Brief Report

MYELOID NEOPLASIA

Somatic activating ARAF mutations in Langerhans cell histiocytosis

David S. Nelson, Willemijn Quispel, Gayane Badalany-Very, Astrid G. S. van Halteren, Cor van den Bos, Judith V. M. G. Bovée, Sara Y. Tian, Paul Van Hummelen, Matthew Ducar, Laura E. MacConaill, R. Maarten Egeler, and Barrett J. Rollins

Introduction

Langerhans cell histiocytosis (LCH) is characterized by the inappropriate accumulation of cells that share phenotypic characteristics with Langerhans cells, the primary antigen-presenting cells of skin and mucosa. The disease has a broad spectrum of clinical behaviors, from a mild self-limited version to an aggressive form associated with a 20% mortality. Treatment has generally been empiric. Mild disease responds to intralesional steroids or systemic therapy with vinblastine plus steroids. Combination antineoplastic therapies have been used with some success against more aggressive forms, but they are associated with substantial toxicity and some patients are refractory.

The discovery of somatic activating BRAF mutations—in particular, but not exclusively, BRAF V600E—in 50% to 60% of patients with LCH has brought the promise of targeted therapeutics, particularly, but not exclusively, BRAF V600E patients are refractory.

Study design

Patients and samples

LCH patients whose materials were analyzed by whole exome sequencing were enrolled at Willem Alexander Children’s Hospital/Leiden University Medical Center, Leiden (patients 1 and 2) or the Emma Children’s Hospital/Academic Medical Center, Amsterdam (patient 3). Clinical characteristics are described in supplemental Materials available on the Blood Web site. Snap frozen LCH-affected tissue biopsies were obtained either at diagnosis (patients 1 and 2) or after LCH reactivation (patient 3). CD1a-positive cells were isolated by fluorescence-activated cell sorter. Buccal swabs (patients 1 and 2) or peripheral blood cells (patient 3) were collected in parallel as a source of germ-line DNA. All LCH patients, or their parents in the case of patients <12 years of age, provided written informed consent for the study.

Key Points

- Whole exome sequencing reveals novel mutations in ARAF that activate the kinase and are inhibitable by vemurafenib in a patient with LCH.
- Requiring the presence of BRAF V600E in LCH to qualify for rat fibrosarcoma inhibitor treatment may be overly exclusionary.

The extracellular signal-regulated kinase (ERK) signaling pathway is activated in Langerhans cell histiocytosis (LCH) histiocytes, but only 60% of cases carry somatic activating mutations of BRAF. To identify other genetic causes of ERK pathway activation, we performed whole exome sequencing on purified LCH cells in 3 cases. One patient with wild-type BRAF alleles in his histiocytes had compound mutations in the kinase domain of ARAF. Unlike wild-type ARAF, this mutant was a highly active mitogen-activated protein kinase kinase in vitro and was capable of transforming mouse embryo fibroblasts. Mutant ARAF activity was inhibited by vemurafenib, a BRAF inhibitor, indicating the importance of fully evaluating ERK pathway abnormalities in selecting LCH patients for targeted inhibitor therapy. (Blood. 2014;123(20):3152-3155)


R.M.E. and B.J.R. contributed equally to this work.

The online version of this article contains a data supplement.
which was approved by the Institutional Review Board of the Leiden University Medical Center. An additional 52 LCH samples for targeted analysis of ARAF and BRAF abnormalities were obtained from the Departments of Pathology at Brigham & Women’s Hospital and Boston Children’s Hospital (discarded specimens) or Leiden University Medical Center, Leiden, and Academic Medical Center, Amsterdam. The latter samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch Society of Pathology (www.federa.org). The samples were handled in a coded (pseudonymized) fashion according to the procedures as accorded by the Leiden University Medical Center ethical board. This study was conducted in accordance with the Declaration of Helsinki.

Exome sequencing and analysis

Sequencing libraries were synthesized from 50 ng of fragmented genomic DNA using an Illumina TruSeq Library kit (Illumina, San Diego, CA). Two libraries were pooled at equimolar concentrations for exome enrichment using the Agilent SureSelect Human All Exon Kit (Agilent Technologies) and plated over base agar (0.5%). Colonies were counted using a dissection microscope after 22 days of culture. Life Technologies) and plated over base agar (0.5%). Colonies were counted using a dissection microscope after 22 days of culture.

Validation assays

Additional samples were analyzed for the presence of mutations in ARAF and BRAF using either Sequenom allelotyping as described previously 5 or a custom-designed Illumina-based targeted sequencing panel described in supplemental Methods.

ARAF and BRAF vectors and mutagenesis

Human ARAF variants were created by restriction site-directed or primer extension mutagenesis and then cloned into pCMV6-Entry containing a C-terminal DDK (FLAG) epitope tag (Origene, Rockville, MD).

In vitro kinase assays

ARAF- and BRAF-containing expression vectors were transfected into HEK293T cells using FuGENE HD Transfection Reagent (Promega, Madison, WI). To maximize rat fibrosarcoma (Raf) kinase activation, cells were cotransfected with activated mutant Harvey rat sarcoma oncogene (H-Ras) (pcDNA3-H-Ras-V12; Addgene, Cambridge, MA) and activated cellular Rous sarcoma virus oncogene (c-Src) Y527F (pcSrc527; Addgene).13 Pooled sample reads were de-convoluted (de-multiplexed) using PICARD tools (http://picard.sourceforge.net/command-line-overview.shtml), aligned to the Human Genome Reference sequence (h37 edition) using bwa (http://bio-bwa.sourceforge.net/bwa.shtml), filtered for duplicate reads, and analyzed for quality using Genome Analysis Toolkit (GATK) (http://www.broadinstitute.org/gsa/wiki/index.php/Base_quality_score recalibration) and PICARD tools. Variant analysis was performed using cancer-specific variant discovery tools (GATK),11 including MuTect12 for somatic base substitutions and indelocator for short insertions and deletions (http://www.broadinstitute.org/cancer/cga/indelocator). Lane performance criteria are described in supplementas Materials.

ARAF and BRAF vectors and mutagenesis

Human ARAF variants were created by restriction site-directed or primer extension mutagenesis and then cloned into pCMV6-Entry containing a C-terminal DDK (FLAG) epitope tag (Origene, Rockville, MD).

Results and discussion

To identify possible genetic drivers of LCH, we sequenced exomes from purified CD1a-positive lesional cells and normal leukocytes or buccal cells of 3 patients. All 6 samples met quality standards for paired-end library synthesis, and 80% of all targets were covered to a depth of at least 30×; mean target coverage was 196× (range, 178-227×). Massively parallel sequencing revealed 18 non-synonymous base changes in the 3 LCH samples that were not present in matched germ-line DNA (supplemental Table 1). BRAF V600E was identified in the lesional DNA of patient 1 with an allelic fraction of 45%. BRAF exons were wild type in the lesional DNA of patients 1 and 3. No other obviously relevant variants were observed in the DNA from patient 3. However, the LCH cells from patient 1 contained a somatic single nucleotide variant in ARAF with an allele frequency of 63%: g.47426808C>G [NM_001654.2(ARAF_v001):c1053C>G],
which is predicted to result in a substitution of leucine for phenylalanine at amino acid position 351 (F351L). This sample also showed a 6 nucleotide deletion at position 47 426 794 [NM_001654.4(ARAF_v001):c.1044_1049del], which is predicted to result in an in-frame loss of amino acids 347 and 348 (Q347_A348del). Neither alteration appears in any human sequence database. Informatics analysis indicated that the single nucleotide variant and the deletion were only observed on the same chromosome, indicating that both abnormalities occurred in the same allele. This inference is consistent with the fact that ARAF maps to the X chromosome and that patient 1 was male. Both abnormalities were validated using an orthogonal allelotyping technology, namely Sequenom.14 The ARAF abnormalities were not detected by Sequenom genotyping or Illumina sequencing in an additional 44 LCH samples, of which 23 were wild type for BRAF (supplemental Methods).

Because nearly all LCH samples show activation of the ERK signaling pathway regardless of BRAF mutational status, the results from patient 1 suggest that mutant ARAF might be involved in ERK pathway activation. However, even though ARAF mutations have been described in cancer,15,16 none have been reported to enhance its kinase activity. In fact, other ARAF mutations found in cancer inactivate the kinase.17,18 and structural studies suggest that, in contrast to BRAF, mutations in ARAF cannot easily activate its kinase.19,20 Therefore, to determine the likelihood that the ARAF variant found in patient 1 could be a pathogenetic driver of LCH, we tested its MEK kinase activity. ARAF-encoding cDNAs were prepared with the F351L substitution and the deletion of amino acids 347 and 348 both separately and together. After transfection into HEK293T cells, immune precipitates containing ARAF were tested for their ability to phosphorlyate a synthetic MEK substrate in vitro. The double ARAF mutant (containing both F351L and Q347_A348del) had substantial MEK kinase activity, whereas wild-type ARAF’s activity was nearly undetectable (Figure 1A). ARAF containing only the deletion mutant had approximately half the kinase activity of the double mutant and the F351L mutant had low but detectable activity.

RAFT family members form homo- and heterodimers with each other and can influence the kinase activities of their bound partners.18,21,22 Although we did not detect BRAF in ARAF immune precipitates, we nonetheless confirmed that the double mutant’s enhanced MEK kinase activity was an inherent property of the mutant ARAF protein by engineering a “kinase-dead” version of the double mutant (K336M).18 This protein had no detectable MEK kinase activity, suggesting that, in contrast to BRAF, mutations in ARAF cannot easily activate its kinase. Therefore, to determine the likelihood that the ARAF variant found in patient 1 could be a pathogenetic driver of LCH, we tested its MEK kinase activity. ARAF-encoding cDNAs were prepared with the F351L substitution and the deletion of amino acids 347 and 348 both separately and together. After transfection into HEK293T cells, immune precipitates containing ARAF were tested for their ability to phosphorlyate a synthetic MEK substrate in vitro. The double ARAF mutant (containing both F351L and Q347_A348del) had substantial MEK kinase activity, whereas wild-type ARAF’s activity was nearly undetectable (Figure 1A). ARAF containing only the deletion mutant had approximately half the kinase activity of the double mutant and the F351L mutant had low but detectable activity.

RAFT family members form homo- and heterodimers with each other and can influence the kinase activities of their bound partners.18,21,22 Although we did not detect BRAF in ARAF immune precipitates, we nonetheless confirmed that the double mutant’s enhanced MEK kinase activity was an inherent property of the mutant ARAF protein by engineering a “kinase-dead” version of the double mutant (K336M).18 This protein had no detectable MEK kinase activity, suggesting that, in contrast to BRAF, mutations in ARAF cannot easily activate its kinase. Therefore, to determine the likelihood that the ARAF variant found in patient 1 could be a pathogenetic driver of LCH, we tested its MEK kinase activity. ARAF-encoding cDNAs were prepared with the F351L substitution and the deletion of amino acids 347 and 348 both separately and together. After transfection into HEK293T cells, immune precipitates containing ARAF were tested for their ability to phosphorlyate a synthetic MEK substrate in vitro. The double ARAF mutant (containing both F351L and Q347_A348del) had substantial MEK kinase activity, whereas wild-type ARAF’s activity was nearly undetectable (Figure 1A). ARAF containing only the deletion mutant had approximately half the kinase activity of the double mutant and the F351L mutant had low but detectable activity.

Acknowledgments

The authors appreciate the insights and ideas of Dr Mark D. Fleming and technical support provided by Inge Briare and Sarah Ouahoud.

The authors acknowledge support from the Histiocytosis Association, Team Ippolittle/Deloitte of the Boston Marathon Jimmy Fund Walk, Stichting 1000 Kaarsjes voor Juultje, the Say Yes to Education foundation, and Mr George Weiss.

Authorship

Contribution: D.S.N. constructed ARAF mutant cDNAs, designed and performed in vitro kinase assays, and designed and performed soft agar transformation assay; W.Q. purified patient cellular material and extracted genomic DNA; G.B.-V. assisted in optimization of DNA extraction from patient material in the original sequencing set and extracted DNA from patient material in the validation set; A.G.S.v.H. identified patients and oversaw collection and purification of patient material; C.v.d.B. identified patients and provided patient material; J.V.M.G.B. provided archived tissues and marked CD1a-rich areas in these biopsies; S.Y.T. provided technical assistance for the in vitro kinase assays; P.V.H. performed whole exome sequencing and prepared the sequencing report; M.D. performed informatics analysis on sequencing results; L.E.M. oversaw and contributed to sequencing performance and analysis; R.M.E. oversaw and developed capabilities for LCH patient identification and tissue acquisition and storage; and B.J.R. conceived and designed the project.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Barrett J. Rollins, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215; e-mail: barrett_rollins@dfci.harvard.edu.


Somatic activating ARAF mutations in Langerhans cell histiocytosis

David S. Nelson, Willemijn Quispel, Gayane Badalian-Very, Astrid G. S. van Halteren, Cor van den Bos, Judith V. M. G. Bovée, Sara Y. Tian, Paul Van Hummelen, Matthew Ducar, Laura E. MacConaill, R. Maarten Egeler and Barrett J. Rollins

Articles on similar topics can be found in the following Blood collections
- Brief Reports (1932 articles)
- Myeloid Neoplasia (1666 articles)
- Pediatric Hematology (508 articles)
- Phagocytes, Granulocytes, and Myelopoiesis (608 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml