

Bioinformatics characterization of differential proteins in serum of mothers carrying Down syndrome fetuses: combining bioinformatics and ELISA

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Abstract

Introduction: Characterization of novel proteins in maternal serum derived from mothers carrying Down syndrome (DS) fetuses.

Material and methods: Based on last comparative proteomic analysis, five significant differences of expressed proteins in serum from four groups have been confirmed by ELISA. DAVID and GeneGo MetaCore were used to bioinformatically analyze candidate protein markers.

Results: The serum levels of ceruloplasmin (CP) and complement factor B (CFB) were significantly increased in mother carried DS fetuses (346.5 ng/ml and 466.8 ng/ml vs. 248.6 ng/ml and 293.5 ng/ml, $p < 0.05$). Twenty-nine proteins were mainly categorized into binding, catalytic activity and enzyme regulator activity proteins, and their biological roles were involved in biological regulation, metabolic processes, cellular processes, and response to stimuli. The immune response alternative complement pathway was the most significant GeneGo Pathway related to DS.

Conclusions: These 29 proteins have relations with the development of Down syndrome, especially CP and CFB play more important roles.

Key words: Down syndrome, proteomic, serum, bioinformatics, biomarkers, prenatal diagnosis.

Introduction

It is well known that prenatal screening and diagnosis for Down syndrome (DS) are important for prepotency. They have been widely used in pregnant women all around the world in the past 25 years. Nowadays, the main methods of prenatal screening are screening in the second trimester [1], screening in the first trimester [2] and integrated screening [3]. The biomarkers associated with DS which have been reported previously include α -fetoprotein (AFP), unconjugated estriol (uE3), free β subunit of human chorion gonadotrophin (β hCG), pregnancy-associated plasma protein A (PAPP-A), disintegrin and metalloprotease 12 (ADAM-12), superoxide dismutase 1 (SOD1), and so on [4]. However, all of these markers still could not be sufficient, because they only achieved a detection rate of 50–85% at a 5% false-positive rate [5-8]. Not only will many pregnant women carrying DS fetuses miss diagnosis, but also some will be offered

invasive and risky diagnostic procedures. Therefore, prenatal medical experts are increasingly focusing on discovery of new biomarkers which can improve the efficiency of DS screening.

In recent years, proteomic studies have proved to be a powerful platform for biomarker discovery, and the identified new biomarkers will revolutionize diagnostics for many diseases [9-13]. Nagalla *et al.* [14] attempted to identify potential serum biomarkers to detect DS by a comprehensive proteomic analysis firstly. However, similar studies based on maternal blood have been limited [14-17]. The protein biomarkers reported by these studies were not always reproducible at the same time. Hence, it is important to improve the research on application of proteomics in DS.

In our previous study, we used 2-DE and MS to identify the different proteins for DS in maternal serum, and successfully identified 29 protein biomarkers [18]. Moreover, we also found that TF, DES, SERPINA1, CP, APCS, CFB, C4A, CPN1, CFHR1 and PLG were noteworthy for further study, because they showed the most significant changes. Although some reports have indentified some proteins in maternal serum which were considered as new DS markers, these proteins have not been analyzed by bioinformatics methods. Thus we used bioinformatics analysis to determine whether these 29 proteins are related to the aetiology and function of DS.

This work describes the biochemical characteristics of novel proteins in maternal serum from mothers carrying DS fetuses, and explores their complicated interrelations. By ELISA, we aimed to detect the serum concentration of 5 proteins which showed the most significant differential expression, and carry out bioinformatics analysis using DAVID and MetaCore.

Material and methods

The study design and protocol were reviewed and approved by the ethics committee of Changzhou Woman and Children Health Hospital affiliated to Nanjing Medical University.

Samples collection

The maternal serum was collected from 11 pregnant women carrying a DS fetus and 10 controls with a normal fetus. They all entered the system of prenatal screening in Changzhou Women and Children Health Hospital from April 2008 to June 2010. The mothers with DS were prenatally diagnosed by the karyotype analysis of fetal cells from AF obtained by amniocentesis between gestational weeks 16 and 21. Ten women with normal fetuses were selected as the control group. The match conditions of cases and the control group were: 1) age < 1 year, 2) gestational weeks < 7 days.

Eleven DS patients (2 days – 3 months) and 10 control subjects (3 days – 1 month) underwent routine clinical and laboratory evaluation. All DS patients examined were confirmed to possess the chromosome abnormality (trisomy 21). Control subjects had no previous history of neurological deficits or any serious disorder.

Three millimetres blood samples of all the cases were collected by simple needle aspiration. The samples were centrifuged at 3000 rpm for 5 min to remove cells. The serum was stored at –86°C until further analysis.

Methods

2DE-MS and protein identification

Firstly, we depleted high-abundance proteins in serum, including albumin, IgG, and so on. Then, 2-DE and MS were used to identify the proteins for DS in maternal serum. Twenty-nine proteins were identified successfully in the end. The methods of 2-DE, MS and protein identification were performed as in our report [18].

ELISA

Among the 29 protein biomarkers, we selected 5 proteins to detect their serum concentration. They were Ceruloplasmin (CP, P00450), Complement factor H-related protein 1 (CFHR1, Q03591), Complement factor B (CFB, P00751), Desmin (DES, P17661) and Plasminogen (PLG, P00747).

Enzyme-linked immunosorbent assays for these 5 proteins were based on the non-competitive sandwich ELISA method using commercially available kits purchased from R&D, USA. The main protocol was performed as described previously [19].

Bioinformatics analysis

DAVID (<http://david.abcc.ncifcrf.gov/>, version: 6.7) [20, 21] was used to process the bioinformatics analysis of these candidate protein markers, including protein classification (based on Biological Process Ontology and Molecular Function Ontology, respectively), enrichment analysis for significant gene ontology categories, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping and significant pathway computing, and so on. GeneGo MetaCore (<http://www.genego.com>, version: 6.5) [22] was used to analyze the pathways of these proteins and related genes. At the same time, we generated biological association networks with MetaCore.

Statistical analysis

All data were collected and statistically analyzed using SPSS 13.0 software. Results of parameters were expressed as mean and SD. We compared dif-

ferences in serum level of proteins by means of the *t* statistic. A value of *p* less than 0.05 was considered to be statistically significant.

Results

Differential expression of five proteins

A total of 29 proteins were identified successfully in maternal serum coming from DS cases compared with the control group, including 14 proteins that were up-regulated, while 15 proteins were decreased (Table I). More results of the 29 proteins were described in our last study [18]. In the present study, we selected 5 proteins for further analysis, including CP, CFHR1, CFB, DES and PLG. Their entry name, protein name, molecular weight (MW), PI,

score, coverage, expect and the fold change of expression density are shown in Table II.

Serum concentration

In order to verify the results of 5 proteins as identified by MALDI-TOF-TOF/MS, we detected the serum concentrations of them by ELISA. Table III shows the ELISA results of 5 proteins in the four groups.

Compared with women with normal fetuses, the serum levels of CP and CFB were significantly increased in mothers carrying DS fetuses (*p* < 0.05). The mean concentrations were 346.5 ng/ml and 466.8 ng/ml respectively, vs. 248.6 ng/ml and 293.5 ng/ml in the control group, respectively (Figures 1 A, D). There were no significant differences in the amount

Table I. Twenty-nine proteins differentially expressed in serum of mothers with DS fetuses

Gene names	Entry name	Accession	Protein names
A1BG	A1BG_HUMAN	P04217	α1B-glycoprotein
AFM	AFAM_HUMAN	P43652	Afamin
AMBP	AMBP_HUMAN	P02760	Protein AMBP
APCS	SAMP_HUMAN	P02743	Serum amyloid P-component
APOA1	APOA1_HUMAN	P02647	Apolipoprotein A-I
APOH	APOH_HUMAN	P02749	β ₂ -glycoprotein 1
C4A	CO4A_HUMAN	P0C0L4	Complement C4-A
CFB	CFAB_HUMAN	P00751	Complement factor B
CFH	CFAH_HUMAN	P08603	Complement factor H
CFHR1	FHR1_HUMAN	Q03591	Complement factor H-related protein 1
CFHR2	FHR2_HUMAN	P36980	Complement factor H-related protein 2
CFI	Q8WW88_HUMAN	Q8WW88	CFI protein
CLU	CLUS_HUMAN	P10909	Clusterin
CP	CERU_HUMAN	P00450	Ceruloplasmin
CPN1	CBPN_HUMAN	P15169	Carboxypeptidase N catalytic chain
DES	DESM_HUMAN	P17661	Desmin
GC	VTDB_HUMAN	P02774	Vitamin D-binding protein
GRIN1	GRIN1_HUMAN	Q7Z2K8	G protein-regulated inducer of neurite outgrowth 1
HPX	HEMO_HUMAN	P02790	Hemopexin
KLC2	KLC2_HUMAN	Q9H0B6	Kinesin light chain 2
LRG1	A2GL_HUMAN	P02750	Leucine-rich α2-glycoprotein
MASP2	MASP2_HUMAN	O00187	Mannan-binding lectin serine protease 2
PEPD	PEPD_HUMAN	P12955	Xaa-Pro dipeptidase
PLG	PLMN_HUMAN	P00747	Plasminogen
RIMS3	RIMS3_HUMAN	Q9UJD0	Regulating synaptic membrane exocytosis protein 3
SERPINA1	A1AT_HUMAN	P01009	α1-antitrypsin
TF	TRFE_HUMAN	P02787	Serotransferrin
VTN	VTNC_HUMAN	P04004	Vitronectin
ZNF485	ZN485_HUMAN	Q8NCK3	Zinc finger protein 485

Table II. Five proteins differentially expressed in serum of mothers with DS fetuses

Entry name	MW	PI	Score	Coverage	Fold change	Expect
CFAB_HUMAN	86847	6.67	264	43%	17.5/0	2.70E-22
FHRI_HUMAN	38766	7.38	82	31%	1.0/0	0.00045
CERU_HUMAN	122983	5.44	119	21%	5.43	8.60E-08
DESM_HUMAN	32382	4.97	78	39%	7.0	0.001
PLMN_HUMAN	15776	8.73	92	44%	0.7/0	3.90E-05

Table III. Serum concentrations of proteins by ELISA

	<i>n</i>	CP [ng/ml]	DES [nmol/l]	PLG [ng/ml]	CFB [ng/ml]	CFHR1 [ng/ml]
Mothers with DS fetuses	11	346.5 ±111.8 ¹	21.1 ±13.5	2.1 ±1.3	466.8 ±216.4 ¹	109.0 ±35.5
Mothers with normal fetuses	10	248.6 ±78.3	20.1 ±11.7	1.7 ±1.2	293.5 ±75.0	88.6 ±51.9
<i>t</i>		2.301	0.191	0.786	2.400	1.058
P		0.033	0.851	0.442	0.027	0.303
DS patients	11	166.1 ±55.0 ²	6.5 ±2.3 ¹	0.9 ±0.4	174.3 ±55.0 ²	44.6 ±17.4 ¹
Normal babies	10	244.0 ±36.0	12.3 ±7.8	0.9 ±0.4	311.8 ±102.4	67.4 ±15.2
<i>t</i>		-3.873	-2.273	-0.608	-3.776	-3.218
P		0.001	0.035	0.550	0.001	0.005

¹compared with control group $p < 0.05$, ²compared with control group $p < 0.001$

of CFHR1, DES and PLG between the two groups ($p > 0.05$) (Figures 1 B, C, E). However, the levels of CP, CFB, DES and CFHR1 were decreased in DS patients. There were significant difference between DS patients and normal babies ($p < 0.05$). Especially, CP and CFB were significantly reduced ($p < 0.001$). The level of PLG still had no significant changes ($p > 0.05$).

Gene ontology analysis

By DAVID, 29 proteins were mapped to at least one annotation term within the GO molecular function category, including 40 (53.3%) binding proteins, 9 proteins (12.9%) with catalytic activity, 8 proteins (10.7%) with hydrolase activity, 5 proteins (6.7%) with enzyme regulator activity, 5 proteins (6.7%) with transporter activity, and 9 others (12.0%), including proteins with signal transducer activity, receptor activity, motor activity, oxidoreductase activity, structural molecule activity, and ion transmembrane transporter activity, as shown in Figure 2 A. On the other hand, they were also mapped within the GO biological process category: biological regulation (18, 13.5%), metabolic process (18, 13.5%), cellular process (18, 13.5%), response to stimulus (15, 11.3%), establishment of localization (11, 8.3%), localization (11, 8.3%), immune system process (8, 6.0%), multicellular organismal process (8, 6.0%), developmental process (7, 5.3%), cellular component organization (6, 4.5%) and other proteins (13, 9.8%) related to the categories of multi-organism process, biological adhesion, death, cellular component biogenesis, locomotion, reproduc-

tive process, reproduction, and growth, as shown in Figure 2 B.

KEGG pathway analysis

Relating the gene symbol of these proteins to the KEGG GENE ID, a total of 12 proteins corresponded to 15 pathways of KEGG, of which the pathway of Complement and coagulation cascades was the most significantly enriched. There were 7 proteins participating in the pathway: C4A, CFB, CFAH, CFI, MASP2, PLG and SERPINA1.

Enrichment analysis

Enrichment analysis consists of matching gene IDs of proteins in functional ontologies by MetaCore. The probability of a random intersection between a set of IDs the size of the target list with ontology entities was estimated with the p value of the hypergeometric intersection. A lower p value means higher relevance of the entity to the dataset, which shows in higher rating for the entity. All maps were drawn by GeneGo. The height of the histogram corresponded to the relative expression value for a particular gene/protein.

The top three most significant GeneGo Pathway Maps were: 1) Immune response_Alternative complement pathway, 2) Immune response_Lectin induced complement pathway, and 3) Blood coagulation_Blood coagulation (Figure 3 A). Meanwhile, protein activation cascade, complement activation and regulation of response to stimulus were the

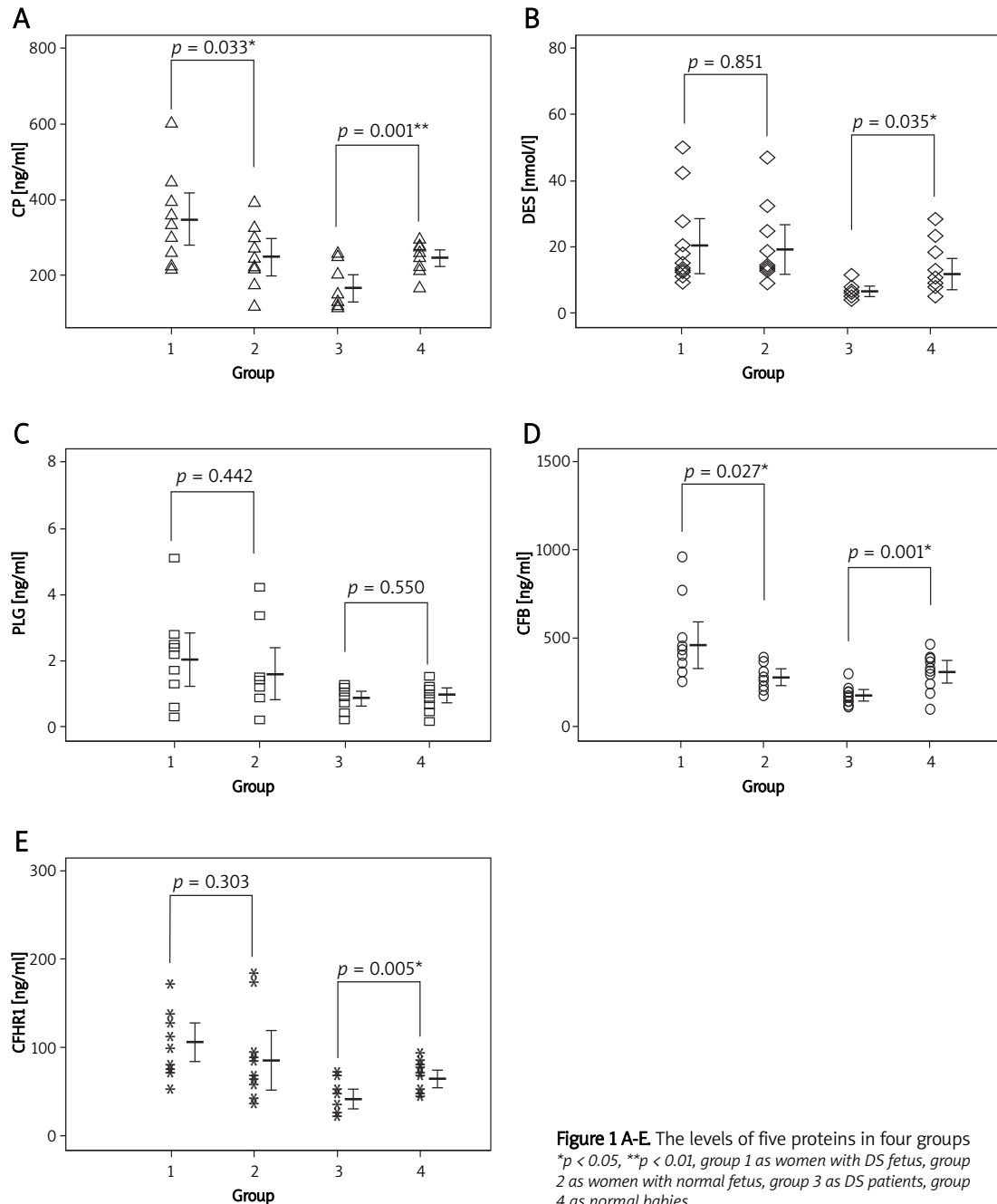


Figure 1 A-E. The levels of five proteins in four groups * $p < 0.05$, ** $p < 0.01$, group 1 as women with DS fetus, group 2 as women with normal fetus, group 3 as DS patients, group 4 as normal babies

most significant enriched GO processes of the proteins (Figure 3 B). With the Disease folders, representing over 500 human diseases annotated by GeneGo, these 29 proteins were mainly related to eye diseases and some kinds of heart diseases (Figure 3 C).

Network connectivity analysis

GeneGo MetaCore was used to generate biological association networks. A total of 15 relevant networks were constructed. The one with the highest score is shown in Figure 4, which was consti-

tuted by 6 proteins with direct interaction. The 6 proteins were PLG, APOH, Vitronectin, Carboxypeptidase N (cat), α 1-antitrypsin and A1M. The PLG was the center of the network.

Discussion

Two-dimensional (2-D) gel electrophoresis and tandem mass spectrometry (MS-MS) have been used to search for new biomarkers, including in DS screening and diagnosis [12, 14-17, 23-27]. But limited studies have been focused on this screening in maternal blood [14-17]. In a past study of this lab,

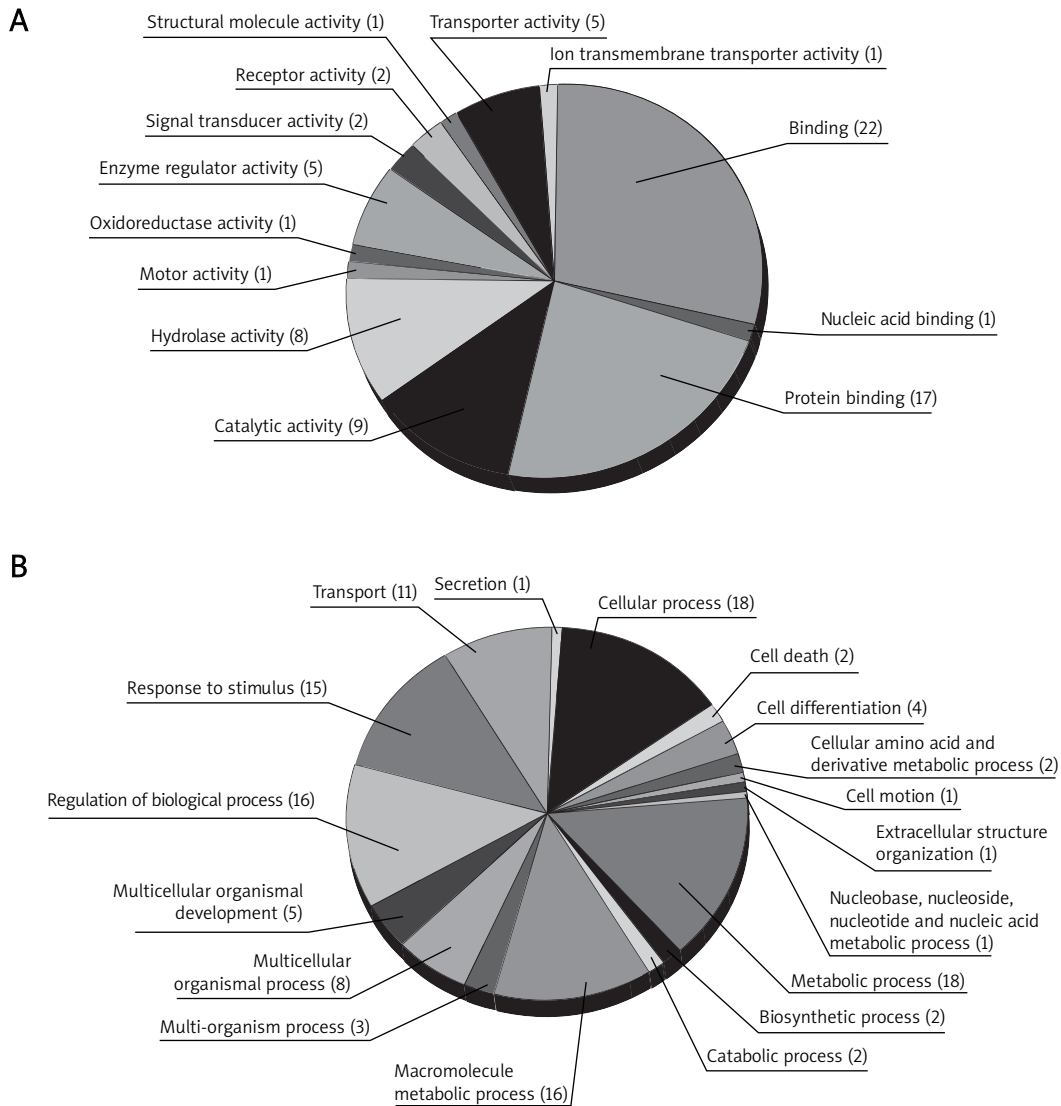


Figure 2. Classification of identified proteins based on GOA: **A** – Molecular Function Ontology, **B** – Biological Process Ontology

29 protein biomarkers for DS in maternal serum were successfully identified by the two methods [18]. The top 5 increased proteins were TF, A1BG, DES, SERPINA1 and CP, while APCS was the most down-regulated one. In the present study, we selected 5 proteins (CP, CFHR1, CFB, DES and PLG) for further analysis (bioinformatics analysis and ELISA). The reasons why we selected them were: 1) the degree of differential expression, 2) biological function of protein, 3) the relationship between protein and disease, 4) learning from published literature. Based on maternal serum, we found that only the serum levels of CP and CFB were significantly increased, while there were no significant differences in the amount of CFHR1, DES and PLG. Some studies have also reported different results between proteomics and ELISA [28, 29]. Perhaps the reasons were: 1) there are differences in the detection sensitivity of the two methods between

MS and ELISA, 2) related to the small number of samples of ELISA tests, 3) some technological limitations of 2-DE and MS. Meanwhile, we found that the levels of CP, CFB, DES and CFHR1 were significantly decreased in DS patients, while PLG still had no changes. Combined with the two ELISA tests, we suggest that CP and CFB play more important roles in DS. They might be used to improve the efficacy of the prenatal screening and diagnosis of DS, and they might relate to the disease phenotype and molecular mechanism of DS. However, the expressions of protein were inconsistent in the serum from mothers and newborn babies. This might be caused by the biosynthesis and metabolism of the proteins, and it is worthy of further research.

The CP is an important iron transport protein for physiological iron homeostasis in the brain and neuronal survival [30], and it is reported to be associated with neurodegenerative disease [31]. Recent-

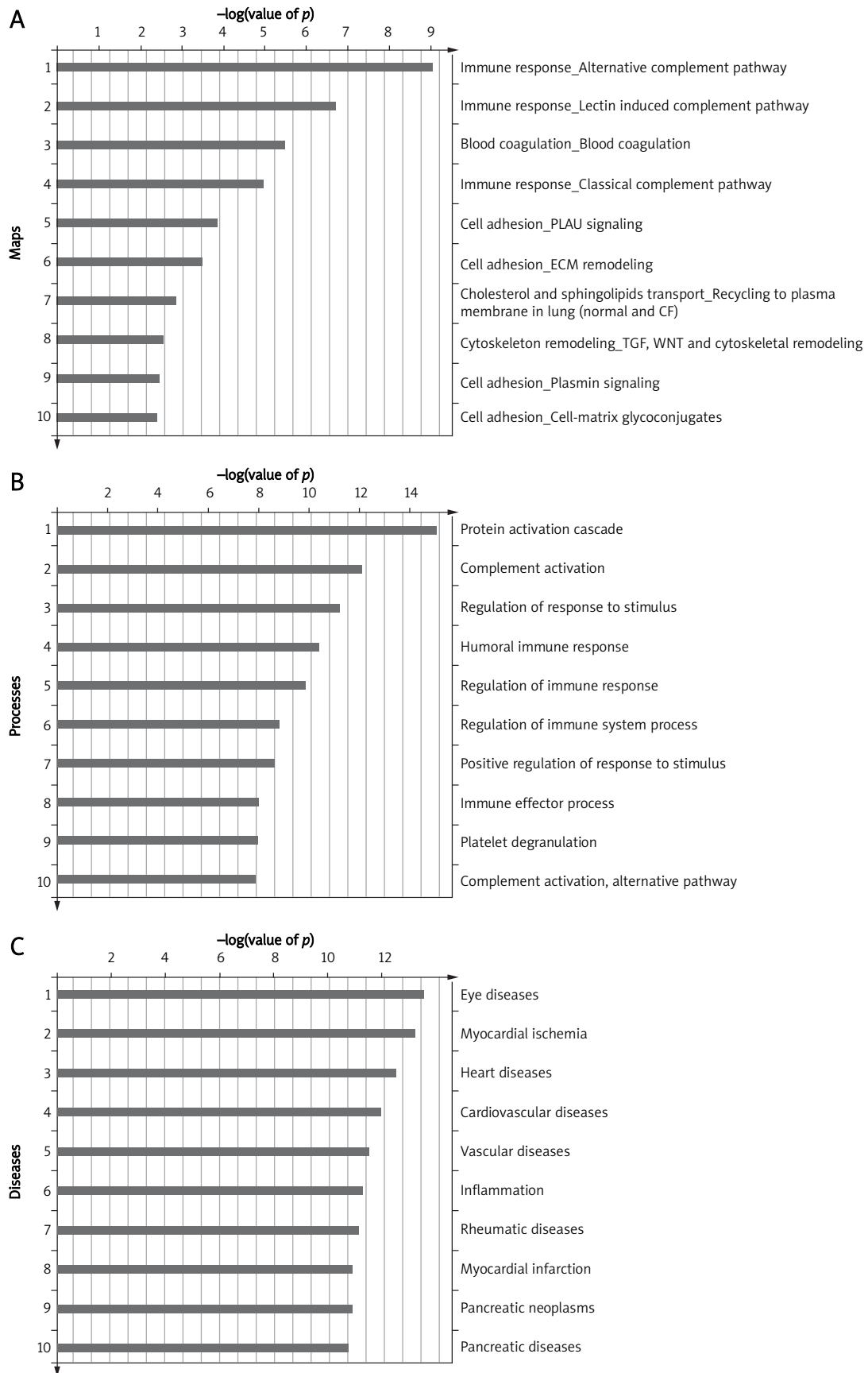


Figure 3. Enrichment analysis of the proteins by GeneGo MetaCore: **A** – GeneGo Pathway Maps, **B** – GO Processes, **C** – GeneGo Diseases (by Biomarkers)

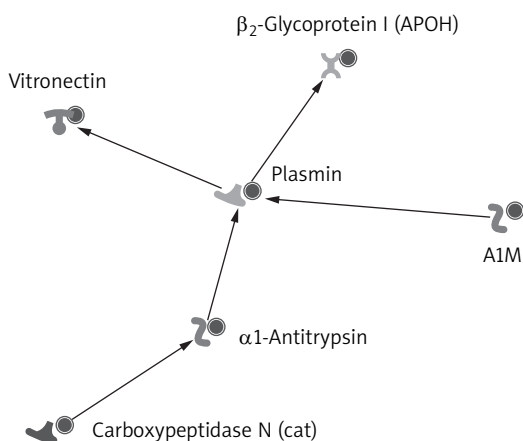


Figure 4. A small network constituted by six proteins with direct interaction

ly, Perluigi *et al.* [32] found that oxidative damage is an early event in DS pathogenesis and might contribute to the development of deleterious DS phenotypes. Moreover, its expression is adjusted by SOD1 [33], which is located on chromosome 21. Complement factor B (CFB, P00751) plays an important role in the alternative pathway for complement activation. Strohmeyer *et al.* [34] reported that CFB was present in the frontal cortex of AD patients, and was significantly increased, indicating alternative pathway activation. It suggested that conditions conducive to chronic alternative pathway activation may exist in the AD brain. Furthermore, as a temporal model for studying the development of AD, the brain in DS often had similar changes as in AD brain [35]. According to our study, both the 2 proteins seemed associated with the brain damage of DS, but the exact mechanisms need further research.

Pathway and network analyses in this study related the immune response alternative complement pathway to DS. Some previous studies have also reported that there were relationships between DS and the complement system [36, 37]. For example, C1q was positive in DS cases and was associated with activated microglia. It provides evidence for antibody-mediated inflammatory factors contributing to the rapid accumulation of neuropathology in DS brain [36]. It was very interesting that we first found CFB also to be associated with DS. Surprisingly, the enrichment analysis of differentially expressed proteins in DS using the MetaCore database showed the most strongly related disease to be eye diseases and some kinds of heart diseases. About 3% of DS newborns are associated with cataract, and the rate of congenital heart disease in DS patients is up to 40%. Thus, these proteins may play important roles in the development of the disease. Fifteen relevant networks were constructed by MetaCore. The highest

scoring network was constituted by 6 proteins with direct interaction. The PLMN was located in the center of the network. However, the serum concentration from both mothers and patients showed no significant change. But the reason for this needs to be more deeply explored in future studies.

Regarding the 29 proteins we found in this study, we think that it needs clinical verification whether these proteins could be used for prenatal screening. Based on the study, we analyzed the characterization of differential proteins by bioinformatics analysis, and found something very interesting. These proteins may play important roles in the development of DS, for example mental retardation. They may help to explain the new mechanism of the disease.

In conclusion, based on comparative proteomic analysis, five significant differences of expressed proteins in maternal serum between normal and DS cases have been confirmed by ELISA. DAVID and GeneGo MetaCore were used for bioinformatics analysis of these candidate protein markers. The combined use of ELISA and bioinformatics analyses proved the different expression of the proteins, and revealed their biological processes and functional network in DS. These 29 proteins have relations with the development of Down syndrome, especially CP and CFB play more important roles.

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