RESEARCH BRIEF

Diverse and Targetable Kinase Alterations Drive Histiocytic Neoplasms

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ABSTRACT

Histiocytic neoplasms are clonal, hematopoietic disorders characterized by an accumulation of abnormal, monocyte-derived dendritic cells or macrophages in Langerhans cell histiocytosis (LCH) and non-Langerhans cell histiocytosis (non-LCH), respectively. The discovery of BRAFV600E mutations in approximately 50% of these patients provided the first molecular therapeutic target in histiocytosis. However, recurrent driving mutations in the majority of patients with BRAFV600E–wild-type non-LCH are unknown, and recurrent cooperating mutations in non-MAP kinase pathways are undefined for the histiocytic neoplasms. Through combined whole-exome and transcriptome sequencing, we identified recurrent kinase fusions involving BRAF, ALK, and NTRK1, as well as recurrent, activating MAP2K1 and ARAF mutations in patients with BRAFV600E–wild-type non-LCH. In addition to MAP kinase pathway lesions, recurrently altered genes involving diverse cellular pathways were identified. Treatment of patients with MAP2K1- and ARAF-mutated non-LCH using MEK and RAF inhibitors, respectively, resulted in clinical efficacy, demonstrating the importance of detecting and targeting diverse kinase alterations in these disorders.

SIGNIFICANCE: We provide the first description of kinase fusions in systemic histiocytic neoplasms and activating ARAF and MAP2K1 mutations in non-Langerhans histiocytic neoplasms. Refractory patients with MAP2K1- and ARAF-mutant histiocytoses had clinical responses to MEK inhibition and sorafenib, respectively, highlighting the importance of comprehensive genomic analysis of these disorders.

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INTRODUCTION
Systemic histiocytic neoplasms consist of Langerhans cell histiocytosis (LCH) and the non-Langerhans forms of histiocytosis (non-LCH), including Erdheim–Chester disease (ECD), juvenile xanthogranuloma (JXG), and Rosai–Dorfman disease (RDD; ref. 1). These are diverse disorders marked by infiltration of tissues with neoplastic histiocytes whose cellular origins have long been debated. The discovery of the \textit{BRAF}^{V600E} mutation in approximately 50% of patients with LCH (2, 3) and ECD (4) led to biologic and therapeutic advances in these disorders. For example, characterization of the cells in the hematopoietic system of patients with LCH at which the \textit{BRAF}^{V600E} mutation arises resulted in the identification of LCH as derived from myelomonocytic precursors (3). Moreover, multiple reports have demonstrated that treatment of adult (5–7) and pediatric (8) patients with \textit{BRAF}^{V600E}, mutant histiocytosis with vemurafenib (a RAF inhibitor that specifically targets \textit{BRAF}^{V600E}) confers clinical responses in >90% of patients marked by a prolonged durability compared with that seen with BRAF inhibition in common \textit{BRAF}^{V600E}, mutant malignancies. The robust efficacy of RAF inhibitor therapy, especially in severe forms of non-LCH, stands in contrast to the relative ineffectiveness of existing treatments for adults with these disorders (9).

More recently, \textit{MAP2K1} mutations have been identified in approximately 25% of patients with \textit{BRAF}^{V600E}-wild-type LCH, reinforcing the notion that LCH is a disease driven by MAP kinase pathway activation (10–12). However, the direct clinical importance of activating \textit{MAP2K1} mutations in LCH is unknown. Moreover, in contrast to LCH, activating kinase mutations in the majority of patients with \textit{BRAF}^{V600E}_\textit{wild-type} non-LCH are undefined. In addition, recurrent alterations that coexist with activating kinase mutations have not been defined for any of these conditions. Finally, despite the clinical and histopathologic differences between LCH and non-LCH, the molecular bases for these differences are not known.

To comprehensively define the genomic alterations in histiocytic neoplasms, we performed unbiased whole-exome and transcriptome sequencing across pediatric and adult LCH and non-LCH patients. In addition, on the basis of novel kinase alterations identified in this study, 3 patients with severe and refractory \textit{BRAF}^{V600E}_\textit{wild-type} non-LCH were successfully treated with targeted therapies, recapitulating the efficacy of BRAF inhibition in \textit{BRAF}^{V600E}-mutated histiocytoses.

RESULTS
Whole-Exome and Transcriptome Sequencing across Histiocytic Neoplasms
To address the above, we performed whole-exome sequencing (WES) of frozen tumor biopsies from 24 patients with LCH (\(n = 10\)) or ECD (\(n = 14\)) paired with peripheral blood mononuclear cells. Thirteen of 24 patients also underwent RNA sequencing (RNA-seq; Supplementary Table S1). Both adult (\(n = 15\); \(n = 2\) with LCH) and pediatric cases (\(n = 9\); \(n = 8\) with LCH) were included. All mutations in activating kinases identified by WES or RNA-seq were validated by droplet-digital PCR (ddPCR), and all others were validated by targeted-capture next-generation sequencing (Supplementary Tables S2 and S3).

Overall, a mean of 7 nonsynonymous mutations per adult patient was identified (range, 1–22) compared with 5 mutations per pediatric patient (range, 4–9; \(P = ns\); Supplementary Fig. S1). The median variant allele frequency (VAF) for the 23 individual known activating kinase mutations identified by WES was 11%, whereas the median VAF for all other somatic mutations was 10% (Supplementary Table S3). Combined WES and RNA-seq revealed mutations or fusions activating MAP kinase signaling in 100% of patients (Fig. 1A; Supplementary Fig. S2A and S2B). In addition, mutations affecting diverse biologic processes coexisted with activating kinase mutations, including recurrent mutations affecting the p38/MAPK and epigenetic regulatory pathways (Supplementary Table S3); however, inspection of the co-occurring genes demonstrated no clear differences in the frequencies of mutated specific genes or pathways of genes affected by mutations between the 24 LCH and non-LCH cases. An analysis of WES data from peripheral blood DNA used as the germline reference for pathologic mutations in a total of 565 cancer-associated genes (including 60 autosomal-dominant cancer predisposition genes, MAP kinase pathway members, as well as all genes reported as mutated in the recent studies of clonal hematopoiesis; refs. 13–15) failed to reveal mutations in any of these genes in blood DNA.

Recurrent \textit{MAP2K1} and \textit{ARAF} Mutations in Non-LCH Neoplasms
WES and RNA-seq revealed \textit{MAP2K1} mutations in both LCH and non-LCH patients and \textit{ARAF} mutations in non-LCH patients (Fig. 1; Supplementary Fig. S3A and S3B). Because neither \textit{MAP2K1} nor \textit{ARAF} mutations have previously been described in ECD, we next interrogated a validation cohort of 37 \textit{BRAF}^{V600E}-wild-type, non-LCH, formalin-fixed, paraffin-embedded (FFPE) tissue cases (Fig. 1B). Exons 2 and 3 of \textit{MAP2K1} and all coding regions of \textit{ARAF} were sequenced, along with regions of recurrent mutations in \textit{NRAS}, \textit{KRAS}, and \textit{PIK3CA}, because rare mutations in these genes have previously been noted in patients with \textit{BRAF}^{V600E}-wild-type non-LCH (16). This revealed recurrent activating mutations in \textit{MAP2K1} (32%; \(n = 12\)), \textit{NRAS} (16%; \(n = 6\)), \textit{KRAS} (11%; \(n = 4\)), \textit{PIK3CA} (8%; \(n = 3\)), and \textit{ARAF} (3%; \(n = 1\); Fig. 1B–D). Expression of 10 of these \textit{MAP2K1} mutants identified activation of MAP kinase signaling over wild-type \textit{MAP2K1} (Fig. 1E). Two non-LCH cases demonstrated concurrent activating \textit{NRAS} and \textit{ARAF} mutations. Eleven cases (30%; \(n = 11\)) did not demonstrate known activating mutations in these kinase genes based on targeted gDNA sequencing (Fig. 1A–D; Supplementary Figs. S2, S3C, and S3D).

Recurrent Kinase Fusions in Non-Langerhans Histiocytic Neoplasms
RNA-seq identified kinase fusions involving \textit{BRAF}, as well as \textit{ALK}, exclusively in \textit{BRAF}^{V600E}-wild-type non-LCH (Fig. 1A; Supplementary Fig. S2). An \textit{RNF11–BRAF} fusion was detected in an infiltrative, non-LCH brain lesion of a 14-year-old child (Fig. 2A; Supplementary Fig. S4A). Because kinase fusions have not been described in histiocytic neoplasms, we first confirmed...
expression by reverse transcription polymerase chain reaction (RT-PCR) with breakpoint-flanking primers, as well as with interphase FISH (Fig. 2A and B). Sequencing of the RNF11–BRAF fusion revealed that this transcript developed from an in-frame fusion of exon 1 of RNF11 to exons 11 to 18 of BRAF. This results in loss of the N-terminal regulatory, RAS-binding domain in BRAF and placement of the BRAF kinase domain under the aberrant regulation of the RNF11 promoter. Although RNF11 is a novel BRAF fusion partner, the RNF11–BRAF fusion has a similar configuration to previously described BRAF fusions (17). Stable expression of RNF11–BRAF in cytokine-dependent, murine pro-B cell Ba/F3 cells, along with an empty vector, wild-type BRAF, or BRAFV600E, revealed activation of ERK and MEK phosphorylation by both

Figure 1. Mutational profiles of systemic histiocytic neoplasm patients and recurrent MAP2K1 and ARAF mutations in non-Langerhans histiocytic neoplasms. A, results of whole exome sequencing of LCH and non-LCH neoplasms. Each patient is represented in one column. Diagnosis (LCH or ECD), age category, and sequencing method are in the first 3 rows. Somatic mutations identified are in the lower rows and subdivided based on mutations known to activate kinases, affect the JNK/p38 MAP kinase pathway, or involve a diverse array of co-occurring pathways (shown on the right). Only mutations identified in >1 sample and selected other mutations are shown. B, mutational analysis of NRAS, KRAS, MAP2K1, ARAF, and PIK3CA from archival, formalin-fixed, paraffin-embedded tissue from non-LCH patients with a spectrum of non-LCH neoplasms. Diagnosis and percent histocyte content per section are shown in the first 2 rows. C, diagram of MAP2K1 mutations identified by WES, RNA-seq, and targeted sequencing approaches in this study. D, diagram of activating ARAF mutations identified by WES, RNA-seq, and targeted sequencing approaches in the study. E, Western blot analysis of pERK1/2, pMEK1/2, and controls in 293T cells transfected with vector, wild-type (WT) FLAG-MEK1, or various FLAG-MEK1–mutant CDKN3a along with HA-tagged ERK2.

Figure 2. Kinase fusions in non-LCH neoplasms. A, illustration of the RNF11–BRAF fusion with Sanger sequencing confirmation. B, BRAF FISH break-apart probes revealing an isolated green signal confirming translocation of BRAF. C, effect of stable expression of BRAF wild-type (WT), BRAFV600E, RNF11–BRAF, or an empty vector on MAP kinase and AKT signaling, and D, cytokine-independent growth of Ba/F3 cells. Mean viable cell number after IL3 withdrawal from a triplicate experiment is shown. Error bars, SD of mean. E, CellTiter-Glo luminescent viability IC_{50} results from three independent experiments of Ba/F3 cells from D exposed to MEK inhibitor GDC-0973, vemurafenib, or sorafenib. Log_{10} IC_{50} values are shown on the y-axis. Error bars, SEM. F, illustration of the CLIP2–BRAF fusion with Sanger sequencing confirmation identified in histiocytic ovarian infiltrates in a patient with non-LCH. G, illustration of the KIF5B–ALK fusion with Sanger sequencing confirmation. H, ALK FISH break-apart probe reveals an isolated red signal confirming the translocation of ALK. I, effect of KIF5B–ALK expression on ALK, STAT3, MEK1/2, ERK1/2, and AKT signaling in serum-starved Ba/F3 cells. J, effect of expression of KIF5B–ALK on cytokine-independent growth in Ba/F3 cells. Mean viable cell number after IL3 withdrawal from triplicate experiment is shown. Error bars, SD of the mean. K, CellTiter-Glo luminescent viability IC_{50} results from three independent experiments of Ba/F3 cells from J exposed to crizotinib or alectinib. Log_{10} IC_{50} values are shown on the y-axis. Error bars, SEM. L, illustration of a second KIF5B–ALK fusion identified in the liver lesions of a 50-year-old patient with ECD involving exons 1 to 24 of KIF5B and 20 to 29 of ALK. M, IHC of NTRK1 (top left) and CD68 (top right) in skin lesions of the LMNA–NTRK1 fusion index patient (400x magnification; scale bar, 50 μm) and illustration of the LMNA–NTRK1 fusion (bottom).
Kinase Fusions and Mutations in Histiocytoses

A. RNF11 BRAF

B. CR3 (kinase domain)

C. Empty vector BRAF WT BRAF V600E RNF11–BRAF BRAF V600E

D. Visible cells (×10^6/mL)

E. IC50 (μmol/L)

F. CLIP2 BRAF

G. KIF5B ALK

H. Viable cells (×10^6/mL)

I. Empty vector KIF5B–ALK

J. Days

K. IC50 (μmol/L)

L. Kinase domain

M. NTRK1 IHC CD68 IHC

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RNF11–BRAF and BRAFV600E proteins (Fig. 2C). Likewise, expression of RNF11–BRAF or BRAFV600E resulted in cytokine-independent growth of Ba/F3 cells (Fig. 2D), indicating that the RNF11–BRAF fusion is an activating event with pathway activity similar to the BRAFV600E mutation. Moreover, RNF11–BRAF expression sensitized Ba/F3 cells to MEK inhibition but not to RAF inhibition by vemurafenib, similar to previous reports analyzing other BRAF fusion genes (18, 19; Fig. 2E).

Targeted RNA-sequencing (RNA-seq) (20) of 9 patients with BRAFV600E wild-type, non-LCH using a panel of 265 genes known to be translocated in cancer detected an additional BRAF fusion. This was a CLIP2–BRAF fusion identified in retroperitoneal lesions from a patient with non-LCH, resulting in juxtaposition of the kinase domain of BRAF (exons 11–18) to the N-terminal domain of the protease CLIP2 (Fig. 2F). Expression was confirmed by RT-PCR/Sanger sequencing. As with the RNF11–BRAF fusion, this is a previously unreported fusion of BRAF.

In addition to BRAF fusions, an in-frame fusion of KIF5B (exons 1–24) to the kinase domain of ALK (exons 19–29) was identified in skin lesions from a 25-year-old with diffuse cutaneous LCH and progressive organ dysfunction. This fusion was confirmed by RT-PCR/Sanger sequencing. As with the RNF11–BRAF fusion, this is a previously unreported fusion of ALK.

Three of the investigated non-LCH patients with refractory disease and progressive organ dysfunction were treated with targeted therapies based on the discovery of novel kinase alterations described above. A MAP2K1R257T mutation was noted from WES of perirenal lesions from a 53-year-old patient with ECD with progressive disease following treatment with both IFNα and anakinra, who was symptomatic by way of inflammatory ascites and renal failure. Treatment was initiated with a MEK inhibitor (single-agent trametinib 2 mg daily), resulting in abrupt cessation of ascitic accumulation and normalization of creatinine. Additionally, there was metabolic resolution of FDG-avid infiltrates in the retroperitoneum (SUV 8.2) and spermatic cords (SUV 6.2) to background SUV (Fig. 4A and B). The patient has been maintained on trametinib single-agent therapy with a sustained clinical response for >180 days. Similarly, treatment of a second patient with ECD with MAP2K1S214A-mutant disease refractory to 4 lines of prior therapy led to resolution of PET-avid disease in renal (SUV 8.4), aortic (SUV 3.9), and maxillary sinus (SUV 9.5) infiltrations to background SUV within a single month of administration of the MEK inhibitor cobimetinib (Fig. 4C).

Further evidence of effective targeted inhibition was found in a patient with ECD with disease in the bones, orbits, cavernous sinuses, and choroid, whose tumor was found to harbor an ARAFR621Q mutation. This patient’s disease had progressed following cladribine, clofarabine, and anakinra. The patient was also symptomatic with visual impairment from macular edema that required ongoing corticosteroids (Fig. 4D; Supplementary Fig. S3A and S3B). Given a recent report of complete response to sorafenib in a patient with NSCLC with an ARAFR621Q mutation (28), we initiated
Figure 3. Gene expression analysis of histiocytic neoplasms by RNA-seq. A, unsupervised hierarchical clustering of the top 1% most differentially expressed genes in 7 LCH and 6 non-LCH lesions presented in a heat map. B, gene expression by RNA-seq of 6 of the 159 genes from A, which encode proteins currently known to differentiate these diseases in clinical diagnosis. C, enrichment plots of gene sets differentially enriched in LCH (n = 4) or non-LCH (n = 3) as detected by gene set enrichment analysis (analysis restricted to those samples with BRAF alterations only). D, eleven lineage-defining genes with enriched expression in LCH (4 cases) or non-LCH samples (3 cases) with BRAF kinase alterations.
Figure 4. Therapeutic efficacy of MEK and RAF inhibition in patients with MAP2K1- and ARAF-mutant systemic histiocytic neoplasms. A, axial FDG-PET scans before trametinib and 4 weeks after trametinib in a patient with MAP2K1K57N ECD with histiocytic infiltration of kidneys (top) and spermatic cord (bottom). B, creatinine and platelet counts in same patient before and after trametinib therapy (green line, boundary of normal values). C, PET scan before cobimetinib and 4 weeks after cobimetinib of a patient with MAP2K1Q56P-mutant ECD with disease infiltration in facial sinuses, heart, and kidneys. D, axial brain MRI of a patient with ARAF S214A-mutant ECD with histiocytic infiltration of retina and optic nerves. MRI images show optic nerve infiltration (arrows) before and 6 weeks after sorafenib (top). Retinal fundoscopic photographs from the same time points (bottom) reveal improvement in retinal infiltrates with sorafenib treatment. E, ratio of concentration of ARAF S1214A: ARAF wild-type in plasma cell–free DNA with sorafenib treatment.
soralenb therapy, titrated up to 600 mg daily. Within 12 weeks, there was regression of lesions in the cavernous sinuses (by post-gadolinium MRI) and retina (as visualized directly by fundoscopy), and the patient was able to taper her steroid dose. This coincided with a >50% decrease in mutant ARAF DNA in plasma cell-free DNA (Fig. 4E).

**DISCUSSION**

Systemic histiocytic neoplasms constitute a broad spectrum of disorders that are characterized by the accumulation of abnormal, mononuclear, phagocyte-derived cells within infiltrative granulomatous lesions in nearly any organ (1). The rarity of histiocytic disorders combined with their protean clinical manifestations has resulted in great uncertainty about their pathogenesis for decades. The discovery of abnormal, mononuclear, phagocyte-derived cells as myeloid-derived disorders driven by activating mutations affecting the MAP kinase pathway (3). In fact, nearly all cases of LCH and ECD have been demonstrated to have the prominent presence of activated ERK within lesional tissue (2). This has led to the hypothesis that histiocytic neoplasms contain mutations affecting the MAP kinase pathway in 100% of patients. Despite this, the cellular heterogeneity of histiocytic lesions combined with their frequent occurrence in sites unamenable to biopsy (such as brain and heart) has presented challenges to identifying the full constellation of genomic alterations in an unbiased manner. As a result, technologies such as RNA-seq, for example, have never been performed in histiocytic neoplasms, and mutations that coexist with MAP kinase alterations are undefined in these disorders.

Through the efforts of an international collaboration to unravel the molecular bases of these disorders in an unbiased manner, we combined WES and RNA-seq of fresh-frozen biopsies from patients with histiocytic disorder and identified a spectrum of activating kinase alterations in 100% of cases. This includes the first description of kinase fusions in systemic histiocytic neoplasms and ARAF and MAP2K1 mutations in non-LCH. The identification of fusions involving BRAF, ALK, and NTRK1 in patients with non-LCH further enriches the number of genomic alterations shared between histiocytic neoplasms and common malignancies such as NSCLC. Moreover, kinase fusions may provide further novel therapeutic targets for patients with histiocytosis. For example, patients with histiocytosis bearing ALK fusions may be amenable to ALK inhibitors, as these have demonstrated efficacy in ALK-rearranged NSCLC (29, 30). In addition, those patients expressing NTRK fusions might be eligible for ongoing clinical trials utilizing novel TRK inhibitors (31).

Although mutations in genes involved in diverse biologic processes and pathways co-occurring with activating kinase alterations were identified here, future efforts with larger sequencing cohorts will be needed to determine if there are differences in mutational patterns between LCH and non-LCH. In addition, due to the heterogeneity of cells in systemic histiocytoses lesions, the overall VAFs of somatic mutations identified here were low, precluding our ability to determine the clonal composition of these tumors. Furthermore, although coexisting activating kinase alterations involving BRAFV600E and ARAF or compound ARAF mutations have been described in LCH (10, 32) and now in non-LCH based on our study, very little is currently known about the mechanistic consequences of ARAF mutations on MAP kinase signaling. Thus, future efforts will be needed to understand the basis for this scenario of ARAF mutations coexisting with additional MAP kinase pathway mutations.

Overall, mutations in MAP2K1 and ARAF were the most common kinase alterations among BRAFV600E–wild-type patients. The clinical responses of MAP2K1- and ARAF-mutant ECD to therapies targeted against these alterations may overhaul the landscape of treatment for severe forms of BRAFV600E–wild-type non-LCH, mandating prospective clinical trials of these agents, as have been performed for BRAFV600E-mutant histiocytoses (33). The rapid and sustained clinical responses to MEK inhibition in this study are particularly significant in light of the fact that single-agent MEK inhibition has not demonstrated robust clinical benefit in the context of other BRAF/RAS-mutant malignancies previously (34–36). It is possible that the relatively small number of mutations per exome in histiocytic neoplasms seen here compared with those in common malignancies, such as melanoma (37) and NSCLC (38, 39), may account for the remarkable and sustained clinical responses of histiocytoses to single-agent RAF or MEK inhibition.

In addition to the identification of kinase fusions, RNA-seq analyses also identified important transcriptional profiles in LCH and non-LCH, which appear to be distinct from one another. These data suggest the intriguing possibility that LCH has a gene expression profile most similar to cDCs and late-stage myeloid progenitor cells and reinforce prior reports that suggest LCH is derived from immature myeloid dendritic cells (3). In contrast, non-LCH lesions appear to share transcriptional profiles more similar to monocytes and earlier hematopoietic stem and progenitor cells. These data represent the first attempt to identify the cell-of-origin of non-LCH using transcriptomic data, which will be important to validate in future efforts, and further refine our knowledge of the cell-of-origin of LCH.

Overall, these findings demonstrate the need for comprehensive genomic analysis of these rare and diverse tumor types, as they may directly affect clinical therapy for patients with histiocytosis. Further efforts to integrate genomic analysis into the clinical care of patients with histiocytic disorder may greatly help in both disease classification and therapeutic decision making for these patients.

**METHODS**

**Patients**

The study was conducted according to the Declaration of Helsinki, and human tissues were obtained with patient-informed consent under approval by the Institutional Review Boards of Memorial Sloan Kettering Cancer Center, St. Jude Children’s Research Hospital, The University of Texas MD Anderson Cancer Center, the National Human Genome Research Institute, and Pitie-Salpetriere Hospital. Excised lesions were either flash-frozen for DNA/RNA extraction and/or fixed in 4% neutral-buffered formalin, embedded in paraffin, and processed by routine histologic methods. For patients undergoing WES, DNA extracted from peripheral blood mononuclear cells...
Targeted DNA Sequencing

The Supplementary Methods. Previously (44), and the methodology and approach are described in Supplementary Tables S1 and S3.

Whole-Exome Sequencing

Analysis of exome sequencing data, which includes mapping, coverage and quality assessment, single-nucleotide variant (SNV)/

indel detection, tier annotation for sequence mutations, and prediction of deleterious effects of missense mutations, has been described previously (40, 41). Approximately 250 ng of DNA from each sample was sheared to an average of 150 bp in a Covaris instrument for 360 seconds (duty cycle, 10%; intensity, 5; cycles/burst, 200). Bar-coded libraries were prepared using the Kapa Low-Throughput Library Preparation Kit Standard (Kapa Biosystems), amplified using the KAPA HiFi Library Amplification Kit (Kapa Biosystems; 8 cycles), and quantified using Qubit Fluorometric Quantitation (Invitrogen) and Agilent Bioanalyzer. An equimolar pool of the 4 bar-coded libraries (300 ng each) was used as input to capture the exome using one reaction tube of the Nimblegen SeqCap EZ Human Exome Library v3.0 (Roche; cat no. 06465684001), according to the manufacturer’s protocol. The pooled capture library was quantified by Qubit (Invitrogen) and Bioanalyzer (Agilent) and sequenced on an Illumina HiSeq 2500 using a paired end, 100 nucleotide in length run mode, to achieve an average of 100× coverage.

Confirmation of Mutations

The following mutations in genes coding tyrosine kinases were confirmed using ddPCR: ARAF S214A, S186R, A225V, and P539H; BRAF V600E and R603Q; MAP2K1 K57N, F53, Q58del, Q58_E62del, and F68L; NRAS Q61R, and PIK3CA E542K, using BioRad probes with FAM for the mutants and HEX for the wild-type sequences, on a BioRad iQ500 real-time PCR system, using the manufacturer’s instructions, starting from 30 ng of template DNA. For the rest of the mutations, we used a custom-designed, TruSeq Custom Amplicon probe to confirm the mutations detected by exome sequencing. Design Studio (illumina) was used to design amplicons covering the regions of interest. The regions were amplified using 250 ng of template genomic DNA, using the manufacturer’s instructions, with 25 cycles of amplification, and were run on an Illumina MiSeq 2 × 250 cartridge.

RNA Sequencing

RNA library construction for transcriptome sequencing was done as per the manufacturer’s instructions using the Illumina TruSeq RNA sample preparation V2. Sequencing was completed on the Illumina HiSeq 2000 as per the manufacturer’s instructions. Analysis of transcriptome sequencing data, which includes mapping, coverage, and quality assessment, SNV/indel detection, tier annotation for sequence mutations, and prediction of deleterious effects of missense mutations, has been described previously (40, 41). For gene expression analyses, transcript expression levels were estimated as fragments per kilobase of transcript per million mapped reads (FPKM); gene FPKMs were computed by summing the transcript FPKMs for gene FPKMs were computed by summing the transcript FPKMs for each gene using Cufflinks2 (42, 43). A gene was considered “expressed” if the FPKM value was ≥0.5 based on the distribution of FPKM gene expression levels. Genes that were not expressed in any sample group were excluded from the final data matrix for downstream analysis. Gene set enrichment analysis (GSEA) was performed as described previously (44), and the methodology and approach are described in the Supplementary Methods.

Targeted DNA Sequencing

We sequenced the regions of known mutations in MAP2K1, NRAS, KRAS, and PIK3CA, as well as all coding exons of ARAF, using Sanger sequencing (primer sequences listed in Supplementary Table S5) and/or hybrid-capture, next-generation sequencing using the MSKCC IMPACT assay as previously described (45) or the Foundation One Assay (Foundation Medicine, Inc.), as previously described (46). Prior to DNA extraction, FFPE samples from all cases were reviewed to confirm that the tissue was of sufficient size to generate a minimum of 50 ng of 20% histiocyte nucleic acid. DNA was isolated from 40-μm-thick sections of FFPE tissue.

Targeted RNA Sequencing

Total RNA extracted from 40-μm-thick sections of FFPE tumor was reverse transcribed with random hexamer primers using the Super-Script III First-Strand Synthesis System (Invitrogen). Double-

stranded cDNA was synthesized with the NEBNext mRNA Second Strand Synthesis Module (New England BioLabs). Hybrid selection of indexed, adaptor-ligated libraries was performed using the cDNA Kinome hybridization kit with 612 transcripts of kinases and kinase-related genes (Agilent SureSelect Human Kinome Kit). Selected libraries were sequenced on the HiSeq-2000 instrument (Illumina) with 49 × 49 paired reads. For RNA sequencing, we used a sequencing approach targeting 612 transcripts of kinases and kinase-related genes. We aimed for a high number of unique read pairs (~50,000,000) per sample (Supplementary Table S2).

Gene Fusion Confirmation

All gene fusions were validated with RT-PCR followed by direct sequencing, interphase FISH, and/or immunohistochemical analysis. RT-PCR was performed from cDNA (primer sequences available upon request), followed by analysis on a Bioanalyzer (Agilent). Specific PCR amplicons were detected only with the appropriate combination of primer and template and not with negative controls. The nucleotide sequence at the fusion site was confirmed with Sanger sequencing. Details of interphase FISH are included in the Supplementary Methods.

Plasma Cell-Free DNA (cfDNA) Collection and Analysis by ddPCR

Blood (10 mL) was collected into Streck tubes, and plasma was then separated from blood using standard techniques. Plasma cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s instructions, and the concentration was assessed using a BioAnalyzer.

The S214A (c.640T>G) mutation for ARAF was evaluated by ddPCR (BioRad QX200) in a custom-designed, allele-specific assay (primer sequences available upon request). The experiments were performed using the following protocol: 1 cycle at 93°C for 10 minutes, 40 cycles at 94°C for 30 seconds and 55°C for 1 minute, 1 cycle at 98°C for 10 minutes, then held at 4°C, all at a ramp rate of 2°C/second. BioRad’s T100 thermal cycler was used for the PCR step. When available, 5 ng of DNA was assessed in a 20 μL PCR reaction, partitioned into approximately 20,000 droplets. A total of two replicates were used per sample. Droplets were quantified using the BioRad QuantaSoft Software (version 7.0) to identify the concentration of ARAF(214S)-mutant copies/mL DNA and of ARAF wild-type copies/mL of DNA. The ratio of the concentration of mutant to wild-type ARAF([Mu]/[WT]) was then calculated for each patient pretreatment and following 1 and 3 months of sorafenib therapy.

Germline DNA Mutational Analysis

An automated analysis of mutations from the WES of germline DNA was performed to search for pathologic mutations in 565 cancer associated genes, including 60 autosomal dominant cancer predisposition genes (ALK, APC, BAP1, BMPRIA, BRAF, BRCA1, BRCA2, CBL, CDC73, CDH1, CDK4, CDKN1C, CDKN2A, CEBPA,
Kinase Fusions and Mutations in Histiocytes

**DISCUSSION OF POTENTIAL CONFLICTS OF INTEREST**

J. Estrada-Veras is a consultant/advisory board member for the Erdheim-Chester Disease Global Alliance. M. Lacouture reports receiving commercial research grants from and is a consultant/advisory board member for Genentech and Roche. P.J. Stephens has ownership interest in Foundation Medicine. V.A. Miller has ownership interest in Foundation Medicine. D.B. Solit reports receiving a commercial research grant from and has ownership interest in Foundation Medicine. D.B. Solit is a consultant/advisory board member for Pfizer. D.M. Hyman is a consultant/advisory board member for Atara Biotherapeutics and Chugai. J.-F. Emile reports receiving commercial research grants from Biocartis, Foundation Medicine, and Trovagene and is a consultant/advisory board member for Trovagene. A. Dogan is a consultant/advisory board member for Cancer Genetics. J.-F. Emile is a consultant/advisory board member for Roche and GlaxoSmithKline. N. Rosen reports receiving a commercial research grant from Chugai and is a consultant/advisory board member for AstraZeneca, Chugai, Kura, and Millennium-Takeda. No potential conflicts of interest were disclosed by the other authors.

**AUTHORS’ CONTRIBUTIONS**


Other (performed original extraction of RNA, quality and quantification of RNA): J. Nakitandwe

Other (support for contributing investigator): W.A. Galil

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