



BRAF Mutations in Pulmonary Langerhans Cell Histiocytosis: A Multicentre Survey

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Abstract

Pulmonary Langerhans cell histiocytosis (PLCH) is a smoking-related condition in which aggregates of Langerhans cells admixed with other inflammatory cells are found in lung tissue. The general view is that PLCH is a reactive condition rather than a neoplasm. However, in small series BRAF c.1799T>A (p.V600E) mutations have been identified which raise the possibility that PLCH may have features of a neoplasm after all. Because the reported cases are few in number, we undertook a multi-institute survey of BRAF mutations in adult smokers. We found BRAF V600E mutations in 57% of our cohort of 61 patients. No significant relation with age or gender and BRAF status was found. Our series confirms the presence of BRAF V600E mutations in a large proportion of PLCH patients.

Keywords

Langerhans cell histiocytosis; Lung neoplasia; BRAF

Introduction

Despite sharing morphological and immunohistochemical features, pulmonary Langerhans cell histiocytosis (PLCH) is considered a distinct disease compared to localized and disseminated forms of non-pulmonary Langerhans cell histiocytosis (nPLCH), which most commonly occur in the pediatric population. While nPLCH is considered at least a proliferative and probable neoplastic process, PLCH is regarded as a reactive proliferation, induced by cigarette smoking [1,2]. There is clinical and scientific support for this supposition, PLCH is almost restricted to smokers (90%) and may stabilize following cessation of smoking [3,4]. This contrasts with the various forms of nPLCH, which very rarely spontaneously regress and require therapeutic intervention, while severe multisystem disease (formerly known as Letterer-Siwe disease) may be fatal in a considerable proportion of cases [5]. In up to 20% of patients PLCH may progress and may lead to progressive pulmonary fibrosis [3]. Although from a clinical point of view PLCH and nPLCH are different

diseases, the hallmark cell, the Langerhans cell (LC), is similar in all forms of LCH with identical morphology, ultrastructural features and immunohistochemical profile. In a previous study by Badalian-Very et al., aimed at identifying cancer-associated aberrations in LCH, BRAF c.1799T>A (p.V600E) mutations were found in 57% of LCH cases [6]. Their series included 12 PLCH cases and 49 nPLCH cases. Five (42%) PLCH and 29 (59%) nPLCH harbored a BRAF mutation.

Given the limited number of cases that have been analyzed to date, we undertook a survey aimed at identifying BRAF mutations by multiplex allele-specific PCR in a large multicenter cohort of PLCH cases.

Materials and Methods

Patient identification

Consent for this study was granted by the Medical Ethics committee (medisch ethische toetsingcommissie; METC) of the Erasmus MC. All patient data was analyzed anonymously. There was no research involving animals performed for this manuscript.

In a retrospective study cases of PLCH were accrued through the Dutch Pulmonary Pathology Club after an invitation to participate in the study was circulated among the group members. Participants were asked to query the local pathology database for PLCH cases, retrieve material (slides and formalin fixed-paraffin wax embedded (FFPE) tissue blocks) and send these for central analysis.

Standard 5 µm HE stained sections were prepared from all blocks, to determine the presence and the extent of a LCH lesion. A single block from each case was selected for further analysis, except in two patients where two blocks with different LCH lesions were analyzed. From the selected blocks, ten consecutive 5µm sections were prepared on microscope slides. The first sections were HE stained and the second and last sections were stained for CD1a (Clone EP3622, Ventana 760-4525 on the Benchmark ULTRA slide staining platform, Ventana, Tuscon, AZ) and used for estimation of the LC content. The intervening slides were used for BRAF molecular analysis. DNA was extracted from microdissected FFPE tissue fragments with a high percentage of LCs by proteinase K digestion for 16h at 56°C in the presence of 5% Chelex 100 resin.

PCR and Sanger sequencing

BRAF mutational analysis was performed by multiplex allele-specific PCR for detection of the hotspot codon 600 mutation (NM_004333: c.1799T>A encoding p.V600E). The analytical sensitivity of this assay was demonstrated to detect at least 5% BRAF mutant cells. The multiplex PCR includes a control primer pair that amplifies another region of BRAF. Primer mixes contain BRAF V600E-Fwd, 5'-GGTGATTTTGGTCTAGCTACG*GA*-3', in which the asterisks indicate allele-specific substitutions, BRAF V600 Rev-FAM, 5'-ATAGCCTCAATTCTTACCACCCAC-3', BRAF CF Fwd-FAM, 5'-TGCAAAGCAAATATGAGAATCC-3' and BRAF CF Rev, 5'-GATGGGGTCTCACTATGTTGAG-3'. Only the presence of a c.1799T>A substitution yields both a mutation-specific product of 101bp and a control fragment of 174 bp; otherwise only the larger control fragment will be obtained. PCR was carried out after initial

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Received: November 28, 2016 Accepted: January 17, 2017 Published: January 24, 2017

denaturation at 95°C for 3min, followed by 35 cycles of 95°C for 30s, 60°C for 45s, and 72°C for 45s were performed and 10 min at 72°C. All WT cases were further analyzed for the presence of a BRAF V600K mutation. In these cases BRAF V600E-Fwd was substituted for BRAF V600K-Fwd, 5'-TTTTTGGTGATTTGGTCTAGCTACG*A*A*-3', yielding a product of 106bp. Fragment analysis was performed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Patient- and tumour characteristics were described using frequencies and percentages. Proportions were compared using χ^2 test for categorical variables. Differences in the mean age between the group of patients with the BRAF V600E mutation and the group of wild-type patients were tested using independent samples T-test. Two-sided P-values < 0.05 were considered statistically significant for all analyses. Data analysis was performed with SPSS version 20.0 (SPSS, Chicago, IL, USA).

Results

FFPE blocks from 61 patients were selected for analysis, in two patients two separate lesions were analyzed, yielding a total of 63 blocks. The median age of the patients was 45 years (range 16-73 years) and 67% was female (Table 1).

The sample tumour cell percentage ranged between 1 and 70%; all but a single case contained more than 5% tumour cells in LCH lesions based on CD1a staining (Figure 1a, 1b); sufficient DNA was extracted from all samples for BRAF V600E testing. Overall tumour cell percentages were evenly distributed in cases with and without a BRAF V600E mutation. A BRAF V600E mutation was identified in 36 samples obtained from 35 patients (57%; Figure 1c), while in the remaining 25 samples no BRAF V600E mutation was detected.

BRAF V600E mutations were more common in female patients (66% versus 40% in male patients), however gender was not significantly related to the presence of a BRAF V600E mutation (p=0, 12) (Table 2). No significant difference in mean age between patients with and without BRAF V600E mutation was observed (p=0.28) (Table 2).

A concordant BRAF V600E mutation was identified in both samples from one patient, while in the second patient a BRAF V600E mutation was found in one sample, but the quality of second sample was insufficient for analysis. No BRAF V600K mutations were identified in LCH cases without a BRAF V600E mutation. In two samples there was insufficient material to test for the BRAF V600K mutation.

Table 1: Results BRAF V600E analysis.

| | PLCH patients, n=61 (%) | |
|----------------------------|-------------------------|---------|
| No. samples | 63 ^a | |
| Median age (Range) | 44 | (16-73) |
| Gender | | |
| Male | 20 | (33) |
| Female | 41 | (67) |
| BRAF V600E mutation | 36 | (57) |

Note: ^aFrom two patients two FFPE tissue blocks from separate lesions were obtained

Discussion

PLCH is a smoking-related nodular-cystic pulmonary disorder, which occurs in young-medium-aged adults (20-40 years of age) with an equal gender distribution [2,3]. The clinical outcome of PLCH is variable but is difficult to establish reliably as many PLCH patients continue to smoke with concurrent other smoking related diseases impacting on prognosis and outcome. While most PLCH patients have a good prognosis [4] lung transplantation may be required for intractable progressive disease [7].

The pathogenesis of LCH remains uncertain. Although nPLCH is categorized as a neoplastic disease by the WHO, this neoplasia view is challenged by some, favouring an “immune-frustrated” reactive process instead, where LCH proliferation is induced by a “cytokine storm” with mediators released from a host of inflammatory cells, which accompany the LCs [1,8]. For PLCH a reactive, smoking-related, etiology is generally deemed more likely, given the strong evidence from both an epidemiological, clinical and pathological point of view [1]. Supportive evidence for a neoplastic etiology may be inferred from a clonal origin of lesional cells [9]. X-chromosome inactivation studies revealed clonality in nPLCH [10-12]. In PLCH a mixed clonal – polyclonal pattern was found with some nodules from one patient showing a clonal LC population and other nodules revealing a polyclonal pattern of X-linked chromosomal inactivation [13]. Further evidence for a neoplastic etiology of a cellular proliferation may be assumed when a non-random genetic mutation is encountered, particularly if this involves a proto-oncogene or tumour suppressor gene. Indications for involvement of possible tumour suppressor genes on chromosomes 9 and 22 in PLCH was found by Dacic et al., who evaluated allelic loss and LOH in microdissected samples of PLCH [14]. The oncogenic V600E BRAF mutation was first demonstrated in LCH in 12 PLCH cases in a survey aimed at detecting mutations in cancer-related genes [6]. Since the identification of BRAF mutations in LCH only a limited number of cases in small series have been investigated (Table 3) [6,15-19].

We sought to survey the prevalence of BRAF mutations in a large set of (adult) PLCH cases, derived from the archives of several Dutch pathology laboratories. In this series we found BRAF mutations in a large proportion of cases, mirroring that of a single smaller series by Chilosi et al. [15].

Although our series comprised twice as many females as males this contrasts with the Dutch population smoking habits, where traditionally male smokers have predominated. Although a selection bias for either patient selection for a biopsy procedure or selection in our study may be considered this is highly unlikely considering the involvement of multiple institutes. We believe the observed gender differences coincidental. In contrast to published smaller series our study showed a high relative proportion of BRAF V600E mutations in female patients (67%), while in previously published series a slight male predominance was reported. Despite the female predominance, gender was not significantly related with the presence of a BRAF V600E mutation. In addition, no difference in age between BRAF V600E mutant and BRAF WT PLCH cases was shown. However, despite our largest series of LCH cases analyzed to date, the gender and age distribution should ideally be studied in a larger cohort. Although we confirmed the presence of BRAF V600E mutations in a significant number of PLCH cases, a proportion of PLCH cases without BRAF mutations may still have BRAF involvement. There is evidence that additional mechanisms leading to hyperactivation of the RAS/RAF/

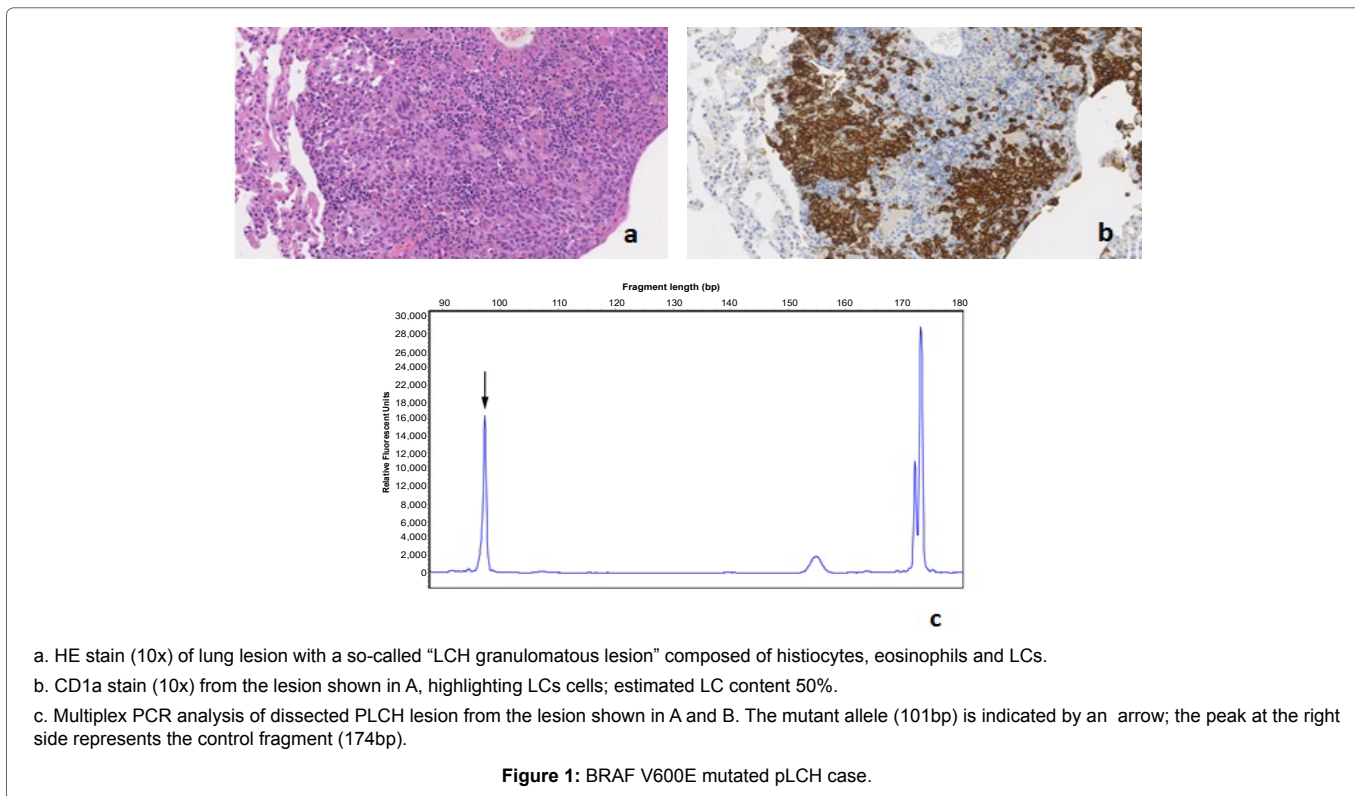


Table 2: Statistical correlation age, gender and BRAF mutation.

| | BRAF V600E n=36 (%) | | Wild type n=25 (%) | | p-value |
|----------------------|---------------------|--------|--------------------|--------|----------------------|
| Mean Age (sd) | 44.4 | (14.6) | 40.32 | (14.1) | 0.28 (t-test) |
| Gender | | | | | 0.12 (χ2) |
| Male | 9 | (25.0) | 11 | (44.0) | |
| Female | 27 | (75.0) | 14 | (56.0) | |

Table 3: BRAF mutations in PLCH.

| Author | Technique | No. of PLCH patients analyzed | BRAF V600E mutation |
|------------------------------|--------------------------------|-------------------------------|---------------------|
| Badalian-Very [6] | Genetic | 12 | 5 (42%) |
| Chilosi [15] | Genetic + immunohistochemistry | 19 | 12 (63%) |
| Roden [18] | Immunohistochemistry* | 25 | 7 (28%) |
| Mourah [16] | Genetic | 26 | 13 (50%) |
| Nelson [17] | Genetic | 30 | 10 (33%) |
| Yousem [19] | Genetic# | 5 | 2 (40%) |
| den Bakker (this manuscript) | Genetic | 61 | 35 (57%) |

Note: *Genetic analysis performed, no case details provided, #Multiple nodules (2-9) from each patient analyzed

MAPK/ERK pathway may underlie BRAF WT cases, as Badalian-Very et al., demonstrated activation of the pathway by immunofluorescent staining of phosphorylated (activated) components of the MAPK pathway [6].

Recently Nelson et al., provided further experimental support for activation of the MAPK pathway in BRAF WT LCH cases by showing that in BRAF WT cases mutation and functional activation of either MAP2K1 or MAP3K1 genes may occur. Furthermore, their experiments showed that inhibition of activated MAPK proteins by a tyrosinase inhibitor (TKI) is possible in vitro, implying that TKI treatment may be an option for LCH patients with severe or progressive disease [17]. Support for alternative activation of the

MAPK pathway in pulmonary BRAF WT LCH cases was provided in work by Mourah et al., in which NRAS mutations (NRAS Q61K/R) were observed 40% of PLCH cases. Furthermore, they showed that BRAF and NRAS mutations were present in different lesions in one patient, suggesting that BRAF and NRAS mutations are mutually exclusive in PLCH [16].

Although the identification of BRAF mutations in PLCH may be seen as evidence for neoplasia, the relationship between clonality, oncogene status and neoplasia is not always clear-cut. For instance Kaposi sarcoma has features of both a reactive condition and neoplasia. It may regress in transplantation patients following lowering of immune-suppression, while on the other hand it has

been shown to be a clonal proliferation [20]. Similarly, nodular fasciitis has traditionally been viewed as a reactive, self-limiting pseudosarcomatous myofibroblastic process with polyclonal composition by X-chromosome inactivation assay, yet has been shown to harbor a non-random gene rearrangement (fusion gene MYH9-USP6), leading to authors to consider nodular fasciitis a form of 'transient neoplasia' [21,22].

Similar mechanisms may underlie the enigmatic semi-neoplastic nature of PLCH as well. Given the inflammatory niche in which LCs reside, it is conceivable that a proliferative advantage afforded by the BRAF V600E mutation is enhanced by the inflammatory cytokine storm. This hypothesis would tie in the observation that PLCH may regress upon cessation of smoking.

In conclusion, we have shown that the presence of the BRAF V600E mutation is probably more prevalent in PLCH than hitherto reported. Taken together with reported findings, activation of the BRAF-associated signal transduction cascade may underlie additional cases of PLCH without the classic BRAF V600E mutation. We propose that PLCH may represent another form of so-called "transient neoplasia" where proliferative advantage through BRAF activation coupled with immune stimulation drives the process. Considering the natural course of PLCH and the effects of smoking cessation, it is unlikely that BRAF mutations impact on the outcome of PLCH.

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