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Original Article

Following up of Surgical Treated Human Liver Cystic Echinococcosis: A Proteomics Approach

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Abstract

Background: Cystic echinococcosis (CE) is one of the most important parasitic zoonosis in the world. Post-surgery follow up in CE patients is an important non-solved problem up to now. Therefore, the investigations on this problematic issue would be very applicable in the view of CE clinical treatment.

Methods: A total of 24 confirmed liver CE patients sera including eight sera before surgery (BS), eight sera three months post-surgery (3MPS), and eight sera six months post-surgery (6MPS) were used in the present study. Proteomics methods including 2DE and LC-MS/MS were performed on the specimens followed by bioinformatics analysis such as Gene Ontology (GO) and Protein-Protein Interaction (PPI) network analysis.

Results: A total of 235 proteins were detected of which 12 differentially expressed proteins (DEP) were identified by LC-MS/MS in all sera. The proteins were presented in BS and suppressed after surgery as follows: HPX, SERPINA1, SERPINC1, CP, HBD, and HBA2. Comparisons of the protein expression in sera of patients BS, 3MPS, and 6MPS revealed that GC, IGJ, AHSG, CD5L, FGG, and APOC3 have been overexpressed in 3MPS and 6MPS. PPI network analysis demonstrated that SERPINC1 and AHSG with more connection in the network could be considered as hub proteins and potential prognostic biomarkers in response to surgical treatment of liver CE.

Conclusion: Application of proteomics methods on patient's sera could be used as a novel biomarker tool for following-up liver CE patients. In this regards, proteomics and, application of bioinformatics analysis including GO and PPI showed that SERPINC1, AHSG and HPX are of more value as a potential follow up biomarkers in response to surgical treatment.

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Introduction

Cystic echinococcosis (CE)/ hydatidosis is one of the most important parasitic zoonosis in the world (1). The human can be infected by eating eggs with different species and genotypes origin, leading to cyst formation especially in the liver followed by lungs and different other organs (2, 3).

Different imaging and serological methods have been used for following up on the treated patients (4–7). However, diagnosis, treatment, and follow-up of patients after treatment are three important challenges about this disease up to now (4).

Usually, the treatment of CE is performed through surgical procedures and drug therapy. The choice of the best medical management option including surgery, in case of hepatic CE needs to be completely monitored through the time using ultrasound (8). Due to the possibility of cyst rupture during surgery, the possibility of forming secondary cysts and recurrence of the disease, follow-up of CE patients at regular intervals after surgery is essential (9). Currently, there are no reliable markers for the evaluation of surgical and drug therapy on CE (10). Antibody assay in sera of treated patients is not recommended for follow-up patients due to persistently high levels of antibodies even after removal of the cyst for several months (4, 11).

Keeping in mind the above limitations and due to the lack of good markers and the relatively low serological efficiency, it is essential to find new methods for monitoring the disease in humans. Therefore, the development of new methods to find a marker or specific markers that can facilitate monitoring and follow up of the disease especially in treated patients is essential (12). Therefore, a new approach such as proteomic that improves the understanding of different aspects, as well as biochemical characteristics of this zoonotic disease, is necessary (13, 14).

The present study using proteomics methods was aimed to find probable biomarker/s in the serum of CE patients in three separate times for following up purposes either after surgical removal of cyst/s. Moreover, bioinformatics methods including Protein-Protein Interaction (PPI) network and Gene Ontology (GO) annotation analysis were used to interpret the proteomics expression experiments.

Materials and Methods

Sample collection

A total of 24 sera were collected from eight liver CE patients who went under surgery at three different times: eight sera before surgery (BS), eight sera three months post-surgery (3MPS), and eight sera six months post-surgery (6MPS). All sera have been kept at -80 °C until use.

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Code: IR.SBMU.RETECH.REC.1396.537). Informed consent was obtained from all individual participants included in the study.

Protein separation (2D gel electrophoresis)

All sera in each group (BS, 3MPS, and 6MPS) were thawed at room temperature, vortexed on ice, and pooled separately. Protein assay was performed on every pooled sera group using the 2D Quant kit (GE Healthcare). Immobilized pH gradient (IPG) 11cm strips (pH 3-10) (BioRad) were used for the first dimension of 2D gel electrophoresis. A diluted sample was made by adding 20μl of each pooled sample in 180μl of rehydration buffer (7M urea, 2M thiourea, 2% (v/v) CHAPS, 0.5% (v/v) ampholite, 40 mM DTT and 0.002% bromphenol blue). The diluted samples were loaded on IPG strips and incubated at room temperature for 20 minutes. The first dimension of 2dimensional gel electrophoresis (2DE) was performed with the

following voltage and time schedule: (150v, 1hrs), (300v, 3hrs), (1000v, 1hrs), (6000v gradient, 2hrs) and (6000v, 2.5hrs). In the next step, the gels were equilibrated twice in equilibration buffers including urea 7.2 gr, SDS 0.4 gr, Tris pH 8.8 1 ml, glycerol 3.428 ml, once by DTT 0.06 Gr and once by iodoacetamide 0.75 gr, for 15 minutes. To run the second dimension of 2DE, IPG strips were transferred into the 2D HPE Double gel 12.5% (SERVA). The gels were transferred into Flat Top tower device using its specific buffers. The second dimension of 2DE was performed by connecting the electric current in four steps containing: (100V, 7mA, 1W, 30 min), (200V, 13mA, 3W, 30min), (300V, 20mA, 5W, 10min) and (1000V, 40mA, 25W, 2hrs) at 15 °C temperature.

Gel staining and gel de-staining

After completion of 2DE, the gels were removed and visualized by Coomassie Brilliant Blue: CBB/ G-250 (Sigma, Germany) (15, 16).

Statistical analysis

A densitometer GS-800 (BioRad) together with Quantity One software was used for simultaneous scanning and image preparation of 2DE gels. The images were analyzed using Progenesis SameSpots software to compare the processed gels for detecting the protein spots, evaluating their color intensity, spots alignment, and statistical analysis. ANOVA and fold changes were used to determine the statistically significant changes in protein expression between groups. Spots with P-values<0.05 and fold>=1.5 are considered as significant spots for further analysis.

Mass spectrometry

The most significant spots were incised and isolated followed by mass spectrometry for protein identification. LC-MS/MS was performed for protein identification at PhenoSwitch Bioscience, the laboratory in Sherbrooke, Canada, using ABSciex TripleTOF 5600 instrument. All runs were analyzed sim-

ultaneously with the Protein Pilot software. Gel bands were dehydrated to remove excess reagents and were rehydrated in 50 mM tris pH 8.0 + 1μg of Trypsin/LysC. The digestion was carried overnight at 37 °C with agitation. Peptides were extracted from the gel and purified by reversed-phase solid-phase extraction before LC-MS/MS analysis.

An ABSciex TripleTOF 5600 (ABSciex, Foster City, CA, USA) which was equipped with an electrospray interface with a 25μm id, capillary, and coupled to an Eksigent μUHPLC (Eksigent, Redwood City, CA, USA) was used for acquisition. Analyst TF 1.7 software was used to control the instrument, data processing, and acquisition. The acquisition step was done in Information Dependent Acquisition (IDA) mode. The separation step was done on a reversed-phase HALO C18-ES column 0.3 μm i.d., 2.7μm particles, 50 mm long (Advance Materials Technology, Wilmington, DE) which was kept at 50 °C. Loop overfilling was used for samples to be injected into a 5μL loop. The mobile phase consisted the following components: solvent A (0.2% v/v formic acid and 3% DMSO v/v in water) and solvent B (0.2% v/v formic acid and 3% DMSO in EtOH) at a flow rate of 10 μL/min.

Bioinformatics analysis

Bioinformatics methods including PPI network and GO annotation were used to analyze proteomics expression experiments (17). The UniProt identifier of differentially expressed proteins (DEP) was extracted from UniProt database (https://www.uniprot.org/) and subjected to STRING online database (https://string-db.org/) to constructing the functional protein association network. The data obtained from STRING were subjected to Cytoscape 3.2.0 software to draw and analyze the whole network for all DEP in the experiments. Cytoscape extracted the centrality parameters including degree, bottleneck, and betweenness centrality, and analyzed the network using special plugins (18). In our study, CytoHubba plugin was used to find the hub

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proteins. Hubs interpret the highly connected nodes in the network, with special biological properties based on centrality parameters. GO annotation was performed for interpretation of the cellular component, biological process, and molecular function of DEP in our study.

The 2DE method, which was used, for separating sera proteins in samples showed a total of 235 protein spots in three groups including BS, 3MPS, and 6 MPS (Fig. 1).
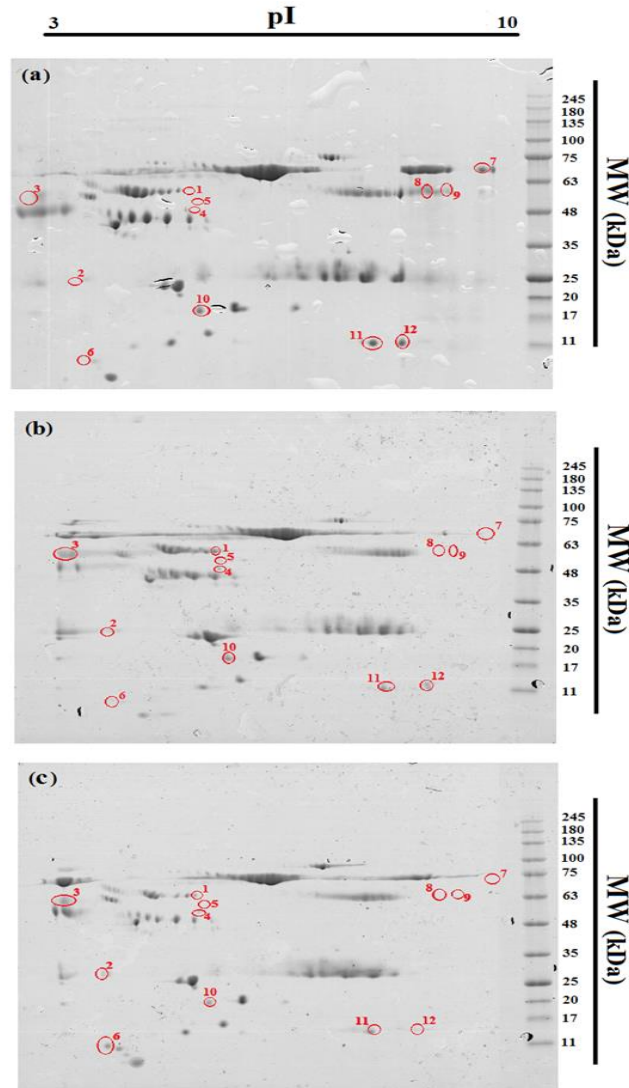
Results

Fig. 1: The 2DE gel scan images of CE patients' sera in three groups: a) BS (Before Surgery), b) 3MPS (3 Months Post-Surgery) and c) 6MPS (6 Months Post-Surgery). These spots indicated with red circles in images were selected for identification by LC-MS/MS

Based on fold changes>=1.5 and p-value<0.05, 129 DEP have been expressed in three groups of sera tested. A total of 82 out of 129 DEP spots had increased in their expression after surgery. Simultaneously, the expression after surgery from a total of 47 out of

129 DEP, were decreased which showed in the form of a line in the expression pattern as a, and, b in purple and dark green color (Fig. 2). The trend of 82 DEP was increased in the following up of the patients after three and six months post-surgery (Fig. 2 a). The trend of 47 DEP was decreased in the following up of the patients after three and six months post-surgery (Fig. 2 b). The expression rate of 82 DEP was more obvious in sera belonged to three months than six months post-surgery.

Based on gels analysis 12 protein spots of DEP with different significant expressions were selected for identification by LC-MS/MS (Fig. 3).
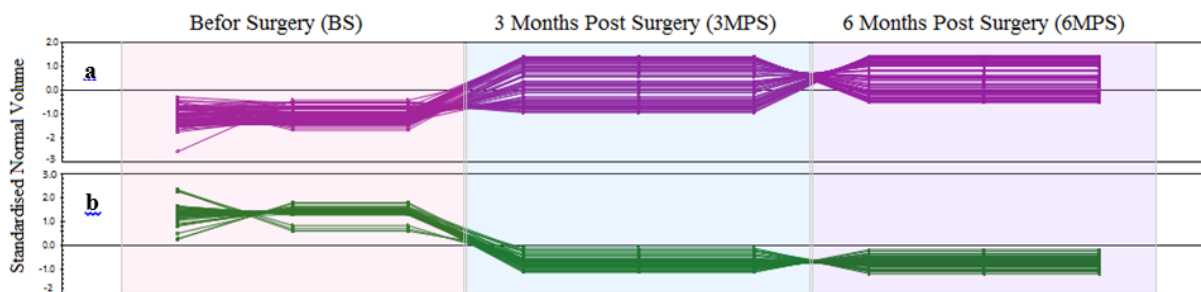

Fig. 2: Expression patterns of (a) increased and (b) decreased proteins in three groups of patients including BS (Before Surgery), 3MPS (3 Months Post-Surgery) and c) 6MPS (6 Months Post-Surgery). Diagram was obtained from Progenesis SameSpots software

Fig. 3: 2DE gels stained with Coomassie Brilliant Blue R250 showing protein spots with different expression in the sera BS, 3MPS and 6MPS patients groups. LC-MS/MS was applied for the spots numbers 1-12. BS: Before Surgery, 3MPS: 3 Months Post-Surgery, 6MPS: 6 Months Post-Surgery

Proteomics analysis

A total of 12 out of 12 protein spots were identified by LC-MS/MS. The identified proteins are shown in Fig.3. In the BS group, a total of six spots with high expression proteins were identified as follows: Hemopexin (HPX), Alpha-1-antitrypsin (SERPINA1), Antithrombin-III (SERPINC1), Ceruloplasmin (CP), Hemoglobin subunit delta (HBD), and Hemoglobin subunit alpha 2 (HBA2). The ex-

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pression of these proteins was decreased after removal of the cyst, as is shown in Fig. 3.

In the 3MPS group, three spots with high expression proteins were identified as follows:

Vitamin D-binding protein (GC), CD5 antigen-like (CD5L), and Fibrinogen gamma chain (FGG). Protein identification on the 6MPS group revealed four proteins, Vitamin D binding protein (GC), Alpha-2-HS-glycoprotein

(AHSG), Immunoglobulin J chain (IGJ), and Apolipoprotein C3 (APOC3) while Vitamin D binding protein (GC) was identified in both 3MPS and 6MPS groups. The protein spots with different significant expression rates identified by LC-MS/MS are shown in Fig. 3. More details about identified proteins are shown in Table 1.

Table 1: Details of different protein spots which identified in patients

No.	Protein name	Gene name	Uni-Prot accession no.	MW(Da)	pI	ANO-VA	Fold	Up/down regulated		
								BS	3MPS	6MPS
1	Vitamin D-binding protein	GC	P02774	52964	5.4	9.707e-007	2.9	Down	Up	Up
2	Immunoglobulin J chain	IGJ	P01591	18099	5.09	3.714e-004	1.5	Down	Down	Up
3	Alpha-2-HS-glycoprotein	AHSG	P02765	39325	5.43	1.852e-007	7.3	Down	Up	Up
4	CD5 antigen-like	CD5L	O43866	38088	5.28	4.057e-007	2	Down	Up	Up
5	Fibrinogen gamma chain	FGG	P02679	51512	5.37	4.162e-007	3.2	Down	Up	Down
6	Apolipoprotein C-III	APOC3	P02656	10852	5.23	6.682e-010	6.1	Down	Down	Up
7	Hemopexin	HPX	P02790	51676	6.55	2.993e-013	4.3	Up	Down	Down
8	Alpha-1-antitrypsin	SERPINA1	P01009	46737	5.37	3.794e-012	4.6	Up	Down	Down
9	Antithrombin-III	SERPINC1	P01008	52602	6.32	2.720e-013	2.5	Up	Down	Down
10	Ceruloplasmin	CP	P00450	122205	5.44	4.162e-007	3.2	Up	Down	Down
11	Hemoglobin subunit delta	HBD	P02042	16055	7.84	6.770e-011	1.5	Up	Down	Down
12	Hemoglobin subunit alpha 2	HBA2	P69905	15258	8.72	2.931e-014	5.7	Up	Down	Down

BS: Before Surgery, 3MPS: 3 Months Post-Surgery, 6MPS: 6 Months Post-Surgery

Protein-protein interaction (PPI) network analysis

The protein-protein interaction network of DEP in the experiment (BS, 3MPS, and 6MPS) was constructed using the STRING database and analyzed using Cytoscape v3.2.0. In the STRING database, the associations between queries are derived from high-throughput experimental data and computational predictions (Fig. 4). The statistical in-

formation about the network showed the network composed of 12 nodes and 30 edges. Details about the PPI network has been described in Table 2. The top 3 hub proteins including SERPINC1, AHSG, and HPX were identified based on the bottleneck, degree, and betweenness algorithms using the CytoHubba plugin (Fig. 5). SERPINC1 was considered the top scorer hub protein by bottleneck, degree, and betweenness algorithms (Table 3).
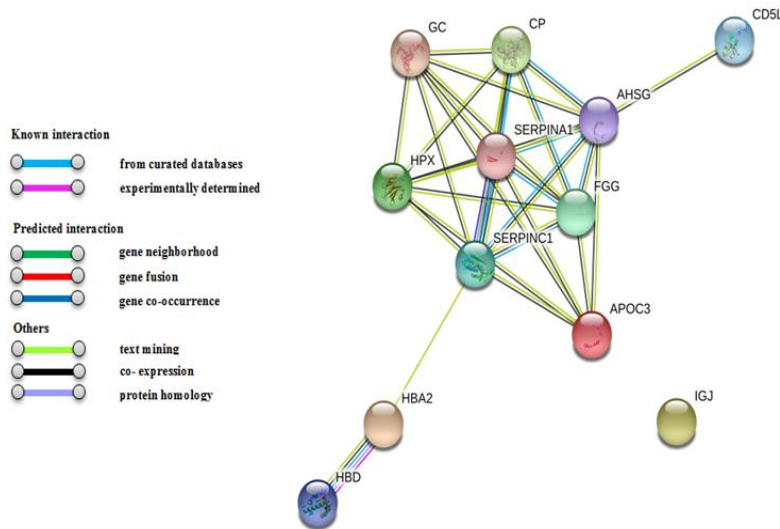

Fig. 4: PPI network obtained from STRING online database. The nodes in the network are proteins and the edges are protein-protein interaction. The sources of interactions between nodes in PPI network are shown by various colors
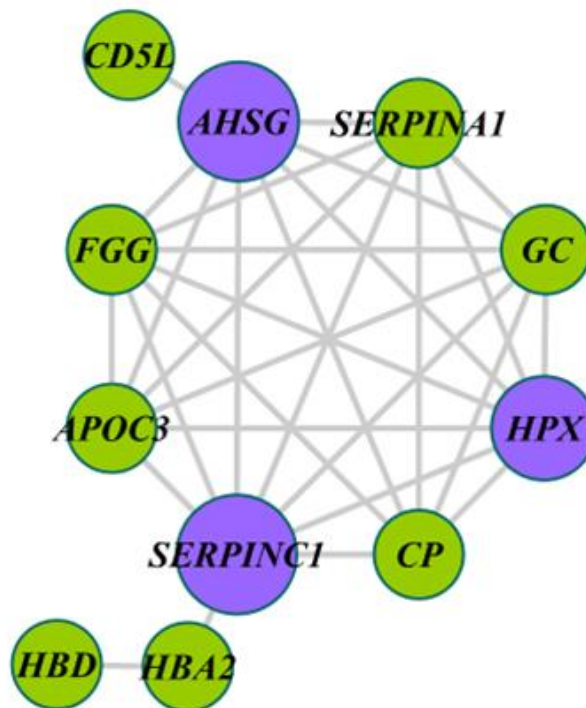

Fig. 5: The detail of PPI network which its hub proteins represented in purple color. Construction and analysis of PPI network performed by Cytoscape 3.2.0 software

Table 2: Topological features of protein-protein interaction network of DEP in hydatidosis patients in BS, 3MPS and 6MPS

Topological parameter	Values
Number of nodes	12
Number of edges	30
Clustering coefficient	0.658
Network centralization	0.311
Network density	0.545
Network diameter	4
Average node degree	5

Table 3: The top 3 hub proteins were identified in PPI network using CytoHubba plugin based on degree, bottleneck and betweenness centrality

Top 3 hub based on degree			Top 3 hub based on betweenness			Top 3 based on bottleneck		
No	Name	Score	No	Name	Score	No	Name	Score
1	SERPINC1	8	1	SERPINC1	32.33	1	SERPINC1	3
2	AHSG	8	2	AHSG	18.33	2	AHSG	2
3	HPX	7	3	HBA2	18	3	HBA2	2

Functional annotation analysis

The classification results are interpreted in three parts including molecular function, biological process, and cellular components, which were illustrated in Fig. 6. The molecular function of proteins was as follows: enzyme inhibitor activity (GO:0004857), endopeptidase inhibitor activity (GO:0004866), oxygen carrier activity (GO:0005344), oxygen binding (GO:0019825), steroid binding (GO:0005496), serine-type endopeptidase inhibitor activity (GO:0004867), protease binding (GO:0002020) and heme-binding (GO:0020037). The GO term of enzyme inhibitor activity and endopeptidase inhibitor activity were the main molecular function of proteins in our experiments.

The results of the biological process were as follows: transport (GO:0006810), post-translational protein modification (GO:0043687), receptor-mediated endocytosis (GO:0006898), endocytosis (GO:0006897), acute inflammatory response (GO:0002526), blood coagulation (GO:0007596), vesicle-mediated transport (GO:0016192), oxygen transport (GO:0015671), platelet degranulation (GO:0002576), negative regulation of hydrolase activity (GO:0051346), response to stress (GO:0006950), inflammatory response (GO:0006954), acute-phase response (GO:0006953), negative regulation of endopeptidase activity (GO:0010951), defense response (GO:0006952), cellular iron ion homeostasis (GO:0006879), regulation of blood coagulation (GO:0030193), immune system process (GO:0002376), regulation of biological quality (GO:0065008), transition metal ion transport (GO:0000041), regulation of vesicle-mediated transport (GO:0060627) and secretion (GO:0046903). The proteins classified into transport and response to stress were highest among biological process annotation.

The results of cellular component annotation of proteins showed the proteins distributed in the extracellular region part (GO:0044421), extracellular region (GO:0005576), extracellular space (GO:0005615), endoplasmic reticulum lumen (GO:0005788), cytoplasmic vesicle lumen (GO:0060205), platelet alpha granule lumen

(GO:0031093), hemoglobin complex (GO:0005833), endocytic vesicle lumen (GO:0071682), cytoplasmic vesicle (GO:0031410), intracellular organelle lumen (GO:0070013), cytoplasm (GO:0005737) and cytoplasmic part (GO:0044444). Based on cellular component results, most proteins are in the extracellular region and cytoplasm.
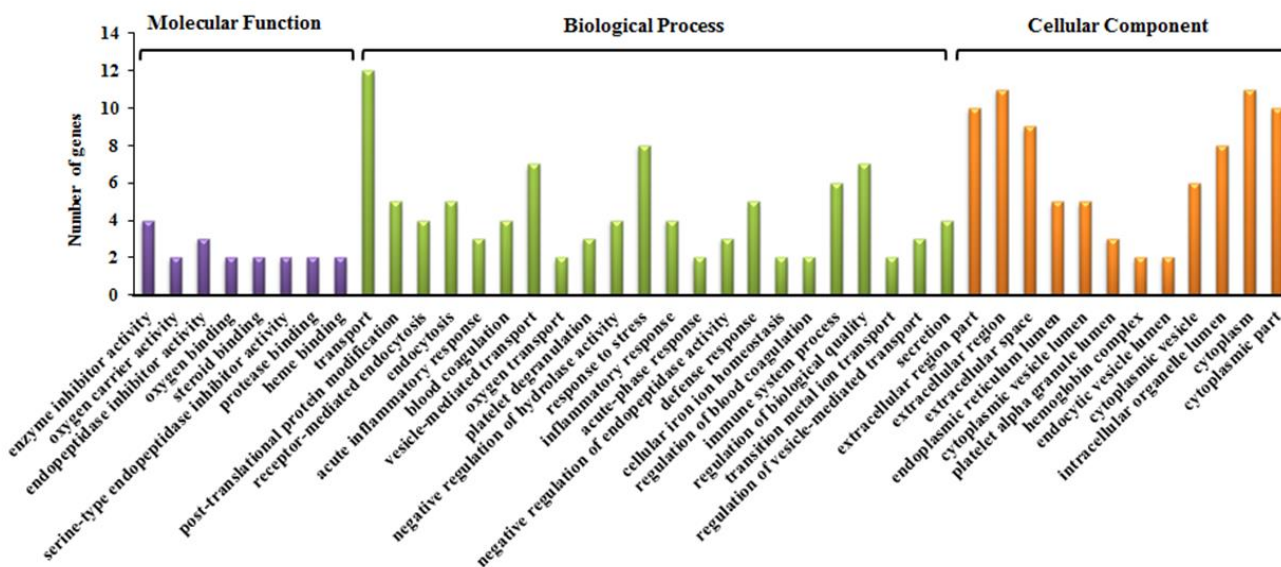

Fig. 6: The GO annotation of DEP in all groups. The information about molecular function, biological process and cellular component obtained from STRING online database

Discussion

Following-up of CE patients at regular intervals after surgery is essential due to the probable rupture of the cyst and the formation of secondary cysts leading to recurrence of the disease (4). Usually follow-up is usually carried out by imaging and serological tools (19). However, imaging examination and serological tests have some drawbacks such as low sensitivity (14, 20). Studies on the recombinant antigens for following up and, monitoring of CE have not also been successful (21). The available tests used for sera based on measurements of antibodies against crude antigens have low sensitivity and specificity and not useful for the follow-up of patients post-treatment (22). In this regard, efforts to develop a reliable world standard based on serology to be used for the diagnosis and monitoring of CE patients is still a need (23). In this regard, the application of new tools especially the pro-

teomic approach should be investigated (12, 14, 20, 24). Serum proteomics investigation on blood components has been used to identify several biomarkers in infectious diseases (25, 26).

In the present study, 12 DEP were identified in BS, 3MPS, and 6MPS groups. Six out of twelve including GC, IGJ, AHSG, CD5L, FGG, and APOC3 were highly expressed after surgery, in 3MPS or 6MPS.

In the meantime, six proteins including HPX, SERPINA1, SERPINC1, CP, HBD, and HBA2 suppressed after the surgery. GO annotation analysis of these expressed and suppressed proteins showed that they can involve in various functions such as transporter activity, response to stress, immune system process, inflammatory responses.

One of the important proteins was vitamin D binding protein (GC) which was overexpressed in both 3MPS and 6MPS. The reduction of vitamin D binding protein has been

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reported in severe hepatic diseases (27). GC is synthesized mainly in hepatic parenchymal cells (27); and its reduction in plasma in severe hepatic diseases is comparable to our results. So elevation of GC three and six months after the removal of hepatic CE could introduce it as a marker for follow up and success of removal hepatic CE. Vitamin D binding protein elevation was also observed in follow-up samples, which is comparable with the results of multiple sclerosis patients where vitamin D binding proteins were identified as a prognostic biomarker for them (28).

Alpha 2-HS-glycoprotein (AHSG) is a protein identified in two follow up groups (3MPS and 6MPS). This protein is synthesized in hepatocytes and is involved in several metabolic disorders (29). Hemopexin protein, which is a protein that tends to bind to the blood proteins (30), was highly expressed in our samples with liver CE and decreased after removal of the cysts. The high expression of hemopexin in human and bovine cysts has been reported in a proteomics study on CE cyst fluid originated from humans, cattle, and sheep cysts (31). Given the similarity of some of the fluid proteins of the hydatid cyst and serum, it seems that removing the cyst could be lead to a decrease for HPX in sera after surgery.

Alpha-1 antitrypsin (SERPINA1), a protease inhibitor encoded by the SERPINA1 gene on chromosome 14, was another protein identified in human sera before surgery with high expression. This glycoprotein is essentially synthesized in the liver and secreted into the bloodstream (32). It is an effective inhibitor of neutrophil elastase, plasmin, thrombin, trypsin, chymotrypsin, and plasminogen activator (33). In this study, we observed that the expression of SERPINA1 was suppressed after the surgery. This observation is similar to the results of Zeghir-Bouteldja and her colleagues on patients' CE fluid (34).

Antithrombin-III (SERPINC1), a plasma protein involved in the acute inflammatory response to antigenic stimuli (25), was identi-

fied in the BS group and suppressed after the surgery. Our finding is with correlation to the studies in Algeria by finding SERPINC1 as one of the host proteins in hydatid cyst fluid (HCF) (34). The permeability of the cyst wall and the exchange of serum and cystic proteins can interpret this finding.

Bioinformatics methods including PPI network and GO annotation analysis, which can interpret the proteomics expression experiments, showed promising results, such that PPI network analysis showed the expression changes of these three hub proteins as a follow-up tool. Hub proteins of the PPI network represent highly connected nodes with special functions, so that AHSG, SERPINC1, and HPX were the most important hubs in this network. They can be applied for CE follow up purposes. Similar work has been done on HCF to find the most important proteins related to the biological function of proteins in CE (35).

The PPI network analysis showed that SERPINC1 and AHSG proteins as the most important hubs with highly centrality parameters.

Conclusion

On following-up, samples of liver CE patients the expression of several proteins was changed significantly either in 3MPS or 6MPS groups in response to treatment and cyst removal. SERPINC1, AHSG, and HPX with high centrality properties may be considered as potential prognostic biomarkers in response to surgical treatment of CE and probably usable for follow-up in CE patients. Such that application of proteomics methods such as 2DE and LC-MS/MS together with computational methods on patients' sera after surgery or removal of cyst could be used as a novel biomarker tool for following up the CE patients.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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