HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS CHARACTERIZATION: CASES FROM BRAZIL

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Introduction

- Hemophagocytic lymphohistiocytosis (HLH) is a rare and fatal childhood immunodeficiency characterized by a hyperinflammatory syndrome;
- It is a consequence of an exacerbated activation of lymphocytes/macrophages and deficient CD8 T lymphocytes / Natural Killer (NK) cells;
- May occur in two different forms: primary (or familiar) and secondary, with similar clinical profiles;
- Familiar HLH is associated with constitutive mutations in lymphocyte cytotoxicity related genes, affecting Perforin or intracellular vesicles transport;
- In this study, we track in our series of cases and identify a PRF1 mutation, present in subtype 2 of the familial HLH.

Objective

To establish a testing algorithm for differential diagnosis of HLH among serious childhood diseases that resemble hematological malignancies.

Methods

Patients: Children aged 0 to 5 years in suspicion of HLH (with at least two of the eight criteria of HLH diagnosis (HENTER, 2007), sent to our laboratory between 2004 and 2019. Exclusion criteria: diagnosis of acute leukemia, aplastic anemia or other hematological disorders.

Molecular characterization: Genomic DNA was extracted through salting-out method from slides, peripheral blood or bone marrow samples. Exons 2 and 3 were amplified by PCR in the thermal cycler GeneAMP PCR System 9700 (Thermo Fisher). Mutations were detected by Sanger sequencing in 3500 Genetic Analyser (Applied Biosystems) and analyzed in Mutation Surveyor v3.00 software (MOLLERAN LEE et al., 2004).

Immunophenotypic characterization: 6 patients were evaluated for leukocyte distribution, through the antibodies: anti-CD3, anti-CD4, anti-CD5, anti-CD8, anti-CD14, anti-CD16, anti-CD19,anti-CD20,anti-CD33,anti-CD56,anti-HLA-DR,andanti-Perforin. Staining was performed on peripheral blood samples collected in EDTA according to previously described (NORONHA et al, 2016).
Monoclonal antibodies were purchased from BD Biosciences and EXBIO. Samples were acquired at the FACSCanto II flow cytometer (BD Biosciences).

NK cells Degranulation Assay: Mononuclear cells were separated by Ficoll-Paque ™ PLUS (GE Healthcare). Cells from patients or healthy control ($2x10^5$) were incubated for 2 hours with K562 ($2x10^5$) in each well. After washing, they were labeled with anti-CD3, anti-CD56, anti-CD19, and anti-CD107a antibodies, according to Bryceson et al (2010), and acquired atthe FACSCanto II flow cytometer (BD Biosciences). Monoclonal antibodies were purchas ed from BD Biosciences.

Results

***PRF1* gene screening:** Thiyty-five patients were selected, being, one with HLH confirmed diagnosis and 34 cases with at least two variables of HLH diagnosis criteria. The majority of children were older than 12 months (74,2%), male (62%) and from northeast region (71,4%). Clinically, the most common hematological findings were pancytopenia with marked platelets low count, followed by hepatosplenomegaly (Table 1).
The genotyping screening identified one patient (frequency of 3%) with a PRF1 mutation associated with HLH. This mutation, identified in codon 272 consists of a cytosine replace for thymine (c.272 C>T) alredy described in the literature as rs35947132 (HOUSE et al., 2015). It is a missense single nuceic variant (SNV) and confers a substitution of alanine for valine at aminoacid 91 (Figure 1).

Table1: Clinical and demographic characteristics of patients screened for the PRF1 gene

Characteristics	N (%) 35 (100)
Age (months)	
< 6	4 (11,4)
6 ~ 12	5 (14,2)
> 12	26 (74,2)
Gender	
Male	22 (62,8)
Female	13 (37,1)
WBC (x10⁹/L)	
< 5	15 (42,8)
5 ~ 10	7 (20)
≥10	12 (34,2)
Hemoglobin	
< 9	4 (11,4)
≥ 9	30 (85,7)
Platelets	
< 100000	25 (71,4)
≥ 100000	9 (25,7)
Hepatosplenomegaly	
Yes	22 (62,8)
No	13 (37,1)
Region	
Northeast	25 (71,4)
Midwest	7 (20)
Southeast	2 (5,7)
South	1 (2,8)

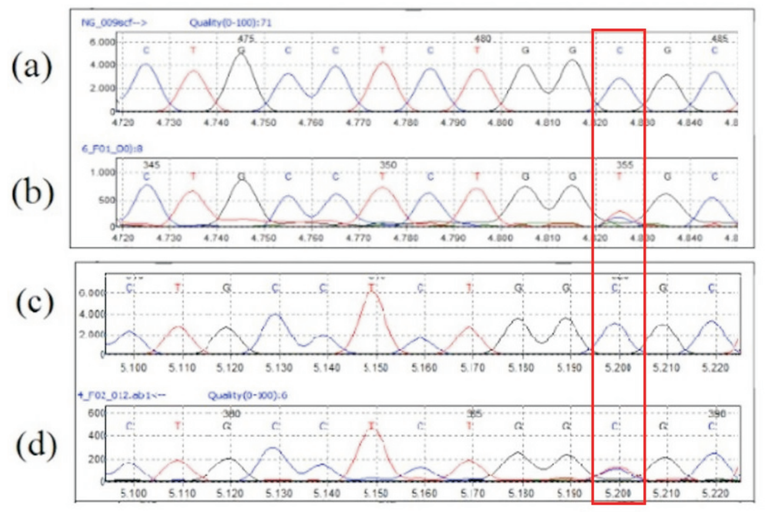

Figure 1: Sanger sequencing of exon 2 in the PRF1 gene, presenting the alteration rs35947132, conferring c.272 C>T and A91V. (a) Reference forward sequence. (b) Patient 1 forward sequence. (c) Reference reverse sequence. (d) Patient 1 reverse sequence.

Imunophenotyping and NK Functional assay: six patients were evaluated, 5 had reduced Perforin expression in NK cells and 4 had reduced Perforin expression in CD8+Tcells (Table 2). Patient P1 presented reduced expression of Perforin in CD8 + and NK T cells, with normal expression of the other cell populations. P2 has low expression of Perforin in CD8+ and NK T cells and normal ranulation capacity compared to healhy control. P3 has a majority of lymphocyte population. He also presented higher percentage of B lymphocyte population, and lower Nk5 population compared to the other patients. There was a reduction in pern expression in NK cells, but with normal expression in CD8+ T lymphocytes. P4 had normal expression of Perforin in CD8+ T lymphocytes and low in NK, with reduced NK degranulation capacity. P6 showed reduced perforin expression in CD8+ T lynphocytes and NK cells, with normal NK degranulation activity compared to healthy control.

Table 2: HLH patients evaluated concerning leukocyte profile, perforin and CD107a expression

	P1	P2	P3	P4	P5	P6
Total lymphocytes	29	NP	56,9	10,5	45	3,4
T lymphocytes (CD3 +)	58	0,9	55,4	52,5	72,1	68,2
CD4+	58,2	NP	50,2	34	24,2	52,8
CD8+	37,3	NP	41,9	57,4	66,1	43
CD4+CD8+	0,4	NP	0,3	2,3	1,1	0,1
CD4-CD8-	3,4	NP	7,6	5,3	8,6	3,9
CD8+ Perforin+	1,5	0,9	61,1	23,9	0,2	1,8
Total NK	17,9	NP	11	13,2	14,4	1
NK1	0,6	NP	7,8	2,9	0,7	NP
NK2	0,4	NP	2,1	0,4	1,1	NP
NK3	2,4	NP	4,6	9,6	7,0	NP
NK4	56,1	NP	78	40,2	60,5	NP
NK5	40,4	NP	7,5	46,9	30,7	NP
NK Perforin+	29,8	64,5	84,7	43,4	0,9	55,6
NK CD107a+	NP	25	NP	7,1	NP	19
B lymphocytes	7,8	NP	34,8	2,2	11,8	29,5
Monocytes	1,7	NP	6	1,5	3,2	0,4
Granulocytes	41,1	NP	23,8	68,2	20,3	53
Eosinophils	2,2	NP	Absent	0,4	0,2	Absent

Partial Conclusion

We were able to identify a *PRF1* mutation in a patient included in our series of cases. We also identified patients with reduced Perforin expression in CD8+ T lymphocytes and NK cells, as well as reduced NK degranulation capacity.

Perspectives

To analyse the patients with reduced perforin expression or NK degranulation regarding *PRF1* mutations;
To study the other molecular alterations in genes *UNC13D, STX11* and *STXBP2*.

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Projeto Gráfico: Área de Edição e Produção de Materiais Técnico-Científicos / Seitec / INCA