New Biomarkers of Kawasaki Disease Identified by Gingival Crevicular Fluid Proteomics

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Research

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Abstract

Background: Kawasaki Disease (KD) is an acute self-limiting systemic vasculitis syndrome which can result in arterial damage especially in coronary artery. To find possible new biomarkers for the diagnosis of KD by Data independent acquisition (DIA) quantitative proteomics.

Methods: Twenty-seven patients with KD were enrolled in the present study, further, gingival crevicular fluid of before IVIG treatment with KD was collected as the experimental group. Meanwhile, 18 healthy volunteers were recruited as the control group.DIA quantitative proteomics mass spectrometry analysis was performed on the GCF samples of each group, and the protein expression profiles of the two groups of GCF were detected. Function enrichment of DEP by KEGG and GO, protein-protein interaction (PPI) analysis for all the DEPs detected, Finally, the multiple reaction monitoring mass spectrometry method was used to verify the selected DEPs.

Results:197 DEPs (174 up-regulated and 23 down-regulated) were detected in the CGF between the KD group and the normal control group. Cellular process and metabolic process, binding and catalytic activity are the most altered biological process and molecular function, respectively. NOD−like receptor signaling pathway, Protein processing in endoplasmic reticulum pathway and influenza pathway are most significant pathway. EIF2AK2, B2M and GBP1 are kernel proteins in PPI network. The results of MRM-MS of 12 DEPs including IFIT3, UB2L6, HP, A1AT, HSP90AA1, HNRPC, HSP90AB1, SAA1, MX1, B2M, FKBP4 and TRAP1 were highly consistent with DIA.

Conclusions: We suggested that 12 proteins in GCF could be used as new biomarkers for early diagnosis of KD. We also found that KD is closely related to gingival inflammation at the molecular level, which provides new ideas and directions for the diagnosis and treatment of KD.

Background

KD is an acute self-limiting systemic vasculitis syndrome which can result in arterial damage especially in coronary artery(1). The arterial damage will be greater if there is a delay in diagnosis, thus timely and accurate diagnosis and treatment for patients with KD may reduce the harm to the cardiac health and life quality. Biomarkers can be used as essential marker for precision diagnose of patients with atypical KD and may reduce the incidence rate of complications with heart. GCF is the bio-fliud that exudate from periodontal tissues composing of serum and locally generated materials, GCF is considered to rich in multiple immnue factors which can be used as diagnostic test for periodontal disease and certain systemic disorders(2, 3). Besides plasma and urine, GCF is also one of the major human bio-fluids that have been focused for clinical diagnosis use over the past few years. GCF is the exudate from periodontal tissues composing of serum and locally generated materials which can be used as diagnostic test for periodontal disease and other systemic disorders such as Sjogren Syndrome and type-2 diabetes(4). GCF proteins have been reported to change significantly in type-2 diabetes compared to healthy control,

diabetic and prediabetic subjects, which indicated that GCF can be used as biomarkers'source for diabetes mellitus(5).

KD is a disease that considered to be closely related to immune status, which may change the GCF protein composation. However, there has no systemic assessment of potential GCF biomarkers for the diagnosis of KD so far. KD usually induce small to medium sized vasculitis which may also occur in gingival tissue and lead to different components in GCF. In order to investigate the different components of GCF between patients with KD and healthy volunteers, mass spectrometry proteomics was applied. Data independent acquisition (DIA) can efficiently determine protein molecules with very low abundance in complex samples, which can make the data high-throughput, accurat and repeatabe(6). Multiple reaction monitoring mass spectrometry (MRM-MS) can identify protein without synthesizing specific antibodies, which has the advantage of accurate simultaneous measurement of at least 100 protein targets per sample(4).

In the present study, we used proteomics in DIA mode to screen the DEPs and MRM-MS to validate the DEPs. As far as we know, this is the first report about the characteristics of GCF proteins in patients with KD with the opinion that uses the biomarkers in GCF for the diagnosis of KD.

Methods

Subjects

All patients are diagnosed according to the American Heart Association (AHA) guidelines of KD. 28 patients diagnosed with KD in Shenzhen Children's Hospital from October to December 2019 were included and 18 healthy volunteers were also recruited as control group. All manipulations were approved by the Ethics Committees (202003802) at Shenzhen Children's Hospital and written informed consents were acquired from the guardians of all donors. There is no statistical differences of average age and male/female ratio between the two groups .

GCF collection

Patients' GCF was collected when KD was diagnosed before immunoglobulin treatment performed. The GCF of healthy children was collected during the physical examination in the children's health department of our hospital. Using a disposable oral examination kit, the tooth surface was dried with a sterile cotton ball, and then a moisture-absorbing paper (Beijing Dayading) tip was inserted into the gingival crevicular area. The paper tip will be taken out after 30s (if there is blood on the moisture-absorbing paper tip, discard it) and put into a EP tube and stored in the -80℃ refrigerator for later inspection(n=3).

GCF protein extraction

A 5mm steel bead was added to all samples, and protein was extracted with Lysis buffer containing 1mM PMSF and 2mM EDTA (final concentration). After 5 min, 10 mM DTT (final concentration) was added to the samples. After centrifugation at 4°C, 25000g, the supernatant was discarded. To reduce disulfide

bonds in proteins of the supernatant, 10 mM DTT (final concentration) was added and incubated at 56°C for 1 h. Subsequently, 55 mM IAM (final concentration) was added to block the cysteines, incubated for 1 h in the darkroom. The supernatant was mixed well with 4x volume of chilled acetone for 2 h at -20°C to precipitate proteins until the supernatant is colorless (repetition of addition if necessary). An appropriate amount of Lysis Buffer 3 was added to the processed sample for precipitation, following by ultrasonication to dissolve the precipitated proteins. After centrifugating, the supernatant was taken for quantification.

Protein Digestion

One hundred μg of total protein was taken out of each sample solution and digested with Trypsin Gold (Promega, Madison,WI, USA) with the ratio of protein : trypsin =30 : 1 at 37°C for 16 hours. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5M TEAB.

High-pH reverse-phase separation

Ten μg of all samples were taken respectively to mix, and 200 μg mixture was diluted with 2 mL of mobile phase A buffer(5% ACN pH 9.8) and injected into the Shimadzu LC-20AB HPLC system coupled with a Gemini high pH C18 column (5 um, 4.6 x 250 mm). The sample was subjected to the column and then eluted at a flow rate of 1 mL/min by gradient: 5% mobile phase B (95% CAN, pH 9.8) for 10 minutes, 5–35% mobile phase B for 40 minutes, 35–95% mobile phase B for 1 minute, flow Phase B lasted 3 minutes and 5% mobile phase B equilibrated for 10 minutes. The elution peak was monitored at a wavelength of 214 nm and component was collected every minute. Components were combined into a total of 10 fractions, which were then freeze-dried.

DIA analysis by Nano-LC-MS/MS

The peptides were separated by liquid phase chromatography were ionized by a nanoESI source and passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher Scientific, San Jose, CA) in DIA (data-independent acquisition) detection mode. Main parameter settings were: ion source voltage was set to 1.9 kV; MS1 scanning range was 400~1250 m/z; resolution was set to 120,000; maximum ion implantation time (MIT) was 50 ms; 400-1250 m/z was equally divided into 50 continuous windows for fragmentation and signal acquisition. The ion fragmentation mode was HCD, the maximum ion implantation time (MIT) was selected as the automatic mode, the fragment ions were detected in Orbitrap, the resolution was set to 30,000, the fragmentation energy was distributed fragmentation: 22.5, 25, 27.5; AGC was set to 1E6.

MRM-MS Verification

Samples were digested as described and spiked with 50 fMol of β-galactosidase for data normalization. MRM analyses were performed on a QTRAP 6500 mass spectrometer (SCIEX, Framingham, MA, USA) equipped with LC-20AD nanoHPLC system (Shimadzu, Kyoto, Japan). The Mobile phase consisted of solvent A, 0.1% aqueous formic acid and solvent B, 98% acetonitrile with 0.1% formic acid. Peptides were separated on a C18 column (0.075 x 150 mm column, 3.6 μm) at 300 nL/min, and eluted with a gradient

of 5%-30% solvent B for 38 min, 30%-80% solvent B for 4 min, and maintenance at 80% for 8 min. For the QTRAP 6500 mass spectrometer, spray voltage of 2400 V, nebulizer gas of 23 p.s.i., and a dwell time of 10 ms were used. Multiple MRM transitions were monitored using unit resolution in both Q1 and Q3 quadrupoles to maximize specificity.

Statistical analysis

DEPs in the KD compared to normal control samples were identified with Abundance Ratio ≥2 or <0.5, and P-value <0.05 was considered significant. GO and KEGG enrichment analysis were performed using Phyper, a function of R. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value < 0.05). Moreover, by using the String database (https://string-db.org/), a protein–protein interaction (PPI) network of DEPs was obtained.

Results are presented as the mean ± standard deviation (SD). To confirm a significant difference between specific groups, group comparisons were performed using the Student's t-test. In all cases, a *P*-value < 0.05 was considered a statistically significant difference.

Results

DEPs in GCF

A total of 3,353 proteins were detected by DIA. We performed Pearson correlation coeffciency test to evaluate biological repeats are. It turned out that all the 3 biological repeats correlates well with least 0.958 (Figure 1.a). After basic analysis, we discovered 197 significant DEPs in the KD group compared to healthy controls(Figure 1.b), of which 174 DEPs were up-regulated and 23 DEPs were down-regulated.

GO and KEGG Enrichment Analysis of DEPs

We performed GO analysis of all the DEPs disvcoverd in the KD group. We found that the DEPs are mainly up regulation involved in the biological process, cellular component and molecular function (Figure 2). Cellular process and metabolic process, binding and catalytic activity are the most altered biological process and molecular function, respectively. Furthermore, cell killing, biological phase, supramolecular complex, synapse, molecular transducer activity, hijacked molecular function, translation regulator activity, antioxidant activity and protein tag are only up regulation GO term.

There were thirty enriched pathways of DEPs in KD compared with normal (Figure 3A). Similar to GO enrichment, 26 pathways contained only up regulation DEPs. In addition, fourteen significantly enriched pathways are showed in Figure 3B, including NOD–like receptor signaling pathway, Protein processing in endoplasmic reticulum pathway and influenza pathway.

Protein-Protein Interaction (PPI) Network Analysis of DEPs

DEPs were subjected to interaction analysis by comparison with the STRING database and network interactions were plotted taking the top 100 interaction relationships with confidence (Figure 4). We can

see that PPI in the KD DEPs are most consist of 2 networks. The bigger network is a complex interaction composed of 42 proteins and the smaller one is an indepent 3 protein triangle network. EIF2AK2, B2M and GBP1 are kernel proteins, able to interact with more proteins.

Validation of protein biomarkers in KD

We choose 12 significant DEPs among the PPI network for validation using MRM-MS, which showed that the results of MRM and DIA tended to be consistent (Figure 5). Combined with $P < 0.05$, it can be considered that the results detected by the two detection methods are positively correlated. $R^2 = 0.9387 > 0.85$ means that the linear regression fitting is good, indicating thecorrelation. Table 1 showed that the validated DEPs were selected by $P < 0.05$ and Fold Change>1.5, which were all up-regulated.

Table 1
Protein markers in GCF of KD verified by MRM

Protein	Comparison	Fold Change	*P* value	adj.pvalue
IFIT3	KD/N	1.695	0.023	0.025
UB2L6	KD/N	2.464	0.000	0.000
HPT	KD/N	2.393	0.001	0.001
A1AT	KD/N	2.329	0.000	0.000
HS90A	KD/N	1.932	0.000	0.000
HNRPC	KD/N	1.630	0.000	0.000
HS90B	KD/N	1.822	0.000	0.000
SAA1	KD/N	16.159	0.000	0.000
MX1	KD/N	2.959	0.000	0.000
B2MG	KD/N	2.619	0.000	0.000
FKBP4	KD/N	1.766	0.000	0.000
TRAP1	KD/N	1.999	0.000	0.000

Discussion

In this study, we extracted GCF samples from KD patients and healthy volunteers, and performed DIA quantitative proteomics identification and MRM validation for DEPs between the two groups. We identified 197 DEPs from a total of 3353 proteins, of which 174 were significantly up-regulated, and 23 were significantly down-regulated. Then, 46 potential alternative protein markers were found in the GCF of KD using PPI network analysis. Subsequently, we performed quantitative protein detection by MRM-MS to validate 12 DEPs, which were consistent with the results we acheived from DIA detection.

The results of GO enrichment analysis showed that the DEPs in GCF of KD were mainly enriched in the biological processes including cell proliferation, immune system process and cellular process. The main signaling pathways involved in DEPs were cell growth and death, post-transport catabolism, cell motility, cardiovascular diseases, infectious diseases: bacteria, immune diseases. Based on previous published data, we found that cell proliferation, migration, death and immune processes played a key role in the pathogenesis of KD and the occurance of vascular injury(7, 8). The presents of related protein in GCF samples suggested that GCF would be used as a biomarker resource for KD diagnose. The GCF could be used as samples to investigate the pathomechanisms of periodontitis(9). The present research revealed that bacterial infection pathway was also enriched in GCF in patients with KD, which indicated that pathogen infection may be involved in the pathogenesis of KD and periodontitis. In order to screen the key protein markers in GCF for KD, we selected 12 DEPs detected by DIA to verify by MRM-MS, which included SAA1,FKBP4, IFIT3, UB2L6, HPT, A1AT, HS90A, HNRPC, HS90B, MX1, B2MG, and TRAP1. Interestingly, these results were all consistent with the data of DIA.

SAA1 is one of the major proteins of amyloid A (AA), which plays an important role in lipid metabolism, bacterial infection, arterial inflammation and tumor(10, 11). Chen et al. reported the association between genetic locus polymorphisms of SAA1 and coronary artery disease in KD, which indicated that SAA1 may be involved in the process of coronary artery injury (12). The Increased levels of SAA1 protein in human periodontal lesion tissues are positively correlated with periodontal inflammation, and SAA1 may induce inflammatory cell infiltration and release of inflammatory factors through Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4)(13). The present study showed that the protein expression of SAA1 was significantly increased in the GCF of patients with KD, which suggests that SAA1 may be closely related to the pathogenesis of periodontal tissue and vessel inflammation.

The dynein-associated immunoaffinity FKBP52 (FKBP4) belongs to the immunoaffinity protein family and plays an important role in immunoregulatory processes, protein folding and trafficking activities associated with heat shock protein 90 (HSP90)(14). FKBP4 interacts with HSP90 to regulate the activity of the steroid receptor axis. Data have showed that FKBP4-deficient mice could develope specialized phenotypes associated with androgen, progesterone, and glucocorticoid insensitivity(15, 16). HSP90 is a chaperone protein that regulates protein maturation, and is involved in the regulation of atherosclerotic lesions through various pathways such as lipid metabolism disorders, vascular smooth muscle cell proliferation and migration, glucocorticoid receptor axis, and oxidative stress(7, 17–20). Xu 's study has confirmed that the traditional Chinese medicine berberine can protect against oxidative stress damage to coronary endothelial cells in Kawasaki disease by inhibiting HSP90B(7). HSP90 has also been demonstrated to be involved in the immune response to periodontal inflammation due to P. gingivalis infection(21, 22). The results of this study suggested that FKBP4 and HSP90A/B expression were synergistically increased in KD, which revealed that FKBP4 and HSP90A/B may involve in the regulatory process of gingival injury in patients with KD.

Interferon-induced protein repeats with tetratricopeptide 3 (IFIT3) is an interferon-inducible protein with antiviral and proinflammatory effects. IFIT3 can be used as a biomarker of macrophage polarizing

proinflammatory phenotype (M1) and is up-regulated in the arterial tissue of atherosclerotic mice(23). MX1 has anti-pathogen and proinflammatory functions, which has been reported to be associated with the depletion of vascular endothelial progenitor cells and endothelial dysfunction(24). Studies have shown that MX1 is also involved in the regulation of pathogen defense mechanisms in gingival tissues(25). IFIT3 and MX1 are important mediators for inflammation and vascular diseases, which may be involved in the pathogenesis of KD and periodontal diseases.

E2 ubiquitin-conjugating enzyme UB2L6 (UB2L6) belongs to the ubiquitin-proteasome system and is used to transport ubiquitin and promote substrate proteins to complete ubiquitin labeling, which is associated with the regulation of both apoptosis and cell cycle(26). The increased expression level of UB2L6 in the present experiment suggested an increased level of ubiquitination in the gingival tissue of KD, but the molecular regulatory mechanism involved was still unclear. Tumor necrosis factor receptor-associated protein 1 (TRAP1, or HSP75) is a major member of the HSP90 family, which can prevent cardiomyocyte injury induced by hypoxia via maintaining mitochondrial activity(27). Increased TRAP1 expression may indicate a protective function in gingival tissue in patients with KD.

Studies have confirmed that the expression of α1-antitrypsin (A1AT) was increased in the plasma in patients with KD,which can inhibit neutrophil elastase activity associated with coronary artery damage induced by KD(28). Published data have also confirmed that A1AT is also associated with periodontal inflammation, which may identify the severity of periodonatal status(29). Data have also shown that A1AT has antiviraland protective effects in lung diseases, while the role of A1AT in the cardiovascular system has not yet been clearly reported(30, 31). In the present study, the expression of A1AT was up-regulated as mentioned above. Further experiments should be performed to investigate the role of A1AT in the pathogenesis of KD and periodontal diseases.

Conclusions

We found that there were 174 DEPs in GCF between control group and KD group, most of which were associated with cell growth and death, post-transport catabolism, cell motility, cardiovascular disease, infectious diseases: bacteria, immune diseases. Among the 174 DEPs, 12 proteins were validated using MRM-MS, including IFIT3, UB2L6, HPT, A1AT, HS90A, HNRPC, HS90B, SAA1, MX1, B2MG, FKBP4, and TRAP1, which could be used as potential biomarkers for KD diagnosis. The clinical utilizability of the present results should be investigated further via furthur basic researches.

Declarations

Authors' contributions

MX conceived and designed the research, reviewed and revised the manuscript. XF wrote the preliminary manuscript. YL performed a preliminary analysis. YL, GX and JJL collected the samples. All authors read and approved the final version of the manuscript.

Availability of data and materials

All data are available in the manuscript and they are showed in figures.

Code availability

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Ethical Standards and consent to participate

This study was approved by the ethics committee of Shenzhen children's Hospital (Ethics No. 201905402) and was performed in accordance with the Declaration of Helsinki. Written informed consent for participation was obtained from all participants.

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

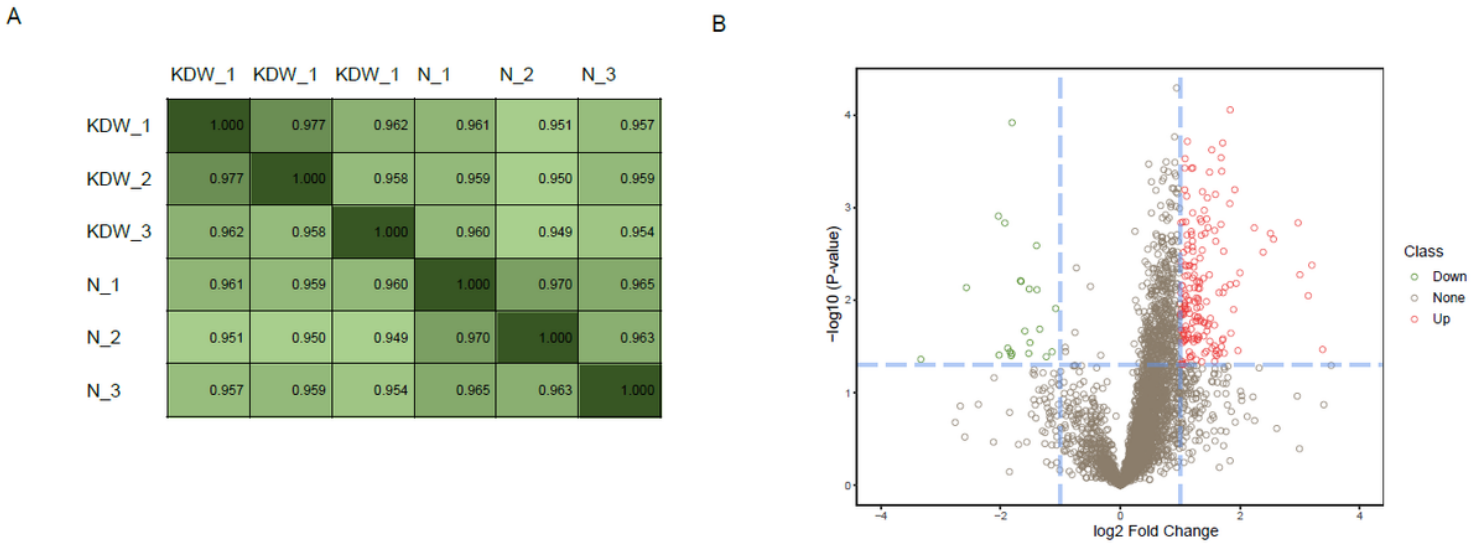

Figure 1

Repeats steadity and differential expressed proteins. a. Pearson coeffiency test of biological repeats. b. Volcano plot of differential proteins. Fold Change (absolute value of fold change) > 2 was the standard screening differential protein based on P < 0.05. Differential proteins significantly up-regulated in the red part and significantly down-regulated in the green part of the figure.

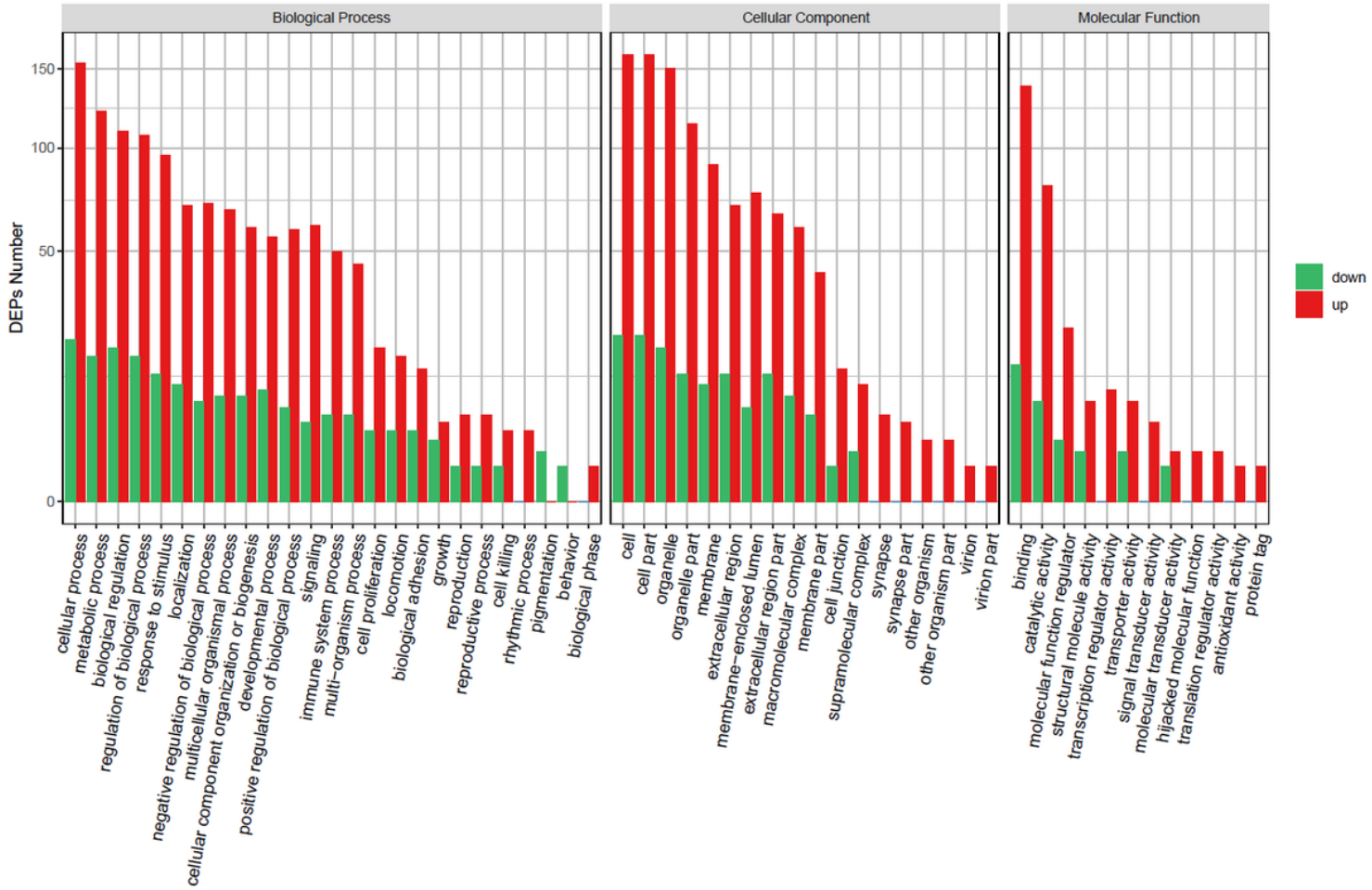

Figure 2

GO enrichment of DEPs. GO enrichment plot of differential proteins. The horizontal axis in the figure is the number of differential proteins and the vertical axis is the GO enriched entries.

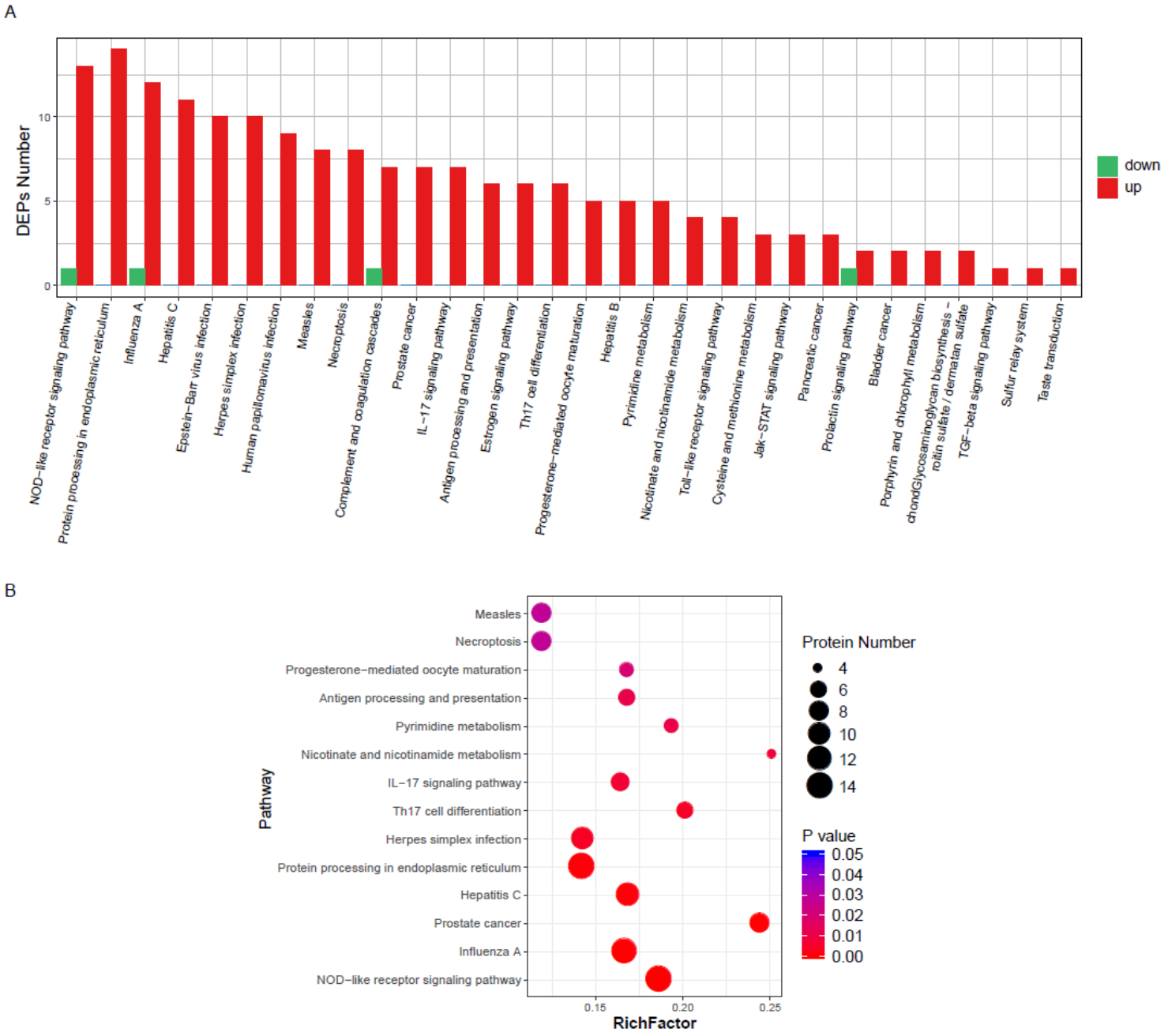

Figure 3

Pathway enrichment of all the DEPs a. Cluster plot of differential protein expression. The horizontal axis in the figure is the pathway name and the vertical axis is the number of DEPs found in the KD group. b. Pathway enrichment of differential proteins. The horizontal axis in the figure is the group name and the vertical axis is the protein ID of the differential protein. The legend on the right shows metrics for differential proteins (converted from P to Fold Change, not added in parentheses).. In the figure, the horizontal axis is the number of differential proteins and the vertical axis is the Pathway enrichment entries.

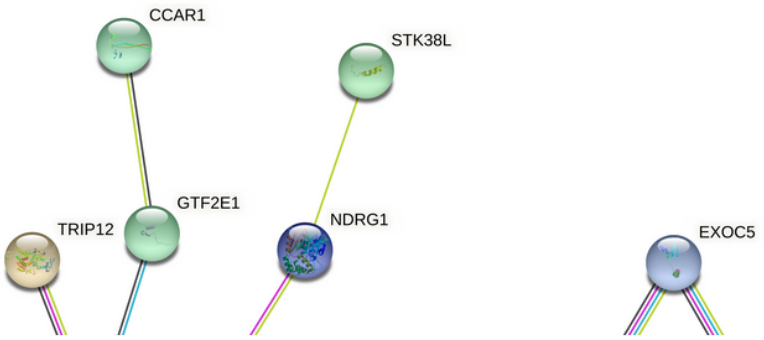

Figure 4

Differential protein interaction network. Red circles indicate protein upregulation and blue circles indicate protein downregulation. The degree of denseness of the junction line represents the degree of tightness of the relationship between this protein and other differential proteins.

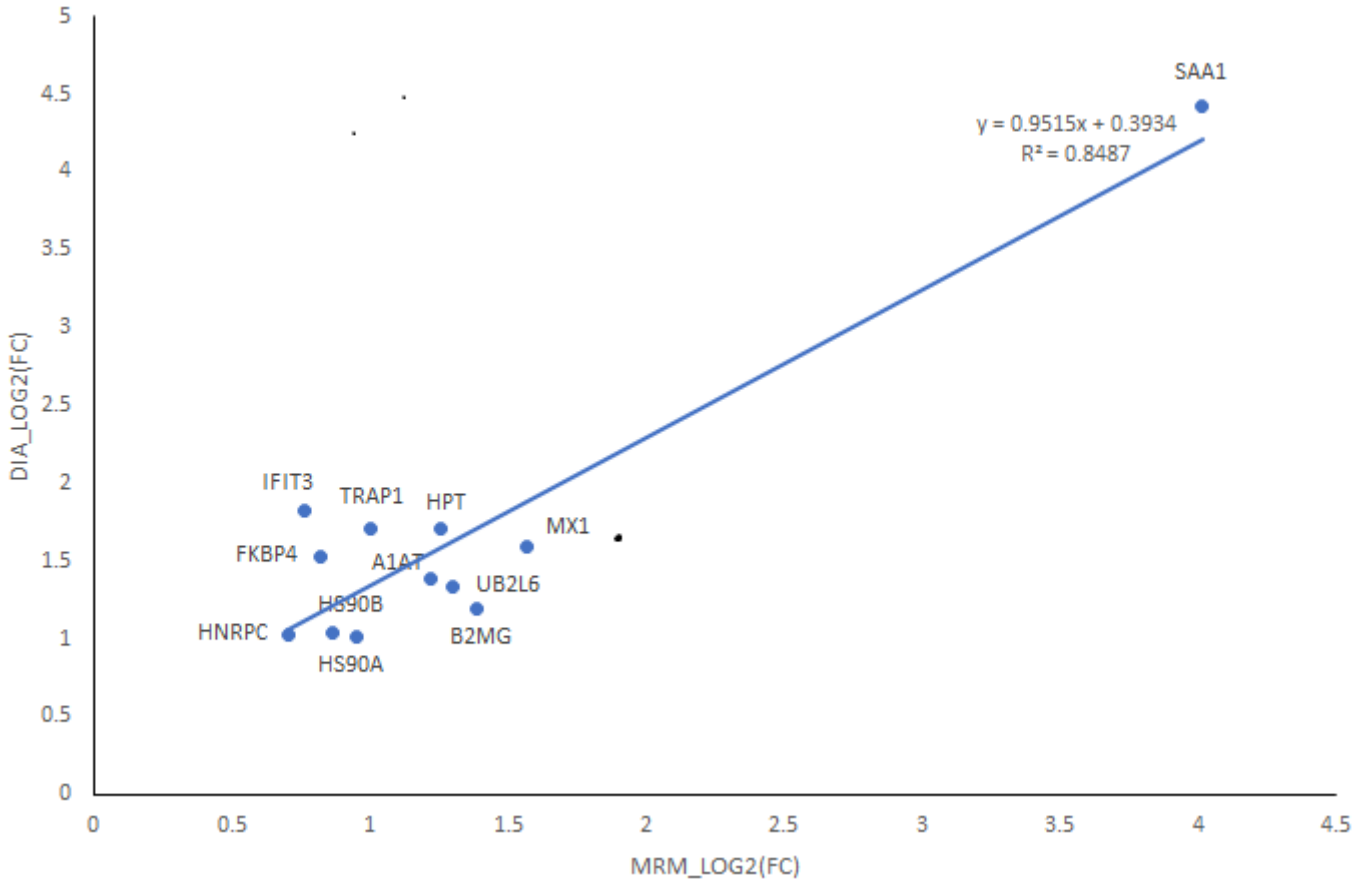

Figure 5

Correlation plot between the expression of alternative protein markers detected by MRM and DIA.

Supplementary Files

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