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Proteomic identification of human serum biomarkers in diabetes mellitus type 2

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ABSTRACT

Discovery of protein biomarkers in different diseases is an important area of research in the field of proteomics. We have described the levels of protein biomarkers specific to diabetes mellitus type 2 in the local population of Pakistan using proteomic technology. Type 2 diabetic patients, age and sex-matched normal healthy controls were recruited from Sheikh Zayed Hospital, Lahore, Pakistan. Plasma proteins were analysed by 2D liquid chromatographic system in which samples were initially fractionated by chromatofocusing and the selected fractions were further analysed by reverse-phase high performance liquid chromatography. The proteins which showed variation between test and control samples were identified by MALDI-TOF analysis. All the samples belonging to the control and diabetic groups were then analyzed by ELISA and estimated four proteins which were found to vary. Levels of apolipoprotein A-I was found to decrease by −6.4% while apolipoprotein E, leptin and C reactive protein (CRP) were increased by +802, +842 and +872%, respectively, in the diabetic patients as compared to the controls. The discovery of these marker proteins might thus provide an adjunctive method for early detection of risk for this disease.

1. Introduction

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by ineffectiveness of the insulin produced [1]. Protein biomarkers are useful for diagnosis and prognosis of many disease states like diabetes mellitus, various forms of cancer and other diseases. Certain proteins can be up- or down-regulated during disease progression. Detection of these differences in protein expression levels, as a function of disease progression is important for prognosis [2].

The protein biomarkers are useful for predicting long-term mortality in patients with diabetes mellitus and as well as coronary syndromes [3]. Cardiac isoform of α-2 macroglobulin was a new biomarker for myocardial infarcted diabetic patients [4]. Some biomarkers of diabetes were associated with oxidative stress and antioxidant status in young diabetic patients with or without sub clinical complications [5]. The serum levels of the biomarker clusterin and apolipoprotein J increased significantly in diabetes type 2 [6]. C-reactive protein was used to monitor the severity and progression of some well-defined cardiovascular diseases [7]. The intramuscular heat shock protein 72 and heme oxygenase-1 mRNA was reduced in patients with diabetes mellitus type 2 [8]. Proteomic analysis of vitreous from diabetic macular edema recorded six proteins including PEDF, ApoA-4, ApoA-1, Trip-11, PRBP, and VDBP and only Apo H was expressed in non-Diabetics [9]. The search for protein biomarkers specific to diabetes mellitus type 2 has been intensively studied just within the past years. Proteomic identification of salivary biomarkers of type 2 diabetes has been done [10]. Five candidate protein biomarkers were identified in type 1 diabetes as alpha-2-Glycoprotein 1 (zinc), corticosteroid-binding globulin, and lumican were 2-fold up-regulated, whereas clusterin and serotransferrin were 2-fold down-regulated in diabetic samples relative to control samples Application of proteomics in the discovery of candidate protein biomarkers in a diabetes [11]. Proteomic analysis for urinary biomarkers in diabetic nephropathy identified 7 proteins that were progressively up-regulated with increasing albuminuria and 4 proteins that exhibited progressive down-regulation. The majority of these potential candidate biomarkers were glycoproteins [12].

2. Experimental

2.1. Sample collection

125 diabetic and 50 controls individuals (age 35–65 years) were initially enrolled in this study using the stringent inclusion and exclusion criteria recommended by the American Diabetes Associ-

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ation for type 2 diabetes between 12 October 2006 and 1 December 2006.

2.2. Ethical approval of the study

Ethical approval for the study was given by the Ethical/Protocol/Synopsis Committee of FPGMI (Shaikh Zayed Federal Postgraduate Medical Institute, Lahore, Pakistan).

2.3. Estimation of biochemical parameters

Blood and 24 h urine samples were collected from the selected control and diabetic Individuals. Relevant biochemical parameters were assessed and described previously in our study [13].

2.4. Sample preparation for proteomic analysis

Total serum proteins and total urinary proteins were determined by Bradford assay [14]. High abundance proteins like albumin and immunoglobulins were removed from the serum samples by the Proteo-extract kit of Calbiochem (Merck Bioscience, Darmstadt, Germany). 1D analysis was performed using 10% SDS-PAGE according to the recommended method of Laemmli (1970) [22].

2.5. 1st dimension analysis: chromatofocusing (CF)

Equilibration of 100 μl of serum sample was done using chromatofocusing (CF) start buffer upto a final volume of 2 ml containing 5 mg of total serum proteins. HPCF 1D Column (2.1 mm × 250 mm, GE Healthcare, USA) was used where mobile phase comprised of start buffer pH 8.5 ± 0.1, eluent buffer pH 4.0 ± 0.1, high ionic Strength wash and HPLC-grade water with flow rate of 0.2 ml/min at ambient temperature. Detection was done at UV280 nm in the ProteomeLab PF2D (Beckman Coulter Inc., USA) using 600–1000 psi back pressure [12]. The fractions at 0.3 pH intervals were collected in a 96 deep-well plate.

2.6. 2nd dimension analysis: reverse-phase high performance liquid chromatography

Selected fractions from the CF column were analyzed in second dimension reverse-phase high performance liquid chromatography (RP-HPLC) using a HPRP 2D column (4.6 mm × 33 mm) packed with C18 silica beads (Beckman coulter, USA) where mobile phase comprised of 0.10% trifloroacetic acid (TFA) in water and 0.08% TFA in acetonitrile (ACN) with flow rate of 0.75 ml/min at 50 °C. Detection was done at UV214 nm using 1500–2000 psi back pressure [15].

2.7. Mass spectrometric (MALDI-TOF) analysis

1 M NH_4HCO_3 and 10 mM DTT were added to each fraction from HPRP column to final concentrations of 100 and 1 mM, respectively. The resulting fractions were incubated at 60 °C for 10 min and then 1 ml of trypsin solution (100 ng/ml) in 50 mM CH_3COOH was added. Digestion was done by incubation in a shaker at a medium speed at 37 °C for 24 h. The tryptic digests were evaporated and then reconstituted in 3 ml HPLC-grade water and purified with a ZipTipc18 (Millipore, Billerica, MA, USA) using the procedure recommended by the manufacturer. The purified peptides were eluted from the ZipTip directly onto a stainless steel MALDI target plate (Bruker Daltonics, Bremen, Germany) with 1 ml of a saturated solution of α-cyanohydroxy cinapinic acid in a mixture 0.1% TFA:ACN (2:1, v/v). The peptide mixture after drying at room temperature was analyzed with a Reflex IV MALDI-TOF TOF mass spectrometer (Bruker Daltonics) and the protein identity was obtained by MASCOT program using Swiss-Prot and NCBInr databases limited to *Homo sapiens* species [16].

2.8. Protein analysis by ELISA

Quantification of the proteins, which were found to vary between the controls and the diabetics, was done in all the diabetes mellitus type 2 patients and the controls by ELISA methods. Levels of C-reactive protein (CRP), apolipoprotein A-1 (apo A-1), apolipoprotein E (apo-E) and leptin were determined. All the assays were based on non-competitive sandwich ELISA method using commercially available kits. Protocols, as recommended by the manufacturers, were used for the assays. CRP was assayed using APLCO Diagnostic kits USA. For apolipoproteins A-1 and E the assay kits were supplied by Mabtech AB, Sweden. Leptin in the samples was assayed using leptin ELISA kit supplied by the Ray Biotech, UK.

2.9. Statistical analysis and software's used

All statistical analysis was done with the SPSS statistical software package (version 17.0). Statistical tests and Significance of difference between various groups were determined using Student's *t*-test and Mann–Whitney U-test, respectively. For protein profiling 32Karat, ProteoVue, DeltaVue and Mascot software's were used for identification and characterization of the biomarker proteins in all the samples.

3. Results and discussion

In this study, the serum samples were initially analyzed by 2D liquid chromatography in which the samples were first analyzed by chromatofocusing and the selected fractions from the first analyses were further analyzed by reverse-phase chromatography. The identity of selected fractions was established by analysis of the intact protein and their tryptic digest by mass spectroscopy. Thereafter the levels of the proteins which were varied in diabetic samples or the samples from diabetic patients were determined by ELISA.

3.1. 1st dimension analysis (chromatofocusing)

The elution profile of the proteins in the various fractions as analysis by the chromatographic columns has been shown (Fig. 1). The protein composition in the fraction range 20–22 seemed to vary between that of healthy control and the samples of the diabetic patients. The protein levels in the fractions 20, 21 and 22 was quite low in the healthy samples whereas in the diabetic samples the significant levels of at least three proteins in these fractions. The fractions 20, 21 and 22 as obtained from the first dimension analysis were analyzed further by reverse-phase chromatography column in the representative samples of all groups of individuals.

3.2. 2nd dimension analysis (RP-HPLC)

Fig. 2 showed the elution profile of the fraction 20, 21 and 22, respectively, from the control sample. The analysis of the diabetic placebo group showed that there were two protein species fractions 20 from 1st dimension but only one protein species in fractions 21 and 22. The fraction 20 of the diabetic sample showed two prominent peaks as compared to only one in case of control sample. Also, fractions 21 and 22 of the diabetic group showed significantly higher level of proteins as compared to the control. This pattern of protein composition of these corresponding fractions seemed very

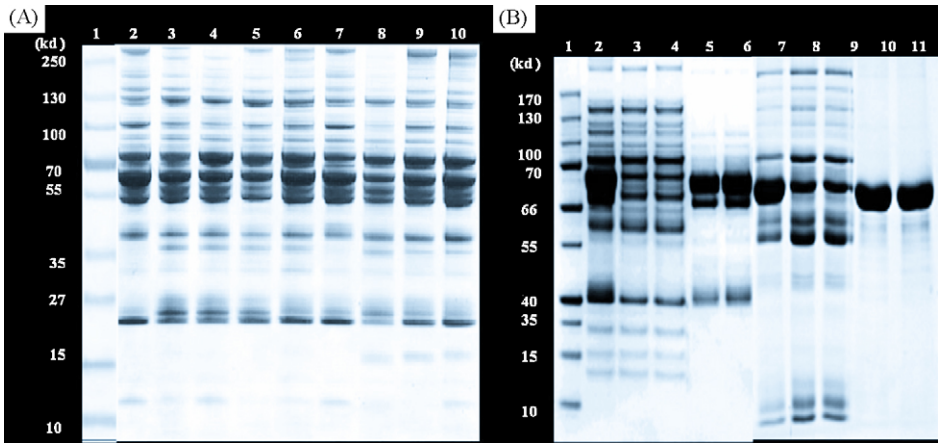

Fig. 1. (a) 10% SDS-PAGE analysis of human serum of control and diabetic individuals. (b) Electrophoresis of human serum, control and diabetic individuals with and without depletion of abundant proteins.

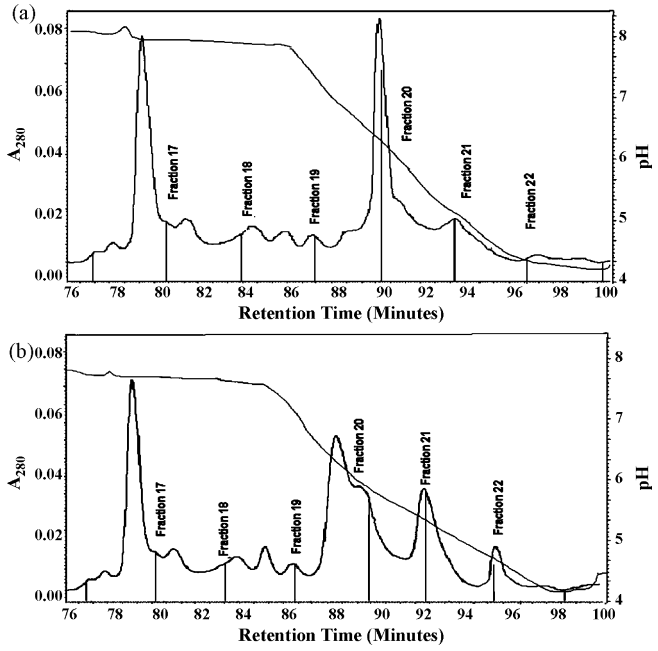

Fig. 2. Analysis of serum proteins of representative (a) samples of normal with comparison of (b) diabetic groups by first dimension chromatography (Chromatofocusing).

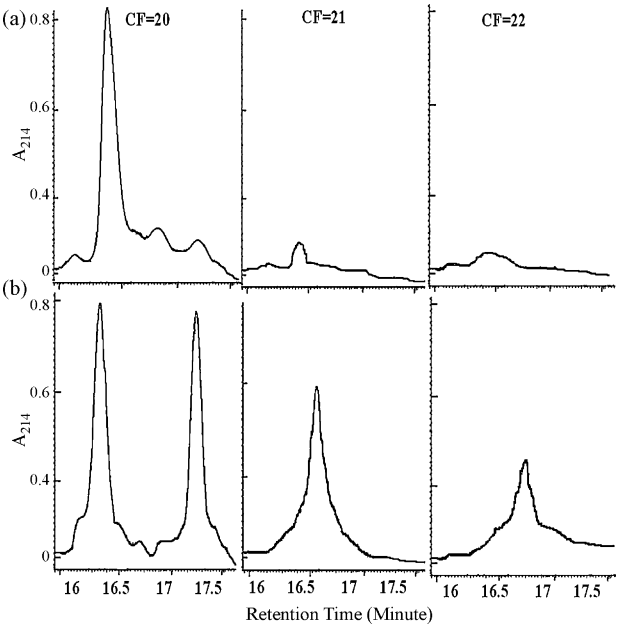

Fig. 3. Second dimension reverse-phase high performance liquid chromatographic analysis of comparison of normal with diabetic group from fractions 20 (a), 21 (b) and 22 (c) obtained from the first dimension analysis.

similar in the diabetic group. The proteins fractions 20, 21 and 22 from the chromatofocusing column contain proteins which were in the p*I* range of 6.05–5.91, 5.91–5.67 and 5.67–5.43, respectively was analyzed by ProteoVue and DeltaVue software's (Table 1).

3.3. Mass spectrometric analysis (MALDI-TOF)

The four protein fractions which were obtained from the reverse-phase chromatography in the representative sample of various groups were analyzed by mass spectrometric analysis in order to confirm their identity. The intact molecular mass of each

Table 1
Identification of protein biomarkers in diabetes type 2 by 2D liquid chromatography and Mass Spectrometric (MALDI-TOF) analysis.

CF fractions p*I*	Protein identified	Accession no.[a]	Observed MW (D)	Theoretical MW (D)/p*I*	Identification method[b]	MALDI MOSWE score	Matched peptides no. (%)	Sequence coverage	% age change
6.05–5.91	Apolipoprotein A-I	P02647	30778.56	30778/5.9	PMF, MS	5.49e+28	66/67(98)	98%	+872
6.05–5.91	Apolipoprotein E	P02649	36054.23	36154/5.9	PMF, MS	6.33e+8	20/23(87)	86%	−6.4
5.91–5.67	C reactive protein	P02741	25038.025	25039/5.7	PMF, MS	4.29e+6	12/24(50)	80%	+802
5.67–5.43	Leptin (precursor)	P41159	18641.30	18641/5.5	PMF, MS	9.56e+10	18/27(67)	70%	+842

[a] Accession number in NCBInr databases.
[b] PMF, peptide mass fingerprinting (MALDI-TOF TOF MS).

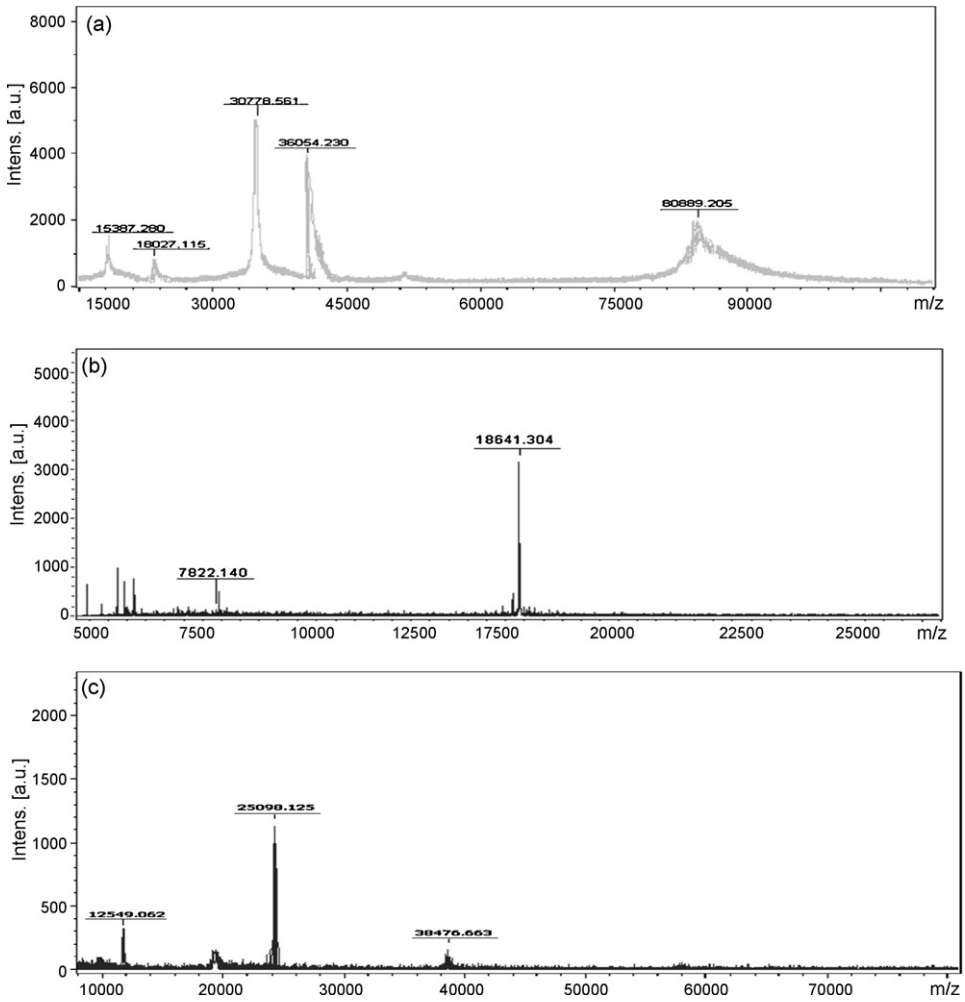

Fig. 4. Mass Spectrometric analysis (MALDI-TOF) of four protein species showing their intact masses, two from the fraction 20 (a) and one each from fraction 21 (b), and 22 (c).

of these proteins as well as the mass profile of tryptic digest of these proteins was also determined. Fig. 3(a) showed the analysis of the two proteins take into together in the same sample which showed the protein mass of the two species 30778.56D and 36054.23D which corresponding to apolipoprotein A-I and apo-E, respectively. The analysis of peptide profile for each of the protein as done by MASCOT software, confirm that these protein were apolipoprotein A-I and E. Similarly Fig. 3(b) showed that mass of the protein present in fraction 21 of 1st dimension chromatography and purified by 2nd dimension chromatography was found to be 25038.025D, which was the same as that of C-reactive protein. Similarly the protein species in fraction 22 of 1st dimension analysis and purified by reverse-phase column in the intact state as shown in Fig. 3(c) was found to be leptin precursor with molecular weight of 18641.30D. On the basis of this data, the protein identified in the four fractions was apolipoprotein A-I, E, CRP and leptin, respectively (Figs. 3 and 4).

3.4. Quantification analysis by ELISA

In order to determine the levels of four proteins as confirmed by mass spectrometric analyses of the levels of these proteins in all the groups of individuals was done by ELISA assay, which is more efficient and economical. There were 872% and 842% increase in the level of CRP and apolipoprotien E, respectively in the diabetic's patients as compared to the controls. The level of leptin was also 280% higher in the diabetic as compared to the controls. The level of apolipoprotien A-I, however was marginally lower in the diabetic individuals. The variations in the level of these proteins in the diabetic groups were significant (Table 2).

In this study, increased levels of C-reactive protein, apolipoprotein E, leptin in diabetic group as compared to the control samples have been observed. The level of apolipoprotein A-1 was down-regulated and E was elevated in the diabetes [17]. Studies indicate that human obesity may be associated with leptin protein and their up-regulation in obese diabetes mellitus [18]. We found the up-regulated levels of apolipoprotein E and leptin in our population that may reflect altered adipocyte function. Apolipoprotein and leptin both are involved in obesity. Our results showed increase in CRP in the diabetic population that actually supports a possible role for inflammation and obesity in diabetogenesis.

Table 2
Quantification of identified biomarker proteins after proteomics by ELISA assay in the control and diabetic groups.

Variable*	Control	Diabetic
C-reactive protein (μg/ml)	6.5 ± 1.30	64.2 ± 28.3[a]
Apolipoprotein A-I (g/l)	1.76 ± 0.67	1.4 ± 0.68
Apolipoprotein E (g/l)	1.05 ± 0.55	9.47 ± 2.56[a]
Leptin (ng/ml)	1.348 ± 0.5	12.7 ± 1.74[a]

*,[a] $P < 0.001$, control baseline compared with diabetic baseline.

These results were in accord with previous hypotheses that in type 2 diabetes mellitus, CRP concentration was also report to increase in obesity and diabetes in a sample population of United States of America. Elevated levels of inflammatory markers like interleukin-6 (IL-6) and CRP were associated with development of type 2 diabetes mellitus in the women of American population [19]. Higher risk of coronary heart disease in diabetes mellitus was associated with hyperglycaemia and plasminogen activator inhibitor-1 [20]. In diabetes cytosolic hyperglycemia in endothelial and mesengial cells leads to accumulation of triose phosphates and increased formation of diacyglycerol *de novo*, activation of protein kinase Cβ, activation of polyol pathway, activation of hexosamine pathway, increased influx from glycerol phosphate shuttle and mitochondrial dysfunction with increased oxygen radical production and increased formation of methylglyoxal and related formation of advanced glycation end products [21]. These inflammatory processes lead to increased formation of CRP an acute phase inflammatory protein from the liver released into the blood. It may be said that CRP is not only predictive marker but also a prognostic marker for many metabolic disorders such as diabetes mellitus [7].

4. Conclusion

Elevated levels of CRP, apolipoprotein and leptin were found to be a powerful independent risk determinant. Apolipoprotein A-I levels also was down-regulated among diabetics. Our data further consolidates the evidence that CRP, apolipoproteins and leptin as protein biomarkers for diabetes mellitus type 2. The discovery of these marker proteins might provide an adjunctive method for early detection of risk for this disease.

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