The isobaric tags for relative and absolute quantification-based quantitative proteomics of fresh tissue-derived secretome in hepatocellular carcinoma

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Abstract

Introduction: Proteomics technology platforms offer an extremely useful tool for the discovery of new cancer biomarkers. Secreted proteins play important roles in signal transduction, cellular growth, proliferation, differentiation, and apoptosis. This study aimed to investigate the molecular signatures of the hepatocellular carcinoma (HCC) by quantitative proteomics using isobaric tags for relative and absolute quantification (iTRAQ) with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Material and methods: In this study, we used an iTRAQ-based quantitative proteomic approach to analyse the secretome of HCC tissues to identify plasma biomarkers. Serum-free conditioned media (CM) were collected from the primary cultures of cancerous tissues, the surrounding noncancerous tissues, and distal noncancerous tissues.

Results: A proteomic analysis of the CM proteins allowed for a total of 5214 identified proteins, of which 190 and 44 proteins were dysregulated in the HCC tissues/distal noncancerous tissues (HCC/DN group) and the adjacent noncancerous tissues/distal noncancerous tissues (AN/DN group) compared with the distal noncancerous tissues. The dysregulated proteins in the HCC/DN group were concentrated in mitogen-activated protein kinase (MAPK) signalling and Janus kinase-signal transducer and activator of the transcription (JAK-STAT) signalling, but the dysregulated proteins in the AN/DN group were more concentrated in the basal material metabolism.

Conclusions: The secretome profile alternations and signalling pathways were associated with HCC incidence and development. The dysregulated proteins in the HCC/DN group were concentrated in the MAPK signalling and JAK-STAT signalling, but the dysregulated proteins in the AN/DN group were more concentrated in the basal material metabolism.

Key words: hepatocellular carcinoma (HCC), iTRAQ, tissue secretome, incidence and development, molecular mechanism.

Introduction

Hepatocellular carcinoma (HCC) is a kind of clinical common malignant tumour with an insidious onset, which is invasively fast-growing
and has a poor prognosis [1]. Although surgical excision was demonstrated to be the first choice for HCC treatment, most HCC is not diagnosed until the advanced stage of the disease, when surgical treatments are not suitable for treating the disease [2]. Therefore, early detection and treatment are key to improving therapeutic outcomes, reducing mortality, and increasing the long-term survival rate in HCC patients.

Alpha-fetoprotein (AFP) was the only widely accepted and applied biomarker in clinical practice because of its practical value for the diagnosis and monitoring of the development of HCC. However, the AFP method experienced insufficient sensitivity and specificity in the early diagnosis of HCC. Meanwhile, the AFP level is also easily affected by other diseases, such as hepatitis during pregnancy and liver regeneration after damage, which increases the inaccuracy of clinical diagnoses [3–5]. There is, therefore, an urgent need to identify new biomarkers with high sensitivity and high specificity for the early diagnosis of HCC.

Proteomics technology platforms are an extremely useful tool for the discovery of new cancer biomarkers. A highly desirable biomarker for cancer screening and monitoring would be a biomarker that can be measured in body fluid samples [6]. Accordingly, blood samples such as serum and plasma have been the ideal targets of proteomics studies aimed at identifying cancer diagnostic and prognostic biomarkers [7, 8]. However, several challenges have hindered the progress of these studies. The main 2 reasons include the complex nature of serum and plasma samples and the large dynamic range between the concentrations of different proteins.

Secreted proteins play important roles in signal transduction, cellular growth, proliferation, differentiation, and apoptosis. They are also important in tumourigenesis, development, invasion, and metastasis of HCC [9]. Therefore, the secretomes of cell lines are also performed during screening. Many researchers have reported the application of secretomes in the screening of diagnostic and prognostic protein biomarkers [10–12]. Essentially, it is well established that any potential biomarker candidates screened from HCC cell lines should be ultimately validated in clinical tissue samples that are closer to tumours than any of the model systems. As a result, it is more direct and convincing to utilise the primary culture of tumour tissues and the proteomic analysis of serum-free conditioned media to search the diagnostic or prognostic biomarkers [13, 14].

We, thus, conducted this study to investigate the molecular signatures of HCC by quantitative proteomics using isobaric tags for relative and absolute quantification (iTRAQ) coupling with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Material and methods

Sample collection and tissue culture in vitro

In our study, the HCC tissue group, the adjacent noncancerous tissue (AN) group, and the distal noncancerous tissue (DN) group were obtained from 2 primary HCC patients who were diagnosed with HCC by post-operative pathological examinations and subjected to standard radical resection. The fresh tissues were collected at the time of surgery from the HCC patients and immediately washed with phosphate-buffered saline in a sterile environment. Subsequently, the tissues were cut into 2 mm3 pieces, washed several times until the tissues became colourless, and then cultured in a Dulbecco’s modified eagle serum-free medium at 5% CO2 for 24 hours. Thereafter, the supernatants were collected for protein extraction. This study was approved by the Ethics Committee of our hospital, and the 2 patients signed informed consent forms.

Protein extraction and digestion

The collected culture supernatant was centrifuged at a low speed (200 g) to remove the cells and tissue debris and then filtered with a 0.22 µm filter membrane to remove the residual cells. Thereafter, the filtrate was concentrated with 3K ultrafiltration until the phenolic red colour was completely removed. The proteins were precipitated by ice-cold acetone, and the protein concentration of the supernatant was determined by bicinchoninic acid assay following the manufacturer’s protocol. Subsequently, 4 µl of a reducing reagent was added to each sample tube and vortex to mix and incubate the tubes at 60°C for 1 hour, and 2 µl of a cysteine blocking reagent was added to each tube and vortex to mix and incubate the tubes at room temperature for 10 minutes. Finally, the proteins were digested by sequence-grade modified trypsin through filter-aided sample preparation.

Isobaric tags for relative and absolute quantification labelling

The peptides from 100 µg proteins per group were labelled according to the Applied Biosystems iTRAQ™ reagent chemistry reference guide. The peptides were labelled as follows: 2 HCC groups became colourless, and then cultured in a Dulbecco’s modified eagle serum-free medium at 5% CO2 for 24 hours. Thereafter, the supernatants were collected for protein extraction. This study was approved by the Ethics Committee of our hospital, and the 2 patients signed informed consent forms.

High pH reversed-phase separation

The dried peptide mixture was fractionated by high pH separation using ekxpert™ ultraLC 100
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Mobile phase A: 20 mM ammonium formate in water, mobile phase B: 20 mM ammonium formate in 80% ACN, the pH was adjusted to 10.0 with ammonium hydroxide. High pH (pH = 10) separation was performed using a 65-min linear gradient as follows: 0–5 min, 0–5% B; 5–30 min, 5–15% B; 30–45 min, 15–38% B; 45–46 min, 38–90% B; 46–54.5 min, 90–90% B; 54.5–55 min, 90–5% B; 55–65 min, 5–5% B. Finally, 40 fractions were collected, and 4 fractions with the same time interval were pooled together to reduce the fraction numbers, such as 1, 2 and 21, 22 and 3, 4 and 23, 24, and so on [15]. Ten fractions at the end were dried in a vacuum concentrator for further usage.

The Nano-LC-MS/MS analysis

The fractions were re-suspended with 30 µl solution A (solution A: 0.1% FA and 2% ACN in water) and 8 µl was loaded on an exigent nano LC-Ultra system nano-LC with a trap column (ChromXP C18-CL-3 µm, 120A, 350 µm × 0.5 µm) with a flow of 2 µl/min. The column flow rate was maintained at 300 nl/min with a 101 min linear gradient as follows: 0–0.1 min, 5–10% B; 0.1–60 min, 10–25% B; 60–85 min, 25–48% B; 85–86 min, 48–80% B, 86–90 min, 80–80% B; 90–91 min, 80–5% B; 91–101 min, 5–5% B (solution B: 0.1% FA and 2% ACN in water). The MS data were collected by the Triple TOF 5600 system. The electrospray voltage of 2.3 kV and 150°C heating at the inlet of the mass spectrometer was used. The resolution was set at 30,000 with the scan range of 300–1500 m/z. The cumulative scanning time was 250 ms in the high-resolution scanning mode, and up to 40 sub-ion scans could be performed each time. Each Fraction was repeated three times with instrumental analysis, and all parent ions were collision-induced dissociation using fluctuating collision energy.

Data analysis

The MS data were processed using ProteinPilot 4.5 (AB SCIX, Foster City, CA, USA) and then searched using Mascot (version 2.2; Matrix Science, London, United Kingdom) search algorithms against the UniProt human database. The enzyme specificity of trypsin was used and up to a maximum of 2 missed cleavages were allowed for protease digestion. Mascot was searched with a parent ion tolerance of 10 parts per million (ppm) and a fragment ion mass tolerance of 0.05 Da. Carbamidomethylation of cysteine, as well as iTRAQ modification of peptide N-terminus and lysine residues, were set as a fixed modification; oxidation of methionine and iTRAQ 8-plex labelling of tyrosine were specified as variable modifications. The proteins were accepted if the protein FDR was < 1%.

To identify proteins whose expression was significantly altered in the 2 different groups, a threshold of the iTRAQ ratios were used to define differentially expressed proteins. The proteins were considered to be differentially expressed if the iTRAQ ratio was > 1.5 or < 0.67 in the 2 different groups with the p-value of < 0.05, which were statistically analysed by a paired T-test. The gene ontology (GO) annotation and pathway enrichment analysis of the differentially expressed proteins were carried out using the online tool DAVID (http://david.abcc.ncifcrf.gov/). The gene ontology annotation contains biological processes, cell components, and molecular functions. The pathway analysis was based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The gene ontology annotations and signalling pathways were ranked in terms of the enrichment or number of the differentially expressed proteins. The protein and protein interaction was performed using the online String database (https://string-db.org/).

Results

The relative quantification of the secretome of the primary hepatocellular carcinoma patients

In this study, total proteins were extracted from the collected tumours, their adjacent noncancerous tissues and their distal noncancerous tissues were taken from patients and analysed using iTRAQ 2D LC-MS/MS, and the workflow as described in Figure 1. In total, we quantified 5214 proteins, of which 190 and 44 proteins were classified as differentially expressed in the HCC tissues/distal noncancerous tissues (HCC/DN) group and the adjacent noncancerous tissues/distal noncancerous tissues (AN/DN) group (Table I and II). As is evident in Figure 2A, the number of differentially expressed proteins identified in the HCC/DN group was much higher than that in the AN/DN group.

When we compared the differences between the 2 groups, we found that among these differentially expressed proteins, 35 proteins altered their expression in both HCC types, while 155 proteins were dysregulated in the HCC/DN group only and 9 proteins were dysregulated in the AN/DN group only (Figure 2B). We then analysed the biological functions and signalling pathways of these proteins, including the proteins differentially expressed in both groups and the proteins differentially expressed individually in 1 group.

The gene ontology analysis of the differentially expressed proteins in primary hepatocellular carcinomas

The gene ontology annotation analysis showed that the cell components of the differentially ex-
pressed proteins either overlapped in the 2 groups or were unique in 1 group and were mostly located in the extracellular exosome (Figure 3). For the biological process analysis, the GO annotation analysis showed that the proteins overlapped in both groups and were the major participants in the protein folding, lipid metabolic process, gluconeogenesis, nucleobase-containing compound metabolic process, and canonical glycolysis (Figure 3 A).

There were 155 dysregulated proteins in the HCC group compared to the distal noncancerous tissues (DN) group, but these proteins were not dysregulated in the adjacent noncancerous (AN) tissues group compared to the DN group. These dysregulated proteins were mainly involved in signal transduction, cell proliferation, protein stabilisation, and negative regulation of the apoptotic process (Figure 3 B).

Interestingly, there were 9 dysregulated proteins in the AN group compared to the DN group, but they were not dysregulated in the HCC group compared to the DN group. The gene ontology results also showed that these dysregulated proteins were mainly involved in desmosome organisation, positive regulation of sister chromatid cohesion, translation, rRNA processing, nuclear-transcribed mRNA catabolic process, translational initiation, and SRP-dependent co-translational protein targeting to the membrane (Figure 3 C).

The Kyoto Encyclopaedia of Genes and Genomes pathway analysis of the differentially expressed proteins

As shown in Figure 4, the pathway of metabolism, genetic information processing, environmental information processing, and cellular was analysed. According to the results of the analysis, the dysregulated proteins in HCC are mostly involved in the Janus kinase-signal transducer and activator of the transcription (JAK-STAT) pathway and mitogen-activated protein kinase (MAPK) pathway. However, the signalling pathway that was only enriched in the AN group comprised mainly basic metabolisms, such as biological oxidations, amino
### Table I. Differentially expressed proteins identified between hepatocellular carcinoma tissues and distal noncancerous tissues

| No. | Accession     | Name                                              | FC     | P-value          |
|-----|---------------|---------------------------------------------------|--------|------------------|
| 1   | sp|P08670|VIME_HUMAN Vimentin OS = Homo sapiens GN = VIM PE = 1 SV = 4 | 4.875285 | 0.00000000486 |
| 2   | sp|P16615|AT2A2_HUMAN Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS = Homo sapiens GN = ATP2A2 PE = 1 SV = 1 | 4.655861 | 0.000000146 |
| 3   | sp|P07602|SAP_HUMAN Prosaposin OS = Homo sapiens GN = PSAP PE = 1 SV = 2 | 4.613766 | 0.00058        |
| 4   | sp|P10809|CH60_HUMAN 60 kDa heat shock protein, mitochondrial OS = Homo sapiens GN = HSPD1 PE = 1 SV = 2 | 4.487454 | 0.000000000382 |
| 5   | sp|P14314|GLU2B_HUMAN Glucosidase 2 subunit beta OS = Homo sapiens GN = PRKCSH PE = 1 SV = 2 | 3.872576 | 0.000281 |
| 6   | sp|P31327|PSM_HUMAN Carbamoyl-phosphate synthase [ammonia], mitochondrial OS = Homo sapiens GN = CPS1 PE = 1 SV = 2 | 3.531832 | 0.000000000162 |
| 7   | sp|Q10471|GALT2_HUMAN Polypeptide N-acetylgalactosaminyltransferase 2 OS = Homo sapiens GN = GALNT2 PE = 1 SV = 1 | 3.43558 | 0.00000579 |
| 8   | sp|Q04695|K1C17_HUMAN Keratin, type I cytoskeletal 17 OS = Homo sapiens GN = KRT17 PE = 1 SV = 2 | 3.11311 | 0.000898 |
| 9   | sp|Q05783|K1C18_HUMAN Keratin, type I cytoskeletal 18 OS = Homo sapiens GN = KRT18 PE = 1 SV = 2 | 3.16278 | 0.0000367 |
| 10  | sp|P32004|L1CAM_HUMAN Neural cell adhesion molecule L1 OS = Homo sapiens GN = L1CAM PE = 1 SV = 2 | 3.133286 | 0.00000598 |
| 11  | sp|P27797|CALR_HUMAN Calreticulin OS = Homo sapiens GN = CALR PE = 1 SV = 1 | 3.076097 | 0.000000882 |
| 12  | sp|P07237|PDI1_HUMAN Protein disulphide-isomerase OS = Homo sapiens GN = P4HB PE = 1 SV = 3 | 3.019952 | 0.000287 |
| 13  | sp|P50584|ECHB_HUMAN Trifunctional enzyme subunit beta, mitochondrial OS = Homo sapiens GN = HADHB PE = 1 SV = 3 | 2.85759 | 0.000323 |
| 14  | sp|P80723|BASP1_HUMAN Brain acid soluble protein 1 OS = Homo sapiens GN = BASP1 PE = 1 SV = 2 | 2.85759 | 0.0000445 |
| 15  | sp|Q16497|GANAB_HUMAN Neutral alpha-glucosidase AB OS = Homo sapiens GN = GANAB PE = 1 SV = 3 | 2.805434 | 0.000569 |
| 16  | sp|Q00839|HNRPU_HUMAN Heterogeneous nuclear ribonucleoprotein U OS = Homo sapiens GN = HNRNUPE PE = 1 SV = 6 | 2.728798 | 0.000193 |
| 17  | sp|Q12931|TRAP1_HUMAN Heat shock protein 75 kDa, mitochondrial OS = Homo sapiens GN = TRAP1 PE = 1 SV = 3 | 2.679168 | 0.0000193 |
| 18  | sp|P14625|ENPL_HUMAN Endoplasmic OS = Homo sapiens GN = HSP90B1 PE = 1 SV = 1 | 2.630268 | 0.000362 |
| 19  | sp|P09394|ECH_HUMAN Trifunctional enzyme subunit alpha, mitochondrial OS = Homo sapiens GN = HADHA PE = 1 SV = 2 | 2.630268 | 0.000143 |
| 20  | sp|P27824|CALX_HUMAN Calnexin OS = Homo sapiens GN = CANX PE = 1 SV = 2 | 2.630268 | 0.000476 |
| 21  | sp|P42704|LPPRC_HUMAN Leucine-rich PPR motif-containing protein, mitochondrial OS = Homo sapiens GN = LRP1PC PE = 1 SV = 3 | 2.558586 | 0.000000421 |
| 22  | sp|P06576|ATP8_HUMAN ATP synthase subunit beta, mitochondrial OS = Homo sapiens GN = ATP5B PE = 1 SV = 3 | 2.558586 | 0.00071 |
| 23  | sp|QRTME1|PO210_HUMAN Nuclear pore membrane glycoprotein 210 OS = Homo sapiens GN = NUP210 PE = 1 SV = 3 | 2.535129 | 0.000341 |
| 24  | sp|P05203|AT1A1_HUMAN Sodium/potassium-transporting ATPase subunit alpha-1 OS = Homo sapiens GN = ATP1A1 PE = 1 SV = 1 | 2.535129 | 0.000341 |
| 25  | sp|P02545|LMNA_HUMAN Prelamin-A/C OS = Homo sapiens GN = LMNA PE = 1 SV = 1 | 2.511886 | 0.000397 |
| 26  | sp|Q3N833|DXX21_HUMAN Nucleolar RNA helicase 2 OS = Homo sapiens GN = DXX21 PE = 1 SV = 5 | 2.511886 | 0.000515 |
| 27  | sp|P02786|TFRI_HUMAN Transferrin receptor protein 1 OS = Homo sapiens GN = TFRC PE = 1 SV = 2 | 2.466039 | 0.000549 |
| 28  | sp|P49792|RB2_HUMAN E3 SUMO-protein ligase RanBP2 OS = Homo sapiens GN = RANBP2 PE = 1 SV = 2 | 2.443431 | 0.000679 |
| No. | Accession | Name | OS | GN | PE | SV | FC | P-value |
|-----|-----------|------|----|-----|----|----|----|---------|
| 29  | sp|Q86U2P|KTN1_HUMAN | Kinectin | Homo sapiens | KTN1 | 1 | 2.421029 | 0.000000491 |
| 30  | sp|Q07065|CKAP4_HUMAN | Cytoskeleton-associated protein 4 | Homo sapiens | CKAP4 | 1 | 2.421029 | 0.00000087 |
| 31  | sp|P11021|GRP78_HUMAN | 78 kDa glucose-regulated protein | Homo sapiens | HSPA5 | 1 | 2.398833 | 0.000000146 |
| 32  | sp|Q9PE2E|RIBP1_HUMAN | Ribosome-binding protein 1 | Homo sapiens | RRP1 | 1 | 2.398833 | 0.000000948 |
| 33  | sp|P52277|HNRPM_HUMAN | Heterogeneous nuclear ribonucleoprotein M | Homo sapiens | HNRP | 1 | 2.208005 | 0.000000866 |
| 34  | sp|P04843|RPNI_HUMAN | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 | Homo sapiens | RPNI | 1 | 2.208005 | 0.000056 |
| 35  | sp|Q9U33S|SRRM2_HUMAN | Serine/arginine repetitive matrix protein 2 | Homo sapiens | SRRM2 | 1 | 2.167704 | 0.00000419 |
| 36  | sp|P08195|F2_HUMAN | 4F2 cell-surface antigen heavy chain | Homo sapiens | SLC3A2 | 1 | 2.108628 | 0.0000497 |
| 37  | sp|Q16891|MIC60_HUMAN | MICOS complex subunit MIC60 | Homo sapiens | IMMT | 1 | 2.108628 | 0.00000733 |
| 38  | sp|Q96RP9|EFGM_HUMAN | Elongation factor G, mitochondrial OS | Homo sapiens | GFM1 | 1 | 2.108628 | 0.000905 |
| 39  | sp|P78527|PRKDC_HUMAN | DNA-dependent protein kinase catalytic subunit OS | Homo sapiens | PRKDC | 1 | 2.089296 | 0.00000377 |
| 40  | sp|Q9NE4|SYIM_HUMAN | Isoleucine-tRNA ligase, mitochondrial OS | Homo sapiens | IARS2 | 1 | 2.070141 | 0.000341 |
| 41  | sp|Q9H0E6|XR2_HUMAN | 5′-3′ exoribonuclease 2 | Homo sapiens | XRN2 | 1 | 2.051162 | 0.00038 |
| 42  | sp|Q9N2M1|MYOF_HUMAN | Myosin | Homo sapiens | MYOF | 1 | 2.013724 | 0.000021 |
| 43  | sp|Q9211|DHK9_HUMAN | ATP-dependent RNA helicase A | Homo sapiens | DHK9 | 1 | 2.013724 | 0.000672 |
| 44  | sp|P38666|SERPINE1_HUMAN | Stress-70 protein, mitochondrial OS | Homo sapiens | HSPA5 | 1 | 1.995262 | 0.00000556 |
| 45  | sp|P5705|ATPAR_HUMAN | ATP synthase subunit alpha, mitochondrial OS | Homo sapiens | ATP5A1 | 1 | 1.995262 | 0.000861 |
| 46  | sp|P13667|PDIA4_HUMAN | Protein disulphide-isomerase A4 | Homo sapiens | PDIA4 | 1 | 1.958645 | 0.0000668 |
| 47  | sp|P06748|NPM_HUMAN | Nucleophosmin | Homo sapiens | NPM1 | 1 | 1.940886 | 0.000862 |
| 48  | sp|P5526S|DSRAD_HUMAN | Double-stranded RNA-specific adenosine deaminase | Homo sapiens | ADAR | 1 | 1.923092 | 0.000118 |
| 49  | sp|Q9UHB6|LMA1_HUMAN | Lim domain and actin-binding protein 1 | Homo sapiens | LMA1 | 1 | 1.923092 | 0.000813 |
| 50  | sp|Q1343|NNTM_HUMAN | NAD(P) transhydrogenase, mitochondrial OS | Homo sapiens | NNT | 1 | 1.853532 | 0.0000358 |
| 51  | sp|Q19Y21|TRAP2_HUMAN | Thyroid hormone receptor-associated protein 3 | Homo sapiens | TRAP3 | 1 | 1.836538 | 0.000124 |
| 52  | sp|Q13263|TIFB_HUMAN | Transcription intermediary factor 1-beta OS | Homo sapiens | TRIM28 | 1 | 1.786488 | 0.000475 |
| 53  | sp|Q1514|PLEC_HUMAN | Plectin OS | Homo sapiens | PLEC | 1 | 1.584893 | 0.0000000695 |
| 54  | sp|Q13813|SPT1_HUMAN | Spectrin alpha chain, non-erythrocytic 1 OS | Homo sapiens | SPTAN1 | 1 | 1.559666 | 0.0000874 |
| 55  | sp|Q9Y490|TLN1_HUMAN | Talin-1 OS | Homo sapiens | TLN1 | 1 | 0.60256 | 0.0000353 |
| No. | Accession | Name | FC | P-value |
|-----|-----------|------|----|---------|
| 56  | sp|Q14315|Filamin-C OS = Homo sapiens GN = FLNC PE = 1 SV = 3 |
| 57  | sp|Q5T457|E3 ubiquitin-protein ligase UBR4 OS = Homo sapiens GN = UBR4 PE = 1 SV = 1 |
| 58  | sp|Q14152|Eukaryotic translation initiation factor 3 subunit A OS = Homo sapiens GN = EIF3A PE = 1 SV = 1 |
| 59  | sp|Q14974|Importin subunit beta-1 OS = Homo sapiens GN = KPNB1 PE = 1 SV = 2 |
| 60  | sp|P41091|Eukaryotic translation initiation factor 2 subunit 3 OS = Homo sapiens GN = EIF2S3 PE = 1 SV = 3 |
| 61  | sp|P3621|Coatomer subunit alpha OS = Homo sapiens GN = COPA PE = 1 SV = 2 |
| 62  | sp|Q16851|UDP-glucose-1-phosphate uridylyltransferase OS = Homo sapiens GN = UGP2 PE = 1 SV = 5 |
| 63  | sp|Q96P70|Importin-9 OS = Homo sapiens GN = IPO9 PE = 1 SV = 3 |
| 64  | sp|Q8W4M4|Programmed cell death 6-interacting protein OS = Homo sapiens GN = PDCD6IP PE = 1 SV = 1 |
| 65  | sp|P46940|Ras GTPase-activating-like protein IQGAP1 OS = Homo sapiens GN = IQGAP1 PE = 1 SV = 1 |
| 66  | sp|Q92973|Transportin-1 OS = Homo sapiens GN = TNP1 PE = 1 SV = 2 |
| 67  | sp|Q92598|Heat shock protein 105 kDa OS = Homo sapiens GN = HSPH1 PE = 1 SV = 1 |
| 68  | sp|Q14204|Cytoplasmic dynein 1 heavy chain 1 OS = Homo sapiens GN = DYNC1H1 PE = 1 SV = 5 |
| 69  | sp|Q92616|eIF-2-alpha kinase activator GCN1 OS = Homo sapiens GN = GCN1 PE = 1 SV = 6 |
| 70  | sp|P35606|Myosin-9 OS = Homo sapiens GN = MYH9 PE = 1 SV = 4 |
| 71  | sp|P35606|Coatomer subunit beta OS = Homo sapiens GN = COPB2 PE = 1 SV = 2 |
| 72  | sp|Q27708|CAD protein OS = Homo sapiens GN = CAD PE = 1 SV = 3 |
| 73  | sp|Q68VP6|Cullin-associated NEDD8-disassociated protein 1 OS = Homo sapiens GN = CAND1 PE = 1 SV = 2 |
| 74  | sp|P20073|Annexin A7 OS = Homo sapiens GN = ANXA7 PE = 1 SV = 3 |
| 75  | sp|Q13228|Selenium-binding protein 1 OS = Homo sapiens GN = SELENBP1 PE = 1 SV = 2 |
| 76  | sp|Q96AC1|Fermitin family homolog 2 OS = Homo sapiens GN = FERMT2 PE = 1 SV = 1 |
| 77  | sp|Q9UQ80|Proliferation-associated protein 2G4 OS = Homo sapiens GN = PA2G4 PE = 1 SV = 3 |
| 78  | sp|P46821|Microtubule-associated protein 1B OS = Homo sapiens GN = MAP1B PE = 1 SV = 2 |
| 79  | sp|P40763|Signal transducer and activator of transcription 3 OS = Homo sapiens GN = STAT3 PE = 1 SV = 2 |
| 80  | sp|P22314|Ubiquitin-like modifier-activating enzyme 1 OS = Homo sapiens GN = UBA1 PE = 1 SV = 3 |
| 81  | sp|Q34932|Heat shock 70 kDa protein 4 OS = Homo sapiens GN = HSPA4 PE = 1 SV = 4 |
| 82  | sp|P62826|GTP-binding nuclear protein Ran OS = Homo sapiens GN = RAN PE = 1 SV = 3 |
| 83  | sp|P23526|Adenosylhomocysteinase OS = Homo sapiens GN = AHCY PE = 1 SV = 4 |
| No. | Accession | Name                                                                 | FC       | P-value            |
|-----|-----------|----------------------------------------------------------------------|----------|--------------------|
| 84  | sp|P27816|MAP4_HUMAN | Microtubule-associated protein 4 OS = Homo sapiens GN = MAP4 PE = 1 SV = 3 | 0.380189 | 0.0000000706 |
| 85  | sp|PODMV9|HS71B_HUMAN | Heat shock 70 kDa protein 1B OS = Homo sapiens GN = HSPA1B PE = 1 SV = 1 | 0.366438 | 0.00000206 |
| 86  | sp|Q99832|TCPH_HUMAN | T-complex protein 1 subunit eta OS = Homo sapiens GN = CCT7 PE = 1 SV = 2 | 0.363078 | 0.000659 |
| 87  | sp|Q99613|EIF3C_HUMAN | Eukaryotic translation initiation factor 3 subunit C OS = Homo sapiens GN = EIF3C PE = 1 SV = 1 | 0.356451 | 0.000267 |
| 88  | sp|P48643|TCP_E_HUMAN | T-complex protein 1 subunit epsilon OS = Homo sapiens GN = CCT5 PE = 1 SV = 1 | 0.353183 | 0.00000384 |
| 89  | sp|P78344|IF4G2_HUMAN | Eukaryotic translation initiation factor 4 gamma 2 OS = Homo sapiens GN = EIF4G2 PE = 1 SV = 1 | 0.353183 | 0.000055 |
| 90  | sp|P23921|IRI_HUMAN | Ribonucleoside-diphosphate reductase large subunit OS = Homo sapiens GN = RRM1 PE = 1 SV = 1 | 0.343558 | 0.00001906 |
| 91  | sp|Q06210|GFPT1_HUMAN | Glutamine–fructose-6-phosphate aminotransferase [isomerizing] 1 OS = Homo sapiens GN = GFPT1 PE = 1 SV = 3 | 0.337287 | 0.000328 |
| 92  | sp|Q9BXJ9|NAA15_HUMAN | N-alpha-acetyltransferase 15, NatA auxiliary subunit OS = Homo sapiens GN = NAA15 PE = 1 SV = 1 | 0.331131 | 0.0000275 |
| 93  | sp|P49368|TCPG_HUMAN | T-complex protein 1 subunit gamma OS = Homo sapiens GN = CCT3 PE = 1 SV = 4 | 0.328095 | 0.000143 |
| 94  | sp|O75083|WDR1_HUMAN | WD repeat-containing protein 1 OS = Homo sapiens GN = WDR1 PE = 1 SV = 4 | 0.322107 | 0.00061 |
| 95  | sp|P55786|PSA_HUMAN | Puromycin-sensitive aminopeptidase OS = Homo sapiens GN = NPEPPS PE = 1 SV = 2 | 0.316228 | 0.000026 |
| 96  | sp|P09960|LKH4A_HUMAN | Leukotriene A4 hydrolase OS = Homo sapiens GN = LTA4H PE = 1 SV = 2 | 0.316228 | 0.000935 |
| 97  | sp|P30520|PUR2_HUMAN | Adenylosuccinate synthetase isozyme 2 OS = Homo sapiens GN = ADSS PE = 1 SV = 3 | 0.316228 | 0.0000165 |
| 98  | sp|QI5691|MARE1_HUMAN | Microtubule-associated protein RP/E/B family member 1 OS = Homo sapiens GN = MAPRE1 PE = 1 SV = 3 | 0.316228 | 0.000408 |
| 99  | sp|P12814|ACTN1_HUMAN | Alpha-actinin-1 OS = Homo sapiens GN = ACTN1 PE = 1 SV = 2 | 0.313239 | 0.000176 |
| 100 | sp|P30044|PRDX5_HUMAN | Peroxiredoxin-5, mitochondrial OS = Homo sapiens GN = PRDX5 PE = 1 SV = 4 | 0.313239 | 0.000749 |
| 101 | sp|P78371|TCPB_HUMAN | T-complex protein 1 subunit beta OS = Homo sapiens GN = CCT2 PE = 1 SV = 4 | 0.304794 | 0.00000242 |
| 102 | sp|P51436|SYRC_HUMAN | Arginine–tRNA ligase, cytoplasmic OS = Homo sapiens GN = RARS PE = 1 SV = 2 | 0.304794 | 0.000287 |
| 103 | sp|P09951|TCPD_HUMAN | T-complex protein 1 subunit delta OS = Homo sapiens GN = CCT4 PE = 1 SV = 4 | 0.304794 | 0.0000303 |
| 104 | sp|P23588|IF4B_HUMAN | Eukaryotic translation initiation factor 4B OS = Homo sapiens GN = EIF4B PE = 1 SV = 2 | 0.304794 | 0.0000105 |
| 105 | sp|P13489|LIN_HUMAN | Ribonuclease inhibitor OS = Homo sapiens GN = RNHI PE = 1 SV = 2 | 0.304794 | 0.000529 |
| 106 | sp|P555263|ADK_HUMAN | Adenosine kinase OS = Homo sapiens GN = ADK PE = 1 SV = 2 | 0.301995 | 0.000591 |
| 107 | sp|P40227|TCPZ_HUMAN | T-complex protein 1 subunit zeta OS = Homo sapiens GN = CCT6A PE = 1 SV = 3 | 0.296483 | 0.000029 |
| 108 | sp|P15559|NQO1_HUMAN | NAD(P)H dehydrogenase [quinone] 1 OS = Homo sapiens GN = NQO1 PE = 1 SV = 1 | 0.296483 | 0.000441 |
| 109 | sp|P50990|TCPQ_HUMAN | T-complex protein 1 subunit theta OS = Homo sapiens GN = CCT8 PE = 1 SV = 4 | 0.291072 | 0.00000345 |
| 110 | sp|P31947|1433S_HUMAN | 14-3-3 protein sigma OS = Homo sapiens GN = SFN PE = 1 SV = 1 | 0.285759 | 0.000365 |
### Table I. Cont.

| No. | Accession | Name                        | FC   | P-value            |
|-----|-----------|-----------------------------|------|-------------------|
| 111 | sp|Q16658|FSCN1_HUMAN | Fascin OS = Homo sapiens GN = FSCN1 PE = 1 SV = 3 | 0.280543 | 0.00000154 |
| 112 | sp|Q01518|CAP1_HUMAN | Adenylyl cyclase-associated protein 1 OS = Homo sapiens GN = CAP1 PE = 1 SV = 5 | 0.275423 | 0.0000322 |
| 113 | sp|P00966|ASSY_HUMAN | Argininosuccinate synthase OS = Homo sapiens GN = ASS1 PE = 1 SV = 2 | 0.272898 | 0.0000194 |
| 114 | sp|P60981|DEST_HUMAN | Destrin OS = Homo sapiens GN = DSTN PE = 1 SV = 3 | 0.272898 | 0.0000395 |
| 115 | sp|P52209|6PGD_HUMAN | 6-phosphogluconate dehydrogenase, decarboxylating OS = Homo sapiens GN = PGD PE = 1 SV = 3 | 0.270396 | 0.0000138 |
| 116 | sp|Q9Y2T3|GUAD_HUMAN | Guanine deaminase OS = Homo sapiens GN = GDA PE = 1 SV = 1 | 0.270396 | 0.000249 |
| 117 | sp|Q7L1Q6|BZW1_HUMAN | Basic leucine zipper and W2 domain-containing protein 1 OS = Homo sapiens GN = BZW1 PE = 1 SV = 1 | 0.265461 | 0.000453 |
| 118 | sp|Q16401|PSMD5_HUMAN | 26S proteasome non-ATPase regulatory subunit 5 OS = Homo sapiens GN = PSMD5 PE = 1 SV = 3 | 0.260615 | 0.00000517 |
| 119 | sp|P16152|CBR1_HUMAN | Carboxyl reductase [NADPH] 1 OS = Homo sapiens GN = CBR1 PE = 1 SV = 3 | 0.260615 | 0.0017 |
| 120 | sp|P9N7K5|OLA1_HUMAN | Obg-like ATPase 1 OS = Homo sapiens GN = OLA1 PE = 1 SV = 2 | 0.258226 | 0.000491 |
| 121 | sp|P11586|CTC_HUMAN | C-1-tetrahydrofolate synthase, cytoplasmic OS = Homo sapiens GN = MTHFD1 PE = 1 SV = 3 | 0.253513 | 0.0000226 |
| 122 | sp|P40925|MDHC_HUMAN | Malate dehydrogenase, cytoplasmic OS = Homo sapiens GN = MDH1 PE = 1 SV = 4 | 0.251189 | 0.0000186 |
| 123 | sp|P95373|IPO7_HUMAN | Importin-7 OS = Homo sapiens GN = IPO7 PE = 1 SV = 1 | 0.244343 | 0.000934 |
| 124 | sp|Q9Y617|SERC_HUMAN | Phosphoserine aminotransferase OS = Homo sapiens GN = PSAT1 PE = 1 SV = 2 | 0.244343 | 0.000026 |
| 125 | sp|P54578|UBP14_HUMAN | Ubiquitin carboxyl-terminal hydrolase 14 OS = Homo sapiens GN = USP14 PE = 1 SV = 3 | 0.235050 | 0.000113 |
| 126 | sp|P36952|SPB5_HUMAN | Serpin B5 OS = Homo sapiens GN = SERPINB5 PE = 1 SV = 2 | 0.235050 | 0.000299 |
| 127 | sp|Q9UG17|TES_HUMAN | Testin OS = Homo sapiens GN = TES PE = 1 SV = 1 | 0.235050 | 0.000587 |
| 128 | sp|P49588|SYAC_HUMAN | Alanine--tRNA ligase, cytoplasmic OS = Homo sapiens GN = AARS PE = 1 SV = 2 | 0.233466 | 0.00000015 |
| 129 | sp|P54577|SYC_HUMAN | Tyrosine--tRNA ligase, cytoplasmic OS = Homo sapiens GN = YARS PE = 1 SV = 4 | 0.231207 | 0.000000451 |
| 130 | sp|Q16719|KYNU_HUMAN | Kynureninase OS = Homo sapiens GN = KNYU PE = 1 SV = 1 | 0.229087 | 0.000151 |
| 131 | sp|P07900|HS90A_HUMAN | Heat shock protein HSP 90-alpha OS = Homo sapiens GN = HSP90AA1 PE = 1 SV = 5 | 0.226986 | 0.0000039 |
| 132 | sp|P23381|SYWC_HUMAN | Tryptophan--tRNA ligase, cytoplasmic OS = Homo sapiens GN = WARS PE = 1 SV = 2 | 0.224906 | 0.0000214 |
| 133 | sp|P50395|GDIB_HUMAN | Rab GDP dissociation inhibitor beta OS = Homo sapiens GN = GDI2 PE = 1 SV = 2 | 0.218776 | 0.000381 |
| 134 | sp|P41266|GSTM3_HUMAN | Glutathione S-transferase Mu 3 OS = Homo sapiens GN = GSTM3 PE = 1 SV = 3 | 0.218776 | 0.0000181 |
| 135 | sp|Q01813|PFKAP_HUMAN | ATP-dependent 6-phosphofructokinase, platelet type OS = Homo sapiens GN = PFKP PE = 1 SV = 2 | 0.216770 | 0.0000109 |
| 136 | sp|P29401|TKT_HUMAN | Transketolase OS = Homo sapiens GN = TKT PE = 1 SV = 3 | 0.214783 | 0.00000818 |
| 137 | sp|Q14980|XPO1_HUMAN | Exportin-1 OS = Homo sapiens GN = XPO1 PE = 1 SV = 1 | 0.214783 | 0.000144 |
| 138 | sp|P35237|SPB6_HUMAN | Serpin B6 OS = Homo sapiens GN = SERPINB6 PE = 1 SV = 3 | 0.214783 | 0.000441 |
| No. | Accession | Name                        | FC   | P-value        |
|-----|-----------|-----------------------------|------|---------------|
| 139 | sp|P26038|MOES_HUMAN | Moesin OS = Homo sapiens GN = MSN PE = 1 SV = 3 | 0.212814 | 0.0000000083 |
| 140 | sp|P60174|TP1S_HUMAN | Triosephosphate isomerase OS = Homo sapiens GN = TP1 PE = 1 SV = 3 | 0.210863 | 0.0000000565 |
| 141 | sp|P17987|TCPA_HUMAN | T-complex protein 1 subunit alpha OS = Homo sapiens GN = TCP1 PE = 1 SV = 1 | 0.210863 | 0.000000355 |
| 142 | sp|P37837|TALDO_HUMAN | Transaldolase OS = Homo sapiens GN = TALDO1 PE = 1 SV = 2 | 0.210863 | 0.00002 |
| 143 | sp|P00491|PNPH_HUMAN | Purine nucleoside phosphorylase OS = Homo sapiens GN = PNP PE = 1 SV = 2 | 0.210863 | 0.000182 |
| 144 | sp|P12429|ANXA3_HUMAN | Annexin A3 OS = Homo sapiens GN = ANXA3 PE = 1 SV = 3 | 0.207014 | 0.000102 |
| 145 | sp|P60842|IF4A1_HUMAN | Eukaryotic initiation factor 4A-I OS = Homo sapiens GN = EIF4A1 PE = 1 SV = 1 | 0.205116 | 0.00039 |
| 146 | sp|P08133|ANKA6_HUMAN | Annexin A6 OS = Homo sapiens GN = ANKA6 PE = 1 SV = 3 | 0.203236 | 0.0000209 |
| 147 | sp|P21102|PUR2_HUMAN | Trifunctional purine biosynthetic protein adenosine-3 OS = Homo sapiens GN = GART PE = 1 SV = 1 | 0.201372 | 0.000000215 |
| 148 | sp|Q16881|TRXR1_HUMAN | Thioredoxin reductase 1, cytoplasmic OS = Homo sapiens GN = TXNRD1 PE = 1 SV = 3 | 0.199526 | 0.0000000739 |
| 149 | sp|P35241|RADI_HUMAN | Radixin OS = Homo sapiens GN = RDX PE = 1 SV = 1 | 0.199526 | 0.0000185 |
| 150 | sp|P30085|KCY_HUMAN | UMP-CMP kinase OS = Homo sapiens GN = CMPK1 PE = 1 SV = 3 | 0.192309 | 0.000245 |
| 151 | sp|P17812|PYRG1_HUMAN | CTP synthase 1 OS = Homo sapiens GN = CTPS1 PE = 1 SV = 2 | 0.188799 | 0.000024 |
| 152 | sp|P49327|FAS_HUMAN | Fatty acid synthase OS = Homo sapiens GN = FASN PE = 1 SV = 3 | 0.183654 | 0.0 |
| 153 | sp|P08238|HS9OB_HUMAN | Heat shock protein HSP 90-beta OS = Homo sapiens GN = HSP90AB1 PE = 1 SV = 4 | 0.183654 | 0.0000176 |
| 154 | sp|P19399|PUR9_HUMAN | Bifunctional purine biosynthesis protein PURH OS = Homo sapiens GN = ATIC PE = 1 SV = 3 | 0.183654 | 0.000000181 |
| 155 | sp|P3687|PGM1_HUMAN | Phosphoglucomutase-1 OS = Homo sapiens GN = PGM1 PE = 1 SV = 3 | 0.183654 | 0.0000167 |
| 156 | sp|P18669|PGAM1_HUMAN | Phosphoglycerate mutase 1 OS = Homo sapiens GN = PGAM1 PE = 1 SV = 2 | 0.183654 | 0.000112 |
| 157 | sp|P14143|G6PD_HUMAN | Glucose-6-phosphate 1-dehydrogenase OS = Homo sapiens GN = G6PD PE = 1 SV = 4 | 0.177011 | 0.00000103 |
| 158 | sp|P17655|CAN2_HUMAN | Calpain-2 catalytic subunit OS = Homo sapiens GN = CAPN2 PE = 1 SV = 6 | 0.177011 | 0.00000121 |
| 159 | sp|P43175|ERA_HUMAN | D-3-phosphoglycerate dehydrogenase OS = Homo sapiens GN = PHGDH PE = 1 SV = 4 | 0.175388 | 0.0000184 |
| 160 | sp|P4075|ALDOA_HUMAN | Fructose-bisphosphate aldolase A OS = Homo sapiens GN = ALDOA PE = 1 SV = 2 | 0.17378 | 0.0000134 |
| 161 | sp|P41250|SYG_HUMAN | Glycine--tRNA ligase OS = Homo sapiens GN = GARS PE = 1 SV = 3 | 0.17378 | 0.000000134 |
| 162 | sp|P75874|DHIC_HUMAN | Isocitrate dehydrogenase [NADP] cytoplasmic OS = Homo sapiens GN = IDH1 PE = 1 SV = 2 | 0.172187 | 0.000000103 |
| 163 | sp|P18206|VINC_HUMAN | Vinculin OS = Homo sapiens GN = VCL PE = 1 SV = 4 | 0.170608 | 0.0 |
| 164 | sp|P31948|STIP1_HUMAN | Stress-induced-phosphoprotein 1 OS = Homo sapiens GN = STIP1 PE = 1 SV = 1 | 0.158489 | 0.000000108 |
| 165 | sp|P53936|ACLY_HUMAN | ATP-citrate synthase OS = Homo sapiens GN = ACLY PE = 1 SV = 3 | 0.157036 | 0.0000000426 |
| 166 | sp|Q9Y266|NUDC_HUMAN | Nuclear migration protein nudC OS = Homo sapiens GN = NUDC PE = 1 SV = 1 | 0.157036 | 0.000000195 |
| No. | Accession | Name | FC   | P-value          |
|-----|-----------|------|------|-----------------|
| 167 | sp|P55060| Exportin-2 OS = Homo sapiens GN = CSE1L PE = 1 SV = 3 | 0.155597 | 0.00000000656 |
| 168 | sp|O43776| Asparagine--tRNA ligase, cytoplasmic OS = Homo sapiens GN = NARS PE = 1 SV = 1 | 0.155597 | 0.0000275 |
| 169 | sp|P13797| Plastin-3 OS = Homo sapiens GN = PL35 PE = 1 SV = 4 | 0.148594 | 0.000000327 |
| 170 | sp|Q14914| Prostaglandin reductase 1 OS = Homo sapiens GN = PTGR1 PE = 1 SV = 2 | 0.143219 | 0.000000538 |
| 171 | sp|P62258| 14-3-3 protein epsilon OS = Homo sapiens GN = YWHAPE PE = 1 SV = 1 | 0.138038 | 0.0000521 |
| 172 | sp|P26639| Threonine--tRNA ligase, cytoplasmic OS = Homo sapiens GN = TAR5 PE = 1 SV = 3 | 0.136773 | 0.0000000522 |
| 173 | sp|P27348| 14-3-3 protein theta OS = Homo sapiens GN = YWHAQ PE = 1 SV = 1 | 0.131826 | 0.0000612 |
| 174 | sp|Q15185| Prostaglandin E synthase 3 OS = Homo sapiens GN = PTGES3 PE = 1 SV = 1 | 0.12942 | 0.0000842 |
| 175 | sp|P00338| L-lactate dehydrogenase A chain OS = Homo sapiens GN = LDHA PE = 1 SV = 2 | 0.128233 | 0.000000672 |
| 176 | sp|P08758| Annexin A5 OS = Homo sapiens GN = ANX5 PE = 1 SV = 2 | 0.128233 | 0.0000000417 |
| 177 | sp|Q15181| Inorganic pyrophosphatase OS = Homo sapiens GN = PPA1 PE = 1 SV = 2 | 0.128233 | 0.0000608 |
| 178 | sp|P07195| L-lactate dehydrogenase B chain OS = Homo sapiens GN = LDHB PE = 1 SV = 2 | 0.122462 | 0.0000861 |
| 179 | sp|P06733| Alpha-enolase OS = Homo sapiens GN = ENO1 PE = 1 SV = 2 | 0.115878 | 0.000000119 |
| 180 | sp|Q06830| Peroxiredoxin-1 OS = Homo sapiens GN = PRDX1 PE = 1 SV = 1 | 0.104713 | 0.000282 |
| 181 | sp|P13639| Elongation factor 2 OS = Homo sapiens GN = EF2 PE = 1 SV = 4 | 0.102802 | 0.0000215 |
| 182 | sp|O00299| Chloride intracellular channel protein 1 OS = Homo sapiens GN = CLC1 PE = 1 SV = 4 | 0.102802 | 0.000245 |
| 183 | sp|P15311| Ezrin OS = Homo sapiens GN = EZR PE = 1 SV = 4 | 0.1 | 0.000000224 |
| 184 | sp|P15121| Aldose reductase OS = Homo sapiens GN = AKR1B1 PE = 1 SV = 3 | 0.099083 | 0.00000144 |
| 185 | sp|P37802| Transgelin-2 OS = Homo sapiens GN = TAGLN2 PE = 1 SV = 3 | 0.095499 | 0.0000496 |
| 186 | sp|P06744| Glucose-6-phosphate isomerase OS = Homo sapiens GN = G6PI PE = 1 SV = 4 | 0.091201 | 0.00000011 |
| 187 | sp|P00558| Phosphoglycerate kinase 1 OS = Homo sapiens GN = PCK1 PE = 1 SV = 3 | 0.089496 | 0.00000202 |
| 188 | sp|P30041| Peroxiredoxin-6 OS = Homo sapiens GN = PRDX6 PE = 1 SV = 3 | 0.079433 | 0.00000231 |
| 189 | sp|Q01581| Hydroxymethylglutaryl-CoA synthase, cytoplasmic OS = Homo sapiens GN = HMGS1 PE = 1 SV = 2 | 0.06792 | 0.000000145 |
| 190 | sp|P12725| Alpha-1-antiproteinase OS = Ovis aries PE = 1 SV = 1 | 0.064269 | 0.0000536 |
Table II. Differentially expressed proteins identified between adjacent noncancerous tissues and distal noncancerous tissues of hepatocellular carcinoma

| No. | Accession   | Name                                                                 | FC     | P-value      |
|-----|-------------|----------------------------------------------------------------------|--------|--------------|
| 1   | sp|P12763|FETUA_BOVIN | Alpha-2-HS-glycoprotein OS = Bos taurus GN = AHSG PE = 1 SV = 2 | 4.37   | 0.000000647 |
| 2   | sp|00299|CLIC1_HUMAN | Chloride intracellular channel protein 1 OS = Homo sapiens GN = CLIC1 PE = 1 SV = 4 | 3.49945211 | 0.00064666 |
| 3   | sp|P78417|GSTO1_HUMAN | Glutathione S-transferase omega-1 OS = Homo sapiens GN = GSTO1 PE = 1 SV = 2 | 3.46736908 | 0.00088023 |
| 4   | sp|P0558|PGK1_HUMAN | Phosphoglycerate kinase 1 OS = Homo sapiens GN = PGK1 PE = 1 SV = 3 | 3.34   | 0.000000494 |
| 5   | sp|P17812|PYRG1_HUMAN | CTP synthase 1 OS = Homo sapiens GN = CTPS1 PE = 1 SV = 2 | 3.28095293 | 0.0009827 |
| 6   | sp|Q01813|PFKAP_HUMAN | ATP-dependent 6-phosphofructokinase. platelet type OS = Homo sapiens GN = PFKP PE = 1 SV = 2 | 3.2210691 | 0.00021282 |
| 7   | sp|P06744|G6PI_HUMAN | Glucose-6-phosphate isomerase OS = Homo sapiens GN = G6PI PE = 1 SV = 4 | 3.16   | 0.00000522 |
| 8   | sp|P04264|K2C1_HUMAN | Keratin. type II cytoskeletal 1 OS = Homo sapiens GN = KRT1 PE = 1 SV = 6 | 3.133286 | 0.00012301 |
| 9   | sp|P21266|GSTM3_HUMAN | Glutathione S-transferase Mu 3 OS = Homo sapiens GN = GSTM3 PE = 1 SV = 3 | 2.88   | 0.0000379 |
| 10  | sp|P30041|PRDX6_HUMAN | Peroxiredoxin-6 OS = Homo sapiens GN = PRDX6 PE = 1 SV = 3 | 2.83139205 | 0.00092527 |
| 11  | sp|P08133|ANXA6_HUMAN | Annexin A6 OS = Homo sapiens GN = ANXA6 PE = 1 SV = 3 | 2.83139205 | 0.00077018 |
| 12  | sp|P31939|PURH_HUMAN | Bifunctional purine biosynthesis protein OS = Homo sapiens GN = ATIC PE = 1 SV = 3 | 2.56   | 0.0000395 |
| 13  | sp|P36871|PGM1_HUMAN | Phosphoglucomutase-1 OS = Homo sapiens GN = PGM1 PE = 1 SV = 3 | 2.49   | 0.00000426 |
| 14  | sp|Q15181|PYR_HUMAN | Inorganic pyrophosphatase OS = Homo sapiens GN = PPA1 PE = 1 SV = 2 | 2.48885703 | 0.00020884 |
| 15  | sp|Q96970|IP9_HUMAN | Importin-9 OS = Homo sapiens GN = IPO9 PE = 1 SV = 3 | 2.46603894 | 0.00095205 |
| 16  | sp|P50395|GDIB_HUMAN | Rab GDP dissociation inhibitor beta OS = Homo sapiens GN = GDIB PE = 1 SV = 2 | 2.38   | 0.0000832 |
| 17  | sp|P00491|PNPH_HUMAN | Purine nucleoside phosphorylase OS = Homo sapiens GN = PNP PE = 1 SV = 2 | 2.37684011 | 0.00012449 |
| 18  | sp|Q96177|SERC_HUMAN | Phosphoserine aminotransferase OS = Homo sapiens GN = PSAT1 PE = 1 SV = 2 | 2.26986504 | 0.00016311 |
| 19  | sp|P55060|XPO2_HUMAN | Exportin-2 OS = Homo sapiens GN = CSE1L PE = 1 SV = 3 | 2.25   | 0.00000809 |
| 20  | sp|P13797|PLST_HUMAN | Plastin-3 OS = Homo sapiens GN = PLS3 PE = 1 SV = 4 | 2.051162 | 0.00014754 |
| 21  | sp|Q01581|HMCS1_HUMAN | Hydroxymethylglutaryl-CoA synthase. cytoplasmic OS = Homo sapiens GN = HMCS1 PE = 1 SV = 2 | 1.95884502 | 0.00053098 |
| 22  | sp|P18206|VINC_HUMAN | Vinculin OS = Homo sapiens GN = VCL PE = 1 SV = 4 | 1.91   | 0.00000266 |
| No. | Accession | Name | FC   | P-value       |
|-----|-----------|------|------|---------------|
| 23  | sp|P49588|SYAC_HUMAN | Alanine-tRNA ligase, cytoplasmic OS = Homo sapiens GN = AARS PE = 1 SV = 2 | 1.87 | 0.00000459 |
| 24  | sp|P49327|FAS_HUMAN | Fatty acid synthase OS = Homo sapiens GN = FASN PE = 1 SV = 3 | 1.85 | 0.000000763 |
| 25  | sp|P14133|G6PD_HUMAN | Glucose-6-phosphate 1-dehydrogenase OS = Homo sapiens GN = G6PD PE = 1 SV = 4 | 1.80301797 | 0.00072658 |
| 26  | sp|Q86U2P|KTN1_HUMAN | Kinectin OS = Homo sapiens GN = KTN1 PE = 1 SV = 1 | 0.60255963 | 0.00045493 |
| 27  | sp|P13667|PDIA4_HUMAN | Protein disulfide-isomerase A4 OS = Homo sapiens GN = PDIA4 PE = 1 SV = 2 | 0.603 | 0.0000523 |
| 28  | sp|P25705|ATPA_HUMAN | ATP synthase subunit alpha, mitochondrial OS = Homo sapiens GN = ATP5A1 PE = 1 SV = 1 | 0.53951061 | 0.00031264 |
| 29  | sp|O75369|FLNB_HUMAN | Filamin-B OS = Homo sapiens GN = FLNB PE = 1 SV = 2 | 0.535 | 0.000967 |
| 30  | sp|P13127|CPSM_HUMAN | Carbamoyl-phosphate synthase [ammonia], mitochondrial OS = Homo sapiens GN = CPS1 PE = 1 SV = 2 | 0.492 | 0.00000077 |
| 31  | sp|Q9P2E9|RBP1_HUMAN | Ribosome-binding protein 1 OS = Homo sapiens GN = RBP1 PE = 1 SV = 4 | 0.488 | 0.0000851 |
| 32  | sp|Q14126|DSG2_HUMAN | Desmoglein-2 OS = Homo sapiens GN = DSG2 PE = 1 SV = 2 | 0.40550849 | 0.00097142 |
| 33  | sp|P30050|RL12_HUMAN | 60S ribosomal protein L12 OS = Homo sapiens GN = RPL12 PE = 1 SV = 1 | 0.38725761 | 0.00062865 |
| 34  | sp|P14049|MDH2_HUMAN | Malate dehydrogenase, mitochondrial OS = Homo sapiens GN = MDH2 PE = 1 SV = 3 | 0.37325019 | 0.00081137 |
| 35  | sp|Q07065|CAP43_HUMAN | Cytoskeleton-associated protein 4 OS = Homo sapiens GN = CAP43 PE = 1 SV = 2 | 0.366 | 0.00000472 |
| 36  | sp|P23246|SFPQ_HUMAN | Splicing factor, proline- and glutamine-rich OS = Homo sapiens GN = SFPQ PE = 1 SV = 2 | 0.36078097 | 0.0001904 |
| 37  | sp|Q8IVZ2|AHN2_HUMAN | Protein AHN2 OS = Homo sapiens GN = AHN2 PE = 1 SV = 2 | 0.36 | 0.000000183 |
| 38  | sp|Q13813|SPTN1_HUMAN | Spectrin alpha chain, non-erythrocytic 1 OS = Homo sapiens GN = SPTAN1 PE = 1 SV = 3 | 0.344 | 0.0000000289 |
| 39  | sp|P19338|NUCL_HUMAN | Nucleolin OS = Homo sapiens GN = NCL PE = 1 SV = 3 | 0.316 | 0.0000007999 |
| 40  | sp|P11021|GRP78_HUMAN | 78 kDa glucose-regulated protein OS = Homo sapiens GN = HSPA5 PE = 1 SV = 2 | 0.27 | 0.00000000617 |
| 41  | sp|P27824|CALX_HUMAN | Calnexin OS = Homo sapiens GN = CANX PE = 1 SV = 2 | 0.258586 | 0.0005111 |
| 42  | sp|P46779|RL28_HUMAN | 60S ribosomal protein L28 OS = Homo sapiens GN = RPL28 PE = 1 SV = 3 | 0.237684 | 0.00034004 |
| 43  | sp|P10809|CH60_HUMAN | 60 kDa heat shock protein, mitochondrial OS = Homo sapiens GN = HSPD1 PE = 1 SV = 2 | 0.215 | 0.00000000379 |
| 44  | sp|P08670|VIME_HUMAN | Vimentin OS = Homo sapiens GN = VIM PE = 1 SV = 4 | 0.175 | 0.00000297 |
Figure 2. Features of the hepatocellular carcinoma secretome dataset from the isobaric tags for relative and absolute quantification shotgun analysis. A – The distribution of differently abundant proteins in 2 groups. B – Venn diagrams show the numbers of the identified proteins and the overlaps of differently abundant proteins in the 2 groups.

Figure 3. The gene ontology (GO) analysis of the differently abundant proteins. A – The GO analysis of differently abundant proteins overlapped in the 2 groups. B – The GO analysis of differently abundant proteins only involved in the hepatocellular carcinoma tissues/distal noncancerous tissues group.
The isobaric tags for relative and absolute quantification-based quantitative proteomics of fresh tissue-derived secretome in hepatocellular carcinoma

The isobaric tags for relative and absolute quantification-based quantitative proteomics of fresh tissue-derived secretome in hepatocellular carcinoma

Molecular function
- Viral transcription
- SRP-dependent cotranslational protein targeting to membrane
- Translation initiation
- Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
- Translation
- Positive regulation of sister chromatid cohesion
- Positive regulation of skeletal muscle contraction by regulation of release of actin
- Desmosome organization
- Extracellular exosome
- Plasma membrane
- Cytoplasm
- Cellular protein agglutination activity
- Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
- rRNA processing
- Translation
- Positive regulation of sister chromatid cohesion
- Positive regulation of skeletal muscle contraction by regulation of release of actin
- Desmosome organization
- Extracellular exosome
- Plasma membrane
- Cytoplasm
- Cellular protein agglutination activity
- Cellular protein agglutination activity
- Cytoplasmic large ribosomal subunit
- Structural constituent of ribosome
- DNA binding
- RNA binding
- Nucleotide binding
- Methylarsonate reductase activity
- Malate dehydrogenase (NADP+) activity
- Glutathione dehydrogenase (ascorbate) activity
- Cell adhesive protein binding involved in bundle of His cell-Purkinje myocyte co-telomeric DNA binding
- RNA polymerase II distal enhancer sequence-specific DNA binding
- Number of proteins

Figure 3. Cont. C – The GO analysis of differently abundant proteins only involved in the adjacent noncancerous tissues/distal noncancerous tissues group

acids metabolism, trichloroacetic acid (TCA) cycle, glucose metabolism, etc.

The String analysis of the differentially expressed proteins

As shown in Figure 5A, in the HCC/DN group, the proteins could be classified into 3 major clusters: proteins in the red region were related to protein translation and post-translation processing, proteins in the blue region were related to protein glycosylation modification, and proteins in the green region were related to biological metabolic functions dominated by glucose metabolism. While in the AN/DN group, the proteins could also be classified into 3 clusters: the red region represented proteins related to immune and metabolic functions, the green region represented proteins related to apoptosis functions, and the blue region represented proteins related to protein binding functions (Figure 5 B).

Discussion

Hepatocellular carcinoma has become the third-most-common cause of cancer-related death worldwide. Most cases of HCC were developed in patients who had already had liver cirrhosis [16]. Therefore, surveillance for the early onset of HCC was recommended. The biomarkers with high sensitivity and specificity were essential for optimising the management of HCC [17]. Zhang et al. used the iTRAQ pipeline to distinguish the proteomic profiles of malignant ascites in HCC patients from those with non-malignant liver cirrhosis and found that Enolase-1 and fibrinogen are potential ascitic fluid-based biomarkers for diagnosis and prognosis of HCC [18]. Guo et al. reported that assaying CD14 levels may complement AFP measurement for the early detection of HCC [19]. Wang et al. suggested that different molecular alterations and specific signalling pathways were indeed involved in different HCC subtypes [20]. Our study aimed to investigate the molecular signatures of the HCC by quantitative proteomics using iTRAQ with LC-MS/MS.

In our study, the number of differentially expressed proteins identified in the HCC/DN group was much higher than in the AN/DN group. These findings indicate that the features between the adjacent noncancerous tissues and distant noncancerous tissues were more similar than those between the HCC tissues and the distant noncancerous tissues, which were accorded with objective existence.

The gene ontology annotation analysis showed that the cell components of the differentially expressed proteins that either overlapped in 2 groups or uniquely in 1 group were mostly located in the extracellular exosome, which indicated that the proteins extracted in this experiment were mainly secreted proteins. For the biological process analysis, the GO annotation analysis showed that the proteins overlapped in both the groups and were the major participants in the protein folding, lipid metabolic process, gluconeogenesis, nucleobase-containing compound metabolic process, and canonical glycolysis. Most of these processes focused on metabolic changes, which
Figure 4. The key signalling pathways involved in the 2 groups. A – The key signalling pathways involved in the hepatocellular carcinoma tissues/distal noncancerous tissues group. B – The key signalling pathways involved in the adjacent noncancerous tissues/distal noncancerous tissues group. The top 10 enriched signalling pathways were displayed in the figures.
Figure 5. The interaction networks of differently abundant proteins in the 2 groups. A – The interaction networks of differently abundant proteins involved in the hepatocellular carcinoma tissues/distal noncancerous tissues group. B – The interaction networks of differently abundant proteins involved in the adjacent noncancerous tissues/distal noncancerous tissues group.
suggested that the changes in the material metabolism were universal, regardless of the transformation from distant cancer to adjacent cancer or the approach of adjacent cancer to HCC. The molecular function of these proteins also focuses on energy metabolism, which also supported the hypothesis [15, 21–23].

There were 155 dysregulated proteins in the HCC group compared to the DN group, but these proteins were not dysregulated in the AN group compared to the DN group. We further analysed that these proteins involved the biological process by GO analysis; the results showed that these dysregulated proteins were mainly involved in signal transduction, cell proliferation, protein stabilisation, and the negative regulation of the apoptotic process. These processes might have been involved in the formation of development of HCC, and it has been reported that these processes are involved in the disturbing of the signal transduction and protein degradation [24–27], apoptotic process [27, 28], and cell proliferation [28, 29] in tumours. The molecular function of these proteins, such as the cadherin binding involved in cell-cell adhesion, protein homodimerisation activity, ubiquitin-protein ligase binding, calcium ion binding, GTP binding, etc., also supported this conclusion.

Interestingly, there were 9 dysregulated proteins in the AN group compared to the DN group but no dysregulation in the HCC group compared to the DN group, and the GO results showed that these dysregulated proteins were mainly involved in desmosome organisation, positive regulation of sister chromatid cohesion, translation, rRNA processing, nuclear-transcribed mRNA catabolic process, translational initiation, and SRP-dependent co-translational protein targeting the membrane. The results also showed that the dysregulated proteins may have affected the incidence and progress of HCC, such as the change of the combination of the protein and the RNA function presenting the disorder of the transcription and translation function, which suggested that the surrounding noncancerous cells might increase the expression of the nucleic acid and enzyme by tumour microenvironment to promote the HCC proliferation and growth [30], and that the changes of telomere and telomerase in the surrounding noncancerous cells revealed the dysregulation on the chromosome stability, repair, and proliferation, which were all closely related to the incidence of HCC development [31, 32]. Similarly, the molecular function of these proteins, such as cadherin binding-involved nucleotide binding, RNA binding, calcium ion binding, chromatin binding, transcription regulatory region DNA binding, identical protein binding, etc., also supported this conclusion.

To further reveal the possible molecular mechanisms of the tumourigenesis and the development of the primary HCC, we applied the KEGG database to analyse the signalling pathways in which the differentially expressed proteins were involved. Our study also analysed the pathway of metabolism, genetic information processing, environmental information processing, and cellular. According to the results of the analysis, the dysregulated proteins in HCC are mostly involved in the JAK-STAT pathway and MAPK pathway. All the above-mentioned signalling pathways are actively associated with cancers [33–36]. It has been reported that the MAPK signalling pathway played an essential role in the development and aggressive behaviour of tumours by enhancing tumour cell proliferation, differentiation, apoptosis, and cell cycle [37, 38]. Therefore, it is not surprising that the MAPK signalling pathway is involved in HCC tissues. Interestingly, the JAK-STAT pathway was only enriched in the HCC group but not in the AN group. JAK-STAT pathway has been regarded as one of the main molecular pathways in HCC progression [39].

However, the signalling pathway only enriched in the AN group comprised mainly basic metabolisms, such as biological oxidations, amino acids metabolism, TCA cycle, glucose metabolism, and so on. All of these processes belong to the material metabolism and illustrate that the primary material changes play an important role in the tumourigenesis and development of HCC. Also, the different pathways in the HCC and the AN group suggest that there might be different molecular mechanisms in the carcinogenesis and development of the primary HCC in the HCC tissue and the surrounding noncancerous tissues. The above-mentioned results that were analysed demonstrate that our quantitative proteomics approach is suitable in studying the overall molecular profile changes of HCC and could give further insight into the possible molecular mechanisms.

In our study, the proteins in the HCC/DN group could be classified into 3 major clusters: proteins in the red region were related to protein translation and post-translation processing, proteins in the blue region were related to protein glycosylation modification, and proteins in the green region were related to biological metabolic functions dominated by glucose metabolism. As is already known, the malignant proliferation of tumour cells was a process of energy consumption, so the hyperactive glucose metabolism in the HCC group might provide the necessary conditions for the progression of HCC [40, 41]. Glycosylation was involved in the folding, aggregation, maturation, and transportation of protein-peptide chains and was a terminal signal on the surface of the cancer cells in carcinogenesis [42, 43]. The incidence, development, and invasion of HCC were accompanied by glycosylation changes of relevant glycoproteins, so the change of the carbohydrate
structure on the surface of the HCC cells played an important role in the occurrence and development progress of HCC [44, 45].

The proteins in the AN/DN group could also be classified into 3 clusters: the red region represented proteins related to immune and metabolic functions, the green region represented proteins related to apoptosis functions, and the blue region represented proteins related to protein binding functions. This indicated that immune and metabolic changes were relatively active in the para cancer tissues, which might be related to changes in the tumour microenvironment [46–49]. All these results suggest that the evolution of the tissues adjacent to HCC promoted the incidence of HCC.

In summary, this study applied the iTRAQ-based qualitative proteomic approach to analyse the secretome of the primary cultures of HCC tumour tissues. The results visibly showed that the secretome profile alternations and signalling pathways were associated with HCC occurrence and development. The dysregulated proteins in the HCC/DN group were concentrated in the MAPK signalling and JAK-STAT signalling, but the dysregulated proteins in the AN/DN group were more concentrated in the basal material metabolism. The different protein expression profiles in the primary HCC tissues, the surrounding non-cancerous tissues, and the distal noncancerous tissues might also reveal different underlying molecular mechanisms. This study provides a valuable resource of the HCC tissue secretome to investigate the molecular mechanism of HCC incidence and development.

In conclusion, the secretome profile alternations and signalling pathways were associated with HCC incidence and development. The dysregulated proteins in the HCC/DN group were concentrated in the MAPK signalling and JAK-STAT signalling, but the dysregulated proteins in the AN/DN group were more concentrated in the basal material metabolism.

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Conflict of interest

The authors declare no conflict of interest.

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