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A novel reactive epitope-based antigen targeted by serum autoantibodies in oligoarticular and polyarticular juvenile idiopathic arthritis and development of an electrochemical biosensor

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ABSTRACT

Currently, there are no specific markers for juvenile idiopathic arthritis (JIA) diagnosis, which is based on clinical symptoms and some blood tests for diseases' exclusion. Aiming to select new epitope-based antigens (mimotopes) that could recognize circulating autoantibodies in most JIA forms, we screened a phage displayed random peptide library against IgG antibodies purified from serum of JIA patients. ELISA assay was carried out to confirm immunoreactivity of selected peptides against sera IgG antibodies from JIA patients, healthy children and patients with other autoimmune diseases. The mimotope PRF + 1 fused to phage particles was able to efficiently discriminate JIA patients from controls, and for this reason was chosen to be chemically synthesized for validation in a larger sample size. The synthetic peptide was immobilized onto bioelectrodes' surface for antibody detection by electrochemical analyses through differential pulse voltammetry. The PRF + 1 synthetic peptide has efficiently discriminated JIA patients from control groups ($p < 0.0001$) with a very good accuracy (AUC > 0.84; sensitivity = 61%; specificity = 91%). The electrochemical platform proved to be fast, low cost and effective in detecting anti-PRF + 1 antibodies from JIA patients compared to healthy controls ($p = 0.0049$). Our study describes a novel and promising epitope-based biomarker for JIA diagnosis that can become a useful tool for screening tests, which was successfully incorporated onto an electrochemical biosensor and could be promptly used in field diagnostics.

1. Introduction

Juvenile idiopathic arthritis (JIA) is the most common inflammatory rheumatic disease of childhood (Ravelli and Martini, 2007). JIA is an autoimmune disease of unknown etiology that begins before 16 years of age and persists for at least 6 weeks. According to the International League Against Rheumatism classification, JIA can be classified in seven distinct subsets, being oligoarticular and polyarticular the most common (Petty et al., 2004). Up to now, JIA diagnosis is based on the combined evaluation of medical history of the patient, physical manifestation, radiographic imaging, and the detection of some serological markers (Petty et al., 2004; Martini

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and Lovell, 2010). Although JIA manifestations are influenced by genetic and environmental factors, studies focused on the identification of autoantigens that can drive the humoral response are of high interest to the clinical area. Currently, only the rheumatoid factor has been used for diagnosis of polyarticular forms of JIA (Duurland and Wedderburn, 2014); however, the lack of consistent biomarkers often delays diagnosis and makes the prediction of disease prognosis difficult. In this context, new biomarkers may be potentially used to guide decisions in the diagnosis and clinical management of JIA. Aiming to select novel peptides as potential serum biomarkers, we screened a phage displayed random peptide library against circulating IgG antibodies purified from JIA patients and submitted a selected peptide to further validation to check sensitivity, specificity and electrochemical properties.

The peptide selected against circulating autoantibodies that was able to discriminate JIA patients in the ELISA assay was successfully immobilized onto an electrochemical platform. This new sensor platform have reached a very good accuracy as a portable, low cost and fast diagnostic tool for autoantibodies detection in JIA.

2. Material and methods

2.1. Patients

For peptides selection, serum samples were obtained from Brazilian donors. A total of 40 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular ($n = 21$), extended oligoarticular ($n = 8$), polyarticular rheumatoid factor negative ($n = 5$) and polyarticular rheumatoid factor positive ($n = 6$), according to the International League of Associations for Rheumatology (Petty et al., 2004), 20 healthy children, and 17 patients with other autoimmune diseases were used. In order to increase specificity of the selected peptides, a subtractive selection step was performed using pooled serum from patients with rheumatic fever ($n = 5$), uveitis ($n = 3$), systemic lupus erythematous ($n = 4$) and Hashimoto's thyroiditis ($n = 5$). For synthetic peptide validation, well-characterized serum samples were obtained from Biobanco-IMM, Lisbon Academic Medical Center (Lisbon, Portugal). A total of 57 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular ($n = 21$), extended oligoarticular ($n = 7$), polyarticular rheumatoid factor negative ($n = 20$) and polyarticular rheumatoid factor positive ($n = 9$), 23 systemic lupus erythematous and 51 healthy children were enrolled in this stage. To minimize potential errors during the data analysis and improve the levels of sensitivity and specificity, samples of patients that presented inaccurate diagnosis at the time of sample collection were discarded.

Informed, written consent according to the declaration of Helsinki was obtained from parents of all study participants before any protocol procedure was carried out. This study was approved by the Research Ethics Committee from Federal University of Uberlândia (CEP-UFU; number 685/09), state of Minas Gerais, Brazil and by the Ethics Committee of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria, Portugal. Demographic and laboratory characteristics of the studied subjects are listed in Table 1.

2.2. Peptides selection by phage display

For peptides selection, a Ph.D.-C7C Phage Display (PD) Peptide Library Kit (New England Biolabs) was incubated with Immunoglobulins G (IgG) from the different groups coupled to magnetic beads according to the manufacturer's recommendations (Dynabeads® Protein G, Invitrogen). This library is based on a 7-mer random peptide combinatorial library fused to the pIII capsid of the M13 phage. For screening the PD library to select JIA mimotopes, we used a subtractive step against purified IgG from healthy children and patients with other autoimmune diseases, followed by a positive selection against purified IgG from JIA subtypes, which is strongly recommended to limit the recovery of target-unrelated peptides (Molek et al., 2011). The biopanning procedure was performed as described elsewhere with some modifications (Barbas et al., 2001). Briefly, 1×10^{11} phage particles of the PD library was incubated for 30 min at room temperature (RT) with beads coupled to IgG purified from serum of healthy children. After magnetic separation, the supernatant containing non-bound phages was transferred to a microtube containing beads coupled to IgG purified from serum of rheumatic fever patients, and incubated for 30 min at RT. The same procedure was performed for each autoimmune disease included in the control group. After subtractive step, the non-bound phages were collected and divided in four microtubes, each one containing beads coupled to IgG purified from serum of four JIA subtypes followed by incubation for 30 min at RT. Non-bound phages were removed after 10 washes with TBS-T 0.1% in the first round, and TBS-T 0.5% in the two subsequent rounds. The eluted phages were amplified in Escherichia coli ER2738 (New England Biolabs) between each round. The peptides selection was performed separately for each JIA subtype due to the fact that our initial goal was to identify biomarkers able to differentiate each subtype of the disease.

2.3. DNA sequencing of selected phage clones

After the third round of biopanning, phage clones were randomly picked up for DNA sequencing. Phages single-stranded DNA were isolated by iodide buffer extraction procedure (Instruction Manual Ph.D.-C7C Phage Display Peptide Library Kit) and analyzed with MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer using 200 ng of primer −96 gIII (5′-OH CCC TCA TAG TTA GCG TAA CG-3′; New England Biolabs). Nucleic acid sequences translation to the corresponding peptide sequences was performed using the ExPASy Translation tool (http://web.expasy.org/translate/) and the biochemical prediction of the PRF + 1 peptide primary structure was performed using the PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/index.html).

2.4. Phage-ELISA with the selected mimotopes

Phage-ELISA assay was first performed to test the reactivity and binding specificity of the selected mimotopes (phage clones) against pooled sera from JIA patients and healthy children (HC). Clones that presented significantly higher absorbance when compared to the HC were individually tested with sera from 40 patients with JIA, 5 with RF, 3 with UV, 4 with SLE, 5 patients with HT and 20 healthy children. Each pooled serum sample was tested against wild M13 phage (without displaying any peptide on its surface) as negative control. For data adjustment, the final optimal density (OD) was adjusted by subtracting the ratio of OD readings obtained by the tested mimotopes to the OD readings obtained by the wild-type M13 phage. All samples were tested in duplicate. Briefly, A ninety-six-well Maxisorp™ microtiter plate (NUNC, NY, USA) was coated with 1×10^{11} phages particles of each one of the selected phage clones diluted in 100 μl of carbonate buffer (0.1 M $NaHCO_3$, pH 8.6) and incubated overnight at 4 °C. The plate was blocked for 1 h at 37 °C with PBS-BSA 5% and then incubated for 1 h at 37 °C with pooled serum (1:250 in PBS-BSA 5%) from the three groups analyzed. The plate was washed 6 times with PBS-T 0.5% followed by incubation with HRP-conjugated goat anti-bovine IgG (Sigma–Aldrich) diluted (1:5000) in PBS-BSA 5% for 1 h at 37 °C. The plate was washed 6 times with PBS-T 0.5%, revealed with OPD SigmaFastTM (Sigma–Aldrich) and read at 492 nm.