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ORIGINAL ARTICLE

Pediatric Langerhans cell histiocytosis: the impact of mutational profile on clinical progression and late sequelae

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Abstract

Langerhans cell histiocytosis (LCH) is a clonal histiocytic disorder with recurrent mutations of *BRAF* and *MAP2K1*, but data on the impact of genetic features on progression and long-term sequelae are sparse. Cases of pediatric LCH with long-term follow-up from our institution were analyzed for mutations in $BRAF^{V600}$ and *MAP2K1* exons 2 and 3 by immunostaining with mutation-specific VE1 antibody, as well as allele-specific PCR and sequencing, respectively. Clinical and follow-up data were obtained from our files and a questionnaire sent to all former patients. Sixteen of 37 (43%) evaluable cases showed $BRAF^{V600E}$, one case a $BRAF^{V600D}$ and eleven (30%) a *MAP2K1* mutation. Nine cases were unmutated for both genes. All cases with risk organ involvement showed either $BRAF^{V600}$ or *MAP2K1* mutation. Patients with $BRAF^{V600}$ mutation excluding Hashimoto-Pritzker cases had a significantly higher risk for relapses ($p = 0.02$). Long-term sequelae were present in 19/46 (41%) patients (median follow-up 12.5 years, range 1.0 to 30.8) with a trend for higher rates in mutated cases (mutated = 9/17, 53% versus non-$BRAF^{V600}$/*MAP2K1* mutated = 2/7, 29%). In addition, 8/9 cases with skin involvement including all Hashimoto-Pritzker cases ($n = 3$) were positive for $BRAF^{V600E}$. Infants below 2 years more frequently had $BRAF^{V600}$ mutations ($p = 0.013$). Despite favorable prognosis, pediatric LCH shows a high frequency of relapses and long-term medical sequelae.

Keywords Langerhans cell histiocytosis · *BRAF* mutation · *MAP2K1* mutation · Long-term sequelae · VE1 immunohistochemistry

Introduction

Langerhans cell histiocytosis (LCH) is a rare histiocytic disorder, histologically characterized by an accumulation of cells with immunophenotypic and ultrastructural characteristics of Langerhans cells, which are defined by the expression of CD1a and CD207/langerin and the presence of Birbeck granules in electron microscopy. LCH cells are accompanied by an inflammatory background dominated by eosinophils, lymphocytes, and giant cells derived from syncytial Langerhans cells [1, 2].

Approximately 4–6 children/10^6 are diagnosed with LCH, a disease with very diverse clinical presentation [3–5]. The most commonly affected organs are bones, skin, lymph nodes, and lung, the latter in isolated form almost invariably associated with smoking. More rarely, lesions are found in the central nervous system or in the risk organs liver, spleen, and/or hematopoietic system. Overall, LCH shows a wide range of mono- or multifocal, uni- or multisystemic organ involvement with a broad spectrum of clinical severity. Therapy for LCH patients is therefore adapted to the pattern of manifestation and the initial response to therapy. It usually consists of prednisolone in combination with cytostatic drugs such as vinblastine, vincristine, cytarabine or mercaptopurine, methotrexate,

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etoposide, cladribine, or clofarabine. In very severe cases, even an allogeneic stem cell transplant can be indicated [3, 5–12]. Depending on the extent and location of lesions and therapy, affected children develop long-term sequelae in 16% to 75%, most commonly involving the endocrine system [4, 6, 10, 13–18].

For a long time, LCH was considered an immunologically triggered inflammatory disease. In 1994, for the first time, evidence was provided for a clonal proliferation in LCH by using X-chromosome inactivation assay [19, 20]. With the detection of somatic mutations in the B-Raf proto-oncogene, serin/threonine kinase (BRAF), and mitogen-activated protein kinase kinase 1 (MAP2K1) of the ERK pathway, LCH was finally established as a neoplastic disorder. About half of the patients showed the *BRAF*^{V600E} and up to 50% of *BRAF* negative cases harbor a *MAP2K1* mutation [21–25]. Additional mutations in the *ARAF*, *ERBB3*, and *MAP3K1* genes are identified in rare cases [24–27]. Despite the establishment of its nature as clonal myeloid neoplasm in at least a significant proportion of cases [28–30], the inflammatory features of LCH are still a focus of research [31]. *BRAF*^{V600E} is also detectable in Erdheim-Chester disease in up to 54% but not in other non-Langerhans cell histiocytoses [32]. Due to the self-limiting course of many cases of LCH despite its clonal nature, several groups have investigated the potential role of oncogene-induced senescence by studying p16^{INK4A} expression. Although a high prevalence of expression was found by all investigators, the published data are contradictory concerning the prognostic relevance of p16 expression [33–37].

As there are only a few prognostic factors known for the marked clinical variability of LCH, we retrospectively examined the correlation between clinical parameters, mutation status, p16 expression, and clinical course, as well as long-term sequelae in a series of pediatric LCH with long-term follow-up, including symptoms originating from the disease itself, such as diabetes insipidus, as well as therapy-related effects.

Materials and methods

Study patients

A total of 62 pediatric LCH patients were identified at the Children's Hospital of the University Hospital Tübingen between 1981 and 2014. All retrospective clinical data from 1981 to 2014 including imaging and laboratory features were taken from medical records of the Department of Pediatrics of the University Hospital Tübingen. At the initiation of the study, all patients received questionnaires (see Online Resource 1) containing polar and open questions with free text fields on recurrences, tumors occurring in first-degree relatives, and long-term medical sequelae. Direct permanent consequences of the disease such as endocrine or orthopedic symptoms as well as therapy-related effects were documented. The study was approved by the ethics committee of the Medical Faculty, University of Tübingen.

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) tissue was available from 37 patients from the Institute of Pathology and Neuropathology, University Hospital Tübingen. All tissues were fixed in 4% buffered formalin. Sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on an automated immunostainer according to manufacturer's instructions (Ventana, Tucson, AZ, USA). Antibodies were used against S100 (1:5000, DAKO, Hamburg, Germany), CD1a (1:50, DAKO), BRAF^{V600E} (VE1) (1:50, DCS, Hamburg, Germany), and p16 (Roche mtm laboratories AG, Mannheim, Germany). P16 expression was evaluated using a semiquantitative score based on the quantity and intensity of positive neoplastic cells compared to histology as described by Sinicrope et al. [38]. The percentage of positive cells was quantified from 0 to 4 (0, ≤ 5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, > 75%) and the intensity from 0 to 3 (0, negative; 1, weak; 2, medium; 3, strong). For better evaluation, the product of both factors was taken for statistical analyses.

Mutation analysis

DNA was extracted with QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, Netherland) according to the manufacturer's instructions from 5 μm FFPE tissue sections with at least 10% tumor cells, if necessary following macrodissection. Sanger sequencing of *BRAF* hotspot region was performed using a protocol supplied by Roche designed for GS Junior sequencing (Roche, Penzberg, Germany). DNA was amplified for the region around codon 600 of the *BRAF* gene with tailed primers containing a universal sequence (CS1 and CS2): Fwd: 5′-TGTAAAACGACGGCCAGTACTGTTTTCCTTTACTTACTACACCTC-3′ and Rev: 5′-CAGGAAACAGCTATGACTCAGTGGAAAAATAGCCTCAA-3′. PCR was performed using 50 ng DNA template in a final volume of 25 μl with 0.05 μM of each primer, 0.2 mM dNTPs, 4.5 mM $MgCl_2$, 5% DMSO, and 1.25 units of FastStart High Fidelity Enzyme Blend (Roche). PCR conditions initially entailed 50 °C for 2 min, 70 °C for 20 min, and 95 °C for 10 min followed by two blocks of PCR cycles (95 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s alternating with 95 °C for 15 s, 80 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s ten, two, eight, two, eight, two, eight, and five times, respectively). In ten cases of low tumor cell content, a locked nucleic acid oligomer 5′-TAGCTACAGTGAAATCTC-3′ (TIB MOLBIOL, Berlin, Germany) was added to suppress *BRAF* wild-type sequence amplification.

For the amplification of *MAP2K1* exons 2 and 3, M13-tailed primers were used: Ex2-Fwd: 5′-TGTAAAACGACGGCCAGTTGACTTGTGCTCCCCACTTT-3′, Ex2-Rev: 5′-CAGGAAACAGCTATGACGTCCCCAGGCTTCTAAGTACC-3′, Ex3-Fwd: 5′-TGTAAAACGACGGCCAGTTCATCCCTTCCTCCCTCTTT-3′, and Ex3-Rev: 5′-CAGGAAACAGCTATGACCTCTTAAGGCCATTGCTCCA-3′. PCR was performed using 0.2 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 0.5 units of Phusion Hot Start DNA Polymerase (Finnzymes, Woburn, MA, USA). Cycling conditions entailed an initial denaturation at 98 °C for 30 s followed by 40 cycles of denaturation (98 °C for 10 s), annealing (56 °C for 45 s), and elongation (72 °C for 30 s), with a final elongation at 72 °C for 7 min. PCR products were purified (AMPure, Beckman Coulter, Brea, CA, USA) and aliquots of 7 μl were used for the sequencing reaction with 1 μM of the universal sequencing primer and 2 μl of GenomeLab DTCS-Quick Start Kit (Beckman Coulter, Brea, CA, USA) in a final volume of 10 μl according to the manufacturer's protocol. Sequencing reactions were purified (CleanSEQ, Beckman Coulter, Brea, CA, USA) and analyzed in a GenomeLab GeXP Genetic Analysis System and evaluated by the GenomeLab GeXP software 10.2 (Beckman Coulter, Brea, CA, USA) to determine the mutation status.

Samples which were positive or equivocal for $BRAF^{V600E}$ immunohistochemistry were additionally investigated by next-generation sequencing with single amplicons using the Ion Amplicon Library Preparation Fusion Method (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol (primer sequence: Fwd: 5′-ACTGTTTTCCTTTACTTACTACACCTC-3′ and Rev: 5′-TCAGTGGAAAAATAGCCTCAA-3′). Amplicons were purified and quantified applying Agencourt® AMPure XP (Beckman Coulter, Brea, CA, USA) magnetic beads and the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific). Amplicons were diluted to 5 pM each and pooled. In the next step, DNA fragments were attached to Ion Sphere Particles (ISPs) and clonally amplified using the Ion PGM™ Hi-Q™ OT2 Kit and the Ion OneTouch™ Instrument. The amount of template-positive ISPs was determined with the Qubit® 2.0 Fluorometer and the Ion Sphere™ Quality Control Kit. Afterwards, the Ion OneTouch™ ES was used to enrich template-positive ISPs. In a last step, sequencing primers were attached to the DNA fragments bound to the ISPs which were subsequently loaded on a semiconductor chip (Ion 318™ Chip Kit). Finally, sequencing was performed using the Ion PGM™ Hi-Q™ Sequencing Kit and the Ion Torrent PGM™ platform. Sequences were visualized and evaluated using the freely available software Integrative Genomics Viewer (IGV, Broad Institute).

To screen for alternative mutations in cases double negative for $BRAF^{V600}$/*MAP2K1* mutations, next-generation sequencing (NGS) was performed using the Ion AmpliSeq Cancer Hotspot Panel (for details, see Online Resource 2).

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 22 using Kaplan-Maier curve, log-rank test, and Pearson's chi-square test. Significance level was set to $p = 0.05$ for all analyses.

Results

Clinical findings

Medical records were available in 55 of 62 pediatric LCH patients identified in the study period. The clinical data are summarized in Table 1. The M/F ratio was 1:2; however, in infants below 2 years was 1:3. Thirty-eight of 55 patients (69%) presented with bone manifestation at initial diagnosis, including scull (16 cases), vertebral column (14 cases), and extremities (8 cases). Nineteen patients (35%) showed skin involvement, including three cases with Hashimoto-Pritzker disease, a self-limited and often congenital form of skin involvement without other manifestations. The lungs were affected in seven patients (13%), and four patients (7%) showed pituitary gland involvement. Six patients (11%) were considered clinically at risk due to the involvement of the hematopoietic system, spleen, and/or liver. Thirty-seven patients (67%) had monosystemic and 18 (33%) had multisystemic disease. Infants younger than 2 years had a significantly increased risk to develop multisystemic disease in comparison to older children (11/21 versus 7/34) ($p = 0.015$) (Fig. 1a). In 21 cases (38%), one or more recurrences occurred, but none of the patients died. Fifteen patients relapsed once, and six children had two or more recurrences. Patients with multisystemic disease had a significantly higher risk for relapse ($p = 0.015$)

Table 1 Summary of 55 patients with available clinical data

	n (%)
Age	< 2 years/ ≥ 2 years 21 (38%)/ 34 (62%)
Age range	1 day–15.6 years, median 3.5 years
Sex	Male/female 35 (64%)/20 (36%)
Stage	Monosystemic/multisystemic 37 (67%)/18 (33%)
Relapse	No/one or more 34 (62%)/21 (38%)

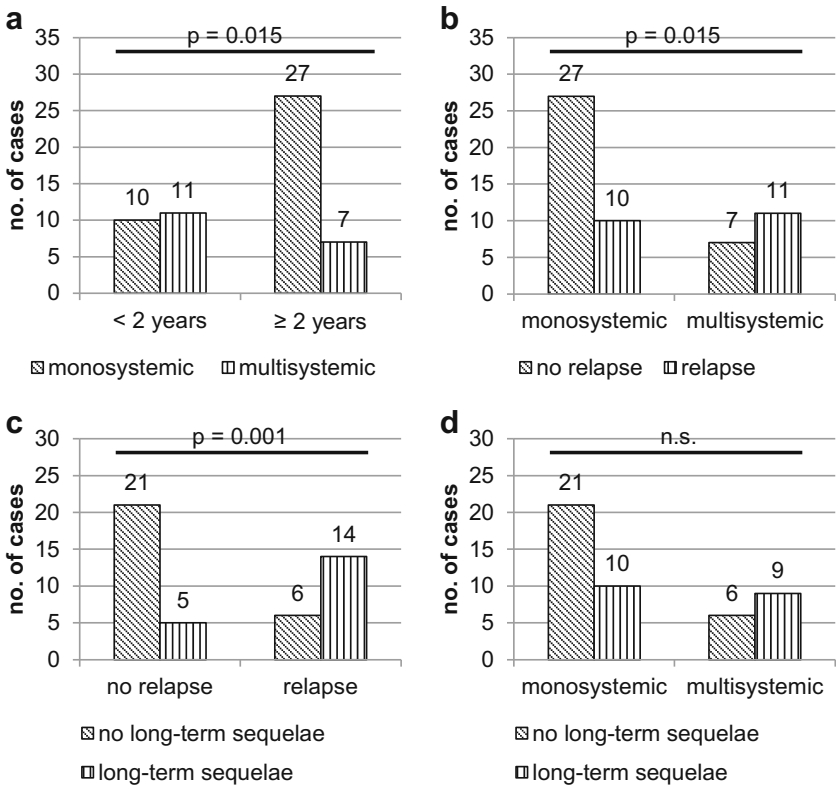

Fig. 1 Correlation of clinical data. **a** Significantly increased frequency of multisystemic versus monosystemic disease for infants younger than 2 years compared to older children at initial diagnosis (11/21 versus 7/34, $p = 0.015$). **b** Significantly increased risk for relapse for patients with multisystemic versus monosystemic disease at presentation (11/18 versus 10/37, $p = 0.015$). **c** Significantly increased risk for long-term sequelae for patients with relapse versus patients with a single disease episode (14/20 versus 5/26, $p = 0.001$). **d** Patients with multisystemic disease showed a trend to more frequent occurrence of long-term sequelae than patients with monosystemic stage (9/15 versus 10/31, $p = 0.073$)

(Fig. 1b). Twenty-seven patients (49%) received systemic therapy including prednisolone alone or in single or multiple combinations with vinblastine, cytarabine, vincristine, mercaptopurine, methotrexate, etoposide, cladribine, or clofarabine. Nineteen patients underwent surgical treatment only, and nine patients all treated before the year 2001 received radiotherapy. Nine infants were followed up without therapy. To obtain a rough estimate of the familial cancer burden in affected children, we asked for tumor occurrence in first-degree relatives. In 7/46 patients (15%), a malignant tumor was reported, range 3 to 65 years with a median of 46 years. Four of the seven cases occurred in subjects younger than 45 years (4/46 patients, 9%).

Long-term sequelae

The incidence and type of long-term sequelae were established in 46 patients, for whom the questionnaires were available with follow-up information. The median follow-up was 12.5 years (range 1.0–30.8 years) within this collective. In the long term, usually permanent sequelae caused by LCH were present in 19 of 46 patients (41%), in particular skeletal abnormalities (12/46, 26%), endocrine disorders (9/46, 20%) including diabetes insipidus, growth hormone deficiency, hypothyroidism, and testosterone deficiency, loss of teeth (4/46, 9%), neurological symptoms (2/46, 4%), and others (7/46, 15%). One patient with multiple relapses at the age of 22 years presented a non-melanocytic skin cancer at the right ear, after local radiotherapy with a dosage of approximately 18 Gy. Patients with recurrent disease showed a significantly increased frequency of long-term sequelae: 14/20 (70%) versus 5/26 (24%) patients with a single disease episode ($p = 0.001$) (Fig. 1c). In addition, there was a trend towards increased long-term complications in patients with multisystemic (9/15, 60%) versus monosystemic (10/31, 32%) LCH ($p = 0.073$) (Fig. 1d). Concerning the distribution of affected organs, most long-term sequelae were seen in patients with pituitary gland involvement (3/4, 75%), followed by risk organ involvement (3/5, 60%), bone involvement (14/31, 45%), and patients with skin involvement excluding two Hashimoto-Pritzker cases with available follow-up (6/14, 43%). The 38 patients without systemic therapy or chemotherapy for less than 1 year had a significantly lower rate (34%) of long-term sequelae compared to 6/8 (75%) patients treated systemically for more than 1 year ($p = 0.033$).

Histological and immunohistochemical findings

All 37 cases with tissue available for immunohistochemical and molecular studies showed the typical morphology and immunophenotype of LCH with Langerhans cells interspersed in an inflammatory background. The Langerhans cells were positive for S100 and CD1a in all cases confirming the diagnosis (Fig. 2a–c) with usually strong and homogeneous CD1a positivity and sometimes a more heterogeneous positivity for S100. P16 staining was positive in 33/34 cases

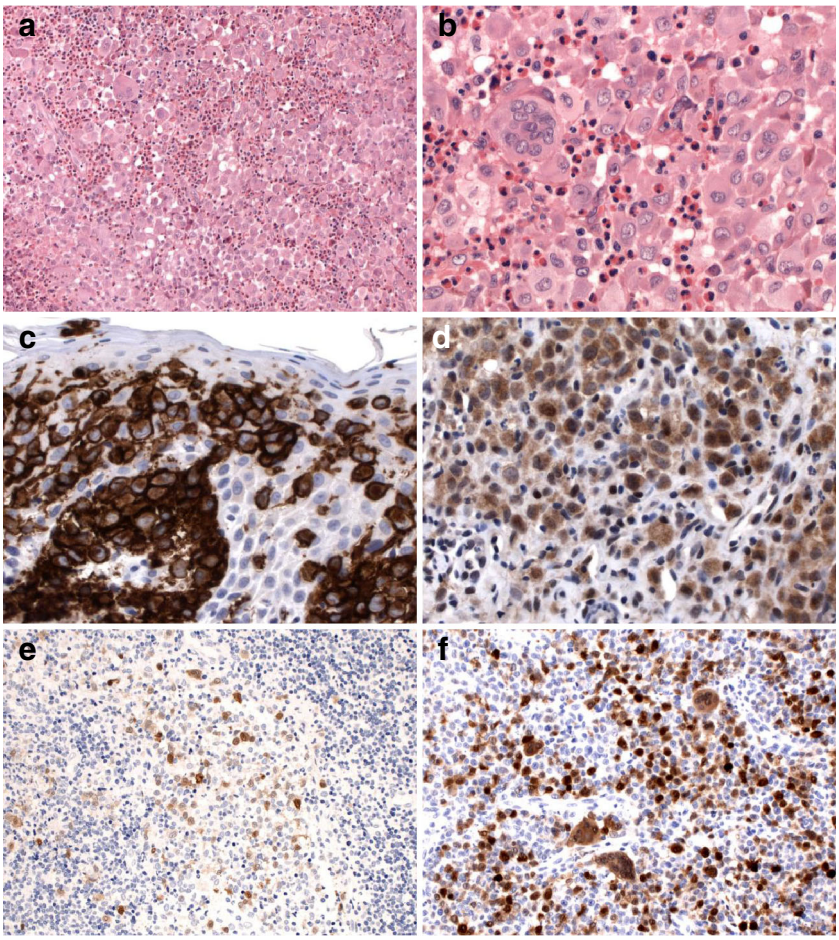

Fig. 2 Histology and immunohistochemistry. **a**, **b** LCH cells admixed with numerous of eosinophilic granulocytes and multinucleated giant cells (hematoxylin and eosin staining, **a** original magnification ×100, **b** original magnification ×400). **c** LCH cells with a strong CD1a positivity (CD1a immunoperoxidase, original magnification ×400). **d** Example for a moderate but specific $BRAF^{V600E}$ positivity of the LCH cells ($BRAF^{V600E}$ immunoperoxidase, original magnification ×400). In **e**, LCH cells with weak positivity for p16 and in **f** with strong positivity for p16 (p16 immunoperoxidase, both original magnification ×200)

(97%), only one case was not evaluable. In 11/33 (33%) cases, staining was weak (Fig. 2e), moderate in 7/33 (21%) cases, and strong in 15/33 (45%) cases (Fig. 2f). Cases with moderate to strong staining in the majority of cells (score 8 and 12) showed a decreased risk for relapse ($p = 0.037$), but there was no association of p16 staining with other clinical parameters.

BRAF (VE1) immunostaining and mutational analysis

$BRAF^{V600}$ status was assessed both by immunohistochemistry with the mutation-specific antibody VE1 as well as by sequencing analysis (Sanger and/or next-generation sequencing as described above) in 37 cases, using wild type suppressing LNA for PCR in a part of the cases. The expression of VE1 antibody in the LCH was sometimes difficult to interpret due to either weak cytoplasmic staining or strong background staining. Sixteen cases were considered positive (Fig. 2d), 10 were negative, and 11 cases were considered equivocal, due to weak cytoplasmic staining with background staining. In cases with a positive $BRAF^{V600E}$ staining, the number of positive cells showed good correlation with CD1a.

Molecular analysis confirmed a $BRAF^{V600}$ mutation in 15 of 35 cases analyzed (43%), two cases with clearly positive VE1 staining documenting presence of $BRAF^{V600E}$ showed insufficient DNA quality for molecular analysis. Fourteen cases had the canonical $BRAF^{V600E}$ mutation in concordance with positive VE1 staining, whereas one case showed the uncommon p.V600D mutation and was negative for VE1 as expected (Fig. 3 and Table 2). Importantly, all cases negative or equivocal in the VE1 stain were negative by sequencing.

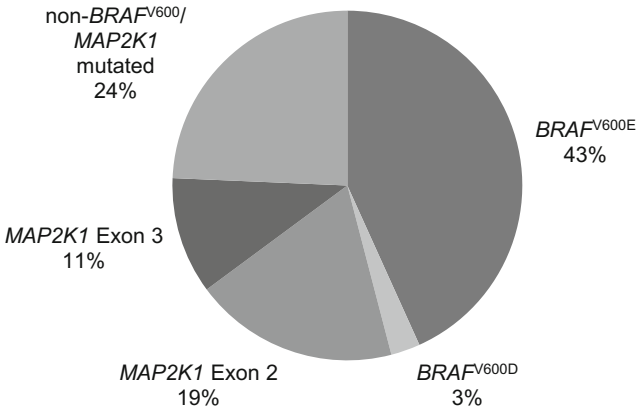

Fig. 3 Distribution of $BRAF^{V600}$ and *MAP2K1* mutation within 37 patients assessed both by immunohistochemistry with the mutation specific antibody VE1 as well as by sequencing

Table 2 Distribution of *BRAF* (Codon 600) and *MAP2K1* (exon 2 and 3) mutations

Mutations	*n* (%)
BRAF	**17 (45.9%)**
$BRAF^{V600E}$	16 (43.2%)
$BRAF^{V600D}$	1 (2.7%)
MAP2K1	**11 (29.7%)**
Exon 2	7 (18.9%)
c.170_184del, p.Q58_E62del	3 (8.1%)
c.168_182del, p.K57_G61del	1 (2.7%)
c.171_185del, p.Q58_E62del	2 (5.4%)
c.173_187del, p.Q58_E62del	1 (2.7%)
Exon 3	4 (10.8%)
c.388T>C, p.Y130H	1 (2.7%)
c.302_307del, p.E102_I103del	1 (2.7%)
c.301_306del, p.L101_E102del	2 (5.4%)
Total mutations	**28 (75.7%)**
non-$BRAF^{V600}$/*MAP2K1* mutated	**9 (24.3%)**

Mutations are specified on coding DNA and protein level

Fractions of main molecular groups are indicated in boldface

The estimated tumor cell content ranged from 10% to 70%, and cases with low tumor cell content were investigated by NGS. Cases sequenced by both methods for control purposes, including the $BRAF^{V600D}$ case, always showed congruent results. In general, there was a fairly good correlation between the estimated tumor cell content and the allele frequencies found by NGS (VAF range 2% to 26%), with the exception of few cases with low allele frequencies.

In 11 cases (11/35, 31%) with wild-type $BRAF^{V600}$, a *MAP2K1* mutation in Exon 2 (7/35, 20%) or Exon 3 (4/35, 11%) was identified; all except one were deletions (Fig. 3 and Table 2).

In summary, mutation-specific immunostaining and mutational analysis demonstrated the presence of a $BRAF^{V600}$ mutation in 17/37 cases and a *MAP2K1* mutation in 11/37 cases. Three wild-type cases with sufficient material were additionally analyzed using the Ion AmpliSeq Cancer Hotspot Panel and showed wild-type sequences for all analyzed genes.

Correlation of mutation status with clinical data

Clinical data were available for 34/37 patients. All cases considered clinically at risk due to involvement of internal organs showed either a $BRAF^{V600}$ or *MAP2K1* mutation. $BRAF^{V600}$ mutation was significantly more frequent in infants under 2 years (10/13, 77% versus 7/21, 33%, $p = 0.013$). This is in part due to the three Hashimoto-Pritzker cases, all of which were $BRAF^{V600E}$ positive (Fig. 4a). Overall, 8/9 patients with skin involvement showed a $BRAF^{V600}$ mutation ($p = 0.007$) (Fig. 4b). Relapses occurred in 2/8 (25%) wild-type cases, 3/9 (33%) *MAP2K1* mutated cases, and 10/17 (59%) $BRAF^{V600}$ mutated cases. After exclusion of the spontaneously regressing Hashimoto-Pritzker cases, the relapse rate for $BRAF^{V600}$ mutated cases (10/14; 71%) was significantly higher in comparison to the other groups ($p = 0.02$) (Fig. 4c). For cases with known follow-up status (excluding Hashimoto-Pritzker cases), long-term sequelae occurred in about one third of the non-mutated cases (2/7, 29%) and half of the patients with mutations ($BRAF^{V600}$ = 7/13, 54%; *MAP2K1* = 2/4, 50%). Patients with $BRAF^{V600}$ mutations showed a 2-year relapse-free survival of 71%, in comparison to 100% without mutation. For patients with relapse, the time to progression was similar for all three groups. Similarly, the median follow-up times for the three molecular groups (non-$BRAF^{V600}$/*MAP2K1* mutated, $BRAF^{V600}$ mutated, and *MAP2K1* mutated cases) showed no significant differences, thus excluding an observational bias.

Discussion

Langerhans cell histiocytosis nowadays is considered a clonal myeloid neoplasm with genetic alterations of the MAPK signaling pathway, most frequently the $BRAF^{V600E}$ mutation, and a significant inflammatory component. The disease shows a very heterogeneous clinical presentation and outcome ranging from benign, self-limiting unifocal manifestations to life-threatening systemic disease, probably influenced by the stage of cell differentiation at which the oncogenic hit(s) are acquired [29, 39]. Our study on pediatric LCH from a single center focused on the clinical features and especially the long-term sequelae and their correlation with mutational data.

Overall, our cohort showed a similar clinical presentation and course of the disease as compared to published reports [3, 5, 12, 14]. Of note, involvement of the pituitary gland was lower in our group in comparison to other studies [10, 12, 14, 40], whereas the vertebral column was more frequently affected [5, 12]. None of our patients died during the observation period. In our series, monosystemic disease was present in 69% and multisystemic LCH in 33% of patients, with a higher risk in infants below 2 years of age. Recurrences occurred in 38% of patients, more commonly in patients with multisystemic LCH.

Only few studies have investigated long-term sequelae of LCH and even less with a comparable long median follow-up of 12.5 years. Although we cannot completely exclude a reporting bias, our use of a questionnaire with a reply rate of 74% probably gives a fairly comprehensive and unbiased view of the true incidence of long-term sequelae, avoiding a bias towards patients with more complicated disease courses. With self-reported long-term sequelae in 41% (19/41) of patients, our data is comparable with published series, with higher rates for studies focusing on patients with multisystemic disease [4, 13–18, 41, 42]. In addition to

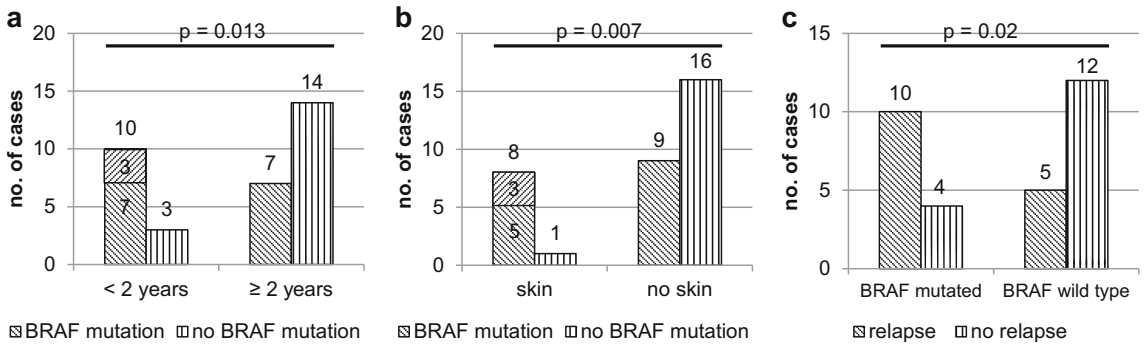

Fig. 4 Correlation of mutation status with clinical data. **a** Significantly higher $BRAF^{V600}$ mutation rate in infants < 2 years in comparison to children ≥ 2 years (10/13 versus 7/21, $p = 0.013$). Upper highlighted part: all three patients with Hashimoto-Pritzker disease were younger than 2 years. **b** Significantly increased frequency for skin involvement versus no skin involvement for $BRAF^{V600}$ mutated cases (8/9 versus 9/25, $p = 0.007$). Upper highlighted part: Hashimoto-Pritzker cases. **c** Patients with $BRAF^{V600}$ mutation excluding the Hashimoto-Pritzker cases had a significantly higher risk for relapse in comparison to *BRAF* wild-type (*MAP2K1* mutated and non-$BRAF^{V600}$/*MAP2K1* mutated) cases (10/14 versus 5/17, $p = 0.02$)

multisystemic LCH, disease recurrence ($p = 0.001$) and involvement of risk organs and pituitary gland were associated with a higher risk for long-term sequelae, in concordance with published data [13, 15, 17, 18, 43].

Of interest, especially given the fact that LCH is a clonal myeloid disorder, is the relative rarity of secondary neoplasms, especially hematopoietic cancer both in our study as well as in published studies, perhaps with the exception of patients treated with high-dose VP-16 [13, 18, 44]. The single case of skin cancer observed in our cohort can be linked to local radiation.

We also looked at the occurrence of cancer cases in the families of our cohort, based on the data obtained from the questionnaire. The tumor burden of the families in our LCH study is with 15% below the German estimated average lifetime cancer prevalence of 20% per first-degree relative, but 4/7 reported cancer cases occurred below 45 years. Nevertheless, it has to be kept in mind that a case-control study would be the appropriate approach for this question. A case-control study on tumor burden in first-degree relatives of 60 LCH cases and 150 control cases showed a significantly increased tumor burden of 30% for the LCH families as compared to 19% for the control cases [45].

The frequencies of *BRAF* and *MAP2K1* mutations in our cohort were comparable to the current literature [21–25]. In addition to the canonical p.V600E mutation, we identified a $BRAF^{V600D}$ mutation in a child with unifocal bone involvement. This mutation has only been described once so far in a LCH case with skin involvement [46]. Although our series is relatively small, we observed several interesting correlations between clinical features and mutational spectrum. *BRAF* mutations had a significantly higher prevalence in children younger than 2 years, whereas *MAP2K1* mutations and non-$BRAF^{V600}$/*MAP2K1* mutated cases were mostly seen in older children. Excluding the universally *BRAF* mutated Hashimoto-Pritzker cases ($n = 3$), seven of ten patients under 2 years had a *BRAF* mutation, in concordance with data by Heritier et al. [14]. The underlying mechanism for this phenomenon could be an age-dependent pattern of driver mutations. This has previously been shown in other tumor entities such as brain tumors [47]. Another observation in our cohort was the high frequency of *BRAF* mutation in cases with skin involvement. In two published studies, 5/10 (50%) and 5/8 (63%) cutaneous cases, respectively, carried the mutation [48, 49]. An association between skin and *BRAF* status was also observed by Heritier et al., but of note, none of their six cases with Hashimoto-Pritzker disease showed a $BRAF^{V600E}$ mutation [14]. This discrepancy cannot be explained at the moment. The fact that benign, self-limiting Hashimoto-Pritzker disease was $BRAF^{V600E}$ positive in all our cases is in support of the theory that the mutation in these cases affects dermal rather than hematopoietic stem cell-derived Langerhans cells. On the other hand, all our cases with risk organ involvement showed either a *BRAF* or *MAP2K1* mutation, also confirming published data [14]. As demonstrated previously, we also observed a statistically significant correlation between increased recurrences and *BRAF* mutation after exclusion of Hashimoto-Pritzker cases [14, 24, 50].

An unresolved issue to date is the relevance of p16 expression as a potential indicator of oncogene-induced senescence in LCH. Whereas Kim et al. observed a greater tendency towards multisystem disease, risk organ involvement, and relapse in the high expression group, Chilosi et al. described a loss of $p16^{INK4A}$ in all their aggressive cases [35, 36]. In accordance with these studies, we observed p16 expression in the vast majority of cases. In our series, we observed an association of moderate to strong positivity in the majority of cells with fewer relapses, but larger series will be required to confirm a potential prognostic impact of p16 expression.

Another aspect of our study was the comparison of immunostaining with the VE1 antibody, which exclusively recognizes $BRAF^{V600E}$ mutated BRAF protein with conventional

molecular techniques. Although we and others have observed a high specificity and sensitivity of VE1 immunostaining for most investigated solid tumors, and some studies on a limited numbers of cases have confirmed these results also for LCH [21, 51–56], we found immunostaining more difficult to interpret in LCH due to weak cytoplasmic staining in $BRAF^{V600E}$ negative cases and thus less reliable, as compared to solid tumors. A lack of sensitivity of molecular detection as explanation for a potential discrepancy was excluded, since all cases with low tumor cell content were re-tested with LNA probes for *BRAF* wild-type suppression and/or NGS. This indicates that the reliability of VE1 staining may depend on tissue type, similar to what has been reported for colorectal carcinomas, where sensitivity (75%) and specificity (93%) were observed in a cohort of 480 cases [57]. Ballester et al. in 2017 showed in 26 pediatric LCH patients a specificity of 100% and a sensitivity of 80% using stringent scoring criteria (≥ 2+ (in a scoring system 0 to 3+) and ≥ 10% positive tumor cells) [58]. Regarding the sensitivity of molecular studies, cases with a tumor cell content below 25% are problematic with conventional Sanger sequencing, but the use of LNA oligomers for suppression of wild-type amplification as in our study can lower the detection threshold. Alternatively, NGS provides an attractive alternative, and especially for hotspots such as $BRAF^{V600}$, detection down to 1% allelic frequency. In our opinion, molecular analysis is more reliable than immunohistochemistry for the detection of *BRAF* mutations in LCH due to the sometimes difficult interpretation of equivocal staining and rare alternative mutations such as $BRAF^{V600D}$.

In conclusion, our single-center study on pediatric LCH is comparable to published data concerning the frequencies of *BRAF* and *MAP2K1* mutations. Despite the generally benign course, long-term sequelae are observed in almost half of the patients and are associated with multisystemic disease, disease recurrence, and the presence of *BRAF* or *MAP2K1* mutations, although these data need to be confirmed in larger series.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

Statement of human rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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