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Biomarkers associated with disease severity in allergic and nonallergic asthma

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ABSTRACT

Asthma is a complex, chronic respiratory disease with a wide clinical spectrum. Use of high-throughput technologies has generated a great deal of data that require validation. In this work the objective was to validate molecular biomarkers related to asthmatic disease types in peripheral blood samples and define their relationship with disease severity. With this purpose, ninety-four previously described genes were analyzed by qRT-PCR in 30 healthy control (HC) subjects, 30 patients with nonallergic asthma (NA), 30 with allergic asthma (AA), and 14 patients with allergy (rhinitis) but without asthma (AR). RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the TRIzol method. After data normalization, principal component analysis (PCA) was performed, and multiple approaches were used to test for differential gene expression. Relevance was defined by RQ (relative quantification) and corrected *P* value (<0.05). Protein levels of IL-8 and MSR1 were determined by ELISA and Western blot, respectively.

PCA showed 4 gene expression clusters that correlated with the 4 clinical phenotypes. Analysis of differential gene expression between clinical groups and HCs revealed 26 statistically relevant genes in NA and 69 in AA. Protein interaction analysis revealed IL-8 to be a central protein. Average levels of IL-8 were higher in the asthma patients' sera (NA: 452.28 ± 357.72, AA: 327.46 ± 377 pg/ml) than in HCs (286.09 ± 179.10), but without reaching statistical significance. Nine genes, especially *MSR1*, were strongly associated with severe NA.

In conclusion, several molecular biomarkers of asthma have been defined, some of which could be useful for the diagnosis or prognosis of disease severity.

1. Introduction

Asthma is an inflammatory disorder of the airways characterized by chronic inflammation and hyperresponsiveness (AHR) as well as symptoms such as recurrent wheezing, cough, and shortness of breath. According to the World Health Organization, asthma is the most common chronic disease in children, affecting more than 300 million children and adults (Mukherjee and Zhang, 2011). Many different clinical phenotypes of asthma have been identified. There are 10%–33% of individuals with nonallergic asthma (NA) or subjects with asthma in whom allergic sensitization cannot be demonstrated. However, allergic mechanisms have been implicated in 50%–80% of asthmatic patients and in approximately 50% of individuals with severe asthma (D'Amato et al., 2014; Holgate., 2012). For this reason, asthma has been associated with type 2 airway inflammation, which is characterized by elevated levels of immunoglobulin E, eosinophils, and several interleukins (IL) such as IL-4, IL-5, IL-13, and IL-9.

Asthma severity reflects the intensity of the disease. The Spanish Guidelines for the Management of Asthma divide asthma into the following categories: intermittent, mild, moderate, and severe

Abbrevations: GEMA, Spanish guide for asthma management; PBMCs, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; PCA, principal component analysis.

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persistent. These categories are assigned according to day and night symptoms, frequency of medication intake, activity limitation, pulmonary function, and exacerbations (GEMA., 2009).

It is well-known that reaction to treatment varies between different asthma patients. Given this clinical heterogeneity, many authors have begun classifying asthma into "endotypes" (Fajt et al., 2015; George et al., 2015; Wenzel., 2012; Anderson, 2008). An endotype consists of several mechanisms that cannot be clearly separated. Their description may rely on biomarkers, which can be the signature of a complex underlying pathway or a key molecule associated with or directly playing a role in a particular disease endotype (Agache et al., 2015). New strategies for discovery and validation of biomarkers such as the *–omics* approaches have been used to explore the mechanisms responsible for the asthma endotypes present in different tissues; these approaches have been used in airway epithelial cells (Woodruff et al., 2009; Woodruff et al., 2007) and in sputum (Baines et al., 2010), but fewer studies have been done in human peripheral blood and, in particular, in peripheral blood mononuclear cells (PBMCs). The studies that have been done have helped to confirm previous results and have identified new genes that need to be investigated (Faiz and Burgess, 2012). Several biomarkers have been described for the Th2 (T-helper 2) endotype, such as exhaled NO, sputum or blood eosinophils, total serum IgE or specific IgE, serum periostin, sputum or epithelial-cell (bronchial or nasal) gene signature, or salivary inflammatory mediator profile. Recently, the first results of molecularly targeted therapies have been reviewed (Fajt et al., 2015). In asthmatic patients with Th2 inflammation, consistent efficacy was observed in therapies targeting the Th2 cytokines, while in patients lacking Th2 biomarkers, biologically targeted therapies are still in their infancy.

Given all this information, our aim was to validate and to define molecular biomarkers that differentiate asthma from allergic diseases and predict disease severity in non-invasive samples obtained from a clinically well-characterized population.

2. Materials and methods

2.1. Subjects

The study population comprised 104 unrelated subjects, 30 healthy control (HC) subjects, 30 patients with nonallergic asthma (NA), 30 patients with allergic asthma (AA) (allergic to airborne allergens), and 14 patients with allergy (rhinitis) but without asthma (AR) (allergic to airbone allergens). The selection criteria appear in Baos et al., submitted.

Informed consent was obtained from each subject. Ethical approval for the study was obtained from the ethical and research committees of the participating hospitals.

2.2. PBMC isolation and RNA extraction

PBMCs were isolated from heparin-containing peripheral blood samples by gradient centrifugation using Lymphoprep (Comercial Rafer, Zaragoza, Spain) following the manufacturer's instructions. Total RNA was isolated from PBMCs (10^6 cells) using the TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA was quantified and its purity was checked by spectrophotometry with the nanodrop (ND-1000 Spectrophotometer) system.

2.3. Gene selection criteria

Ninety-four genes (Table 1, Baos et al., submitted) were selected from previous studies according to the criteria described in Baos et al., submitted.

2.4. Differential gene expression by qRT-PCR

Three hundred nanograms of RNA from each subject was analyzed in the Scientific Park of Cantoblanco (Madrid, Spain) by quantitative real-time PCR using the TaqMan Gene Expression System (Applied Biosystems, Foster City, CA, USA) in 384-well microfluidic cards (Applied Biosystems) that included the 94 selected genes and 18S as a reference gene. Briefly, reverse transcription was performed using "High capacity RNA to cDNA kit" (Applied Biosystems). Real time PCR was performed using Taqman Gene Expression and the HT7900 System (Applied Biosystems) with 40 amplification cycles. The results were analyzed with the SDS software (Applied Biosystems).

Specific mRNA expression from each gene was analyzed in triplicate, normalized into 18S rRNA gene, and calculated according to the cycle threshold (Ct) method. After data normalization, the global gene expression of the samples was checked by an unsupervised statistical procedure called principal component assay (PCA). This method reduces multidimensional data to fewer dimensions to facilitate analysis and visualization. That is, the method groups the data according to a distance without using any external information to organize the groups. PCA is very useful for identifying the most significant patterns of data.

2.5. Measurement of soluble cytokine levels

Levels of IL-8 in the subjects' serum were analyzed using the human IL-8 ELISA kit by Diaclone (Besançon Cedex, France) following the manufacturer's protocol.

2.6. MSR1 protein analysis

MSR1 determination was analyzed in 9 HCs and 18 NA patients (8 severe NA patients and 10 with moderate-mild diagnosis) by Western blot. Specific details are summarized in Baos et al., submitted.

2.7. Statistical analysis

Multiple comparisons were used to test for differential gene expression by using the StatMiner program (http://www.integromics.com/StatMiner). This program follows a simple, step-by-step analysis workflow guide that includes parametric, non-parametric, and paired tests for relative quantification of gene expression, as well as 2-way ANOVA for two-factor differential expression analysis. Significance was defined by RQ (relative quantification) <–2 or >2 and corrected P value (<0.05) adjusting the P value with the Benjamini-Hochberg FDR method. DAVID software v6.7 was used for functional gene analyses (http://david.abcc.ncifcrf.gov/home.jsp) and the STRING v10 free program (http://string-db.org/) was used for the analysis of protein interactions.

3. Results

3.1. Subjects

The demographic and clinical parameters of the population studied are summarized in Table 1. Mean age was slightly different among the groups: the NA subjects were significantly older than the other groups, and the AR subjects were the youngest, with statistically significant differences when compared to the HC and NA groups.

The mean levels of total IgE were increased in the 2 groups of allergic subjects ($P < 0.05$) compared with both the HC and NA groups.