% of patients whose pulmonary involvement worsened during the study, in which patients were followed for a 6-year median time, was a similar proportion to that we previously observed at 5 years in a smaller retrospective multicentre study [40]. In contrast, airflow obstruction at diagnosis was a predictive factor of worsening of lung function during follow-up in this large cohort study, in which the data were prospectively registered.

Although our study population was representative of all histologically confirmed adult LCH cases seen at our centre, it may not reflect patients with isolated mild PLCH for whom a surgical lung biopsy is not always performed. However, these patients with a presumptive diagnosis retained on a clinical and imaging basis usually do not progress [2]. Thus, the findings of the present study are clinically meaningful. Similarly, because the LCH recruitment of our centre is orientated towards PLCH, the lack of clinical impact of the *BRAF*^{V600E} mutation found in this study needs to be confirmed in a series of adult LCH cases without lung involvement.

In summary, MAPK alterations are present in most lesions from adult LCH patients, particularly in PLCH. The search for alterations involved in MAPK pathway activation, including *BRAF*^{N486_P490} deletion, is important to guide the choice of targeted treatment in selected patients with refractory progressive LCH.

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