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Serum protein profiling of adults and children with Crohn's disease

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Abstract

Objectives—Crohn's disease (CD) and ulcerative colitis (UC), known collectively as inflammatory bowel disease (IBD), are chronic immuno-inflammatory pathologies of unknown etiology. Despite the frequent utilization of biomarkers in medical practice, there is a relative lack of information regarding validated paediatric biomarkers for IBD. Further, biomarkers proved to be efficacious in adults are frequently extrapolated to the paediatric clinical setting without considering that the pathogenesis of many diseases is distinctly different in children. In the current study, proteomics technology was employed in order to monitor differences in protein expression among adult and children CD patients, in order to identify a panel of candidate protein biomarkers that might be used to improve prognostic-diagnostic accuracy and to advance paediatric medical care.

Methods—Male and female serum samples from 12 adults and 12 children with active CD were subjected to two-dimensional gel electrophoresis. Following the relative quantitation of protein spots exhibiting a differential expression between the two groups by densitometry, the spots were further characterized by MALDI-TOF-MS. Results were confirmed by Western blot analysis.

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Results—Clusterin (CLUS) was found to be significantly over-expressed in adults with CD, whereas ceruloplasmin (CERU) and apolipoprotein B-100 (APOB) were found to be significantly over-expressed in children indicating that the expression of these proteins might be implicated in the onset or progression of CD in these two sub-groups of patients.

Conclusions—Interestingly, we found a differential expression of several proteins in adults versus paediatric CD patients. Undoubtedly, future experiments using a larger cohort of CD patients are needed to evaluate the relevance of our preliminary findings.

Keywords

Crohn's disease; ulcerative colitis; proteomics; paediatric; inflammatory bowel disease

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are known collectively as inflammatory bowel diseases (IBD), and have become increasingly common in both children and adult populations worldwide [1]. The factors that determine the age of onset remain unexplained, and the precise aetiology is not yet understood [2]. It has been thought that IBD pathogenesis is the consequence of an overly aggressive cell-mediated immune response to commensal enteric bacteria in a genetically susceptible host [3]. Different studies suggest that patterns of IBD (including location, clinical behaviour and progression) in childhood differ due to a large extent from adult onset disease [4–6]. However, data collected so far appear to be contradictory, since several studies suggested that paediatric CD behaviour seems to parallel that of adult CD [7,8]. Considering the evolution of disease phenotype and behaviour in patients with a paediatric or adult onset-CD, differences might be expected in the respective contributions of genetics, the host immune system, microbiota and environmental factors [9]. Regarding the genetics, several studies suggest that the role of genetic factors is greater in paediatric-onset than in age-related late-onset IBD, indicating the existence of CD heterogeneity according to age of onset [10–13]. Despite the frequent utilization of biomarkers in medical practice, there is a relative lack of information regarding validated paediatric biomarkers [14]. Frequently, biomarkers found to be efficacious in adults are extrapolated to the paediatric clinical setting without considering that the pathogenesis of many diseases like CD, may be distinctly different in children. Even though genetic determinants have long been considered important in the pathogenesis of early-onset IBD, recently investigators have identified genetic polymorphisms specific to this age group, including *IL-10RA*, *IL-10RB*, *IL-10*, *XIAP*, *ADAM17* and the reduced nicotinamide adenine dinucleotide phosphate oxidase genes *NDF2/RAC2* and *NCF4* [15–18].

As it is known that the biological and functional output of cells is governed primarily by proteins, characterization at the level of the proteome is necessary to resolve the crucial changes that occur at different stages of IBD onset [19]. Current proteomic approaches are beginning to have a profound impact on the way and capacity by which we profile protein expression and post-translational modifications, functional interactions between proteins and disease biomarkers [19–25]. Very recently, Piras et al [26], investigated the serum proteomic profile of early and advanced CD in order to identify differentially expressed proteins in acute CD and during the disease course. Inflammatory proteins and complement 3 chain C

(C3c) were over-represented during early CD, while clusterin, retinol binding protein, α 1-microglobulin and transthyretin were under-represented. Nevertheless, to the best of our knowledge, there are no published data available concerning proteomic paediatric CD biomarkers. In the current study, proteomics technologies were employed in order to monitor differences in serum protein expression between adult and child CD patients. Our aim was to identify a panel of candidate protein biomarkers that could be used to improve prognostic-diagnostic decisions and to advance paediatric medical care.

Materials and methods

Patients

Adult patients attended the “Aretaieio” University Hospital, and children attended the First Department of Paediatrics of Athens University, “Aghia Sophia” Children’s Hospital. Patients consisted of those who were newly diagnosed with moderate to severe CD and participation was voluntary. Informed consent was provided by each adult or parent of a child who were personally asked to participate in this study. The study was approved by the ethical committee in the participating hospitals. CD was diagnosed based on a combination of standard clinical, endoscopic, radiological and histological criteria [27,28], and represented a consensus among treating physicians. With respect to age of onset, disease classification was accomplished according to the Montreal system [29], and disease activity was assessed with a Harvey–Bradshaw index (HBI). The main clinical characteristics of the CD patients are detailed in Table 1.

Proteomic analysis

A blood sample was taken from each patient at the time of diagnosis. Following centrifugation, sera were collected and stored at -80°C until use. The sera protein content was determined using the Bioanalyzer Automated Electrophoresis Station (Agilent Technologies Inc., Waldbornn, Germany) combined with a Protein 200 plus kit (Agilent Technologies Inc., Waldbornn, Germany) as previously described [30]. Two dimensional gel electrophoresis (2-DE) was performed as previously described [31,32]. More specifically, 1 mg of total protein from each sample was applied to an 18 cm immobilizer (pH 3–10) and a 17 cm (pH 4–7) linear gradient IPG strip (Bio-Rad Lab, Hercules, CA) at their basic and acidic ends, respectively, using sample cups. Prior to isoelectric focusing, IPG strips were rehydrated overnight in 500 μL of rehydration solution consisting of 8 M urea, 2% CHAPS and 0.4% dithioerythritol (DTE) in rehydration trays. To ensure maximal reproducibility in 2-DE experiments and to prevent variations due to technical factors, all 2-DE gel experiments were carried out simultaneously under the same electrophoretic conditions. First dimensional electrophoresis focusing started at 250 V and the voltage was gradually increased to 5000 V at 3 V/min, where it was kept constant for 25 h (approximately 80,000 Vh in total). Prior to conducting the second dimension, strips were equilibrated in 50 mM Tris–HCl (pH 6.8), containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 30 mM dithiothreitol (DTT) for 15 min and then in the same buffer containing 0.23 M iodoacetamide. The second dimensional electrophoresis was performed in 12% SDS-polyacrylamide gels ($180\times 200\times 1.5\text{ mm}^3$) with a run of 40 mA/gel, using a PROTEIN-II multicell apparatus (Bio-Rad). After vertical electrophoresis, proteins were

fixed in 50% ethanol containing a 10% acetic acid solution for 2 h. The fixative solution was washed-off by agitation in distilled water for 45 min. Protein spots were visualized by application of a Coomassie Blue G-250 staining solution (Novex, San Diego, CA) to 2-DE gels for 12 h. Gel images were scanned in a GS-800 Calibrated Densitometer (Bio-Rad) and stored on a PC for further analysis [20].

Image analysis

Protein spots from all gels analysed were detected, aligned, matched and quantified using the PD-Quest v8.0 image processing software (Bio-Rad) according to the manufacturer's instructions. Manual inspection of the spots was used to verify the accuracy of matching. Spot volume was used as the analysis parameter to quantify protein expression. Normalization of each individual spot was performed according to the total quantity of the valid spots in each gel, after subtraction of the background values. Optical density (O.D.) level (%) of each protein from the CD groups was determined separately and calculated as the sum of the volume % of all spots from all gels containing the same protein. Selection of protein spots or entire gel regions for MS analysis was based upon the O.D. alterations observed between the two groups. A minimum of 1.5-fold change in the expression level was used as a selection criterion at the $p < 0.05$ level.

Protein identification by mass MALDI-TOF-MS

For Matrix-Assisted Laser Desorption Tandem TOF Mass Spectrometer (MALDI-TOF-MS) analysis, protein spots of interest were manually annotated using Melanie 4.02 software and excised from 2-DE gels using a Proteiner SPII instrument (Bruker Daltonics, Bremen, Germany). Gel pieces were then placed into 96-well microtiter plates, destained with 150 μ L of 30% ACN (acrylonitrile) in 50 mM ammonium bicarbonate and dried in a speed vacuum concentrator (MaxiDry Plus, Heto, Allered, Denmark). In-gel digestion was performed with 0.01 μ g/ μ L trypsin (Roche Diagnostics, Basel, Switzerland) for 16 h at room temperature. Next, 10 μ L of 50% CAN containing 0.3% trifluoroacetic acid (TFA), were added to each dried gel piece and digested peptides were extracted. Tryptic peptide mixtures (1.5 μ L) were applied on an anchor chip MALDI plate with 1 μ L of matrix solution, consisting of 0.08% CHCA (α -cyano-4-hydroxycinnamic acid, Sigma) and the internal standard peptides des-argbradykinin (Sigma, 904.4681 Da) and adrenocorticotrophic hormone fragment 18–39 (Sigma, 2465.1989 Da) in 65% ethanol, 50% ACN and 0.1% TFA. Peptide mixtures were analyzed in a MALDI-TOF mass spectrometer (Ultraflex, Bruker Daltonics). Laser shots of intensity between 40% and 60% were collected and summarized and the peak list was created using the Flexanalysis v2.2 software (Bruker). Smoothing was performed with the Savitzky–Golay algorithm (width 0.2 m/z, cycle number 1). S/N was calculated by SNAP algorithm and a threshold ratio of 2.5 was allowed. Peptide matching and protein searches were automatically performed with use of MASCOT Server 2 (Matrix Science). Peptide masses were compared with the theoretical peptide masses of all available proteins from homo- sapiens in the SWISS-PROT and TReMBL databases. Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of four matching peptides. A probability score with $p < 0.05$ was used as the criterion for affirmative protein identification. Monoisotopic masses were used, and one missed trypsin-cleavage site was calculated for proteolytic products. Search

parameters included potential residue mass modification for carbamidomethylation and oxidation. Any redundancy of proteins that appeared in the database under different names and accession numbers was eliminated. If more than one protein was identified under one spot, the single protein member with the highest protein score was singled out from the multiprotein family.

Western blot analysis

Total proteins (10 µg) of control and CD patient group samples were separated by 10% SDS-PAGE under reducing conditions and electroblotted to Hybond-ECL NC membranes (Amersham Biosciences, Uppsala, Sweden). After blocking with 5% non-fat dried milk in TBST solution (20 mM Tris/pH 7.6, 137 mM NaCl, 0.1% Tween20) for 1 h at room temperature, membranes were washed with TBST and incubated overnight at 4 °C with the appropriate primary antibodies against clusterin (CLUS), (sc-56079, dilution 1:200) and ceruloplasmin (CERU) (sc-365206, dilution 1:1000). Next, membranes were washed with TBST and incubated with an anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000). After a final wash with TBST solution proteins were detected by an ECL, West Pico (Pierce) detection system. Western blots were scanned with a GS-800 calibrated densitometer (Bio-Rad). Band quantification was performed with the Quantity One image processing software (Bio-Rad). Human IgG protein was used as internal control to ensure equal sample loading. All antibodies were purchased from Santa Cruz Biotechnology (CA).

Statistical analysis

To ensure confidence in our experimental approach, we employed a design which involved duplicate 2-DE gels per sample (i.e., to determine analytical variation) and separate preparations for each replicate sample per experiment (i.e., to determine biological variation). Comparisons were performed between samples (childhood-onset CD vs adult-onset CD). Mean densitometry values of all spots corresponding to a specific protein from each group were first checked for normal distribution using the unequal variances. Means of spot intensities for proteins with not normally-distributed values were compared for statistical significance with the Mann–Whitney nonparametric test (GraphPad Instat 3 software, GraphPad software Inc., La Jolla, CA). Statistical significance (alpha-level) was defined as $p < 0.05$. To control the False Discovery Rate (FDR), individual α -levels for each spot were adjusted following the FDR correction procedure [33]. In Western Blot experiments; mean protein quantification was performed by three independent experiments for each protein analyzed. Optical density means of the bands for each protein were compared with two sample t-test assuming unequal variances of the Microsoft Excel 2007 software. A $p < 0.01$ was considered statistically significant.

Results

Patient demographic and clinical characteristics are given in Table 1.

Proteomic analysis

To detect those serum proteins differentially expressed between the childhood-onset and adult-onset groups, we separated each protein sample by 2-DE (Fig. 1). Statistical analysis

of resultant 2-DE gels revealed 416 protein spots differentially expressed between the groups. Seventeen proteins corresponding to the 416 spots were identified, since more than one spot was related to the same protein. Most of the proteins identified in the present study were similarly expressed in both children and adult samples. Table 2 summarizes the identified proteins that were significantly differentially expressed in all samples of each patient group. This table provides their identities, theoretical pI, molecular weights, MASCOT scores, protein coverage, and expression levels as calculated with the PDQuest 8.0 software. An expression level > 1 indicates overexpression and < 1 , under-expression. A protein spot identified as clusterin (CLUS) was found to be significantly up-regulated in adult CD patients compared to paediatric CD patients, whereas ceruloplasmin (CERU) and apolipoprotein B-100 (APOB) were found to be significantly over-expressed in children compared to adult CD patients. There was no statistically significant association between the differentially expressed proteins and disease location or clinical behaviour.

Western blot analysis

The differential expression of CLUS and CERU was further confirmed by Western Blot analysis using the appropriate antibodies for children and adult samples (Fig. 2). Optical density measurements of the bands revealed that there was an approximately 2-fold increase in the amount of CLUS in adult samples compared to children and an approximately 2-fold increase in the amount of CERU in the samples from children compared to adults.

Discussion

To date, most proteomic studies have focused on an investigation of biomarkers, as well as determining the effects of drugs on various proteomes. The majority of proteomic studies refer to adults, with very limited research in the pediatric population. In children, proteomics has been used to examine changes in expression levels of proteins that occur during cardiopulmonary bypass surgery [34] and to investigate a number of disease states such as sickle cell disease [35], leukemia [36] and cystic fibrosis [37]. Regarding IBD, several protein markers have been reported to help in the differential diagnosis of UC and CD [22,24], the early and advanced stages of CD [26], and to predict the response to Infliximab (IFX) in IBD [23,30]; according to our knowledge, however, there are no studies attempting to mine the entire proteome of serum for candidate biomarkers to predict early onset of CD in children. Due to the relative lack of information regarding validated proteomic biomarkers in children, we have characterized several potential biomarkers in this report using MALDI-TOF-MS that discriminate between onset of CD in children and adults. Despite the small number of patients tested and the potential influence of selection bias, we have obtained reproducible results among our experiments suggesting that select serum proteomic markers might help to understand the differences between children and adult CD onset, and perhaps define new markers that can predict early CD onset. Most of the proteins identified in the present study were similarly expressed among children and adults, however, CERU and APOB were found to be up-regulated in the children CD patients compared to adult-onset, whereas CLUS was found to be up-regulated in the adult CD patients. Even if several studies support the concept that APOB levels were significantly lower in CD patients than in healthy controls [38,39], when we compared CD paediatric and adult populations we

observed higher protein levels of APOB in sera from children indicating that serum APOB might be a primary predictor of inflammatory markers in early-onset disease [40]. It is known that the production of APOB is affected by transcription, mRNA stability, translation, posttranslational processing, secretion and reuptake [40]. Recently, Song et al [41] reported that APOB levels were significantly higher among early-onset subjects with type 2 diabetes compared to those with a later-onset disease. Interestingly, Yokoyama et al suggested that IL-6 induced a marked increase in APOB mRNA levels, and previous studies have demonstrated elevated serum levels of IL-6 in individuals with IBD [42]. Thus our findings suggest that the cytokine network might be differentially involved in the metabolism of APOB under certain conditions of inflammation such as seen in paediatric- and adult-onset CD.

Concerning CERU, it is an acute-phase plasma protein produced by activated macrophages is a ferroxidase enzyme with bacteriocidal activity [43]. Gitlin [44] suggested that the serum CERU levels nearly doubles in response to inflammation or infection. Deshmukh et al [45] has examined serum CERU levels under different types of acute and chronic experimentally-induced inflammatory conditions in rats and concluded that an increase in serum CERU level during an induced inflammatory condition suggests the involvement of serum CERU as one of the body's built-in defensive mechanisms against noxious compounds or inflammation. Recently, Bakhautdin et al [46] reported that macrophages recruited to the inflammatory site are the primary source of colon CERU, and that an increase in plasma levels of CERU during the acute-phase reaction suggests it has an anti-inflammatory function.

CLUS was found to be specifically up-regulated in adult CD patients. It is important to notice that Piras et al [26] recently found that CLUS is under-represented in early stage CD. Gassler et al [47] suggested that enhanced expression of CLUS by crypt epithelia might reflect its cytoprotective function in order to prevent further injury of the intestinal mucosal barrier in CD. Additionally, other reports suggested the presence of a differential CLUS expression in subtype of T cells, the regulation of CLUS expression by proinflammatory cytokines and other molecules, and that the regulation of expression and function of CLUS depended upon its subcellular localization and the interaction of CLUS with nuclear and intracellular proteins [48]. It is important also to notice that while Ignjatovic et al [49] was studying plasma samples taken from healthy neonates through to adults, found a decreased CLUS expression in both neonates and children compared to adults. The expression of CLUS is known to be also induced by processes such as oxidative stress and apoptotic stimuli. Considering that these processes are stimulated by ageing, Ignjatovic et al [49] suggested that the observed age-related increase in abundance of CLUS implicates this protein in the process of ageing.

Finally, our results provide evidence that there is a differential serum protein expression of APOB and CERU proteins in children compared to adults with CD. Our studies show the feasibility of disclosing and identifying potential biomarkers associated with children CD onset using proteomics. The findings also suggest that children and adult CD patients may exhibit different immunological responses to the disease. Several studies suggest that immunological defenses are incompletely developed in children whereas adults are

characterized by immunosenescence [50,51]. However, the functional implications of these changes remains to be determined, and likely have major implications for our understanding of the role of these proteins in a wide range of physiological functions during CD development. Undoubtedly, experiments in larger number of subjects are needed to evaluate the relevance of our preliminary results.

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Figure 1.

Serum samples were analyzed by 2D gel electrophoresis. The gels were stained by coumassie blue. In the gel images [adult (A) and children (B) with CD] each spot represented a protein. Differential expressed proteins were detected by the method described in Material and Methods. The corresponding spots were excised from the gel and their protein content was identified by mass spectrometry.

Figure 2. Confirmation of the differentiated expression of CLUS and CERU by Western blot analysis. Quantification of protein content was performed using scanning densitometry. Each bar represents the mean optical density $\pm$ SD of three independent experiments. Differences were significant at the level of $p < 0.01$.

Table 1

Clinical characteristics of patients with Crohn's disease

	Childhood-onset CD (n = 12)	Adult-onset CD (n = 12)
Sex (Male/Female)	6/6	8/4
Age of diagnosis (yr)	8.25 ± 2.72	40.17 ± 11.05
Family history in first degree relatives	2	0
Localization of the disease		
L1	2	2
L2	4	2
L3	6	8
Disease features		
B1	8	6
B2	2	2
B3	1	2
Extraintestinal manifestations	1	2
H-BI (mean ± SD)	4.86 ± 1.08	6.27 ± 2.29

Table 2
Proteins differentially present in the serum of childhood- and adult- onset Crohn's disease.

Protein name	Protein symbol	MW	pI	Mascot score	Coverage	Expression levels	
						childhood	adult
Clusterin	CLUS_HUMAN	53031.00	5.90	96	34	5.55	54.3 *
Apolipoprotein B-100	APOB_HUMAN	516651.00	6.60	352	29	471.1 *	21.6
Ceruloplasmin	CERU_HUMAN	122983.00	5.40	102	26	123 *	17.3

Differentially expressed proteins in all samples of each patient group present with their name and symbol, theoretical pI and molecular weight calculated using the CalPI/MW available on the Swiss-Prot Web site, MASCOT search score, and coverage of each identification are also listed. Score is $-10 \log(p)$, where p is the probability that the observed match is a random event. Scores N55 indicate identity or extensive homology at the $p < 0.05$ level. Expression levels were calculated in relation to control densities. Expression level >1 indicates overexpression, expression level <1 indicates suppression.

* $p < 0.005$.