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Diverse and Targetable Kinase Alterations Drive Histiocytic Neoplasms

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Author Contributions

E.L.D., B.H.D., J.H., T.A.G., and O.A.-W. conceptualized the research program and designed the experiments. E.L.D., J.H., S.A.P., F.C.-A., P.C., C.G., J.E.-V., M.L., W.A.G., S.R.B., O.F., J.B., S.H., J.D., D.M.H., J.B., F.J., Z.A., A.D., and J.-F.E. were involved in sample collection and clinical interpretation. B.H.D., J.C., J.B., J.F.-E., C.Y.P., and A.D., reviewed pathology. B.H.D., Z.Y., Z.W., E.K., S.C.-W.L., Y.G., J.-B.M., B.S., O.A., P.Z., J.N., J.D., D.E., D.B.S., and N.R. conducted laboratory experiments. B.H.D., J.M., Z.W., M.W., I.D., O.A., A.H., P.J.S., V.A.M., J.S.R., S.M.A., B.S.T., and O.A.-W. analyzed the high-throughput sequencing data. B.H.D., E.L.D., J.H., D.W.E., T.A.G., and O.A.-W. supervised data generation. B.H.D., E.L.D., D.M.H., N.R., T.A.G., and O.A.-W. wrote the manuscript.

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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 (LCH) and non-Langerhans (non-LCH) histiocytoses, respectively. The discovery of *BRAFV600E* mutations in ~50% of these patients provided the first molecular therapeutic target in histiocytosis. However, recurrent driving mutations in the majority of *BRAFV600E*-wildtype, non-LCH patients 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 *BRAFV600E*-wildtype, non-LCH patients. In addition to MAP kinase pathway lesions, recurrently altered genes involving diverse cellular pathways were identified. Treatment of *MAP2K1*- and *ARAF*-mutated, non-LCH patients using MEK and RAF inhibitors, respectively, resulted in clinical efficacy demonstrating the importance of detecting and targeting diverse kinase alterations in these disorders.

Introduction

Systemic histiocytic neoplasms consist of Langerhans Cell Histiocytosis (LCH) and the non-Langerhans forms of histiocytosis including Erdheim-Chester Disease (ECD), Juvenile

Xanthogranuloma (JXG), and Rosai-Dorfman Disease (RDD) (1). These are diverse disorders marked by infiltration of tissues with neoplastic histiocytes whose cellular origin(s) have long been debated. The discovery of the *BRAFV600E* mutation in ~50% of patients with LCH (2, 3) and ECD (4) led to biological and therapeutic advances in these disorders. For example, characterization of the cells in the hematopoietic system of LCH patients at which the *BRAFV600E* 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 *BRAFV600E*-mutant histiocytosis with vemurafenib (a RAF inhibitor that specifically targets *BRAF V600E*) confers clinical responses in >90% of patients marked by a prolonged durability compared to that seen with BRAF inhibition in common *BRAFV600E*-mutant malignancies. The robust efficacy of BRAF 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, *MAP2K1* mutations have been identified in ~25% of *BRAFV600E*-wildtype LCH patients, reinforcing the notion that LCH is a disease driven by MAP kinase pathway activation (10–12). However, the direct clinical importance of activating *MAP2K1* mutations in LCH is unknown. Moreover, in contrast to LCH, activating kinase mutations in the majority of *BRAFV600E*-wildtype, non-LCH patients are undefined. In addition, recurrent alterations that co-exist with activating kinase mutations have not been defined for any of these conditions. Finally, despite the clinical and histopathological 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 *BRAFV600E*-wildtype non-LCH were successfully treated with targeted therapies, recapitulating the efficacy of BRAF inhibition in *BRAFV600E*-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), while all others were validated by targeted-capture next-generation sequencing (Supplementary Tables S2–S3 and Supplementary Methods).

Overall, a mean of 7 non-synonymous 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% while 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–B). In addition, mutations affecting diverse biological processes co-existed 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 (13–15)) failed to reveal mutations in any of these genes in blood DNA (Supplementary Methods).

Recurrent *MAP2K1* and *ARAF* Mutations in Non-LCH Neoplasms

WES and RNA-seq revealed *MAP2K1* mutations in both LCH and non-LCH patients and *ARAF* mutations in non-LCH patients (Fig. 1; Supplementary Fig. S3A–B). Since neither *MAP2K1* nor *ARAF* mutations have previously been described in ECD, we next interrogated a validation cohort of 37 *BRAFV600E*-wildtype, non-LCH, FFPE tissue cases (Fig. 1B). Exons 2–3 of *MAP2K1* and all coding regions of *ARAF* were sequenced, along with regions of recurrent mutations in *NRAS*, *KRAS*, and *PIK3CA*, since rare mutations in these genes have previously been noted in *BRAFV600E*-wildtype, non-LCH patients (16). This revealed recurrent activating mutations in *MAP2K1* (32%; n=12), *NRAS* (16%; n=6), *KRAS* (11%; n=4), *PIK3CA* (8%; n=3), and *ARAF* (3%; n=1) (Fig. 1B–D). Expression of 10 of these *MAP2K1* mutants identified activation of MAP kinase signaling over wildtype *MAP2K1* (Fig. 1E). Two non-LCH cases demonstrated concurrent activating *NRAS* and *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 Fig. S2 and Supplementary Fig. S3C–D).

Recurrent Kinase Fusions in Non-Langerhans Histiocytic Neoplasms

RNA-seq identified kinase fusions involving *BRAF*, as well as *ALK*, exclusively in *BRAFV600E*-wildtype non-LCH (Fig. 1A; Supplementary Fig. S2). An *RNF11-BRAF* fusion was detected in an infiltrative, non-LCH brain lesion of a 14-year-old child (Fig. 2A; Supplementary Fig. S4A). Since 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–B). Sequencing of the *RNF11-BRAF* fusion revealed that this transcript developed from an in-frame fusion of exon 1 of *RNF11* to exons 11–18 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, wildtype *BRAF*, or *BRAFV600E* revealed activation of ERK and MEK phosphorylation by both *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-seq analysis (20) of 9 *BRAFV600E*-wildtype, non-LCH patients 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 non-LCH patient, 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 *BRAFV600E*-wildtype ECD (Fig. 2G; Supplementary Fig. S4B). Expression was confirmed by RT-PCR/Sanger sequencing, interphase FISH, and immunohistochemistry (IHC) (Fig. 2G–H; Supplementary Fig. S4C). Although *KIF5B-ALK* fusions have been described in non-small cell lung cancer (NSCLC) (21), this particular case harbored a unique breakpoint. In both tumor types, the kinase domain of *ALK* is fused to the N-terminal coiled-coil domain of *KIF5B* resulting in inappropriate *ALK* expression, as well as constitutive *ALK* activation. Introduction of this patient's *ALK* fusion into Ba/F3 cells resulted in clear *ALK*, MAP kinase, STAT3, and PI3K-AKT pathway activation (Fig. 2I), as well as cytokine-independent growth (Fig. 2J). *KIF5B-ALK*-expressing cells, but not empty vector controls, were exquisitely sensitive to *ALK* inhibition (Fig. 2K). Interestingly, targeted RNA-seq analysis identified a second *KIF5B-ALK* fusion in the liver lesions of a 50-year-old ECD patient (Fig. 2L). Finally, an *LMNA-NTRK1* fusion was identified in the skin lesions of a 27-year-old ECD patient (Fig. 2M; Supplementary Fig. S4D). *LMNA-NTRK1* fusions have been identified in Spitzoid neoplasms (22) and result in aberrant *NTRK1* expression with consequent MAP kinase and PI3K-AKT pathway activation.

Gene Expression Analysis of Langerhans and Non-Langerhans Histiocytic Neoplasms

Given the presence of *BRAFV600E*, *MAP2K1*, and *ARAF* mutations in both LCH and non-LCH, we next performed gene expression analysis to understand potential biological differences in these clinically distinct disorders. Unsupervised hierarchical clustering of the top 1% of differentially expressed genes (Supplementary Table S4; Fig. 3A) revealed that samples clustered first by clinical diagnosis (LCH or ECD) then by kinase alteration. Genes differentially expressed between LCH and ECD included all the genes known to encode proteins used to discriminate LCH from ECD histologically (1) (Fig. 3B). Moreover, restriction of gene expression analyses to those LCH and non-LCH samples harboring *BRAF* mutations revealed the presence of a core set of genes discrepant between LCH and ECD (Fig. 3C–D; Supplementary Fig. S5A–B). Firstly, this included strong enrichment of genes expressed in LCH samples from this study with prior gene expression data from purified

CD207+ LCH tumor cells (23). In addition, LCH samples harbored strong enrichment of gene sets upregulated in late-stage myeloid progenitor cells and granulocyte-monocyte progenitors (GMPs), as well as multiple gene sets from classical dendritic cells (cDCs) (Fig. 3C and Supplementary Fig. S5). In contrast, non-LCH demonstrated enrichment of multiple gene sets upregulated in hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and monocytes. These differences in gene set enrichments were largely due to upregulation in LCH samples of genes known to be highly expressed in cDCs (24) (including *IRF7*, *RUNX3*, *GPR82*, and *CCR7*) (Fig. 3D). In contrast, genes known to be upregulated in HSCs such as *BAALC* (25) and *IDI1* (26), as well as core macrophage-associated genes (such as *CEBPA* (27)) were upregulated in non-LCH samples compared with LCH (Fig. 3D). In addition to gene sets related to hematopoietic ontogeny, gene sets involved in cell cycle regulation and IL1 signaling were found to be enriched in LCH but not ECD. Conversely, genes involved in lipid metabolism and adipogenesis were uniquely enriched in ECD but not LCH (Supplementary Fig. S5). Although this result is consistent with the histologic appearance of ECD lesions, these data highlight a potential role of adipogenesis not previously recognized in this disorder.

Therapeutic Targeting of *MAP2K1*- and *ARAF*-mutant Refractory Histiocytoses

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 *MAP2K1*K57N mutation was noted from WES of peri-renal lesions from a 53-year-old ECD patient with progressive disease following treatment with both interferon-alpha and anakinra, who was symptomatic by way of inflammatory ascites and renal failure. Treatment was initiated with a MEK inhibitor (single-agent trametinib 2mg 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–B). The patient has been maintained on trametinib single-agent therapy with a sustained clinical response for >180 days. Similarly, treatment of a second ECD patient with *MAP2K1*Q56P-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 an ECD patient, with disease in the bones, orbits, cavernous sinuses, and choroid, whose tumor was found to harbor an *ARAF*S214A 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–B). Given a recent report of complete response to sorafenib in a NSCLC patient with an *ARAF*S214C mutation (28), we initiated sorafenib therapy, titrated up to 600mg 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 *BRAFV600E* mutations in LCH (2, 3) and ECD (4), followed by identification of *MAP2K1* mutations in LCH (10–12), however, resulted in a new understanding of histiocytic neoplasms 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) have 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 histiocytic disorder patients 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 non-LCH patients 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 histiocytosis patients. For example, histiocytosis patients bearing *ALK* fusions may be amenable to *ALK* inhibitors as have demonstrated efficacy in *ALK*-rearranged NSCLC (29, 30). In addition, those expressing *NTRK* fusions might be eligible for ongoing clinical trials utilizing novel TRK inhibitors (31).

Although mutations in genes involved in diverse biological 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 co-existing with additional MAP kinase pathway mutations.

Overall, mutations in *MAP2K1* and *ARAF* were the most common kinase alterations amongst *BRAFV600E*-wildtype 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*-wildtype 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 to that 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 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 reinforcing 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 impact clinical therapy for histiocytosis patients. Further efforts to integrate genomic analysis into the clinical care of histiocytic disorder patients may greatly help in both disease classification, as well as 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's Children's Research Hospital, MD Anderson Cancer Center, the National Human Genome Research Institute, and Pitié-Salpêtrière 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 histological methods. For patients undergoing whole exome sequencing (WES), DNA extracted from peripheral blood mononuclear cells was utilized as a paired germline control. In total, specimens from 64 patients were analyzed, and the clinical, histological, and genetic characteristics are summarized in Supplementary Tables S1 and S3.

Whole Exome Sequencing

Analysis of exome 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 have been described previously (40, 41). Approximately 250ng 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). Barcoded 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 Fluorimetric Quantitation (Invitrogen) and Agilent Bioanalyzer. An equimolar pool of the 4 barcoded libraries (300ng 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 # 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 100X 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 wildtype sequences, on a BioRad QX200 ddPCR system, following the manufacturer's instructions, starting from 30ng 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, San Diego, CA) was used to design amplicons covering the regions of interest. The regions were amplified using 250ng 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 manufacturer's instructions using the Illumina TruSeq RNA sample preparation V2. Sequencing was completed on the Illumina HiSeq 2000 as per 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 have 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 each gene using Cuffdiff2 (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 Cambridge, MA) 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 50ng of 20% histiocyte nucleic acid. DNA was isolated from 40- μ m-thick sections of FFPE tissue.

Targeted RNA Sequencing

Total RNA extracted from 40- μ m-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 fluorescence *in situ* hybridization (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 only detected 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 fluorescence *in situ* hybridization (FISH) are included in the Supplementary Methods.

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

10mL of blood 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; Germantown, MD) 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 Hercules, CA) in a custom-designed, allele-specific assay (primer sequences available upon request). The experiments were performed using the following protocol: 1 cycle at 95°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 1 cycle at 4°C infinite, all at a ramp rate of 2°C/second. Bio-Rad's T100 thermal cycler was used for the PCR step. When available, 5ng of DNA was assessed in a 20 μ l 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 *ARAFS214A* 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*, *BMPR1A*, *BRAF*, *BRCA1*, *BRCA2*, *CBL*, *CDC73*, *CDH1*, *CDK4*, *CDKN1C*, *CDKN2A*, *CEBPA*, *DICER1*, *EPCAM*, *FH*, *GATA2*, *HRAS*, *KRAS*, *MAP2K1*, *MAP2K2*, *MAX*, *MEN1*, *MLH*, *MSH2*, *MSH6*, *NF1*, *NF2*, *NRAS*, *PALB2*, *PAX5*, *PHOX2B*, *PMS2*, *PRKARIA*, *PTCH1*, *PTEN*, *PTPN11*, *RAF1*, *RB1*, *RET*, *RUNX1*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *SHOC2*, *SMAD4*, *SMARCA4*, *SMARCB1*, *SOS1*, *STK11*, *SUFU*, *TMEM127*, *TP53*, *TSC1*, *TSC2*, *VHL*, and *WT1*), as well as all genes previously reported to be mutated in individuals with clonal hematopoiesis associated with a somatic mutation (*ASXL1*, *DNMT3A*, *FLT3*, *GNAS*, *GNB1*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *NPM1*, *SF3B1*, *SRSF2*, *TET2*, *U2AF1*, and others (13–15, 47)). With the exception of variants that matched those contained within the IARC *TP53* database, only novel, non-silent single nucleotide variants (SNVs) or SNVs with <0.1% population frequency in the NHLBI ESP database received an automatic classification. In-frame indels that matched dbSNP did not receive a classification. Indels present in 1000 Genomes or with multiple submissions in dbSNP were also excluded.

To identify pathologic lesions, variants were classified into three tiers, named gold, silver, and bronze. Gold is reserved for truncation mutations in tumor suppressor genes, matches to truncation mutations and hotspot mutations in somatic mutation database, and perfect matches to highly-curated locus-specific databases, including the IARC *TP53* database, NHGRI *BRCA1* and *BRCA2* database for records marked clinically important, ARUP *MEN2* database for mutations in *RET*, ASU database for *TERT* mutations, LOVD for mutations in *APC* and *MSH2*, and the *RB1* mutation database. Silver is used for variants of less certain significance, and bronze for variants more likely to be benign. Additional databases that were utilized for classification included HGMD, ClinVar, and UMD, matches to which generally received a silver classification. Truncation mutations located close to the C-terminus of the protein that do not map to a functional domain were examined manually and consulted with external gene experts for their pathogenicity.

Drug Studies

Vemurafenib, sorafenib, GDC-0973 (cobimetinib), crizotinib, and alectinib were purchased from Selleckchem (Houston, TX, USA). Drug studies were conducted *in vitro* using FACS-sorted, DAPI⁻ eGFP⁺ Ba/F3 cells that stably expressed the MIGII-EV, MIGII-*BRAF* V600E, MIGII-*RNF11-BRAF*, and MIGII-*KIF5B-ALK* constructs using the CellTiter-Glo® Luminescent Cell Viability Assay from Promega Corporation (Madison, WI, USA), according to the manufacturer's instructions. The MIGII-*BRAF* V600E, MIGII-*RNF11-BRAF*, and MIGII-*KIF5B-ALK* FACS-sorted Ba/F3 cells were maintained in RPMI + 10%

Fetal Bovine Serum (FBS) + penicillin and streptomycin media without murine IL-3 while MIGII-EV was maintained in RPMI + 10% FBS + penicillin and streptomycin with recombinant murine IL-3 (1 ng/mL).

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed as described previously³. The dataset was converted to gene symbols, and the gene expression signatures were analyzed using the java GSEA package. The most differentially expressed genes for each comparison were used to generate a signature for GSEA analysis. The input motif gene sets were extracted from the Molecular Signature Database, version 4 (MSigDBv4). Gene sets with a $p < 0.05$ and a false discovery rate (FDR) q -value $< 25\%$ were considered to be significantly enriched in LCH and non-LCH cases evaluated by RNA-seq.

Accession Codes

Whole exome ($n=24$ patients) and transcriptome ($n=13$) sequencing data for the patients from the discovery cohort have been deposited in the Sequence Read Archive (SRA) and Gene Expression Omnibus (GEO), respectively. The SRA accession number is SRP065600, and the GEO accession number is GSE74442.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of 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.

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 and transcriptome sequencing of Langerhans and non-Langerhans cell histiocytic (non-LCH) neoplasms. Each patient is represented in one column. Diagnosis (Langerhans Cell Histiocytosis or Erdheim-Chester Disease), 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 (as shown on right hand label). 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 *BRAF*V600E-wildtype patients with a spectrum of non-LCH neoplasms. Diagnosis and percent histiocyte content per section is 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, wildtype FLAG-MEK1, or various FLAG-MEK1 mutant cDNAs along with HA-tagged ERK2.

Figure 2. Kinase fusions in non-Langerhans cell systemic histiocytic (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* wildtype, *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 post-IL-3 withdrawal from a triplicate experiment is shown. Error bars indicate standard deviation of mean. (E) CellTiter-Glo luminescent viability IC50 results from 3 independent experiments of Ba/F3 cells from (D) exposed to MEK inhibitor GDC-0973, vemurafenib, or sorafenib. Log10 IC50 values are on y-axis. Error bars indicate standard error of mean. (F) Illustration of the *CLIP2-BRAF* fusion with Sanger sequencing confirmation identified in histiocytic ovarian infiltrates in a patient with Erdheim-Chester Disease. (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 post-IL-3 withdrawal from triplicate experiment is shown. Error bars indicate standard deviation of mean. (K) CellTiter-Glo luminescent viability IC50 results from 3 independent experiments of Ba/F3 cells from (J) exposed to crizotinib or alectinib. Log10 IC50 values on y-axis. Error bars indicate standard error of mean. (L) Illustration of a second *KIF5B-ALK* fusion identified in the liver lesions of a 50-year-old ECD patient involving exons 1–24 of

KIF5B and 20–29 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 μ m) and illustration of the *LMNA-NTRK1* fusion (bottom).

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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 out 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.

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Figure 4. Therapeutic efficacy of MEK and RAF inhibition in patients with *MAP2K1*- and *ARAF*-mutant systemic histiocytic neoplasms

(A) Axial FDG-PET scans pre-trametinib and 4-weeks post-trametinib in a *MAP2K1*K57N ECD patient with histiocytic infiltration of kidneys (top) and spermatic cord (bottom). (B) Creatinine and platelet counts in same patient pre- and post-trametinib therapy (green line indicates boundary of normal values). (C) PET scan pre-cobimetinib and 4-weeks post-cobimetinib of a *MAP2K1*Q56P-mutant ECD patient with disease infiltration in facial sinuses, heart, and kidneys. (D) Axial brain MRI of *ARAF*S1214A- mutant ECD patient with histiocytic infiltration of retina and optic nerves. MRI images show optic nerve infiltration (arrows) pre- and 6-weeks post-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* wildtype in plasma cell-free DNA with sorafenib treatment.