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Original Article

Unusual central nervous system (CNS) Herpesvirus-6 infection in a patient with ZAP-70 deficiency due to a homozygous mutation

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ABSTRACT

ZAP-70 deficiency constitutes an autosomal recessive inherited form of T+B+NK+ Combined Immunodeficiency (CID). Clinical features consistent with severe CID (SCID) could be present. Absence of CD8+T cells but normal number of non-functional CD4+T cells are phenotypic characteristics. After primary infection, human Herpesvirus-6 (HHV-6) induces a lifelong latent infection in humans. Even though it is a neurotropic virus encephalomyelitis is an uncommon clinical manifestation in immunocompetent individuals. We describe a 11 month-old girl with a ZAP-70 deficiency, presented as a SCID. The patient presented T lymphopenia, extremely low CD8+T-cells (0.25%-6/mm^3) and low naïve CD4+CD45RA+ (18%) lymphocytes. Zap-70 protein expression was undetectable by flow cytometry. Mutational analysis by Sanger sequencing identified a homozygous frameshift variant (NM_001079 c.1510_1522 delAAGTGGTACGCAC) in *ZAP-70* gene affecting the kinase domain, confirming the diagnosis. The patient developed in the course of her illness many meningeal signs that required several brain image studies and cerebral spinal fluid (CSF) punctures to reach the diagnosis of encephalopathy due to a HHV-6 infection. To sum up, we present an unusual Zap-70-deficient SCID patient who developed a life threatening CNS HHV-6 infection (seizures and progressive deterioration of the sensorium). HHV-6 is a certain possibility of severe CNS infection in primary immunodeficiencies (PID) and should be searched thoroughly, especially when neurological manifestations are present.

KEYWORDS: ZAP-70, failure to thrive, combined immunodeficiency, HHV-6, encephalitis.

1. INTRODUCTION

Herein we report an infant with a ZAP-70 deficiency and human Herpesvirus-6 (HHV-6) encephalitis. Loss of expression or function of ZAP-70 (zeta-associated protein of 70 kDa) in humans leads to an autosomal recessive inherited form of T+B+NK+ Combined Immunodeficiency (CID). Patients characteristically present absence of CD8+T cells but normal number of non-functional CD4+T cells. Eventually, they may show clinical features consistent with severe CID (SCID) such as opportunistic, life-threatening infections and, sometimes, autoimmunity or malignancy. Patients are usually affected by infiltrative erythematous skin lesions on the face and extremities. Hematopoietic stem cell

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transplantation (HSCT) is the only curative treatment for this defect [1].

ZAP-70 is a non-receptor tyrosine kinase normally expressed in T and NK cells. Structurally, it consists of two SH2 (Src homology-2) domains separated by an interdomain-A and a carboxy-terminal kinase domain connected by an interdomain-B. Functionally, it is required for T cell receptor (TCR) signaling, and plays a critical role in development and activation of T cells (Figure 1c).

Following TCR engagement, ZAP-70 becomes associated with the CD3 complex through the SH2 domains and is phosphorylated by CD4-associated Lck. Active ZAP-70 phosphorylates the downstream scaffold proteins LAT (linker for the activation of T cells) and SLP-76 (SH2-leukocyte phosphoprotein of 76 kDA) that, in its turn recruit other molecules, lead to T cells activation. ZAP-70 is indispensable for differentiation into CD8+T cells. Most ZAP-70 deficient patients carry homozygous biallelic mutations located in the kinase domain that abolished protein expression and result in a SCID phenotype [1, 2].

HHV-6, consisting of two HHV-6A and HHV-6B related species, is a beta-herpesvirus that exhibits a wide cell tropism and induces a lifelong latent infection in humans. Primary infection with HHV-6B generally occurs within the first 2 years of life. Salivary gland acts as the main virus reservoir and latency is established in monocytes/macrophages as well as in brain tissue [3]. Although HHV-6 is a neurotropic virus, encephalomyelitis is an uncommon clinical manifestation in immuno-competent individuals. In immunocompromised hosts, such as human immunodeficiency virus (HIV) positive patients or transplant recipients, virus reactivation may be the cause of serious complications such as encephalitis [4].

2. METHODS

2.1. Flow cytometry (FC)

2.1.1. Phenotypic analysis of lymphocyte populations

Whole blood aliquots from the patient were stained by four color FC standard methods using fluorochrome-conjugated monoclonal antibodies, from Becton Dickinson (BD), San Jose, CA, USA, and Immunotech from Beckman Coulter, Marseille France. Samples were acquired in a FACSCanto II cytometer and analyzed by FACS Diva software, all from BD.

2.1.2. ZAP-70 expression

Cytoplasmic ZAP-70 expression was evaluated in the patient and a healthy control with the anti ZAP-70 Clon 1E7.2 PE Cytognos # CYT-ZAP703 using a previously described optimized staining procedure. Briefly, aliquots of peripheral blood were first incubated at room temperature with anti-CD3 PercCP Cy5.5, anti-CD19 PE-Cy7, anti-CD56 APC and anti-CD45V500-conjugated antibodies (Becton Dickinson). Fixation and permeabilization was performed with Intrastain (DAKO #Nr. K2311). Cells were incubated for 30 min at room temperature with anti-ZAP-70 antibody. Absence of ZAP-70 expression on B-cells is used as an internal negative control while normal expression in T cells and NK cells from healthy control is referenced as positive control.

2.2. Lymphocyte functional assay

Lymphocyte proliferation was assessed as previously described on cultured PBMC using the [^{3}H] thymidine (Amersham International, Buckinghamshire, UK) incorporation method. Briefly, 2 x 10^5 cells PBMC per well were incubated at 37 ºC in 5% CO_2 atmosphere with optimal concentrations of the indicated mitogens for 3 days: phytohemagglutinin (PHA) at 1/50 final dilution (GIBCO), anti-CD3 at 3.5 mg/ml (OKT3, Orthoclone), Staphylococcus enterotoxin B (SEB) at 1 mg/ml, PMA at 0.01 mg/ml (Sigma) and Ionomicine (Io) at 1 mg/ml (Sigma). PBMC were also incubated with specific antigens for 6 days: Purified Protein Derivative (PPD, Malbrán Institute, Buenos Aires, Argentina) 2 mg/ml of 1/50 final dilution and tetanus toxoid (TT) (Statens Serum Institute) 944 Lf/ml of 1/50 final dilution.

2.3. Molecular studies

ZAP-70 coding exons and adjacent splicing sites were PCR-amplified and sequenced by the Sanger technique.

3. PATIENT PRESENTATION AND RESULTS

The index case was the 2nd child born to self-reported non-consanguineous parents sharing a common Argentine aboriginal ethnic ancestry. Her elder sister was healthy. History of PID was not documented in her family except for a second cousin who had been diagnosed with GATA2 haploinsufficiency. The patient was first seen at our hospital at the age of 13 months. She had no history of perinatal disease and received a complete immunization schedule without any complications, including anti-BCG vaccine. At birth, her head circumference was within normal levels at -1SD, as were her weight and height at the 50th and 10th percentiles, respectively. At the age of 4 months failure to thrive was detected; thrush and recurrent multifocal pneumonias were diagnosed at 6 and 7 months, both requiring hospitalization. At 10-months she suffered an episode of sepsis with respiratory origin. Bronchoalveolar lavage was positive for *Stenotrophomonas maltophilia* and anti-Chlamydia antibodies were detected. While septic she developed neutropenia, hepatomegaly with elevated transaminases that normalized with the episode resolution. She was referred to our institution at 13-months of age. Growth retardation was patent, and dysmorphic features were also documented: microcephaly (-6SD), hirsute forehead, pointed fontanel, spread ears with risen lobe and retrognathia.

A first, laboratory evaluation (Table 1) showed T cell lymphopenia with virtually absence of CD8+T cells (0.25%-6/mm^3) (Figure 1a) and a marked reduction of the CD45RA+CD4+T naïve compartment. Hypogammaglobulinemia (all isotypes) was detected together with variable B cell counts (from low to normal) but with complete absence of the memory subset and accumulation of immature transitional B cells (CD10+). *In vitro* lymphocyte proliferative responses to both mitogens and recall antigens were absent except for PMA/Io. Thus, SCID diagnosis was suspected and the typical phenotypic findings led us to evaluate ZAP-70 expression by flow cytometry. None of the patient's lymphocyte subsets expressed the ZAP-70 protein (Figure 1b). Mutational analysis by Sanger sequencing identified a homozygous frameshift variant (NM_001079 c.1510_1522 delAAGTGGTACGCAC) in ZAP-70 gene affecting the kinase domain, confirming the diagnosis (Figure 1c). This was a previously reported deleterious mutation and both parents were heterozygous for the same change, strongly suggesting identity by descent. A search for a matched unrelated donor was initiated in order to schedule a HSCT.

During follow up she presented with erythematous and pruritic micropapular exanthema in face, neck and extremities and mycotic intertrigo, but no cultures were obtained. She also developed fever, seizures and subsequently a status epilepticus that required hospitalization. Brain computed tomography (CT) without contrast was initially reported as normal and 48 hours later a minimal enlargement of the cortical subarachnoid area was evidenced.

A first, cerebral spinal fluid (CSF) evaluation showed a normal cytochemical profile and negative microbiological cultures for common bacteria, meningococci, fungi and mycobacteria. In addition, PCR virus scanning for enteroviruses, Epstein-Barr virus and JC virus were also negative. A combined antimicrobial therapy was administered including meropenem, vancomycin, amphotericin-B, Sulfamethoxazole-trimethoprim, acyclovir and anti-tuberculosis drugs. In parallel, IVIg supplementation was started. Initially, her mental status was unaffected; however within a seven days period, sensory alterations became apparent with hipo-reactivity and difficult to control fevers. Finally the patient deteriorated due to apnea and required admission in the intensive care unit.

A second CSF sample was evaluated 48 hours later to expand the virus search. In parallel, a brain Magnetic Resonance Imaging (MRI) with contrast showed thickening and reinforcement of leptomeninges, compromising the anterior Sylvian cistern and the inferior frontal cortical arachnoid space, consistent with an inflammatory infectious process (Figure 1d). CSF analysis showed abnormal glucose (26 mg%) and protein (225.6 mg%) levels, plus abundant leukocytes (231/mm^3 with a 70/30 neutrophils/lymphocytes rate). A positive HHV-6 PCR result was reported and ganciclovir treatment was initiated. Despite the antiviral and

Table 1. Laboratory evaluations at 11 and 13 months of age.

		Units	Patient's age (months)		References values
			11	13	
Serum immunoglobulins					
	IgG	(mg/dl)	123 (*744)	295	442 - 890
	IgM	(mg/dl)	<6.6	6.4	27 - 77
	IgA	(mg/dl)	38	34	19 - 55
	IgE	(UI/ml)	<1UI	nd	Up to 119[$]
Antibody response					
	TT	UI/ml	negative	nd	<0.02 UI/ml
	HBs	UI/ml	negative	nd	<10 UI/ml
Lymphocytes		(/mm^3)	2240	3326	1700-6900
	T cells	%(/mm^3)	63 (1411)	42 (1396)	59.0 - 78.0[#] (3668 - 4760)
CD3	CD4	%(/mm^3)	61.5 (1378)	41.3 (1373)	33.0 - 45.0[#] (1741 - 3402)
	CD8	%(/mm^3)	**0.25 (6)**	**0.4 (133)**	18.5 - 20.8[#] (810 - 1351)
	HLA-DR	%	12	5	9.7 - 20.6[#]
	TCR αβ	%	nd	41.6	50.0 - 75.0[#]
	TCR λδ	%	nd	0.4	2.0 - 3.0[#]
CD4	CD45RA	%	nd	18	65.0 - 80.6[#]
	CD45RO	%	60	88	9.7 - 20.6[#]
	B cells	%(/mm^3)	14 (314)	31 (1031)	15.0 - 30.0[#] (900 - 1540)
CD19	CD27(+)	%	0	ND	12.5 - 32.3
	IgD	%	96	ND	5.4 - 23.0
	IgM	%	99	ND	70.4 - 90.3
	IgG	%	0	ND	4.8 - 14.2
	CD10	%	54	ND	5.4 - 23.0
	NK cells	%(/mm^3)	23 (515)	27 (898)	6.0 - 14.0[#] (336 - 860)
Lymphocyte proliferations [^{3}H]T assay					
Mitogens	Medium	cpm	nd	3370	150 - 5851
	PHA	cpm	nd	1885	86235 - 188129
	OKT3	cpm	nd	1774	85038 - 176294
	PMA/Io	cpm	nd	28424	13.060 - 71254
	SEB	cpm	nd	2988	10191 - 56558
Antigens	Medium	cpm	nd	1156	Up to 5000
	PPD	cpm	nd	869	13080 - 56558
	TT	cpm	nd	4586	15190 - 56558

*Gammaglobulin supplemented, [#]age matched % p25-75, [$]mean ± 1 SD.

TT: Tetanous Toxoide; HBs: surface Hepatitis B.

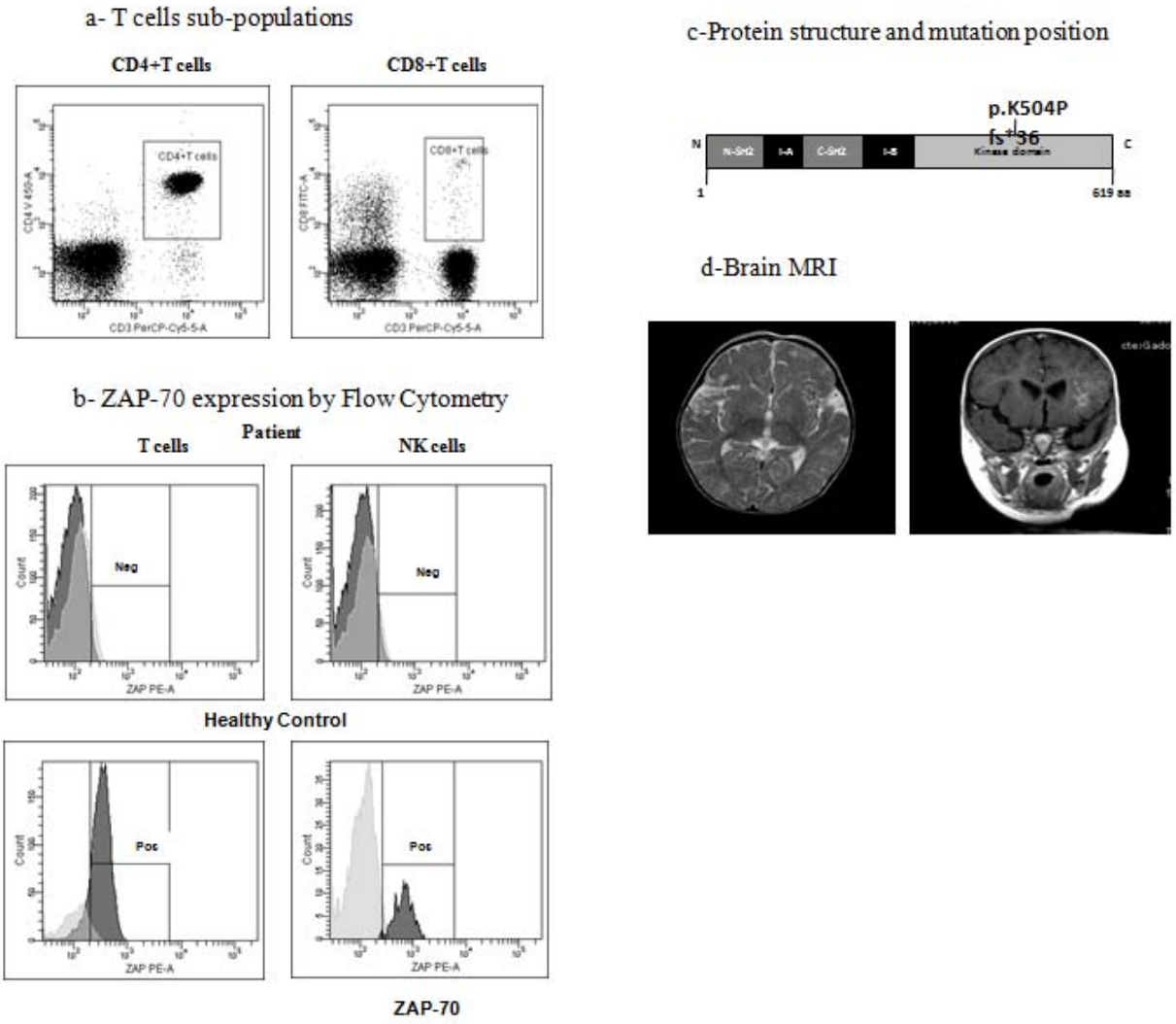

Figure 1. a) T cell sub-populations showing typically low CD8+T cells. **b**) ZAP-70 expression among lymphocyte subsets in the patient and a healthy control: ZAP-70 expression analyzed by flow cytometry in T and NK cells (Black histogram). Lack of ZAP-70 expression in normal B cells is used as an internal negative control (Grey histogram). **c)** Schematic ZAP-70 protein structure and mutation (p.K504Pfs*36) position at the kinase domain. **d)** Brain MRI with gadolinium contrast showing leptomeningeal thickening enhancement affecting the anterior Sylvian cistern and the inferior frontal cortical arachnoid space.

support treatment, the patient did not improve and died 10 days later in the intensive care unit.

A brain CT scan performed just before her death showed hypodensity of the encephalic parenchyma with loss of differentiation between white and gray matter.

To summarize our findings, we identified a 13-month-old patient with a deleterious 13 bp deletion in the ZAP-70 kinase coding domain homozygosity. This mutation completely abrogates protein expression, thus accounting for ZAP-70 deficiency, clinically presenting as SCID. Even though CD4+T cells were moderately low, most of them were CD45RA negative and nonfunctional, assessed through both mitogen and antigen stimulus response. A pronounced reduction of CD8+T cells was the starting hint for the initial suspicion of a ZAP-70 defect, finally confirmed by absence of the protein by flow cytometry and mutational analysis. The patient developed multiple episodes of pneumonia requiring hospitalization

in her first months of life and a lethal CNS-HHV-6 infection documented as her cause of death.

4. DISCUSSION

HHV-6 primary infection usually happens with waning of maternal protective antibodies. However, vertical transmission has been described in up to 1 to 2% of all births. This could arise from either a germline transmission of HHV-6 DNA integrated into the chromosomes' subtelomeric region (ciHHV-6) or transplacental passage of the virus reactivated in the mother [4]. Chromosomally integrated HHV6 was not tested in our patient and even though a vertical transmission was a possibility, the absence of typical cerebral calcification or CNS manifestations make it less likely. In immunocompetent children, HHV-6-associated clinical complications, when present, are malaise, gastrointestinal and respiratory tract infections as well as tympanitis. More severe complications associated with neurological disease, such as meningoencephalitis, have been reported but are infrequent and account for the virus neurotropism. Infections of the central nervous system may happen during primary infection or following reactivation from a latent state.

In immunocompromised individuals, reactivation of infection is markedly more common. Particularly, 50% of patients receiving HSCT reactivate HHV-6 infection but, even when the risk of HHV-6 encephalitis is elevated, the presence of neurological complication is uncommon [4]. Regarding PID, very few publications reported cases describing life-threatening HHV-6 illness and even less with encephalopathy. In more detail, five publications describe 6 PID patients with HHV-6 infections [5-9]. In two cases the virus was involved in pulmonary affectation: a 31 years old patient with a non-genetically characterized hypogamma-globulinemia [5] and a 2-years-old patient with CD3-gamma chain deficiency [6]. In other two, detection of ciHHV-6 was associated to hemophagocytic lymphohistiocytosis (HLH): a 2-month-old child with X-linked gamma chain [7] defect, where the reactivation of a ciHHV-6A was assumed to be associated to HLH and thrombotic microangiopathy, and a 7-years-old boy with an X-linked lymphoproliferative type-2 (XIAP deficiency) [8]. The other two cases indeed presented with a severe HHV-6 neurological affectation. One of them is a 5-month-old girl with a non-genetically characterized CID who initially presented with seizures that progressed to a status epilepticus and finally developed encephalitis with a post-mortem confirmation of a severe brain and spinal cord tissue destruction [6]. The other one, a 17-month-old boy with MHC class II deficiency that manifested meningitis, ankle clonus and hyperreflexia previous to transplantation in whom HHV-6 infection was detected by PCR in a brain tissue examination. This case is one-out-of-8 patients identified with CNS of different viral etiology within a total of 71 SCID patients that were scheduled for receiving HSCT [9]. Particularly, in a recent study describing clinical and immunological aspects on 49 patients with ZAP-70 deficiency, among infective complications, viruses were the most common agents. Only one of these patients suffered from virus encephalitis (varicella-zoster virus) [1].

5. CONCLUSION

Altogether, this data highlights how infrequent HHV-6-associated infection is detected overall in PIDs and even in its most severe clinical form, SCID. Moreover, to our knowledge, our patient would be the first associated with ZAP-70 deficiency. Furthermore, this case evidences that, among the wide spectrum of viruses that may be present in patients with T cell deficiencies, HHV-6 is a certain possibility and should be searched thoroughly, especially when neurological manifestations are present. Noteworthy, we could not certainly establish if the CNS illness in this case was due to primary or reactivation of HHV-6 infection.

The aim of this report is also to create awareness, considering that an early certification of this virus infection associated with CNS compromise or not, would lead to an earlier, more specific antiviral treatment with either foscarnet or ganciclovir.

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CONFLICT OF INTEREST STATEMENT

We declare no conflict of interest.

ABBREVIATIONS

ZAP-70	:	zeta-associated protein of 70 kDa
HHV-6	:	human Herpesvirus-6
CID	:	Combined Immunodeficiency
SCID	:	Severe Combined Immunodeficiency
SH2	:	Src homology-2
HSCT	:	Hematopoietic stem cell transplantation
ciHHV-6	:	chromosomes' subtelomeric region
CSF	:	cerebral spinal fluid
CT	:	computed tomography
MRI	:	Magnetic Resonance Imaging

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