

## MYELOID NEOPLASIA

Recurrent *RAS* and *PIK3CA* mutations in Erdheim-Chester disease

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## Key Points

- *PIK3CA* and *NRAS* mutations are recurrent in *BRAFV600E* wild-type ECD patients.
- 57.5% (46/80) of ECD patients have a *BRAFV600E* mutation, and an additional 10.9% and 3.7% have *PIK3CA* and *NRAS* mutations, respectively.

Erdheim-Chester disease (ECD) is a rare histiocytic disorder that is challenging to diagnose and treat. We performed molecular analysis of *BRAF* in the largest cohort of ECD patients studied to date followed by *N/KRAS*, *PIK3CA*, and *AKT1* mutational analysis in *BRAF* wild-type patients. Forty-six of 80 (57.5%) of patients were *BRAFV600E*-mutant. *NRAS* mutations were detected in 3 of 17 ECD *BRAFV600E* wild-type patients. *PIK3CA* mutations (p.E542K, p.E545K, p.A1046T, and p.H1047R) were detected in 7 of 55 patients, 4 of whom also had *BRAF* mutations. Mutant *NRAS* was present in peripheral blood CD14<sup>+</sup> cells, but not lymphoid cells, from an *NRASQ61R* mutant patient. Our results underscore the central role of RAS-RAF-MEK-ERK activation in ECD and identify an important role of activation of RAS-PI3K-AKT signaling in ECD. These results provide a rationale for targeting mutant RAS or PI3K/AKT/mTOR signaling in the subset of ECD patients with *NRAS* or *PIK3CA* mutations. (*Blood*. 2014;124(19):3016-3019)

## Introduction

Erdheim-Chester disease (ECD) is a histiocyte proliferation with frequent multiorgan involvement,<sup>1</sup> and aggressive phenotypes in ECD may lead to death despite treatment.<sup>2</sup> Recently, ECD and the related disorder Langerhans cell histiocytosis (LCH) have been identified to have *BRAFV600E* mutations in 40% to 70% of patients.<sup>3-6</sup> The discovery of *BRAFV600E* mutations in ECD and LCH has provided an important therapeutic target, and treatment of *BRAFV600E*-mutant ECD and LCH patients with vemurafenib has demonstrated dramatic therapeutic efficacy in pilot studies.<sup>7</sup> Therefore, accurate identification of *BRAFV600E* mutations in ECD and LCH is critical. However, the heterogeneous nature of ECD and LCH lesions frequently presents a challenge to the accurate identification of *BRAFV600E* mutations in lesional tissue.<sup>8</sup> In addition, several groups have noted that a larger proportion of LCH and ECD lesions have activation of ERK signaling than that demonstrated to have the *BRAFV600E* mutation.<sup>3,8,9</sup> Interestingly, Cangi et al recently identified that 18 of 18 ECD patients had a *BRAFV600E* mutation in DNA from whole lesional tissue if an

ultrasensitive methodology was used, suggesting that current data underestimate the true mutational frequency of *BRAFV600E* mutations in ECD.<sup>8</sup> Concurrently, we recently identified an *NRASQ61R* mutation in an ECD patient who was definitively *BRAF*-wild-type.<sup>10</sup>

Given this, we analyzed 80 ECD patients for the *BRAFV600E* mutation followed by interrogation of 25 ECD thought to be *BRAF*-wild-type using a variety of sensitive techniques for repeat *BRAF* mutational analysis. In parallel, we analyzed these samples for *NRAS*, *KRAS*, *PIK3CA*, and *AKT1* mutations.

## Methods

## Patients

Eighty ECD patients were included in this study, approved by the Ethics Committee Ile de France III (#2011-A00447-34) and the Institutional Review

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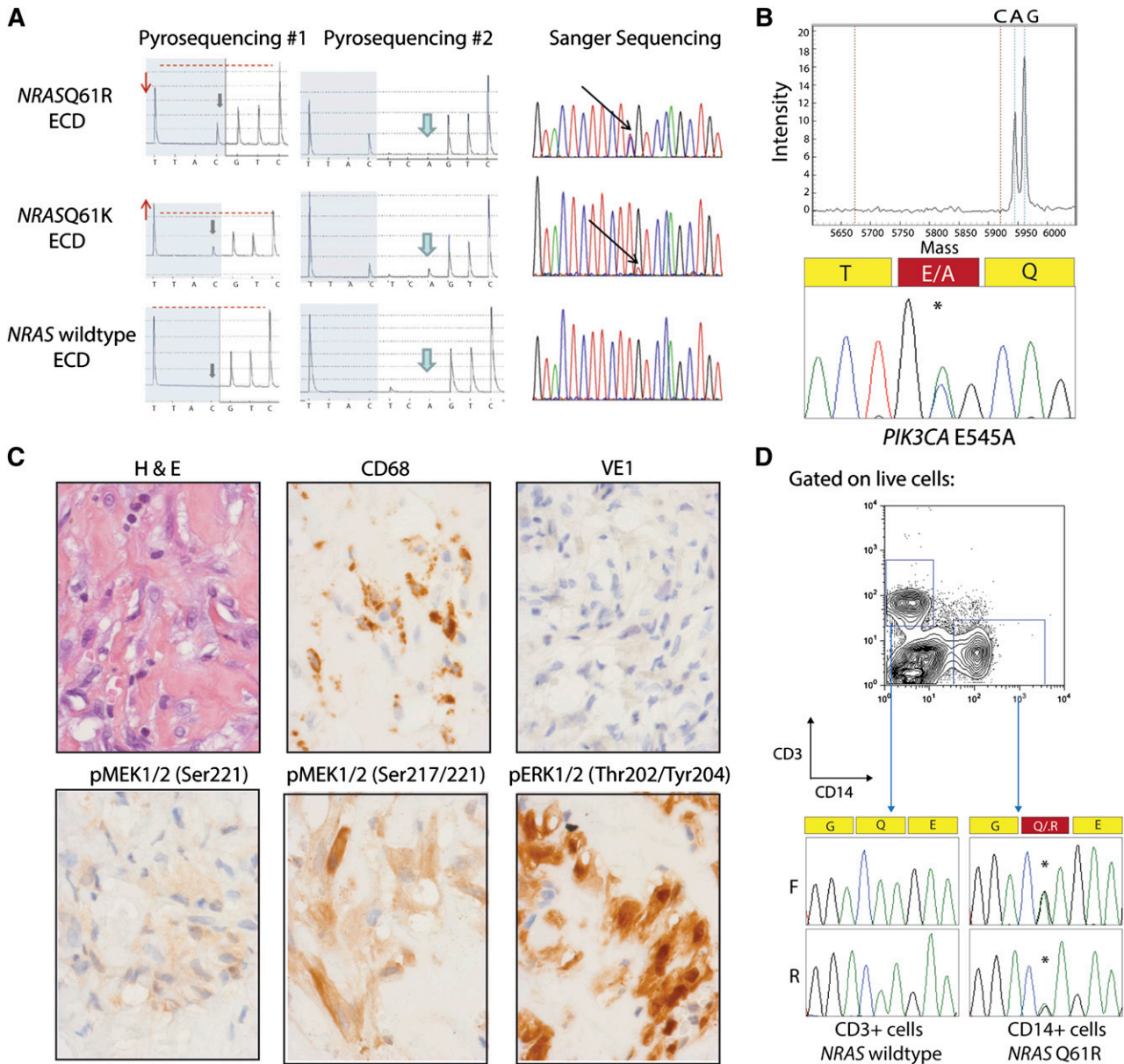
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The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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**Figure 1. NRAS and PIK3CA mutations in ECD histiocytes and CD14<sup>+</sup> cells from peripheral blood.** (A) Detection of NRAS p.Q61 mutation by pyrosequencing (first 2 columns) and confirmation by Sanger sequencing (third column). The lower pyrogram corresponds to an NRAS-wild-type ECD case, and the 2 others correspond to patients with NRAS mutations. Mutants are detectable with the appearance of a new peak at the first C injection (gray arrows), and size variation of the peak at the first T injection compared with the second C injection (red arrows and dashed line). Pyrosequencing with another sequence of injection (ESGTTACTCAGTCAGCT) was used to further identify the mutations (middle column) as c.181C>A mutations. (B) Detection of PIK3CA E545A mutation by Sequenom and Sanger sequencing in an NRAS/KRAS/BRAF-wild-type ECD patient. (C) Phosphorylated MEK (pMEK) and ERK (pERK) detected by immunohistochemistry in BRAFV600E-wild-type, NRAS-mutant ECD. Histiocytes (noted by hematoxylin and eosin and CD68 stain) failed to stain for BRAFV600E VE1 monoclonal antibody but had high cytoplasmic expression of pMEK1/2 with 2 different antibodies as well as cytoplasmic and nuclear expression of pERK1/2 (original magnification ×600). (D) Genotyping of CD14<sup>+</sup> monocytes and CD3<sup>+</sup> T cells purified from the peripheral blood of an NRASQ61R-mutant ECD patient with double-FACS sorting reveals the presence of NRAS mutation in CD14<sup>+</sup> cells but not in T cells.

Board at Memorial Sloan-Kettering Cancer Center. Informed consent was provided according to the Declaration of Helsinki.

**Genetic analyses**

Genomic DNA was extracted from formalin-fixed, paraffin-embedded samples after histologic review and enrichment by macrodissection to ≥10% histiocytes. All samples derived from patients before any therapy. Workflow of genetic analysis is depicted in supplemental Figure 1 (available on the Blood Web site). Detection of BRAFV600 and NRASQ61 mutations was performed by pyrosequencing.<sup>11</sup> BRAF status of 41 of the 80 patients was

already reported (BRAFV600E mutational frequency was 51% in this initial series).<sup>5,6,12</sup> Fifty-eight cases, in which 25 initially did not have BRAF p.V600 mutation detected, were further analyzed for BRAF mutations with other methods including multiplex picodroplet digital polymerase chain reaction (PCR)<sup>13</sup> (Raindance Technologies; details in the supplemental Methods). Screening for mutations in other genes was performed with Sequenom mass spectrometric-based genotyping assays as previously described<sup>14</sup> (NRAS, KRAS, PIK3CA, and AKT1 hotspot mutations), and next-generation targeted sequencing analysis for regions of mutations in BRAF, N/KRAS, and PIK3CA by Illumina MiSeq as described in the supplemental Methods.

**Table 1. Characteristics of *NRAS*- and *PIK3CA*-mutant ECD patients and those not identified as having *BRAF*, *NRAS*, or *PIK3CA* mutations\***

| Age† (y/Gender) | Principal organs involved by histiocytosis | Tissue biopsy site | % Histiocytes in biopsy | <i>BRAF</i> p.V600 | Other genes            | Follow up (mo, status at last follow-up) |
|-----------------|--|--------------------|-------------------------|--------------------|------------------------|--|
| 67/M            | Heart, aorta, RPF, pleura                  | Peritoneum         | 70                      | Wild-type          | <i>NRAS</i> p. G12D    | 43, DoD                                  |
| 56/M            | Aorta, RPF, pleura, bone                   | Pleura             | 70                      | Wild-type          | <i>NRAS</i> p. Q61K    | 108, AwD                                 |
| 65/M            | Aorta, pleura                              | Pleura             | 40                      | Wild-type          | <i>NRAS</i> p.Q61R     | 26, AwD                                  |
| 32/M            | Aorta, paraparesis                         | Paravertebral      | 10                      | Wild-type          | <i>PIK3CA</i> p.E542K  | 100, AwD                                 |
| 32/M            | CNS, visual loss, bone                     | Bone               | 10                      | Wild-type          | <i>PIK3CA</i> p.E545K  | Not available                            |
| 29/M            | RPF, bone                                  | Bone               | 20                      | Wild-type          | <i>PIK3CA</i> p.H1047R | 50, AwD                                  |
| 62/M            | CNS, aorta, bone, sinus                    | Perirenal          | 30                      | p.V600E            | <i>PIK3CA</i> p.A1046T | 60, AwD                                  |
| 52/M            | DI, bone, RPF                              | Skin               | 60                      | p.V600E            | <i>PIK3CA</i> p.H1047L | 24, AwD                                  |
| 58/M            | Lung, CNS, X, aorta                        | Skin               | 50                      | p.V600E            | <i>PIK3CA</i> p.H1047L | 15, AwD                                  |
| 42/M            | E, bone, aorta, RPF                        | Orbital            | 60                      | p.V600E            | <i>PIK3CA</i> p.H1047L | 30, AwD                                  |
| 53/M            | CNS, bone                                  | Bone               | 10                      | Wild-type          | None detected          | 17, AwD                                  |
| 49/M            | RPF, DI, sclerosing cholangitis, sinus     | Perirenal          | 20                      | Wild-type          | None detected          | 48, AwD                                  |
| 56/M            | Lung, bone                                 | Bone               | 40                      | Wild-type          | None detected          | 44, AwD                                  |
| 81/F            | Aorta, X, skin                             | Skin               | 50                      | Wild-type          | None detected          | 52, AwD                                  |
| 70/M            | Aorta, RPF, pleura, skin, arthritis        | Skin               | 30                      | Wild-type          | None detected          | 18, AwD                                  |
| 65/M            | Lung, bone                                 | Bone               | 40                      | Wild-type          | None detected          | 72, AwD                                  |
| 55/M            | Heart, RPF, lung, bone, X                  | Skin               | 70                      | Wild-type          | None detected          | 33, AwD                                  |
| 54/M            | Mesenteric                                 | Mesentery          | 40                      | Wild-type          | None detected          | 27, AwD                                  |
| 39/M            | CNS, E, skin                               | Skin               | 90                      | Wild-type          | None detected          | 134, AwD                                 |
| 42/F            | Not available                              | Bone               | 40                      | Wild-type          | None detected          | Not available                            |
| 30/M            | Heart, aorta, paraparesis, liver           | Paravertebral      | 30                      | Wild-type          | None detected          | 20, AwD                                  |

AwD, alive with disease; CNS, central nervous system; DI, diabetes insipidus; DoD, dead of disease; E, exophthalmos; RPF, retroperitoneal fibrosis; X, xanthelasma.

\*The 42 ECD patients with *BRAF* p.V600E mutations, but without *PIK3CA* mutation, are not included in this table. For 17 other *BRAF*V600E/*NRAS*-wild-type ECD patients, *PIK3CA*, *KRAS*, and *AKT1* hotspot mutations were not investigated, because biopsies and tumor DNA samples were exhausted.

†Age at time of diagnosis.

## Immunohistochemistry

Details of immunohistochemical methods and analyses are provided in the supplemental Methods.

## Results and discussion

A *BRAF* c.1799T>A, p.V600E mutation was initially detected in 38 of 80 (47.5%) ECD patients using direct pyrosequencing of DNA obtained from lesional tissue enriched for histiocytes. Of note, there was no correlation between *BRAF*V600E allele burden from pyrosequencing and percent of histiocytes in these samples. As was mentioned earlier, recent work from our group identified a single ECD patient as having an *NRAS*Q61R mutation without any evidence of *BRAF*V600E mutation based on pyrosequencing, Sanger sequencing, or locked nucleic acid PCR followed by pyrosequencing.<sup>10</sup> Given this result, we screened with pyrosequencing all patients for mutations in *NRAS*Q61 and detected 2 of 80 *NRAS*Q61-mutated cases (Figure 1 and Table 1).

Although a *BRAF*V600E mutational frequency of 47.5% is roughly consistent with the frequency of *BRAF*V600E mutation demonstrated in ECD previously,<sup>5,6</sup> given the results of Cangi et al,<sup>8</sup> we performed further detailed analyses for *BRAF*V600E mutations in the subset of ECD patients thought to be *BRAF*-wild-type based on direct pyrosequencing analysis. Using both allele-specific real-time PCR, next-generation sequencing of lesional DNA (mean depth of 66×), and multiplex picodroplet digital PCR in a subset of 25 samples without initial *BRAF*V600E mutation detection, we were able to identify an additional 8 patients as being *BRAF*V600E-mutant, increasing the frequency of *BRAF*V600E mutations in ECD to 57.5% (46/80) (supplemental Figure 1). We

cannot exclude the possibility that the frequency of *BRAF*V600E mutations found here (46/80) differs from that of Cangi et al (18/18)<sup>8</sup> because of technical differences in modalities used for *BRAF*V600E mutation detection between the 2 studies.

Because both the PI3K/AKT/mTOR and RAF/MEK/ERK pathways are downstream effectors of RAS signaling, we also screened for mutations in commonly mutated genes in the PI3K/AKT pathway using a combination of mass spectrometric-based genotyping as well as next-generation sequencing analysis of frequently mutated regions of *NRAS*, *KRAS*, *PIK3CA*, and *AKT1*. We identified a third patient as having an *NRAS* mutation (Table 1). Immunohistochemical analysis for phosphorylated MEK1/2 (pMEK1/2) and ERK1/2 (pERK1/2) in *NRAS*-mutant ECD revealed that histiocytes were clearly positive for pMEK1/2 Ser217 and Ser217/221 as well as pERK1/2 Thr202/Tyr204 (Figure 1C). Positive staining with pERK1/2 was detected in 11 cases without *BRAF*, *NRAS*, or *PIK3CA* mutations as well as 3 *BRAF*V600E and 3 *NRAS* mutant samples (supplemental Figure 2).

Analysis of regions of recurrent mutations in *PIK3CA* in a subset of 30 *BRAF*V600E-mutant ECD revealed 7 of 58 ECD patients overall with *PIK3CA* mutations, of whom 4 were *BRAF*-mutated (Figure 1 and Table 1). This finding is consistent with data demonstrating a frequent overlap of *PIK3CA* mutations with mutations in the mitogen-activated protein kinase pathway.<sup>14</sup>

The cell-of-origin for histiocytic disorders including LCH and ECD has been long debated, and recent work from both Berres et al<sup>4</sup> and Cangi et al<sup>8</sup> demonstrated the presence of *BRAF*V600E in peripheral blood CD14<sup>+</sup> cells in a proportion of *BRAF*V600E-mutant LCH and ECD patients, respectively. Genetic analysis of CD14<sup>+</sup> cells purified by double fluorescence-activated cell sorting (FACS) of peripheral blood mononuclear cells from an *NRAS*Q61R-mutant ECD patient clearly revealed the presence of the *NRAS* mutation in CD14<sup>+</sup> cells but not in CD3<sup>+</sup> cells (Figure 1D). These

data further suggest that the histiocytic proliferations in ECD are derived from genetically aberrant circulating myeloid hematopoietic cells.

The discovery of recurrent *BRAF*V600E mutations in ECD as well as the consistent activation of ERK regardless of *BRAF* mutational status confirms the central role of RAF/MEK/ERK activation in ECD. The identification of recurrent *NRAS* and *PIK3CA* mutations in ECD further affirms activation of both the PI3K-AKT and RAF/MEK/ERK pathways in a proportion of ECD patients. Although larger series of patients are necessary to determine whether the clinical characteristics of *NRAS*- or *PIK3CA*-mutated ECD patients differ from *BRAF*-mutated or *BRAF/NRAS/PIK3CA*-wild-type ECD patients, several of the *NRAS*- and *PIK3CA*-mutant patients here had multiorgan disease requiring treatment (Table 1). We suspect that ECD patients with *RAS* mutations may benefit from targeted anti-MEK therapy, as have benefited *RAS* mutant metastatic melanoma patients.<sup>15-17</sup> Likewise, therapeutic targeting of a PI3K/AKT/mTOR pathway may be important for *BRAF*-wild-type/*PIK3CA*-mutant ECD patients. Although pharmacologic targeting of this pathway has been pursued for advanced cancer patients<sup>14,18,19</sup> and LCH,<sup>20</sup> this treatment approach has only recently been initiated in ECD with a study of sirolimus for ECD patients (ACTRN12613001321730).<sup>21</sup> Correlation of clinical response to *PIK3CA* mutational status may be critical to interpreting results of this ongoing study and in future studies targeting this pathway in ECD.

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## Authorship

Contribution: J.-F.E., E.L.D., Z.H.-R., F.C.-A., O.A.-W., and J.H. designed the study, collected the data, contributed to data interpretation, wrote the manuscript, and approved the manuscript; and F.C., D.M.H., E.K., R.R., M.P., S.A., C.G., Z.A., G.F., C.L.G., K.L., M.C., J.-E.K., S.T., P.N., J.D., and V.T. collected the data, contributed to data interpretation, and approved the manuscript.

Conflict-of-interest disclosure: J.-F.E. received honoraria from Roche and GlaxoSmithKline for counseling on patients with melanomas on the diagnosis and/or treatment with *BRAF* inhibitors. J.H. received honoraria from GlaxoSmithKline and Roche for counseling on targeted treatments of patients with histiocytosis. The remaining authors declare no competing financial interests.

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