EVALUATION OF DIAGNOSTIC MARKERS OF AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

PhD thesis

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Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a rare immunodeficiency with an unknown incidence, characterized by non-malignant lymphoproliferation, lymphadenopathy, splenomegaly and hepatomegaly. The underlying cause of this disease is the inherited or the acquired damage of the extrinsic apoptotic pathway, which plays an important role in the negative selection of lymphocytes. Thus, the disruption of this pathway leads to non-malignant lymphoproliferation. The condition can manifest in autoimmune disorders, mainly multilineage cytopenia or less frequently, nephritis, hepatitis, uveitis, arthritis or colitis. The symptoms of ALPS show similarities to other lymphoproliferative diseases; therefore, the diagnosis relies on the monitoring of different laboratory parameters characteristic to this disorder.

The ALPS is caused by germline or somatic mutations of genes encoding proteins of the extrinsic apoptotic pathway. The *FAS* gene codes the Fas receptor protein responsible to initiate the apoptosis upon binding its ligand. Approximately 70% of ALPS patients develop the disease due to the germline or somatic mutation of *FAS*. The majority of germline *FAS* mutations are inherited in an autosomal dominant manner, yet rarely the autosomal recessive inheritance is observed. The *FASLG* gene encodes the Fas-ligand, the main activator of the Fas receptor. *FASLG* mutations are the rarest cause of ALPS accounting for around 1% of all ALPS cases. Caspase 10 in part of the intracellular signal transduction of the apoptotic pathway and is encoded by the gene *CASP10* and the mutations of this gene accounts for around 20-30% of ALPS cases. Mutations of other genes that encode proteins that contribute

to the extrinsic apoptotic pathway, namely FADD and CASP8 are considered separate diseases.

The diagnostic categorisation of ALPS is based on the affected genes. The first system categorised the disease into ALPS-I where the affected genes are either *FAS* or *FASLG*, ALPS-II category for patients with *CASP10* mutation and ALPS-III for unidentified background. However, for simplicity this categorisation was renamed. The revised format classifies the disease as ALPS-FAS for germline FAS mutation, ALPS-sFAS for somatic FAS mutation, ALPS-FASL for FASLG mutations, ALPS-CASP10 for CASP10 mutations and ALPS-U for unidentified cause.

The symptoms of ALPS are unspecific and similar to other lymphoproliferative diseases. The diagnosis of this disease relies on a handful of laboratory diagnostic parameters. As ALPS has a marked effect on T-cell selection and development, the increased ratio of CD3+, TCR $\alpha\beta^+$, $CD4^-$, $CD8^-$ double negative T-cells (TCR $\alpha\beta^+$ DNT) in all T-cells is a diagnostic biomarker for this disorder. TCR $\alpha\beta^+$ DNT is around 1% in healthy controls, while it accounts for more than 2.5% of all T-cells in ALPS patients, yet this threshold differs from laboratory to laboratory as it is reliant on the gating strategy. Another important laboratory analysis diagnostic for ALPS is the assessment of the in vitro Fas-mediated apoptosis, which is performed by collecting peripheral blood mononuclear cells (PBMCs) from the patient and a healthy control. PBMCs are incubated with an activating anti-Fas antibody, and the induced rate of apoptosis is assessed by staining against apoptosis-specific proteins detected with flow cytometry. ALPS patients show reduced induction in apoptosis rate after the anti-Fas antibody treatment compared to healthy

individuals, but the cut-offs differ between laboratories due to the different protocols.

Other important laboratory markers of ALPS are the following: the elevation of soluble Fas-ligand (sFasL), elevation of cytokine levels Interleukin-10 (IL-10) and Interleukin-18 (IL-18), elevation of vitamin B12 level and the elevation in γ-immunoglobulin levels. These parameters however, have different diagnostic powers, thus diagnostic protocols are dividing them into major and minor criteria of ALPS. In this study the revised diagnostic criteria for ALPS published in 2010 were applied which categorizes diagnostic results into major and minor criteria.

Objectives

1. Our first aim was to collect and evaluate clinical and laboratory data of patients referred with potential ALPS to the Laboratory of Immunology of Great Ormond Street Hospital between 2008 to 2018.
2. Our second aim was to categorize the patients into definite ALPS, suspected ALPS and unlikely ALPS groups depending on the fulfilled criteria and to compare the diagnostic power of biomarkers between these groups.
3. Our third aim was to assess the results of the genetic studies of these patients and to evaluate the diagnostic power of the laboratory markers according to the affected genes.

Methods

Data of patients referred with the clinical suspicion of ALPS between 2008 to 2018 were collected (132 males and 83 females; median age, 12.3 years; range, 1 month to 76 years) at the Immunology Laboratory of the Great Ormond Street Hospital (London, United Kingdom). Clinical data was collected including presence of chronic nonmalignant, noninfectious lymphadenopathy and/or splenomegaly, cytopenia, and immunoglobulin G (IgG) and vitamin B12 levels. The Immunology Laboratory assessed the TCR αβ+ DNT, in vitro Fas mediated apoptosis function and measured sFasL, IL-10 and IL-18 levels. Genetic analysis was performed in the North East Thames Regional Genetics Laboratory (London, United Kingdom). All data were collected retrospectively. This study was approved by the Bloomsberry Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

The TCR αβ+ DNT levels were assessed with flow cytometry and a level of >1.8% of all T-cells was considered pathogenic. In vitro Fas-mediated apoptosis assay was performed from PBMC sample from the patient and a healthy control. PBMCs were stimulated for 6-7 days with anti-CD3 and IL-2, then activating anti-Fas antibody was added to the samples. Apoptotic cell count was assessed by flow cytometry using apoptosis-specific cell surface membrane staining (Annexin V). Soluble Fas ligand, IL-10 and IL-18 levels were measured by ELISA technique. Normal levels were defined as: sFasL < 200 pg/ml; IL-10 < 40 pg/ml, and IL-18 < 500 pg/ml.

Genes affecting the extrinsic apoptotic pathway (FAS, FASLG, CASP10) and an additional 79 genes included in the targeted immunodeficiency and gastrointestinal enrichment panel were analysed by next-generation

sequencing (NGS). We considered pathogenic, likely pathogenic and variants of unknown significance (VUS), classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines as genetic variants that possibly alter function.

Patients were retrospectively categorized into ALPS diagnostic groups utilizing the ALPS-2010 diagnostic criteria. This diagnostic protocol recognizes chronic non-malignant lymphoproliferation (6 months) with splenomegaly and/or lymphadenopathy, TCR αβ+ DNT elevation, defective in vitro Fas-mediated apoptosis, and recognizable genetic pathogenic mutation (in FAS, FASLG, and CASP10 genes) as major criteria and multilineage cytopenia, elevated levels of IgG, IL-10, IL-18, or vitamin B12 in serum, and increased sFASL level in plasma as minor criteria. In this study we classified patients into three diagnostic groups: (i) definite ALPS patients who fulfilled at least 3 major criteria or at least 2 major and 2 minor criteria; (iii) suspected ALPS patients are who fulfilled at least 2 major and 1 minor criteria; whereas (iii) patients with less criteria fulfilled were classified as unlikely ALPS. The results of at least 2 major and 2 minor criteria needed for a patient to be considered evaluable.

Results

From the 215 patients referred with clinical suspicion for ALPS 140 had sufficient available data for evaluation. From this 140 patient, 85 were categorised as unlikely ALPS (60.7%), 17 were suspected for ALPS (12.1%) and 38 patients had definite ALPS diagnosis (27.1%). Chronic lymphoproliferation with or without lymphadenopathy/splenomegaly occurred more frequently in definite (97.1%, n=33/34) and suspected (87.5%, n=14/16) ALPS groups than in unlikely ALPS patients (55%, n=22/40, ****P<0.0001). Multilineage cytopenia was observed in 69% (n=20/29) of definite, in 56.3% (n=9/16) of suspected and in 56.6% (n=30/53) of unlikely ALPS population (P= 0.5168).

Abnormally high DNT were observed in all definite (n=34) and in all suspected ALPS patients (n=14). In the unlikely ALPS population 35 out of 68 (51.5%) cases showed DNT elevation. There was significant difference between definite ALPS and unlikely ALPS groups (****P<0.0001) and between suspected ALPS and unlikely ALPS groups (*P=0.0496). Regarding the in vitro Fas-mediated apoptosis function assay, in the definite ALPS group (n=33) 15 patients had an abnormal apoptosis functional test (45.5%), 11 had equivocal (33.3%) and 7 had a normal result. While in the suspected ALPS group (n=16), two (12.5%) patients had abnormal, two (12.5%) had equivocal and 12 patients had normal apoptosis function. In the subgroup of patients with unlikely ALPS (n=80), 3 and 8 patients (3.8 and 10%) had abnormal or equivocal apoptosis functional test results (definite ALPS versus suspected ALPS **P=0.0019; and definite ALPS versus unlikely ALPS, ****P<0.0001). However, there was no significant difference between the suspected ALPS group and the

unlikely ALPS group (P=0.895). The sFASL level was higher in definite compared to unlikely ALPS (**P=0.0013; definite ALPS: median 195, range: 44->1000 pg/ml; suspected ALPS: median 154, 23.5-939 pg/ml; unlikely ALPS: median 139, range: 35->1000 pg/ml). IL-10 level showed tendency of being elevated in definite ALPS (median 41.8, range: 19-169.2 pg/ml) compared to suspected ALPS (median 24.2, 20.5-27.9 pg/ml) and unlikely ALPS (median 23.8, range: 8-137.6 pg/ml, P=0.0624). A tendency towards the increase of IL-18 level was also observed between the groups (definite ALPS: median 909, range: 265-3255 pg/ml vs. suspected ALPS: median 642, 157-1127 pg/ml, unlikely ALPS: median 398, range: 206-1375 pg/ml, P=0.0615).

Optimal biomarker combinations were tested by setting up groups where both chosen markers were positive or either of them was negative; the marker combinations were compared between definite and unlikely ALPS groups. The combination of DNT and abnormal in vitro apoptosis functional test was positive in 79.3% in definite ALPS patients and negative in 93.7% of unlikely ALPS patients; therefore, the sensitivity of this biomarker combination was 79.3% and the specificity was 93.7%. The in vitro apoptosis functional test and sFASL combination was positive in 36.7% of definite ALPS, while it was negative in 96.4% of unlikely ALPS patients; therefore the positive predictive value of this combination was 36.7% and the negative predictive value was 96.4%.

Genetic results were available for 87 patients. *FAS* gene variants were identified in 21 patients, 17 of them were considered as having a potentially functional variant (14 definite, 1 suspected ALPS, two cases with unevaluable clinical/laboratory data). From these FAS variants, 4

pathogenic, 5 likely pathogenic, 2 VUS and 6 benign variants were identified. Eight of the pathogenic, likely pathogenic or VUS FAS variants have not been previously published as underlying condition for ALPS. Three different CASP10 variants were found in 5 patients (2 definite, 1 suspected, and 2 non-evaluable), but two variants were considered as likely benign and one as benign variant. *FASLG* and *CASP8* pathogenic mutations were not identified in our definite or suspected patient cohorts.

The diagnostic parameters were compared between patients with known FAS mutations (ALPS-FAS) and patients with unknown genetic reason (ALPS-U). In the combined suspected and definite ALPS groups, ALPS-FAS patients (n=13/17) had significantly higher DNT levels (median 7.5%, range: 4.5-23%) compared to ALPS-U patients (n=32/35) (median 2.7%, range: 1.8-11%; ****P< 0.0001). The in vitro apoptosis functional test was more impaired in ALPS-FAS (n=14/17; median: 1.6, range: 0.4-3.5) than in ALPS-U (n=31/35; median 3.1, range: 1.3-18.1, ****P< 0.0001). Even though sFASL proved to be a highly predictive biomarker for ALPS-FAS (n=16/17; median >1000 pg/ml; range: 128.9->1000 pg/ml), in ALPS-U the vast majority of patients showed normal or moderately elevated biomarker level (n=29/35; median 152 pg/ml; range:23.5-486 pg/ml; ****P< 0.0001).

Examining the marker combinations, DNT and in vitro apoptosis functional tests showed the highest sensitivity (90.9%) to differentiate between ALPS-FAS and ALPS-U with a negative predictive value of 93.8%. On the other hand, the in vitro apoptosis functional test and sFASL combination showed the highest specificity of 92% with the highest positive predictive value of 83.3% for ALPS-FAS.

Conclusions

We have evaluated the data of 215 patients referred with potential ALPS. From this cohort, 140 patients had sufficient data for categorisation. We have found 38 patients with definite and 17 patients with suspected for ALPS.

According to the available data, lymphoproliferation was a strong diagnostic criterion of ALPS, in this cohort, definite ALPS patients had a 97.1% frequency of lymphoproliferation while patients who are unlikely to had ALPS had a 55% frequency, while multilineage cytopenia was a less reliable marker. In conclusion, lymphoproliferation is a stronger indicator of ALPS compared to multilineage cytopenia.

Regarding laboratory findings, our data uncovered that elevated DNT levels, deficient in vitro apoptosis and elevated soluble FAS ligand levels were all significantly more likely to be found in ALPS patients, compared to patients with unlikely ALPS diagnosis.

We evaluated the specificity and sensitivity of the biomarker combination. We found that DNT in combination with in vitro apoptosis functional test had the highest sensitivity, and the abnormality in the in vitro apoptosis function in combination with soluble FAS ligand levels had the highest specificity for this disease.

We identified11 *FAS* gene variants (including 8 novel variants) with functional relevance. Elevated DNT, altered in vitro apoptosis and elevated soluble FAS ligand were more likely to be associated with ALPS with FAS mutation compared to ALPS with an undetermined background.

In conclusion our results supported the efficacy of the currently available diagnostic criteria. Furthermore, these results might help in the optimisation of the diagnostic algorithm of ALPS in the clinical practice.

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