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Proteomics of bronchial biopsies: Galectin-3 as a predictive biomarker of airway remodelling modulation in omalizumab-treated severe asthma patients

Pierluigi Mauri[a,1], Anna Maria Riccio[b,1], Rossana Rossi[a], Dario Di Silvestre[a], Louise Benazzi[a], Laura De Ferrari[b], Roberto Walter Dal Negro[c], Stephen T. Holgate[d], Giorgio Walter Canonica[b,*]

[a] Institute for Biomedical Technologies (ITB-CNR), Milan, Italy
[b] Allergy and Respiratory Diseases Unit, Dpt. of Internal Medicine, University of Genoa, IRCCS-IST AOU San Martino, Genoa, Italy
[c] CMS, Verona, Italy
[d] Inflammation, Infection and Immunity Division, Sir Henry Wellcome Laboratories, Mail Point 810, Level F, South Block, Southampton General Hospital, Southampton SO16 6YD, UK

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ABSTRACT

Asthma is a chronic inflammatory disease. Reticular basement membrane (RBM) thickening is considered feature of airway remodelling (AR) particularly in severe asthma (SA). Omalizumab, mAb to IgE is effective in SA and can modulate AR. Herein we describe protein profiles of bronchial biopsies to detect biomarkers of anti-IgE effects on AR and to explain potential mechanisms/pathways. We defined the bronchial biopsy protein profiles, before and after treatment. Unsupervised clustering of baseline proteomes resulted in very good agreement with the morphometric analysis of AR. Protein profiles of omalizumab responders (ORs) were significantly different from those of non-omalizumab responders (NORs). The major differences between ORs and NORs lied to smooth muscle and extra cellular matrix proteins. Notably, an IgE-binding protein (galectin-3) was reliable, stable and predictive biomarker of AR modulation. Omalizumab down-regulated bronchial smooth muscle proteins in SA. These findings suggest that omalizumab may exert disease-modifying effects on remodelling components.

1. Introduction

Asthma is a chronic inflammatory disease of the airways characterised by a variable narrowing of the airways. Asthma is a major health problem worldwide that affects over 300 million patients [1]. Severe asthma affects a minority of the patients with this disease; however, most treatment resources are directed to these patients [2]. Allergies are one of the most common and well-known causes of asthma [3] Omalizumab, an anti-IgE monoclonal antibody, has been approved for the treatment of "severe, uncontrolled allergic asthma", and its effectiveness has been confirmed in several controlled and real-life clinical trials [4–6].

Personalised medicine [7] and targeted therapies are modern methods of properly treating patients with the most cost-effective approach [8]. There is an absolute need to pursue these approaches and to make them feasible in the identification of biomarkers of clinical response [7].

In recent years, several biomarkers of clinical response have been identified for severe asthma [9]. Among these, periostin is particularly noteworthy, as high blood levels of periostin have been demonstrated to be a reliable biomarker of clinical response to some biotechnology-based treatments, such as lebrikizumab [10] and omalizumab [11]. Reticular basement membrane (RBM) thickening is considered a feature of airway remodelling [12] with increasing prominence in severe asthma [13] with a defined cut-off value.

We recently published a report on the significant effect of omalizumab treatment on bronchial remodelling modulation after 12 months of treatment, which was determined by means of a histological evaluation, according to ATS/ERS guidelines for its use in severe corticosteroid refractory disease. Bronchial biopsies at

* Corresponding author at: Allergy and Respiratory Diseases Unit, Dpt. of Internal Medicine, University of Genoa, IRCCS-IST AOU San Martino Pad. Maragliano L.go R. Benzi, 10 16132 Genoa, Italy. Tel.: +39 0105554890; fax: +39 0105556307.
E-mail addresses: canonica@unige.it, rina.miriello@unige.it (G.W. Canonica).
[1] These authors contributed equally to this work.

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baseline and after therapy were analysed [14], and all patients were found to have clinically benefited from the treatment. However, regarding the modulation of airway remodelling, we surprisingly identified two groups: responders (i.e., reduction of RBM thickening) and non-responders (i.e., increasing or stable RBM). No correlation of remodelling reduction with any clinical or functional parameters was detected [14]. Although a relationship between the smooth muscle bronchial component of remodelling and pulmonary function reduction is known [15], evidence of any muscle reduction upon omalizumab or indeed, any other treatment is lacking.

To improve upon the previous study and in the context of the need to understand the molecular pathways in asthma and relationship to specific phenotypes [16], we further investigated the bronchial specimens that were previously analysed, with the aim of identifying candidate biomarkers of response (in terms of bronchial remodelling reduction). For this purpose, we performed a proteomic analysis of the specimens according to a strategy described by Braido [8] and Wheelock [17]. In particular, we used the multidimensional protein identification technology (MudPIT) proteomic approach, a high-throughput methodology that allows the simultaneous identification of hundreds/thousands of proteins from a single complex sample [18], an evaluation of differential abundance [19,20], and a characterisation of the involved molecular pathways [21].

The protein profiles obtained from the MudPIT analysis of bronchial biopsies were useful for identifying predictive biomarkers and pathways of anti-IgE effects on remodelling and understanding the potential mechanisms involved.

2. Methods

2.1. Subjects

Eight patients with severe, persistent atopic asthma (7 non-smokers; 3 females; range age 40–62 years; mean age 47.0 ± 9.7; mean body mass index (BMI) 23.8 ± 3.1; mean total plasma IgE 309.4 IU/l ± 218.2; mean FEV1 56.2% pred ± 14.5%; ACT score 11.1 ± 2.9) were enrolled in this study.

All the patients were treated with omalizumab according to the AIFA (Italian Drug Agency) prescription rules. The clinical features of the patients were described in a previous report [14]. The study was approved by the local ethics committee.

Two biopsies were obtained from each patient using a flexible bronchoscope (Pentax FB19-TX, Langley, UK) before and 12 months after omalizumab treatment. Bronchial biopsies were obtained from the right middle lobe.

2.2. Morphometric analysis

The reticular basement membrane (RBM) thickness was measured by performing light microscopy image analysis of all the biopsies before and after treatment according to the ERS/ATS recommendations [22] regarding lung structure, as described in a previous study.

2.3. Immunohistochemistry (IHC) and image analysis

Tissue specimens were fixed in 10% formaldehyde at 4 °C for 4 h and were embedded in paraffin. The paraffin sections (3 μm) were dewaxed, hydrated, and pretreated with an antigen-unmasking solution for 30 min. The samples were then treated with blocking solution (BLOXALL Blocking Solution, Vector Laboratories) to inactivate endogenous peroxidases, pseudoperoxidases, and alkaline phosphatase. The samples were then incubated for approximately 10 min in a working solution of blocking serum (normal goat serum).

The sections were incubated overnight at 4 °C with a primary antibody (anti-human GAL-3 rat monoclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 in phosphate-buffered saline (PBS). Subsequently, the sections were incubated with a secondary antibody (biotinylated immunoglobulin G diluted 1:200 in PBS) for 10 min. After several washes with PBS, all the slides were incubated with a concentrated labelled enzyme (biotin–streptavidin–peroxidase complex) for 5 min at room temperature. The peroxidase was developed with 0.04% 3,3-diaminobenzidine in 50 mmol/L Tris–HCl buffer containing 0.03% hydrogen peroxide for 10 min. After being rinsed with PBS, the slides were counterstained with haematoxylin, coverslipped with Eukitt, and examined by light microscopy.

The image analysis was performed using the Leica Q500 MC Image Analysis System (Leica, Cambridge, UK). For each biopsy analysed, the optical density of the signals was quantified using a computer. The video images were generated by a charge-coupled device (CCD) camera (Leica) connected by a frame grabber to a computer. Single images were digitised for image analysis at 256 grey levels. The imported data were quantitatively analysed using Q500MC Software-Qwin (Leica). The tissue was selected by the operator using the cursor, and the positive areas were automatically estimated. A constant optical threshold and filter combination was used. The signal quantisation was expressed as the percent positive area in pixels per square micron [23].

2.4. Protein extraction, estimation, and tryptic digestion for proteomic analysis

The dewaxed tissues (3-μm-thick section from each sample) were dried in a vacuum centrifuge and resuspended in 100 μL of 0.1 M NH_4HCO_3 pH 7.9 buffer with 10% acetonitrile. The tissues were then homogenised and extracted by adding Rapigest™ SF reagent (Waters, Milford, MA, USA) at a final concentration of 0.2% (w/v). The resulting suspensions were incubated under stirring, first at 100 °C for 20 min and then at 80 °C for 2 h. Subsequently, the protein concentration was assayed using the SPN™ Protein Assay Kit (G-Biosciences, Maryland Heights, MO, USA), and 5 ± 0.5 μg of protein from each sample was digested by adding sequencing-grade modified trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:50 (w/w) and incubating it overnight at 37 °C. An additional 0.5 μg aliquot of trypsin was added the next morning, and the digestion continued for 4 h. The addition of 0.5% trifluoroacetic acid (TFA) stopped the enzymatic reaction, and a subsequent incubation at 37 °C for 30 min completed the RapiGest acidic hydrolysis [24]. The water-immiscible degradation products were removed by centrifugation at 14,000 × *g* for 10 min. The tryptic digest mixture was desalted using PepClean™ C18 spin columns (Pierce Biotechnology, Rockford, IL, USA) and was resuspended in 0.1% formic acid at a concentration of 0.4 μg/μL.

2.5. MudPIT analysis

The trypsin-digested samples were analysed using two-dimensional microliquid chromatography coupled online with tandem mass spectrometry (2DC-MS/MS, also referred to as Multidimensional Protein Identification Technology, MudPIT) (Thermo Fisher, San José, CA, USA). Briefly, 5 μL of each digested peptide mixture was first loaded onto a strong cation exchange column (Biobasic-SCX column, 0.32 i.d. × 100 mm, 5 μm, Thermo Fisher) using a Micro AS autosampler (Thermo Fisher). The peptide mixture was then eluted stepwise by applying an eleven-step ammonium chloride concentration gradient (0, 10, 20, 30, 40, 80, 120, 200, 400, 600, and 700 mM). Each salt step was directly loaded onto