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Original Article

Expression and Clinical Correlation of PD-1/PD-L1 and VE1(BRAFp.V600E) in Pediatric Langerhans Cell Histiocytosis

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Abstract. ***Background And Objectives*: Langerhans cell histiocytosis (LCH) is an inflammatory myeloid neoplasm with a wide spectrum of clinical presentations. Programmed Cell Death-1 (PD-1) receptor and its ligand (PD-L1) are overexpressed in LCH, but their clinical significance is unknown. We performed a clinical correlation study of PD-1/PD-L1 and VE1(BRAFp.V600E) expression in 131 children with LCH.**

***Methods*: A total of 111 samples were tested for PD-1/PD-L1 and 109 for VE1(BRAFp.V600E) mutant protein by immunohistochemistry.**

***Results*: PD-1, PD-L1 and VE1(BRAFp.V600E) positivity was observed in 40.5%, 31.53% and 55%, respectively. PD-1/ PD-L1 expression showed no significant effect on the rate of disease reactivations, early response to therapy or late sequelae. The 5-year EFS was not statistically different between patients with PD-1 positive compared to those with PD-1 negative tumours (47.7% vs.58.8%, p=0.17). Similar 5-year EFS rates were also seen in those who were PD-L1 positive compared to PD-L1 negative cases (50.5% vs.55.5%, p=0.61). VE1(BRAFp.V600E) positivity was associated with a significantly higher frequency of risk-organ involvement (p=0.0053), but no significant effect on early response to therapy or rates of reactivations or late sequelae.**

***Conclusions*: Our study showed no significant correlation between VE1(BRAFp.V600E) expression, PD-1 and PD-L1 and clinical outcome in pediatric LCH.**

Keywords: Histiocytosis; Pediatric; Langerhans cell histiocytosis; PD-1; PD-L1; VE1; BRAF-V600E.

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Introduction. Langerhans cell histiocytosis (LCH) is a rare disorder characterized by the accumulation of CD1a+/CD207+ dendritic cells with an inflammatory infiltrate in many organs including bone, skin, lungs, liver, spleen, bone marrow, pituitary gland and the central nervous system (CNS).[1-4] LCH has a widely

variable clinical presentation ranging from single indolent lesions to severe multisystem (MS) disease. Over the past two decades, the survival of children with MS-LCH has improved to nearly 90%.[5] Nevertheless, there remains significant long-term morbidity in both high and low-risk patients, with late sequelae like diabetes insipidus (DI), anterior pituitary dysfunction and neurodegenerative disease (CNS-ND) being increasingly challenging to treat.[6]

A major breakthrough in the understanding of LCH pathogenesis came with the discovery[7] and validation[8] of recurrent *BRAF-V600E* mutations in over 50% of LCH lesions.[7,8] Subsequently, additional *MAPK* pathway gene mutations in *MAP2K1, ARAF, NRAS, KRAS* and in-frame deletions, fusions and duplications of *BRAF* have been reported in LCH.[9,10] Thus, LCH is now considered a myeloid neoplasm with a strong inflammatory component with a median of 8% cells as Langerhans cells in lesions and the remainder being inflammatory infiltrate.[11,12] These discoveries have provided scope for targeted therapy of LCH and other histiocytic disorders with *BRAF* and *MEK* inhibitors.[13, 14]

The programmed cell death-1 (PD-1) receptor and programmed cell death-ligand (PDL-1) immune checkpoint pathway has been implicated in the pathogenesis of different malignancies. Cancer-intrinsic inflammation is involved in cancer progression via recruitment and activation of inflammatory cells. The PD-1/PDL-1 pathway normally inhibits T-cell function thereby resulting in reduced activation and cytokine production by T cells. Tumor-associated T-cells and NK-cells secrete cytokines like IFN-γ which leads to increased PDL-1 expression on tumor cells.[15] Increased infiltration of regulatory T cells (Tregs) as well as PD-L1 expression on $CD207^{+}$ Langerhans cells have been previously reported in patients with LCH.[12,16,17] In addition, PD-1 blockade and targeted *MAPK* inhibition were found to be synergistic in a recent LCH mouse model.[18]

In the current study, we explored the expression and clinical correlations of PD-1/PD-L1 and VE1(BRAF p.V600E) mutant protein in archived pathology samples of 131 children with LCH.

Methods. We conducted an exploratory, single-centre retrospective study with chart and pathology review of all cases of LCH treated at the Hospital for Sick Children, Toronto from the year 2000 until 2018. The study was approved by our institutional ethics board. During the study period, 164 children were treated for LCH at our center; most of these were diagnosed locally while few were referred from other centers. Thirty-three patients were excluded from the study due to unavailable pathology samples, and the remaining 131 patients with available samples were enrolled. Biopsy sites in these cases were bone 69(52.7%), skin 35(26.7%), lymph node 6(12.2%), bone marrow 5(3.8%) and others 16(12.2%). Among the 131 cases, 22 and 20 were unable to be tested for VE1(BRAFp.V600E) and PD-1/PD-L1, respectively, due to either missing pathology samples (slides returned to outside referral centers), insufficient samples or tissue exhausted or degraded from decalcification. Therefore, 111 and 109 samples were successfully tested for PD-1/PD-L1 and VE1(BRAFp.V600E) mutant protein, respectively (**Figure 1**).

PD-1, PD-L1 and VE1(BRAFp.V600E) mutant proteins were tested using immunohistochemistry (IHC) and reported by 2 staff pathologists at our institution (BY-N, CH). VE1 antibody was used to detect BRAFp.V600E expression. The anti -V600 BRAF antibody immunostaining procedure was validated using tissues that is known to contain the BRAF

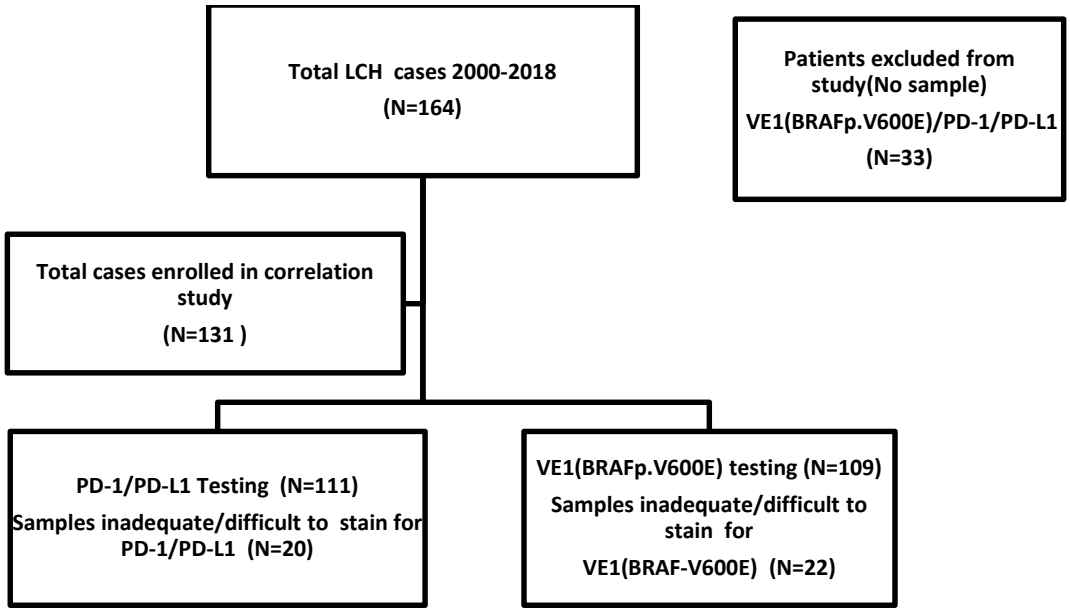

Figure 1: Study cohort for enrolment.

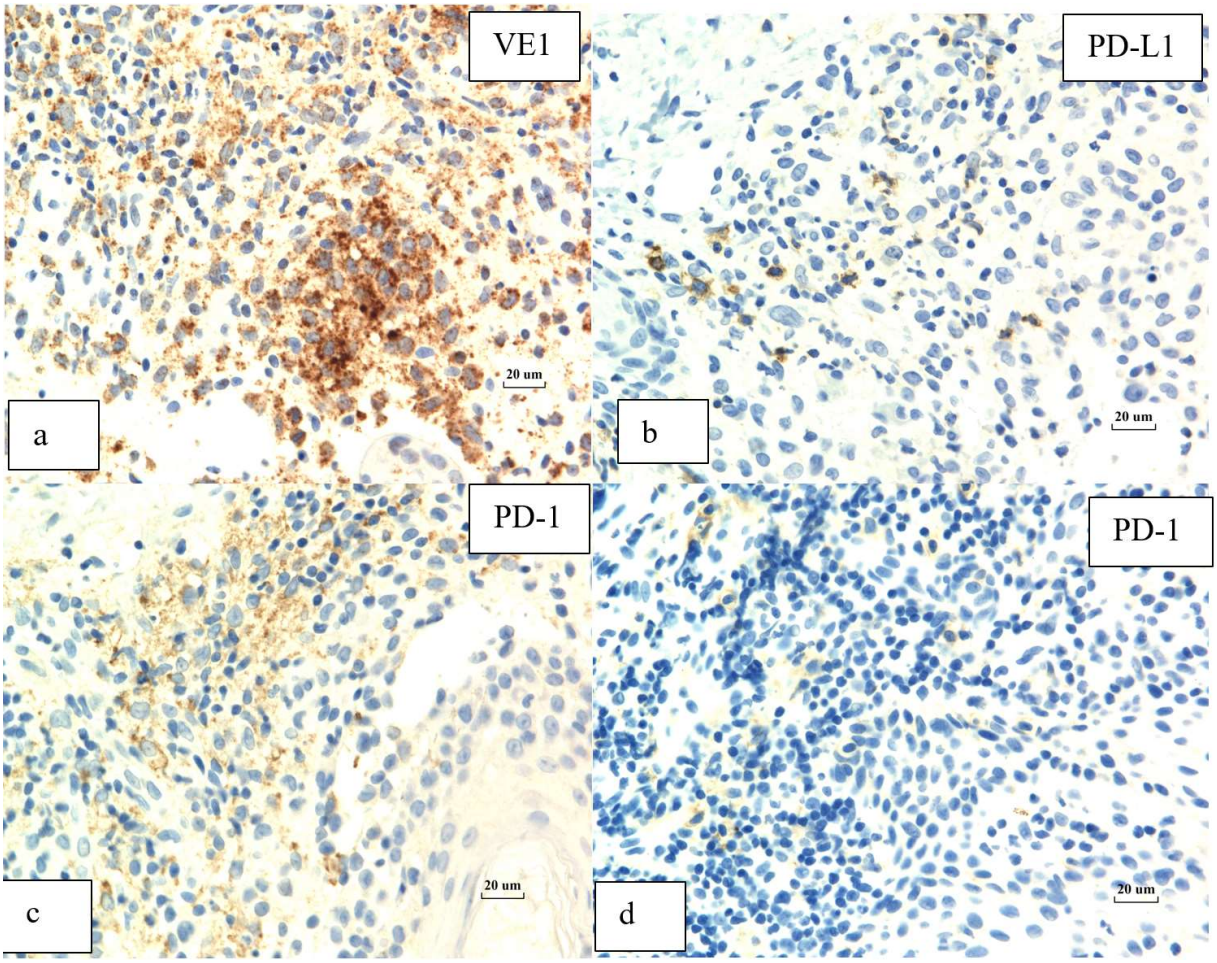

Figure 2. a: Skin biopsy from LCH patient shows strong (4+) immunostaining for VE1(BRAFp.V600E), as indicated by the positive brown color cytoplasmic stain reaction. The counter stain of the nuclei of the stained cell with hematoxylin shows the immune-positive cells have the classical nuclear features of Langerhans cells of LCH. **b:** Skin biopsy shows moderate to strong (3+ to 4+) immunostaining result for PD-L1, as indicated by the positive brown color cytoplasmic stain and some membranous reaction. The counter stain of the nuclei of the stained cell with hematoxylin shows the immune-positive cells have the classical nuclear features of Langerhans cell of LCH. **c:** Skin biopsy shows moderate (3+) immunostaining result for PD-1, as indicated by the positive brown color cytoplasmic stain and some membranous reaction. The counter stain of the nuclei of the stained cell with hematoxylin shows the immune-positive cells have nuclear features of medium size lymphocytes. Note Langerhans cell of LCH that have histiocytic nuclei with nuclear grooves are abundant in this area. **d:** This photomicrograph shows a peripheral area of the skin biopsy that contain only a sparse infiltrate of Langerhans cells of LCH as indicated by the presence of an occasional LCH cells that have folded nuclear contours that are typical of Langerhans cells. There are numerous PD1 positive lymphocytes in this field. In this study positive PD-L1 immunostain stained lymphocytes (indicated by the 2+ to 3+ brown color cytoplasmic and some membranous staining) were usually observed in the peripheral areas of the LCH lesion.

mutation. The percentages of PD-1^{+} infiltrating lymphocytes and PD-L1^{+}-Langerin^{+} tumour cells and staining intensity were evaluated for each sample. Staining intensity was scored considering 0 as negative or trace, 1 as weak, 2 as moderate and 3 as high. Similarly, to previous studies,[13,20] all cases with staining intensity ≥2+ in ≥5% of tumour cells were considered as positive.[13,20] To identify the significance of PD-1+ infiltrating lymphocytes, the presence of any stained PD-1 cells in a selected field were counted by the same pathologists. VE1(BRAFp.V600E) mutant protein was considered either positive or negative by IHC irrespective of staining intensity. Data recorded included patient demographics (age, sex), disease classification using the criteria defined by the Histiocyte society,[21] and treatment protocols. Disease was classified as single system (SS) or multi-system (MS) LCH. Risk organ (RO) involvement was used to classify MS LCH as high risk (RO+) or low risk disease (RO-). Response to treatment at weeks 6 and 12, disease reactivation and long-term sequelae were also captured. Slow early response (SER) at week 6 of therapy was defined as active disease intermediate or worse as per standard response criteria.

PD-1/PD-L1 and VE1(BRAFp.V600E) Immunostaining. Immunostains were performed on deparaffinised tissue slides (**Figure 2 a-d**). For PD-1 and PD-L1

immunostaining, an automated staining system from Dako Omnis (Agilent, Santa Clara, USA) was used. Slides were prepared and stained following procedure protocols and reagents from the supplier. Antibody staining reaction was detected using the Envision Flex detection kit from Dako. Antibody for PD-1 (Clone NAT105, applied at 1/75 dilution) was purchased from Cell Marque, Netherlands, (marketed by Cedarlane, Ontario, Canada). For PD-L1, antibody (Clone 26.6, applied at 1/500 dilution) was purchased from AbCam, Ontario, Canada. Immunostaining for VE1(BRAFp.V600E) was performed using the Ventana Benchmark XT automated staining system (Roche Diagnostics, USA). Staining conditions were used as recommended by the supplier and staining reaction was detected by using the Optiview Amplifier, supplied by Roche diagnostics. Antibody was purchased from Spring Bioscience, clone VE1 (via distributor AbCam, Ontario, Canada). 1/800 dilution of this antibody was used. Immunostain results were evaluated by light microscopy and images were captured with digital camera (Infinity 3) supplied with a calibration software supplied by Lumenera, Ontario Canada.

Statistical Analyses. Overall Survival (OS) was defined as survival from diagnosis time until last follow-up time, and Event Free Survival (EFS), being the primary outcome measure, was defined as absence of reactivations, late sequelae or death. Differences in the OS and EFS between the two groups with or without PD-1 or PD-L1 expression or VE1(BRAFp.V600E) expression were tested using Cox proportional hazards regression and the hazard ratios, 5-year survival rate, and p-values presented. For the categorical outcomes, RO+, disease reactivation, and slow early response (SER) differences were assessed using Fisher's exact test and the odds ratios and p-values presented. Results with a p-value <0.05 were considered significant.

Results.

Patient Characteristics. Among the 131 enrolled patients, the median age at diagnosis was 4 years (range,1.62-8) with a male: female ratio of 1.5:1. SS-LCH was diagnosed in 73% (n=95) patients and 27% (n=36) had MS disease. The majority of MS patients (64%) were RO+ (corresponding to 17% of the total cohort), of whom liver involvement was seen in 9, spleen in 10 and bone marrow in 4 patients. Bone was the commonest site (76.3%) followed by skin (32%). Slow early response at week 6 of therapy was seen in 26% and disease reactivation in 21.4% of patients; median time to reactivation was 1.68 years (range, 0.88-2.28 years). Detailed patient characteristics are shown in **Table 1**.

Treatment strategies. Over the 18-year study period, a number of different therapies were used upfront and for disease reactivation or refractory disease. The commonest were LCH II/III protocols as upfront therapy, and cladribine-cytarabine, vincristine-cytarabine, or clofarabine for reactivations and refractory disease. *BRAF* inhibitor (Dabrafenib) and/or MEK inhibitor (Trametinib) were used as salvage therapy in 5(3.8%) and 2(1.5%) patients, respectively.

Table 1. Patient characteristics at diagnosis and outcomes (N=131).

Characteristics	Total (%)
Gender	
Male	79 (60)
Female	52 (40)
Median Age, years/range	4 (1.6-8)
Disease involvement	
Bone	100 (76.3)
Skin	42 (32)
Lymph node	13 (9.9)
Bone marrow	4 (3.1)
Liver	9 (6.9)
Spleen	10 (7.63)
Lung	8 (6.1)
CNS (+Pituitary)	10 (7.63)
Gut	3 (2.3)
Special site Vertebra plana (18) Odontoid process (2)	20 (15.3)
Risk group (RO)	
Low risk	114 (87)
High risk	17 (13)
Disease stage	
SS	95 (73)
MS	36 (27)
Median follow-up, years/ range	5.34 (2.7-9.9)
5-year EFS	56% (95% CI 0.47-0.67)
5-year OS	96% (95%CI 0.92-0.96)
Late Sequelae	37 (28.2%)

Survival outcomes. The median follow-up duration of our cohort was 5.34 years (range, 2.76-9.93years). The 5-year EFS and OS were 56% (95% CI 0.47-0.67) and 96% (95%CI 0.92-0.96) respectively. The 5-year EFS was not statistically different between PD-1 positive compared to PD-1 negative tumours (47.7% vs.58.8%, p=0.33). Similar 5-year EFS rates were also seen in those who were PD-L1 positive compared to PD-L1 negative cases (50.5% vs.55.5%, p=0.61) (**Figure 3 a, b**). Further, there was no statistically significant difference in the 5-year OS and EFS of the VE1(BRAFp.V600E) negative compared to the VE1(BRAFp.V600E) positive patients with a hazard ratio of 2.3 (95% CI 0.24, 22.1, p=0.47), and 1.67 (95% 0.907, 3.057, p=0.10) respectively

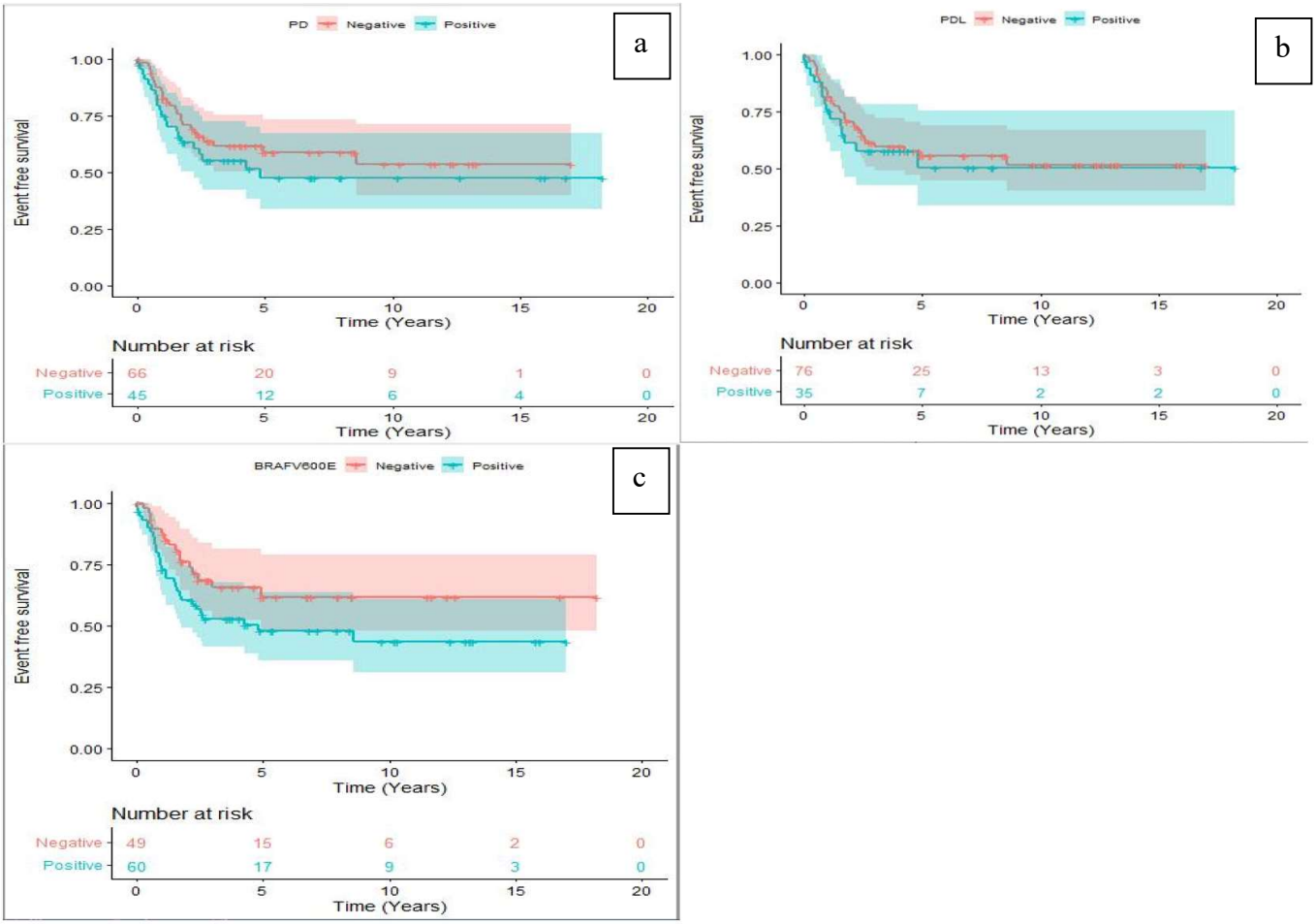

Figure 3. Kaplan Meir curves showing EFS of the cohort in relation to PD-1 (**a**), PD-L1 (**b**) and VE1(BRAFp.V600E) (**c**) positive and negative status. Cox Proportional model HR for EFS for PD-1 1.33 (0.746, 2.381), p>0.05, PD-L1 1.178 (0.627, 2.211), p>0.05 and BRAFp.V600E 1.66 (0.907, 3.057), p>0.05.

(**Figure 3c**).

PD-1/PD-L1 status and clinical correlations. Of the 111 samples successfully tested for PD-1/PD-L1; PD-1 positivity was seen in 45 (40.5%) and PD-L1 in 35 (31.53%) patients. There was no significant difference in the expression of PD-1/PDL-1 between bony and non-bony LCH cases. RO+ was not different in those who were PD-1 positive compared to those who were negative (15.6% vs. 13.6%, p>0.05). Similarly, RO+ did not correlate with PD-L1 positivity (17.1% vs 13.2%, p>0.05). Neither PD-1 nor PD-L1 positivity were correlated with disease response at week 6 of therapy (**Table 2**). Disease reactivation was seen in 31.1% and 28.6% of those who were PD-1^{+} and PD-L1^{+}, respectively, and was not significantly different compared to PD-1/PD-L1 negative patients.

VE1(BRAFp.V600E) status and clinical correlations. Of the total 109 patients tested for VE1(BRAFp.V600E), 60 (55%) were positive and 49 (45%) were negative. Among the VE1(BRAFp.V600E) + cases, 45% had MS LCH, and RO+ was significantly higher compared to the VE1(BRAFp.V600E) negative cases (23.3% vs.4.1%, p=0.0053; OR=7.207, 95% CI: 1.517-69.18) (**Table 2**). Cranio-facial bone involvement was seen in a large proportion of those who were VE1(BRAFp.V600E) positive 18/60 (30%). There was no statistically significant difference in the rates of SER at week 6 (31.7% vs 16.3%; p= 0.073; OR=2.357, 95% CI: 0.865-6.964) or disease reactivation (30% vs 16.3%; p>0.05; OR=2.181, 95% CI: 0.795-6.476) in the VE1(BRAFp.V600E) + vs. VE1(BRAFp.V600E) negative patients.

Late Sequelae and correlation with PD-1/PDL-1 and VE1(BRAFp.V600E) status. Late sequelae related to LCH were observed in 37(28.2%) patients, and were more frequent in those with MS-LCH and in the VE1(BRAFp.V600E) positive cases (36.7% vs.20.4%, p=0.0551). CNS-ND was seen in 7(19%) patients; median duration from LCH diagnosis to the onset of CNS -ND was 3 years (range, 0.1-14 years); 6 of the 7 (86%) had MS disease. CNS-ND was noted to be higher in those with VE1(BRAFp.V600E) (24% vs. 5.8%) (p=0.195) and PD-1 (18.8% vs. 12.5%) (p=1.0) positivity compared those who were negative. Endocrine complications noted were DI 11(29.7%),

Table 2. Disease characteristics of the cohort.

Characteristics	VE1 (BRAFp.V600E)+ (N=60/109)	VE1 (BRAFp.V600E)- (N=49/109)	P-value	PD-1+ (N=45/111)	PD-1- (N=66/111)	P-value	PD-L1+ (N=35/111)	PD-L1- (N=76/111)	P-value
SS	33 (55.0%)	43 (87.8%)	<0.001	27 (60.0%)	51 (77.3%)	0.059	21 (60.0%)	57 (75.0%)	0.122
MS	27 (45.0%)	6 (12.2%)		18 (40.0%)	15 (22.7%)		14 (40.0%)	19 (25.0%)	
RO+	14 (23.3%)	2 (4.1%)	0.005	7 (15.6%)	9 (13.6%)	0.789	6 (17.1%)	10 (13.2%)	0.572
Cranio-facial bone	18 (30.0%)	4 (8.2%)	0.007	12 (26.7%)	10 (15.2%)	0.152	10 (28.6%)	12 (15.8%)	0.130
SER at 6 weeks	19 (31.7%)	8 (16.3%)	0.078	11 (24.4%)	17 (25.8%)	>0.900	8 (22.9%)	20 (26.3%)	0.815
Disease reactivation	18 (30.0%)	8 (16.3%)	0.117	14 (31.1%)	12 (18.2%)	0.170	10 (28.6%)	16 (21.1%)	0.470
Late Sequelae	22 (36.7%)	10 (20.4%)	0.090	13 (28.9%)	19 (28.8%)	>0.900	9 (25.7%)	23 (30.3%)	0.659
Death	3 (5.0%)	1 (2.0%)	0.625	1 (2.2%)	3 (4.5%)	0.645	1 (2.9%)	3 (3.9%)	>0.900
5-year EFS	47.8%	61.7%		47.7%	58.8%		50.5%	55.5%	

short stature 5(13.5%) and delayed puberty 1(2.7%). Of those developing DI, 10(91%) had MS disease. VE1(BRAFp.V600E) + (24%, p=0.70) and PD-1+(25%, p=0.54) expression were associated with increased risk of DI. The median time for the onset of DI from LCH diagnosis was 1.6years (range, 0-4 years). Sclerosing cholangitis was noted in 6(16.2%) cases; 5 had MS disease, 5 were VE1(BRAFp.V600E) +, 3 were PD-L1+ and 4 subsequently underwent a liver transplant. Hearing loss was seen in 4(10.8%). Musculoskeletal complications were seen in a significant proportion of our patients 28(75.6%); scoliosis 1(2.7%), kyphosis 1(2.7%), facial asymmetry4(10.8%), and pathological fracture 4(10.8%). These tended to be more prevalent in patients who were VE1(BRAFp.V600E) +. Overall, there was no statistically significant correlation between the incidence of late sequelae and VE1(BRAFp.V600E) (p=0.0551) or PDL-1(p=0.708) positivity.

Discussion. The involvement of the PD-1/PDL-1 pathway in the pathogenesis of LCH is not well defined. We assessed the expression and prognostic impact of PD-1/PDL-1 molecules and VE1(BRAFp.V600E) protein, using IHC, in childhood LCH.

A recent study showed a PD-1 positivity in 5% to 20% and PDL-1 positivity in 5% of pulmonary LCH.[19] A study by Gatalica et al. demonstrated a PD-L1 positive rate of 88% in LCH samples, and showed that both PD-L1 and VE1(BRAFp.V600E) proteins co-localized to the same multinucleated Langerhans cell.[16] Another study detected PD-L1 expression in 20% of LCH cases (3/15), in 18% of Rosai-Dorfman cases (2/11) and in 50% of histiocytic sarcoma cases (7/14) .[20] Another report suggested that PD-1/PDL-1 pathway may have some role in the microenvironment and pathogenesis of bone LCH; however, the study had a small sample size of 6 patients and PD-1/PDL-1 positivity was quite low (16.6%).[21] In the current study, we observed 40.5% (45/111) positive rate for PD-1, and 31.5% (35/111) positive rate for PDL-1.

A recent report, including 97 children and adults with LCH, showed that *BRAF-V600E* mutation correlated with higher levels of PD-L1 expression, and that both proteins were independent prognostic factors of poor outcomes.[17] In addition, accumulating evidence shows that PD-L1 expression is frequently upregulated in tumours by activation of key oncogenic pathways such as the class A phosphoinositide 3-kinases *(PI3KCA)–AKT and RAS– RAF– MAPK* pathways.[20] This has therapeutic implications for LCH, especially in the MS/relapsed-refractory settings where conventional chemotherapy could lead to significant toxicity. An LCH mouse model showed a decrease in the size of LCH lesions with the use of anti-PD-1 monoclonal antibodies via reduction in the lymphoid component; further, combination therapy with a *MEK* inhibitor proved synergistic in reducing the size of the lesion as well as restored T-cell effector function.[18] Other studies have shown that *BRAF-V600E* expression results in immune suppression in melanoma and papillary thyroid carcinoma via expression of PD-L1 and forkhead box protein 3 (FOXP3), which translates into disruption of endogenous host immune surveillance and tumour immune escape.[16,22,23]

In the present study, there was no significant association between risk category, early disease response or late sequelae with PD-1/PD-L1 expression, and EFS in those who were PD-1 positive vs. PD-1 negative cases

(47.7% vs.58.8%, p>0.05). The reactivation rates in PD-1 positive (29%) and PD-L1 positive cases (25%) were not different from the overall cohort and were similar to those reported by Gadner *et al.* on the LCH-III trial.[5]

VE1(BRAFp.V600E) mutant protein expression was associated with a 30% reactivation rate, a 31.7% resistance to frontline therapy (**Table 2**), lower EFS in VE1(BRAFp.V600E)+ cases, 36.7% rate of late sequelae, and was more frequent in high-risk RO+ patients (p=0.0053). Our results are similar to the BRAFV600E mutated French cohort[24.]

CNS -ND occurred in a higher proportion of children VE1(BRAFp.V600E) positive vs. VE1(BRAFp. V600E) negative ones (24% vs. 5.8%), which is comparable to the published literature.[24-26] This could be related to migration of *BRAF*-V600E positive myeloid cells to particular regions of the brain via perivascular accumulation and parenchymal infiltration.[27] CNS-ND LCH has inferior outcomes and can have devastating sequelae in the long-term, affecting the clinical outcomes as well as quality of life of both patients and their families.[25-26] Previously, these were not reversible with chemotherapy,[28] but a recent report[29] suggested that *BRAF* inhibitor therapy may improve CNS-ND symptomatology. This has implications for prognosis and could warrant more aggressive follow-up of patients who are *BRAF-V600E* positive, as well as the potential to have a lower threshold for using targeted agents in such patients. Successful targeted therapy against *BRAF-V600E* mutation has been shown in patients with relapsed/refractory LCH across various case reports or series,[30, 31] including cases of CNS-ND.[14]

To the best of our knowledge, the present study is the largest one to analyse the clinical significance of PD-1/PD-L1 expression in a pediatric LCH cohort, mostly treated with LCH-III like protocols. Further, the long-term follow-up of 18 years allowed the capture of early and late reactivations as well as late sequelae. However, our study has few limitations. Firstly, although we have analysed a large cohort, this study is retrospective and there is a potential for selection bias. Secondly, 30% of the archived bone samples could not be tested, due to difficulties in IHC staining of bone biopsies for PD-1/PD-L1. Thirdly, only 17% of our patients were RO+, which could contribute to the lack of correlation between PD-1/PD-L1 expression with EFS and OS. Lastly, *BRAF-V600E* status was examined by IHC as opposed to genotyping, and IHC may not be as sensitive as genotyping in the detection of BRAFp.V600E; however, previous studies have shown a strong correlation between IHC and PCR testing of *BRAF*-V600E.[32]

Conclusions. Our study did not find a significant correlation between VE1(BRAFp.V600E) mutation, PD-1, PD-L1 expression and clinical outcomes in pediatric LCH. Thus, it remains to be determined whether checkpoint inhibitors with or without *MAPK* inhibition might be effective in high-risk patients with LCH, such as refractory or relapsed RO+ cases. The expression and prognostic impact of PD-1/PD-L1 should be explored in all types of pediatric LCH, including MS disease, in large prospective clinical trials.

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Author Contributions. All authors contributed to the study design, data collection and interpretation, writing and reviewing the final manuscript. Oussama Abla, Sneha Tandon, Bo Yee Ngan conceptualized the study and design, initial data collection, interpretation of data and critically revising the manuscript. Bo- Yee Ngan and Cynthia Hawkins provided, BRAFp.V600E(VE1), PDL-1 and PD-1 antibodies, reported the immunohistochemistry on samples, and critically reviewed the manuscript. Brooklyn Joyce did initial data collection and interpretation. Derek Stephens, Bryan McGuire helped with study design, data analysis and interpretation and critical review of the manuscript. James Whitlock and Sheila Weitzman made substantial contributions to study design and critical review of the manuscript for important intellectual content.

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