Diagnosing XLP1 in patients with hemophagocytic lymphohistiocytosis

Raffaella Meazza, PhD,[a] Claudia Tuberosa, PhD,[a,b] Valentina Cetica, PhD,[c,d] Michela Falco, PhD,[e] Silvia Parolini, PhD,[f] Sam Grieve, PhD,[g] Gillian M. Griffiths, PhD,[g] Elena Sieni, MD,[c] Stefania Marcenaro, PhD,[e] Concetta Micalizzi, MD,[e] Davide Montin, MD,[h] Franca Fagioli, MD,[i] Alessandro Moretta, MD,[b] Maria C. Mingari, PhD,[a,b] Lorenzo Moretta, MD,[e] Luigi D. Notarangelo, MD,[j] Cristina Bottino, MD,[b,e] Maurizio Aricò, MD,[c,d] and Daniela Pende, PhD[a] *Genoa, Florence, Brescia, and Turin, Italy, Cambridge, United Kingdom, and Boston, Mass*

Background: Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening, heterogeneous, hyperinflammmatory disorder. Prompt identification of inherited forms resulting from mutation in genes involved in cellular cytotoxicity can be crucial. X-linked lymphoproliferative disease 1 (XLP1), due to mutations in *SH2D1A* (Xq25) encoding signaling lymphocyte activation molecule–associated protein (SAP), may present with HLH. Defective SAP induces paradoxical inhibitory function of the 2B4 coreceptor and impaired natural killer (NK) (and T) cell response against EBV-infected cells.

Objective: To characterize a cohort of patients with HLH and XLP1 for SAP expression and 2B4 function in lymphocytes, proposing a rapid diagnostic screening to direct mutation analysis.

Methods: We set up rapid assays for 2B4 function (degranulation or ^{51}Cr-release) to be combined with intracellular SAP expression in peripheral blood NK cells. We studied 12 patients with confirmed mutation in *SH2D1A* and some family members.

Results: The combined phenotypic/functional assays allowed efficient and complete diagnostic evaluation of all patients with XLP1, thus directing mutation analysis and treatment. Nine cases were SAP^-, 2 expressed SAP with mean relative fluorescence intensity values below the range of healthy controls (SAP^{dull}), and 1, carrying the R55L mutation, was SAP^+. NK cells from all patients showed inhibitory 2B4 function and defective killing of B-EBV cells. Carriers with *SH2D1A* mutations abolishing SAP expression and low percentage of SAP^+ cells showed neutral 2B4 function at the polyclonal NK cell level. Three novel *SH2D1A* mutations have been identified.

Conclusions: Study of SAP expression is specific but may have insufficient sensitivity for screening XLP1 as a single tool. Combination with 2B4 functional assay allows identification of all cases. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *HLH, XLP1, SAP expression, 2B4 function, NK cells*

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal hyperinflammatory syndrome triggered by common pathogens and characterized by excessive activation of macrophages and T and natural killer (NK) cells.[1,2] In a proportion of cases, defined as "primary" or "familial" hemophagocytic lymphohistiocytosis (FHL), this results from constitutional defects involving cellular cytotoxicity; although it is usually diagnosed in children, later onset is increasingly reported, including rare cases of adult patients.[3] Among the inherited forms, different genetic subtypes have been identified so far: FHL types 2-5, Griscelli syndrome type 2, Chediak-Higashi syndrome, and Hermansky-Pudlak syndrome, caused by mutations in genes (*PRF1*, *UNC13D*, *STX11*, *STXBP2, RAB27A*, *LYST*, and *AP3B1*, respectively) encoding proteins required for lymphocyte cytotoxicity.[2,4] Furthermore, patients with X-linked lymphoproliferative disease (XLP) may present with HLH. XLP2 results from mutations in the gene encoding the X-linked inhibitor of apoptosis (XIAP). XLP1 (Duncan disease, OMIM#308240) is a rare congenital immunodeficiency caused by mutations in *SH2D1A* (Xq25), the gene encoding the signaling lymphocyte activation molecule–associated protein (SAP).[5,6] The estimated incidence of XLP1 is 1 to 3 per million in males. In 1978, an XLP registry was established[7] and as of the year 2000, 309 males with XLP phenotypes from 89 unrelated families had been registered.[8]

SAP is a cytoplasmic adaptor protein that is recruited by members of the signaling lymphocyte activation molecule family, among which is 2B4 (CD244), a coreceptor surface molecule

From [a]Istituto di Ricovero e Cura a Carattere Scientifico Azienda Ospedaliera Universitaria San Martino-Istituto Nazionale per la Ricerca sul Cancro, Genoa; [b]Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, Genoa; [c]Dipartimento Oncoematologia Pediatrica, Azienda Ospedaliero-Universitaria Meyer, Florence; [d]Pediatric Oncology Network, Istituto Toscano Tumori (I.T.T.), Florence; [e]Istituto Giannina Gaslini, Genoa; [f]Dipartimento di Medicina Molecolare e Traslazionale, Università degli Studi di Brescia, Brescia; [g]the Department of Medicine, Cambridge Institute for Medical Research, Cambridge; [h]Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università degli Studi di Torino, Turin; [i]Oncoematologia Pediatrica e Centro Trapianti, Ospedale Infantile Regina Margherita, Turin; and [j]the Division of Immunology, Boston Children's Hospital, Boston.

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Corresponding author: Maurizio Aricò, MD, Istituto Toscano Tumori (I.T.T.), ITT Bldg 27/B – Cubo 3, Viale G. Pieraccini, 6 - 50139 Firenze, Italy. E-mail: maurizio.arico@ittumori.it.

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Abbreviations used
FHL: Familial hemophagocytic lymphohistiocytosis
HLH: Hemophagocytic lymphohistiocytosis
MRFI: Mean relative fluorescence intensity
NK: Natural killer
PID: Primary immune deficiency
R-ADCC: Reverse antibody-dependent cellular cytotoxicity
SAP: Signaling lymphocyte activation molecule–associated protein
UPN: Unique patient number
XIAP: X-Linked inhibitor of apoptosis
XLP: X-Linked lymphoproliferative disease

expressed in NK and T lymphocytes.[9] In healthy subjects, 2B4, on recognition of CD48, which is upregulated in EBV-infected B cells, recruits SAP and delivers activating signals. In NK cells, these support the function of the activating receptors NKp46, NKp30, and NKp44 (collectively termed natural cytotoxicity receptors), involved in the recognition of B-EBV cells. In the absence of SAP binding, as in XLP1, 2B4 associates with protein tyrosine phosphatases and delivers inhibitory signals that result in impaired NK and $CD8^+$ T-cell–mediated cytolytic responses against EBV infections.[10,11] This leads to B-cell accumulation and persistence of reactive inflammatory responses.[12-14] Thus, in patients with XLP1, EBV infection may have a fulminant course or alternatively induce B-cell lymphomas, lymphoproliferation, dysgammaglobulinemia, or an HLH clinical picture.[15-18]

Among patients with newly diagnosed HLH, rapid identification of cases with a genetic defect is crucial to direct treatment including indication to hematopoietic stem cell transplantation, the only curative treatment available so far.[19,20] Screening methods have been developed over the last decade to select patients for mutation analysis, which is time-consuming and costly.[21,22] Flow-cytometry evaluation of SAP expression has been proposed as a rapid diagnostic assay to screen patients with XLP1; a rat (KST-3)[23,24] and, more recently, a murine (1C9)[14] anti-SAP mAb have been made available. However, the reliability of flow cytometry alone could be hampered by the existence of *SH2D1A* mutations that, rather than disturb SAP transcription or translation, shorten the half-life of the protein or affect its function.[6]

We have developed a rapid phenotypic/functional diagnostic protocol effective in diagnosing XLP1 and the carrier status (see Fig E1 in this article's Online Repository at www.jacionline.org). It combines the analysis of the intracytoplasmic SAP expression by flow cytometry and the analysis of 2B4 function in cytolytic assays. To verify the sensitivity and specificity of our diagnostic approach, we reviewed and updated our experience gained in patients with genetically documented XLP1. We provide evidence that flow-cytometry study of SAP expression is very specific but may have insufficient sensitivity as a single tool for screening XLP1; in contrast, the 2B4 dysfunction turned to be highly predictive of XLP1 in all cases.

METHODS

Patients

Starting from 1989, we established a registry for HLH[19] (www.orpha.net) and a biobank (currently located in Florence, Italy) that centralize patient information and biologic samples from all Italian centers of pediatric hematology-oncology and confirm the clinical diagnosis by immunologic and genetic studies. HLH is defined by the diagnostic criteria established by the Histiocyte Society.[20] Furthermore, information and samples from additional patients are received from other countries, including England, India, Egypt, Belgium, and Spain.

Out of 900 patients referred to the registry between 1985 and 2013, encoded by unique patient number (UPN), 787 were diagnosed as suffering from HLH, and in 585 the genetic study could be performed. In 242 patients (41%), we identified a genetic marker, and among them, we selected 12 cases (5%) (from 7 different centers) on the basis of an algorithm for diagnostic approach to XLP1 (Fig E1). Of these, 3 patients were exclusively studied for mutation analysis because we had received DNA only; EDTA-peripheral blood samples of 9 patients were shipped at room temperature (24 hour-express courier) to the registry site (Florence) for mutation analysis and to the reference immunology laboratory (Genoa, Italy) for phenotypic and functional studies. Furthermore, we included 3 patients diagnosed as suffering from XLP1 at the reference laboratory for pediatric primary immune deficiencies (PIDs) (University of Brescia, Italy) that had been previously described.[10,25] They were selected because of the availability of frozen samples.

Participants gave written informed consent. Institutional review boards at the A.O.U. Meyer (Florence) and the Gaslini Institute (Genoa) approved the study.

Rapid combined cytofluorimetric and functional assay

To evaluate intracellular SAP expression, PBMCs were stained with anti-CD3-PerCP (SK7, IgG_1) and anti-CD56-APC (NCAM16.2, IgG_{2b}), fixed, permeabilized (cytofix/cytoperm, BD Bioscience, San Jose, Calif), and stained with anti-SAP (1C9, IgG_{2a}, Abnova, Taipei, Taiwan) or isotype-matched control mAb, followed by antimouse IgG_{2a}-PE (SouthernBiotech, Birmingham, Ala). Perforin, CD107a, and XIAP intracellular stainings were performed as described.[22] Resting NK ($CD3^-CD56^+$) and T ($CD3^+$) lymphocytes were evaluated. Mean relative fluorescence intensity (MRFI) indicates the ratio between the mean fluorescence intensity of cells stained with the relevant mAb and that of cells stained with the isotype-matched negative control. iNKT cells were identified using 6B11 mAb (Miltenyi Biotec, Bergish Gladbach, Germany). Data were acquired on a FACSCalibur cytometer (BD) or MACSQuant Analyzer (Miltenyi Biotec), and analyzed using FlowJo (version 8.8.6, TreeStar, Ashland, Ore).

To evaluate 2B4 function, PBMCs cultured for 3 to 5 days in the presence of rIL-2 (600 IU/mL) (Proleukin, Chiron Corp, Emeryville, Calif) were used as effectors in reverse antibody-dependent cellular cytotoxicity (R-ADCC) assays against ^{51}Cr-labelled P815 ($Fc\gamma Rc^+$) murine targets, in the presence of functional-grade purified anti-2B4 mAb (PP35, e-Bioscience, San Diego, Calif), alone or in combination with the anti-NKp46 mAb (9E2, Miltenyi Biotec; or BAB281).[26] All mAbs were used at a final concentration of 0.5 μg/mL. In addition, the same R-ADCC was evaluated as degranulation assay using rIL-2 overnight-activated PBMCs and measuring by cytofluorimetric analysis surface CD107a (H4A3-PE, BD Bioscience) expression on NK cells ($CD3^-CD56^+$).

Samples of patients and healthy donors were analyzed in the same daily session of phenotypic and functional assays.

Statistical analyses

Statistical analyses were performed using Graphpad software (version 6.0). The used tests are indicated in the figure legends. Not significant (n.s.); $****P < .0001$; $***P < .001$; $**P < .01$; and $*P < .05$.

Mutation analysis, multiplex ligation-dependent probe amplification analysis, assays on expanded activated NK and T cells, analysis of SH2D1A transcript, HEK-293T transient transfections, and immunoblotting can be found in this article's Online Repository available at www.jacionline.org.

RESULTS

SH2D1A mutation analysis and characterization of the mutations

Twelve patients with XLP1 were identified among 242 patients with HLH in whom a genetic marker could be assigned (Table I). Ten different *SH2D1A* mutations were identified in patients

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