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RESEARCH LETTER

Autoantibody reactivity profile of primary autoimmune hypophysitis patients: preliminary results

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Primary autoimmune hypophysitis (PAH) is a complex and an emerging disorder [1]. PAH is considered an autoimmune disease, as the pituitary gland typically shows an inflammatory infiltration with CD4+ and CD8+ lymphocytes, granulocytes, and monocytes/macrophages [2, 3]. Moreover, a high prevalence of DQ8 and DR54 human leukocyte antigen (HLA) haplotypes were reported in PAH [4, 5]. Several possible PAH antigens have been described, as alpha-enolase, proopiomelanocortin, growth hormone (GH), corticotroph-specific transcription factor, pituitary gland 1a- and 2-specific factor, and rabphilin-3A [6–10]. We aimed to investigate the humoral autoimmune response in our monocentric series of PAH patients, for increasing the knowledge on PAH pathogenesis and the set-up of a reliable diagnostic approach.

Patients and methods

A retrospective study was conducted on 19 PAH patients that entered this study if their sera (collected at the moment of PAH diagnosis) were available. This cohort of patients was clinically described in our previous studies [11–13]. PAH diagnosis was conducted according to clinical criteria, as previously described and as detailed in Supplementary material [13, 14]. The study was approved by the ethical committee of the Università Cattolica del Sacro Cuore, in Rome. All patients involved in the study signed an informed consent. The results of the experiments conducted on sera of PAH patients were compared to those achieved in 19 normal donors (NDs) and in seven cases of non-functioning pituitary adenoma (NFPA). To investigate the humoral response of PAH, we performed immunoblotting (IB) on pituitary gland extracts, mass spectrometry analysis on bands of immunoreactive proteins, IB on recombinant proteins of putative antigens, and ELISA, as reported in Supplementary material.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12020-021-02937-1.

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Table 1 PAH sera reacted with several proteins by immunoblotting on pituitary gland extracts

Patients	Gender	Hypoadrenalism	Hypogonadism	Hypothyroidism	GH deficiency	Insipidus diabetes	Commonly recognized and PAH-specific bands	Other not PAH-specific bands
PAH1	F	No	No	No	Yes	Yes		20, 50, 150
PAH2	F	No	Yes	No	No	No	15, 40	20, 37, 50, 60
PAH3	F	No	No	No	No	No	15, 25, 40	50, 60
PAH4	F	Yes	No	No	No	No	15, 25, 40	47, 50
PH5	F	Yes	Yes	Yes	No	No	15, 25, 40	20, 50
PAH6	M	Yes	Yes	No	No	No		27, 37, 50
PAH7	F	Yes	No	No	No	No		20, 50
PAH8	M	Yes	Yes	No	Yes	No	25, 40	50
PAH9	M	No	No	No	No	Yes		20, 50
PAH10	M	Yes	Yes	Yes	No	No		20, 27, 50
PAH11	F	Yes	Yes	No	No	Yes	25, 40	50
PAH12	M	Yes	Yes	No	No	Yes		18, 50
PAH13	F	Yes	Yes	Yes	Yes	Yes		20, 50, 150
PAH14	F	No	Yes	No	No	No		37, 50
PAH15	F	Yes	No	No	No	No	15, 25	50, 150
PAH16	M	No	No	No	No	Yes		20, 50, 100
PAH17	M	Yes	No	No	No	No		20, 50, 70, 100
PAH18	F	Yes	No	No	No	No	15	50, 60
PAH19	M	No	No	No	No	Yes		50

Results

The demographic and clinical data of PAH patients are summarized in Table 1. Each PAH patient showed a specific reactivity profile and some bands were shared by several patients (molecular weights: 15, 20, 25, 40, and 50 kDalton, kDa) (Table 1 and Supplementary Fig. 1). In parallel, the IB analysis performed on 19 ND sera also showed several bands (the most present: 50 kDa). Our attention was focused on the reactivity profile specifically recognized by patients' sera in comparison with ND sera. In particular, 15, 25, and 40 kDa antigens were recognized by six, six, and six PAH patients, and two, four, and two ND sera, respectively.

Total proteome analysis of bands corresponding to ~15, ~25, and ~40 kDa

Proteomic analysis of the 15 kDa band by high-resolution MS/MS analysis revealed a unique protein (mitochondrial carrier homolog 2) that was identified with high-confidence peptide, although it has a score of zero (Supplementary Table 1).

As a comprehensive detection of peptides and proteins, total proteome analysis of the ~25 kDa band with high-resolution MS/MS proteomics and the adoption of stringent protein identification criteria (FDR of 1% and at least three unique peptides match per protein) resulted in the characterization of 479 peptides corresponding to 64 proteins (Supplementary Table 2). Among these proteins, GH had the highest score and coverage index and its peptides were the most abundant in the band, as confirmed by the highest quantitative abundance index. Interestingly, superoxide dismutase (SOD2) had the high indexes for score, coverage and abundance. Total proteome analysis of the ~40 kDa band resulted in the characterization of 829 peptides corresponding to 94 proteins (Supplementary Table 3). Among these proteins, fructose-bisphosphate aldolase A (ALDOA) had the highest score and coverage index and its peptides were the most abundant in the band. In addition, cytoplasmic actin 1 also termed actin beta protein (ACTB) had high indexes for score, coverage and abundance. In conclusion, considering literature data and proteomic analysis of the selected bands we considered GH, SOD2, ALDOA, and ACTB as putative PAH antigens and conducted further investigations.

Hypophysitis patient sera react with GH, SOD2, ALDOA, and ACTB recombinant proteins

All the four proteins were recognized by a majority of PAH sera. The frequency of reactivity on GH, ACTB, and SOD2 was comparable between patients' and controls' sera (GH: 94% PAH vs 79% ND; ACTB: 94% PAH vs 86% ND and

SOD2: 94% PAH vs 94% ND) (Supplementary Fig. 2). Interestingly, 15 out of 18 PAH sera analyzed (83%) recognized recombinant ALDOA, with respect to 10 out of 18 ND sera (56%), pointing to a higher specificity although not statistically significant (Supplementary Fig. 3).

PAH patient sera specifically react with ALDOA by ELISA

To investigate the IgG reactivity of PAH sera against native antigens and obtain a more specific assay, two ELISA tests based on ALDOA and GH recombinant proteins were established. The target antigens were chosen as two of the four antigens less recognized by normal controls by IB (GH 79% of ND and ALDOA 56%). Only one PAH patient serum specifically reacted to GH and, of note, 3 out of 18 PAH sera were reactive to ALDOA by ELISA, while none of ND and NFPA sera bound the antigen with a specificity of 100% (Fig. 1). This assay confirms that, even if not commonly shared, ALDOA is a specific target antigen of circulating IgG of PAH patients.

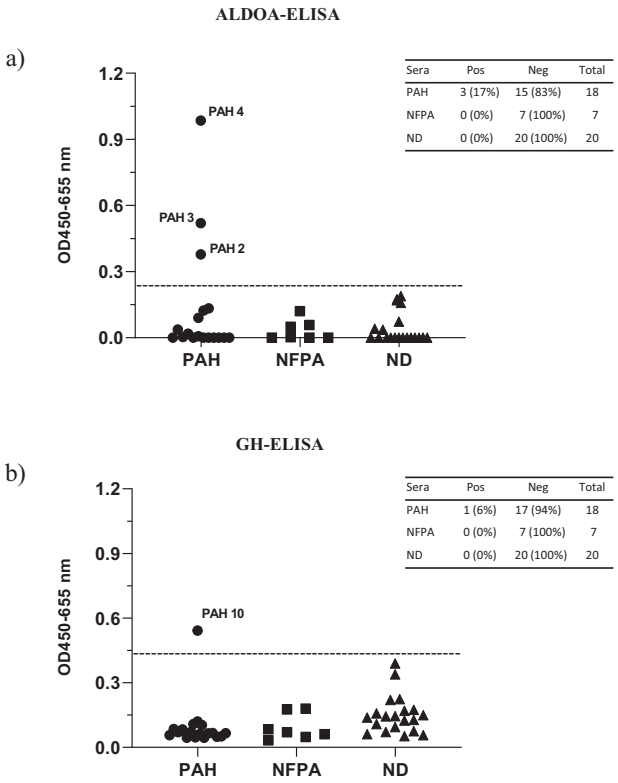

Fig. 1 Scatter plot representation of ALDOA (**a**) and GH (**b**) ELISA test results. **a** Plotted dots refer to the difference of the OD (450–655 nm) readings obtained with GST-ALDOA and GST proteins for each serum sample. **b** Plotted dots refer to the OD (450–655 nm) readings obtained with GH protein for each serum sample. Dashed line indicates the cut-off value, set at mean ± 3 standard deviation of the OD reading from ND sera. The tables (top inset) summarize the ELISA results. PAH sera exceeding the cut-off value are indicated. ELISA, enzyme-linked immunosorbent assay; ND, normal donors; NFPA, non-functioning pituitary adenoma; OD, optical density; PAH, primary autoimmune hypophysitis

Discussion

In this study, we investigated the immune profile in PAH patients demonstrating a specific, although not frequent, reactivity to ALDOA by ELISA. ALDOA may represent a promising marker of autoimmune damage. In fact, recently, the ALDOA was described as an auto-antigen of Alzheimer's disease, being identified in over 50% of 45 patients [15]. In addition, Privitera et al. reported ALDOA as the antigen recognized by autoantibodies in 7 of 14 patients affected by hyperkinetic movement disorders (41%) and in 4 of 30 patients affected by Parkinson's disease (13%) [16].

In contract to data on ALDOA, in this study, we found that the reactivity for GH, SOD2, and ACTB antigens was similar among sera of PAH patients and NDs. Possible explanations for reactivities in NDs could be that (i) eukaryotic post-translational modifications were essential for epitope/s specifically recognized by PAH but not by ND, (ii) cryptic epitopes unveiled with denaturation of proteins by SDS-PAGE could be at the base of ND reactivity, (iii) antibodies originally directed towards other antigens such as viruses or bacteria could cross-react with analyzed putative antigens, and (iv) T and B cells specific for self-antigens could be present also in ND, being not sufficient to induce an autoimmune disease, for mechanisms that prevent their activation. In fact, only the GH is secreted in the blood as peptide by somatotropic cells within the lateral wings of the anterior pituitary gland. The other investigated antigens (SOD2, ALDOA, and ACTB) are not exposed to the immune system in physiological condition, being localized inside the cells. In general, the B-cell tolerance is profound to antigens expressed in the serum or on cells surface, while B cells specific for intracellular proteins often escape clonal deletion.

Our data are in accordance with previous studies in showing that several auto-reactivities are poorly specific in PAH patients and suggesting a secondary role of these antigens in the autoimmune process. Moreover, it could be conceivable that other factors such as a genetic predisposition can induce a chronic autoimmune disease in PAH.

The high specificity of ALDOA ELISA in PAHs and its absence in NDs and in NFPAs are important features for a non-invasive diagnostic tool. Moreover, future studies are advocated to investigate the ELISA sensitivity by expression of ALDOA in eukaryotic cells. In conclusion, our preliminary data suggest that ALDOA may be considered among the putative antigens of PAH, maybe playing a role as target of an epitope spreading phenomenon. However, additional studies

are advocated to validate our results also involving larger series of PAH patients.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethics approval The study was approved by the ethical committee of the Cattolica del Sacro Cuore University, in Rome. All patients involved in the study sign an informed consent.

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