RESEARCH

Quantitative analysis of the mitochondrial proteome in human ovarian carcinomas

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Abstract

Mitochondria play important roles in growth, signal transduction, division, tumorigenesis and energy metabolism in epithelial ovarian carcinomas (EOCs) without an effective biomarker. To investigate the proteomic profile of EOC mitochondrial proteins, a 6-plex isobaric tag for relative and absolute quantification (iTRAQ) proteomics was used to identify mitochondrial expressed proteins (mtEPs) in EOCs relative to controls, followed by an integrative analysis of the identified mtEPs and the Cancer Genome Atlas (TCGA) data from 419 patients. A total of 5115 quantified proteins were identified from purified mitochondrial samples, and 262 proteins were significantly related to overall survival in EOC patients. Furthermore, 63 proteins were identified as potential biomarkers for the development of an EOC, and our findings were consistent with previous reports on a certain extent. Pathway network analysis identified 70 signaling pathways. Interestingly, the results demonstrated that cancer cells exhibited an increased dependence on mitophagy, such as peroxisome, phagosome, lysosome, valine, leucine and isoleucine degradation and fatty acid degradation pathways, which might play an important role in EOC invasion and metastasis. Five proteins (GLDC, PCK2, IDH2, CPT2 and HMGCS2) located in the mitochondrion and enriched pathways were selected for further analysis in an EOC cell line and tissues, and the results confirmed reliability of iTRAQ proteomics. These findings provide a large-scale mitochondrial proteomic profiling with quantitative information, a certain number of potential protein biomarkers and a novel vision in the mitophagy bio-mechanism of a human ovarian carcinoma.

Key Words

- ▶ ovarian carcinoma
- ▶ mitochondrial proteomics
- ▶ iTRAQ
- ▶ TCGA
- ▶ mitophagy
- ▶ biomarker

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Introduction

Epithelial ovarian carcinoma (EOC) is the cause of more deaths than any other female genital tract cancers, and nearly accounts for 6% of all cancers among women (Sakhuja *et al.* 2017). Detection of early-stage EOCs is challenging since this disease is clinically asymptomatic

in early stage, with being progressing toward the development of metastasis (Gadducci *et al.* 2007). In China, an increasing trend in mortality was observed for three of the ten most common cancers (breast, cervix and ovary), with stable trends for colorectum, lung, uterine

and thyroid cancers (Chen *et al.* 2016). Even through tumor biomarkers CA125 and HE4 were widely used in clinical practice (Cymbaluk-Ploska *et al.* 2018), and more combined determinations would result in significantly improved sensitivity and efficiency, which might lead successfully to achieve a significant reduction in mortality.

Mitochondria are involved in various cellular processes, from regulation of metabolic flux to apoptosis. Mitochondrial dysfunctions have been proposed as a cause of cancer, are a biomarker for the early-stage detection of a cancer and are a therapeutic target for a cancer (Kim *et al.* 2017). Moreover, those mitochondrial ribosomal protein-encoding genes could be anti-oncogenes, which can even be new therapeutic targets or prognostic biomarkers. MRPL41, known as bcl-2-interacting mitochondrial ribosomal protein L41, indicated that the differential expression of MRPL41 in carcinomas is reflected by the various epigenetic states together with different responses and promoter methylation through the estrogen receptor (Kim *et al.* 2013). In addition, mitochondria could be 'fuel' to a cancer metabolism. Higher expression of MRPS15 in epithelial breast cells was revealed in an analysis of paired adjacent stromal tissues and neoplasm tissues (Sotgia *et al.* 2012). Those studies showed that the neoplasm-relevant communication pathways were linked with mitochondrial proteins. The role of mitochondrial ribosomal protein S23 (MRPS23) in carcinoma cell proliferation could be a potential therapeutic target, in case of interference of hepatocellular cancer proliferation, oncogenesis and metastasis (Pu *et al.* 2017). The expression of COX1 was involved in endometrial cancers (Ksiezakowska-Lakoma *et al.* 2017), esophageal adenocarcinoma (Huhta *et al.* 2017) and lung cancer (Michalak *et al.* 2016). Taken all together, these examples support the notion that mitochondria contribute to the angiogenetic, tumorigenic, proliferation, invasive and metastatic features of cancer cells. However, no large-scale quantitative reference map of a human EOC mitochondrial proteome was reported previously. Moreover, only focusing on single molecule biomarker is a narrowest form for cancer prediction, prevention and treatment (Cheng & Zhan 2017, Zhan *et al.* 2017a). Cancer, by definition, is a kind of gene disease or protein disease, and results in a series of molecular alterations (Gonzalez-Angulo *et al.* 2012). Multiple biomarkers could provide a novel approach to predict, prevent and personalize the treatment of an EOC. Furthermore, newly discovered proteins could be used as potential targets or biomarkers (Wang *et al.* 2015). However, the proteomic profile of EOC mitochondrial proteins has not been elucidated. It is necessary to find a series of mitochondrial biomarkers

on EOC in terms of detection technology. Proteomics has developed as a powerful approach to investigate novel biomarkers and drug targets (Ray *et al.* 2011), and it has mainly applied in the identification and quantification of proteomic components including post-translational modifications (Zhan *et al.* 2017b,c). Due to great improvement in mass spectrometry (MS) analysis, peptide identification and protein sequence coverage showed a preferable consistency in complex samples (Riley & Coon 2018). The commonly used quantitative proteomic methods include gel-based such as 2D gel electrophoresis (2DGE) and 2D difference in-gel electrophoresis (2D DIGE) and gel-free-based (Hu *et al.* 2013), to allow highly sensitive and high-throughput identification of proteins/peptides and post-translational modifications (van der Wal *et al.* 2018). In general, gel-free methods are able to break through restrictions of gel-based methods that are inefficient in resolving proteins that are insoluble, lowly abundant or large proteins (>200 kDa) (Pasing *et al.* 2017). Multi-dimensional liquid chromatography-tandem mass spectrometry (MDLC-MS/MS)-based proteomics techniques were developed rapidly, and isobaric tag for relative and absolute quantification (iTRAQ) was featured with the advantages of strong separation ability and analysis range. A few of the advantages of the use of iTRAQ to reveal biomarkers or molecular mechanisms of different cancers have been reported (Zhou *et al.* 2017). Even 2DGE-MS with isotopic labeling and the application of high-sensitivity MS enables the quantification of a much larger part (estimated up to at least 500,000 protein species) of the human proteome as assumed before (Zhan *et al.* 2018).

This study used iTRAQ proteomics to identify and quantify mitochondrial expressed proteins (mtEPs) in EOCs. A total of 5115 proteins was identified and quantified. Moreover, Kyoto encyclopedia of genes and genomes (KEGG) analysis found a variety of signaling pathways such as peroxisome, phagosome, lysosome, valine, leucine and isoleucine degradation, and fatty acid degradation pathway, and they might play an important role in EOC mitophagy. The Cancer Genome Atlas (TCGA) database and mtEPs data were integrated and analyzed to investigate the gene ontology (GO) functional enrichments, protein-protein interactions and gene coexpression. Thus, 63 proteins as potential biomarkers for the development of EOCs were identified; and ERBB2, PTBP1 and H2AFX as biomarkers confirmed with previous reports were significantly related to overall survival in EOCs. These findings provide a certain number of proteins with the potential biomarkers and a novel vision

in the mitophagy bio-mechanism of human EOCs. More importantly, we propose the use of the multi-biomarkers strategy to predict, prevent and personalize the treatment of an EOC.

Materials and methods

EOC tissue specimen

Ovarian tissues provided by Department of Gynecology, Xiangya Hospital (Changsha, China) were obtained from 18 female patients (EOC: $n=7$ and control: $n=11$) and were approved by the Xiangya Hospital Medical Ethics Committee of Central South University, and informed consent was obtained from the participation. Seven EOC patients were diagnosed as high-grade, poorly or moderately differentiated carcinoma cells. Eleven control ovaries were with benign gynecologic diseases, such as fibroids, adenomyosis, ovary serous cystadenoma, cervical intraepithelial neoplasia, atypical hyperplasia of endometrium and pelvic organ prolapse. Each obtained tissue was quickly put into liquid nitrogen and then stored in -80°C .

Preparation of mitochondria

Ovarian tissue samples were divided into EOC ($n=7$) and control ($n=11$) groups. The mitochondrial isolation buffer was prepared with 210mM mannitol, 70mM sucrose, 100mM potassium chloride (KCl), 1mM diamine tetraacetic acid (EDTA), 50mM Tris-HCl, 0.1mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 1mM phenylmethanesulfonyl fluoride (PMSF) protease inhibitor, 2mM sodium orthovanadate (V), 0.2% bovine serum albumin (BSA) and pH 7.4. (i) The EOC tissues (1.5g) were fully minced (1mm³ pieces) and homogenized (2min; 4°C) in 13.5mL mitochondrial isolation buffer that contained 0.2mg/mL Nacarse. The tissue homogenates were well mixed with another 3mL mitochondrial isolation buffer, followed by centrifugation (1300g, 10min and 4°C) to remove crude nuclear fraction. The supernatant was re-centrifuged (10,000g, 10min and 4°C) to remove microsomes in the supernatant. The debris was re-suspended in 2mL mitochondrial isolation buffer, followed by centrifugation (7000g, 10min and 4°C) to obtain crude mitochondria in the debris (Jiang *et al.* 2004). The extracted crude mitochondria were dissolved in 12mL 25% Nycodenz (Sigma), and then a discontinuous Nycodenz gradient was made through filling with 5mL of 34%, 8mL of 30%, 12mL of 25% (contained crude

mitochondria), 8mL of 23%, and 3mL of 20% Nycodenz from bottom to top in a tube, followed by centrifugation (52,000g, 90min, 4°C) (Schonenberger & Kovacs 2017). After centrifugation, the purified mitochondria were contained at the interface of 25–30%, which was collected in a clean tube. The collected mitochondria were diluted with mitochondrial isolation buffer to three-fold volume, and centrifuged (15,000g, 20min, 4°C). The debris was re-suspended in 2mL mitochondrial isolation buffer, followed by centrifugation (15,000g, 20min, 4°C). The final debris was the purified mitochondria. (ii) For the mitochondria purified from the control tissues, the above procedure (i) was modified as the following: prior to homogenization, 8mL of 0.05% trypsin/20mM EDTA in PBS solution was added to the minced control tissues and digested for 30min at room temperature, followed by centrifugation (200g, 5min). The discontinuous Nycodenz gradient was made through filling with 8mL of 38%, 5mL of 34%, 8mL of 30%, 12mL of 25% (contained crude mitochondria), 8mL of 23% and 3mL of 20% Nycodenz from bottom to top in a tube, followed by centrifugation (52,000g, 90min, 4°C). After centrifugation, the purified mitochondria were contained at the interface of 25–30% to the interface of 34–38%. The other procedure is the same as the procedure (i).

All purified mitochondria from EOC and control tissues were put together, respectively. Then, proteins were extracted from purified EOC and control mitochondria, respectively.

iTRAQ quantitative proteomics and bioinformatics

The purified EOC and control mitochondria were digested with trypsin. (i) iTRAQ labeling: The tryptic peptides were labeled with a 6-plex iTRAQ Multiplex Kit according to the manufacturer's instructions (Applied Biosystems iTRAQ Reagents–Chemistry Reference Guide, P/N 4351918A). Briefly, the peptides were dissolved in 100mM tetraethyl ammonium bromide solution (pH 8.5) before the labeling reagent was added. After 2-h incubation, the reaction was quenched by adding an equal volume of water. Six labeled peptide samples were mixed equally and dried with a speed-vac (Qi *et al.* 2016). (ii) Strong cation exchange (SCX) fractionation: The labeled peptide mixture was fractionated with SCX chromatography. (iii) LC–MS/MS: Each fractionation was subjected to LC–MS/MS analysis on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60min. MS/MS spectra were acquired and used to search protein database with

MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4. The identified proteins were used for KEGG pathway analysis with Cytoscape (two-sided hypergeometric test, Kappa score >0.9, adjusted *P* value <0.01 corrected with Benjamini-Hochberg), and further for KEGG pathway enrichment with DAVID Bioinformatics Resources 6.7 (<https://david.ncifcrf.gov/home.jsp>). Heat map was plotted by Multiple Experiment Viewer (<https://sourceforge.net/projects/mev-tm4/files/mev-tm4/>). GO biological process (BP), molecular function (MF) and cellular component (CC) were analyzed with Cytoscape ClueGO (two-sided hypergeometric test, adjusted *P* value <0.05 corrected with Benjamini-Hochberg). GO CC was further enriched with PANTHER (<http://www.pantherdb.org/>). Biomarkers that had been reported were checked by CoolGen (<http://ci.smu.edu.cn/CoolGen/Home.php>).

TCGA data of EOC patients

TCGA data portal provides a platform for researchers to search, download and analyze datasets generated from TCGA database (<http://cancergenome.nih.gov/>) (Zhang *et al.* 2016). Level 3 RNA-seq V2 and clinical data were obtained from the TCGA data of 419 EOC patients. The gene expression missing value (expression=0) more than 20% was excluded after the pretreatment. Overall survival analysis of genes in EOCs was calculated by the Kaplan–Meier method, and compared to the log-rank test with R 3.4.2 version (<https://www.r-project.org/>). The *P* value less than 0.01 was considered as statistically significant.

Prediction of protein–protein interaction

The overlapped proteins of iTRAQ-identified proteins and TCGA overall related genes were analyzed by STRING 10.0 (<http://string-db.org/cgi/input.pl>) with a high confidence of parameter (>0.700).

Construction of mRNA–mRNA network of the overlapped proteins

The Pearson correlation test was used to determine whether the expression levels of overlapped proteins of iTRAQ identified proteins and TCGA overall related genes were correlated with each other, respectively. The mRNA–mRNA correlations of those overlapped proteins with *P* values <0.05 were corrected with Benjamini-Hochberg. Extracted data with negative correlation

($r < -0.5$) or positive correlation ($r > 0.7$) from were used to construct circus chart by Package RCircos. Each line denoted one mRNA–mRNA pair. Each region along the circle represented one of the 24 chromosomes (Zhang *et al.* 2013). Chromosomal location was obtained by Ensembl (<http://asia.ensembl.org/index.html>).

Cell lines and cell culture

EOC cell line TOV-21G cells and control cell line IOSE80 cells were purchased from Keibai Academy of Science (Nanjing, China). TOV-21G cells were cultured in RPMI-1640 medium, and IOSE80 cells were cultured in DMEM medium (Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). All these cells were maintained with 5% CO₂ atmosphere at 37°C.

RNA extraction and quantitative real-time PCR (qRT-PCR) analyses

Total RNAs were extracted from cell lines with TRizol Reagent (Invitrogen) according to the manufacturer's instructions. For the detection of SNHG3 and target genes, total RNAs were reversely transcribed into cDNAs and then used to perform qRT-PCR with SYBR Premix Ex Taq (TaKaRa). β -actin was used as an internal control for mRNA quantification.

Western blotting

Equal amounts of proteins were separated by 10% SDS-PAGE gels and blotted onto nitrocellulose membranes. The blotted proteins on the membrane were incubated with primary antibodies against GLDC, PCK2, IDH2, CPT2 and HMGCS2 (1:1000; Abcam) and β -actin (1:2000; Santa Cruz Biotechnology) at 4°C overnight. The membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rat secondary antibody (1:5000; Santa Cruz Biotechnology) at room temperature.

Statistical analysis

Data were expressed as the mean $\pm$ s.d. of triplicates. Each experiment was repeated at least three times. Statistical analyses were performed using SPSS 13.0 (SPSS Inc.). The Student's *t*-test was used to assess the between-group differences of *in vitro* studies with a statistical significance level of $P < 0.05$. Some cases were corrected with Benjamini-Hochberg (FDR) for multiple testing.

Table 1 63 proteins identified as biomarkers in ovarian cancers.

Swiss-Prot accession	Description	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
P0C055	Histone H2A	31.3	2	22	13.54	10.6	0.25	0.00081850
P16104	Histone H2AX	21.7	1	35	15.14	10.7	0.33	0.00011480
P55083	Microfibril-associated glycoprotein 4	14.5	4	23	28.63	5.6	0.36	0.00056068
Q53TN4	Cytochrome b reductase 1	7.7	2	3	31.62	8.8	0.44	0.00246457
Q13884	Beta-1-syntrophin	5.4	1	3	58.03	8.6	0.45	0.00355956
P84157	Matrix-remodeling-associated protein 7	27.0	3	6	21.45	4.3	0.45	0.00258059
A7YME7	IASPP short isoform	5.7	1	1	44.08	4.9	0.47	0.00426621
Q5VY30	Retinol binding protein 4, plasma, isoform CRA_b	5.0	1	2	22.96	6.1	0.48	0.00014748
Q9NQG1	Protein MANBAL	23.5	2	4	9.46	9.2	0.49	0.00204730
Q4KMQ2	Anoctamin-6	7.8	4	6	106.10	7.8	0.50	0.00026427
A6NLN1	Polypyrimidine tract binding protein 1, isoform CRA_b	12.7	5	14	56.48	9.4	0.50	0.00094192
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	19.3	6	20	37.41	9.0	0.51	0.00126949
O75954	Tetraspanin-9	4.6	1	1	26.76	7.7	0.52	0.00345367
E9PIY1	Protein kinase C and casein kinase substrate in neurons protein 3	13.8	4	5	37.19	6.9	0.53	0.00852630
A0A052Z4B5	Serine/threonine-protein phosphatase (Fragment)	1.8	1	1	57.62	6.3	0.54	0.00095765
P30050	60S ribosomal protein L12	44.9	5	12	17.81	9.4	0.57	0.00161750
F8W150	Ankyrin repeat domain-containing protein 13A (Fragment)	14.5	1	1	9.37	6.8	0.58	0.00239321
H0YMA2	Tropomodulin-2 (Fragment)	12.7	2	7	12.38	9.4	0.59	0.00074392
O43491	Band 4.1-like protein 2	30.1	20	38	112.52	5.4	0.60	0.00039823
P07355	Annexin A2	61.7	21	110	38.58	7.8	0.61	0.00087133
F6RFD5	Destrin	44.4	6	20	15.39	8.6	0.62	0.00045171
B7ZLW0	LPP protein	10.6	4	7	65.73	7.4	0.63	0.00184847
P04004	Vitronectin	21.3	9	28	54.27	5.8	0.63	0.00052603
Q16186	Proteasomal ubiquitin receptor ADRM1	6.1	2	4	42.13	5.1	0.63	0.00219031
J3KTF8	Rho GDP-dissociation inhibitor 1 (Fragment)	25.4	4	7	21.50	5.5	0.64	0.00621335
Q86SR1	Polypeptide N-acetylgalactosaminyltransferase 10	12.4	5	7	68.95	8.6	0.64	0.00045129
P25398	40S ribosomal protein S12	53.8	7	21	14.51	7.2	0.65	0.00153508
K7ES31	Eukaryotic translation initiation factor 3 subunit K	20.4	2	4	15.86	6.7	0.65	0.00562823
F8W8H5	Ras-related protein Rab-24	5.2	1	1	19.66	5.4	0.66	0.00776930
O75369	Filamin-B	26.6	44	95	277.99	5.7	0.66	0.00535785
E9PKG6	Nucleobindin-2	41.4	11	26	40.34	5.2	1.51	0.00014496
Q14554	Protein disulfide-isomerase A5	26.6	12	25	59.56	7.9	1.51	0.01206170
Q9BZF1		9.5	8	10	101.13	7.0	1.52	0.00131055
Q6ZNC8	Lysophospholipid acyltransferase 1	2.8	1	1	56.52	9.2	1.53	0.04319160
Q04837	Single-stranded DNA-binding protein, mitochondrial	55.4	7	78	17.25	9.6	1.53	0.00187904
Q9Y5M8	Signal recognition particle receptor subunit beta	44.3	11	37	29.68	9.0	1.55	0.01881880
A0A087WVA1	Selenoprotein T	10.3	2	2	22.33	8.8	1.59	0.01394870
Q9NPJ3	Acyl-coenzyme A thioesterase 13	25.0	3	8	14.95	9.1	1.62	0.00447179
I3L3P5	Protein disulfide-isomerase (Fragment)	57.7	1	34	16.87	4.8	1.67	0.00135696
C5HTZ1	Amiloride-sensitive sodium channel subunit alpha	4.5	1	1	28.38	6.7	1.68	0.00519917
Q14696	LDLR chaperone MESD	34.6	8	13	26.06	7.8	1.68	0.00845485
O75396	Vesicle trafficking protein SEC22b	29.3	6	19	24.58	6.9	1.69	0.00132399
Q8IU55	Epoxide hydrolase 4	13.0	4	4	42.30	8.4	1.72	0.00500565
Q9HD23	Magnesium transporter MRS2 homolog, mitochondrial	14.0	5	7	50.29	6.9	1.72	0.00200604

K7EIE9	Tyrosine-protein phosphatase non-receptor type 2 (Fragment)	7.3	1	1	17.74	9.7	1.75	0.04562050
H0YDQ1	Probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1, 3-glucosyltransferase (Fragment)	10.3	1	2	9.78	10.1	1.77	0.00484252
O43295	SLIT-R	0.8	1	1	124.43	6.7	1.79	0.01316100
Q8N4H5	Mitochondrial import receptor subunit T	27.5	2	4	6.03	9.7	1.79	0.00695095
P13667	Protein disulfide-isomerase A4	61.7	42	268	72.89	5.1	1.81	0.00452458
Q96RP9	Elongation factor G, mitochondrial	36.2	25	56	83.42	7.0	1.88	0.00092116
Q4G148	Glucoside xylosyltransferase 1	8.2	3	5	50.53	8.6	1.92	0.00044414
P54687	Branched-chain amino acid aminotransferase, cytosolic	5.4	2	2	42.94	5.3	1.92	0.00195883
Q15084	Protein disulfide-isomerase A6	58.2	18	91	48.09	5.1	1.96	0.00742499
G3V5T0	Maleylacetoacetate isomerase	28.7	1	7	22.60	7.7	2.07	0.00081986
Q16822	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	35.0	1	31	70.68	7.6	2.18	0.00448436
P50591	Tumor necrosis factor ligand superfamily member 10	4.3	1	1	32.49	7.4	2.38	0.00972657
Q16880	2-hydroxyacylsphingosine 1-beta-galactosyltransferase	5.9	3	3	61.40	9.5	2.38	0.01062680
P09758	Tumor-associated calcium signal transducer 2	26.6	7	10	35.69	8.9	2.41	0.00111629
P55145	Mesencephalic astrocyte-derived neurotrophic factor	42.3	8	23	20.69	8.7	2.48	0.00286699
Q6P1M0	Long-chain fatty acid transport protein 4	11.7	5	19	72.02	8.5	2.51	0.00846281
Q9UBX3	Mitochondrial dicarboxylate carrier	34.5	1	12	31.26	9.5	2.54	0.01059370
Q96CP7	Calfacitin	3.2	1	1	28.53	9.5	2.57	0.00684684
Q9BSE5	Agmatinase, mitochondrial	11.4	2	3	37.64	7.6	3.40	0.00074157

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/M), Ratio of tumors to normal controls.

Results

Quantitative proteome map of EOC mitochondria

The iTRAQ-SCX-LC-MS/MS quantitative analysis of isolated mitochondrial samples identified a total of 5115 proteins that were present in EOC and control tissues (Supplementary Table 1, see section on supplementary data given at the end of this article). Each protein was identified with at least one peptide sequence matches (PSMs). Those proteins identified in EOC and control tissues were distributed within a range of molecular weight (MW) 2.6–1158.2 kDa and pI 3.81–12.25, which was consistent with the pI distribution pattern of the previously identified proteins in the mitochondrial fraction reported by Rezaul *et al.* (Rezaul *et al.* 2005). In addition, iTRAQ proteomic analysis also obtained protein quantitative information, including 2565 (50.14%) upregulated proteins and 2550 (49.86%) downregulated proteins in EOC relative to control tissues. The top 10 upregulated proteins were androgen-induced 1/Golgi SNAP receptor complex member 1 variant 1 fusion protein (AIG1), pituitary tumor-transforming gene 1 protein-interacting protein (PTTG1IP), protein S100-A14 (S100A14), agmatinase mitochondrial (AGMAT), normal mucosa of esophagus-specific gene 1 protein (NMES1), tetraspanin-1 (TSPAN1), protein-glutamine gamma-glutamyltransferase K (Fragment) (TGM1), MHC class II antigen (Fragment) (HLA-DPA1), uncharacterized protein C1orf53 (fragment) (C1orf53) and MHC class I antigen (Fragment) (HLA-A). The top 10 downregulated proteins were collagen alpha-1(II) chain (COL2A1), nanospan (NSPN), SUN domain-containing protein 2 (fragment) (SUN2), pumilio homolog 1 (Fragment) (PUM1), ubiquitin C (UbC), Xin actin-binding repeat-containing protein 1 (XIRP1), cytochrome c oxidase subunit 4 isoform 2 (COX4I2), histone H2A type 2-B (HIST2H2AB), collagen, type V, alpha 2, isoform CRA_b (COL5A2) and collagen alpha-1 (X) chain (COL10A1). Comparative proteomics analysis focuses on those statistically significantly differentially expressed proteins (DEPs) that might be the real potential biomarkers. However, one could not ignore those no-expression-level-changed proteins, which might have experienced post-translational modifications, such as phosphorylation, glycosylation, acetylation, methylation, nitration, ubiquitylation, sumoylation, succinylation, sulfation, myristoylation, palmitoylation, deamidation, prenylation and hydroxylation (Cleland 2018, Khan *et al.* 2018, Sloutsky & Naegle 2018) to result in different proteoforms or they might be hub-molecules in a molecular network with less changes

Figure 2

The interaction networks of identified proteins. (A) The protein-protein interactions (PPIs) network of 262 overlapped proteins. (B) The mRNA-mRNA pair analysis of 262 overlapped proteins constructed circos chart by R package. Upregulated proteins by iTRAQ in red letters and downregulated proteins in green letters. Red line represents positive correlation and green line represents negative correlation. (C) Cancer cells exhibit an increased dependence on mitophagy, such as peroxisome, phagosome, valine, leucine and isoleucine degradation, fatty acid degradation pathway. D-F. Kaplan-Meier (KM) survival curve of ERBB2, PTBP1 and H2AFX in an epithelial ovarian carcinoma (EOC). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

relative to the boundary molecules in a given condition (Zhan *et al.* 2017b).

GO enrichment analysis

To ascertain how the identified proteins promoted phenotype in an EOC tissue, GO analysis was performed

using PANTHER. A total of 5115 proteins were classified according to the CCs. Figure 1A showed variable CCs: cell part (42.7%), organelle (28.2%) and macromolecular complex (17.8%). Moreover, 1108 overall survival-related genes were obtained by TCGA database (Supplementary Tables 2, 3 and 4). There were 262 significant proteins (Supplementary Table 5) obtained when overlapping

Table 2 Mitophagy adaptors and regulatory molecules involved the identified proteins in ovarian cancer biological system.

Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
Q8IVP5	FUN14 domain-containing protein 1	FUNDC1	10.97	1	1	17.16	8.62	1.16	0.048224
H0YBC7	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (Fragment)	BNIP3L(NIX)	9.19	1	2	20.08	5.71	0.77	0.002312
Q96HS1	Serine/threonine-protein phosphatase PGAM5, mitochondrial	PGAM5	32.53	10	37	31.98	8.68	1.49	0.003318
E7EU96	Casein kinase II subunit alpha	CSNK2A1 (CK)	25.45	6	8	45.28	7.94	0.84	0.011967
O60313	Dynamitin-like 120 kDa protein, mitochondrial	OPA1	51.15	44	130	111.56	7.87	1.19	0.000372
Q99623	Prohibitin-2	PHB2	81.61	24	220	33.28	9.83	1.26	0.000444
AAO52Z5I6	Optineurin isoform 3	OPTN	7.94	2	2	28.30	5.29	0.62	0.010129
B4E164	cDNA FLJ56613, highly similar to Serine/threonine-protein kinase TBK1 (EC 2.7.11.1)	TBK1	2.42	1	1	56.96	6.77	1.25	0.011201
B4E3V2	cDNA FLJ52854, highly similar to Sequestosome-1	p62	10.47	1	1	31.81	5.41	1.10	0.19687
B7Z737	cDNA FLJ52784, highly similar to Bcl-2-like 13 protein	Bcl2-L13	13.17	2	2	21.62	4.39	0.81	0.039927

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N), Ratio of tumors to normal controls.

analysis was performed between 5115 identified proteins and TCGA database (Fig. 1C). Moreover, 262 overlapped proteins were classified according to the BP, CC and MF. As shown in Fig. 1D, E and F, the less *P* value and more significant enrichment were shown with the greater node size. The same color indicated the same function group. Among the groups, a representative was chosen of the most significant term and lag highlighted. The overlapped proteins were mainly distributed in endoplasmic reticulum, cellular amino acid metabolic process, viral process, regulation of cellular response to heat and response to heat according to BP (Supplementary Table 6). The localization of overlapped proteins was also differentially distributed in peroxisome, COP9 signalosome, intracellular ribonucleoprotein complex, cell-substrate adherens junction and mitochondrial matrix according to CC (Supplementary Table 7). A series of MFs were involved in 262 overlapped proteins, including GTP binding, unfolded protein binding, oxidoreductase activity (acting on CH-OH group of donors), intramolecular oxidoreductase activity and aminoacyl-tRNA ligase activity (Supplementary Table 8). Compared to previous studies, 992 proteins of 5115 proteins have been reported to relate with ovary (Supplementary Table 9). For example, VTCN1 was overexpressed in early-stage EOCs and was independent of CA125 expression (Simon *et al.* 2007, Fortner *et al.* 2018) and CDKN2A was the similar biomarker for early-stage EOCs (Jiang *et al.* 2017). CD44, PLAT and PTBP1 showed prognostic value in EOC patients (Borgfeldt *et al.* 2003, Zhang *et al.* 2010, Bartakova *et al.* 2018). Correlation between tumor mesothelin (MSLN) expression and serum MSLN in EOC patients was fine (Hanaoka *et al.* 2017). In our findings, 63 proteins were identified as novel biomarkers in EOCs (the fold change ≥ 1.5) (Table 1). Our findings were consistent with previous reports on a certain extent such that ERBB2, PTBP1 and H2AFX were not only biomarkers but also significantly related to overall survival (Fig. 2D, E and F).

The protein-protein and mRNA-mRNA interactions of 262 overlapped proteins

The 262 overlapped proteins were uploaded to STRING for protein-protein interaction analysis. The combined scores of nodes ranged from 0.700 to 0.999. Some key proteins were identified in the part of the common proteins between 5115 identified proteins and TCGA database's RNAs, such as VCP (fold change=1.04, *P*=0.05), RHOA (fold change=0.88, *P*=0.04), RPL7A (fold change=1.12, *P*=0.03), HSPA8 (fold change=0.90, *P*=0.08),

KEGG pathway analysis mapped the identified proteins to 70 signaling pathways (Fig. 1B; Supplementary Table 13). Interestingly, the results demonstrated that cancer cells exhibited an increased dependence on mitophagy, such as peroxisome, phagosome, lysosome, valine, leucine and isoleucine degradation and fatty acid degradation pathway, which might play an important role in EOC

Table 3 Peroxisome pathway involved the identified proteins in ovarian cancer biological system.

Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
Q9Y2Q3	Glutathione S-transferase kappa 1	GSTK1	66.37	13	129	25.48	8.41	2.13	4.24E-07
P09110	3-ketoacyl-CoA thiolase, peroxisomal	ACAA1	46.23	4	50	44.26	8.44	2.70	0.010033
H7C131	3-ketoacyl-CoA thiolase, peroxisomal (Fragment)	ACAA1	56.21	1	38	30.31	8.63	0.92	0.363631
P04040	Catalase	CAT	42.13	19	58	59.72	7.38	1.33	0.002223
P43155	Carnitine O-acetyltransferase	CRAT	21.09	13	22	70.81	8.44	0.87	0.008569
Q99489	D-aspartate oxidase	DDO	6.16	1	1	37.51	7.91	0.98	0.801381
Q9B7Z2	Dehydrogenase/reductase SDR family member 4	DHRS4	29.5	7	19	29.52	8.56	1.34	0.001585
Q95822	Malonyl-CoA decarboxylase, mitochondrial	MLYCD	27.79	9	23	54.97	8.95	1.17	0.007631
A8MXV4	Nucleoside diphosphate-linked moiety X motif 19	NUDT19	17.87	5	11	42.21	7.64	1.55	0.006403
Q9BY49	Peroxisomal trans-2-enoyl-CoA reductase	PECR	26.4	7	10	32.52	8.81	1.57	0.000414
Q92968	Peroxisomal membrane protein PEX13	PEX13	2.48	1	2	44.10	8.05	1.61	0.003575
O75381	Peroxisomal membrane protein PEX14	PEX14	14.85	5	7	41.21	4.94	1.99	0.000651
P56589	Peroxisomal biogenesis factor 3	PEX3	8.85	3	4	42.11	8.15	1.22	0.013231
Q06830	Peroxisomal biogenesis factor 1	PRDX1	56.28	8	66	22.10	8.13	0.99	0.687097
Q7Z7M4	Superoxide dismutase (Fragment)	SOD2	57.28	10	97	23.66	7.31	1.49	0.000523
P48735	Isocitrate dehydrogenase [NADP], mitochondrial	IDH2	56.64	27	355	50.88	8.69	2.02	0.002074
P00441	Superoxide dismutase [Cu-Zn]	SOD1	29.87	3	11	15.93	6.13	0.76	0.002799
E7ETN3	Uncharacterized protein	NUDT12	17.58	15	25	123.94	6.80	1.27	0.023697
Q9ULC5	Long-chain-fatty-acid--CoA ligase 5	ACSL5	7.47	3	5	75.94	6.92	1.20	0.033585
Q9HC35	Echinoderm microtubule-associated protein-like 4	EML4	6.32	5	7	108.84	6.40	0.86	0.040138
P28288	ATP-binding cassette sub-family D member 3	ABCD3	19.88	12	41	75.43	9.36	1.73	0.003973
Q969G5	Protein kinase C delta-binding protein	PRKCD8P	18.77	5	8	27.68	6.43	0.60	0.002111
Q99424	Peroxisomal acyl-coenzyme A oxidase 2	ACOX2	23.05	14	19	76.78	7.56	1.33	0.001417
O96011	Peroxisomal membrane protein 11B	PEX11B	35.14	8	19	28.41	9.85	1.42	0.003051
Q96CP5	PMPCB protein (Fragment)	PMPCB	24.17	11	22	53.48	6.71	1.41	0.003341

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N), Ratio of tumors to normal controls.

Figure 4

Phagosome pathway altered in an ovarian cancer. Green rectangle with red mark means the identified proteins. Green rectangle without red mark means species-specific enzymes. White rectangle means reference pathway. The solid line means molecular interaction. The dot line means indirect effect. The circle means mostly chemical complex. The pathway node in the right panel corresponds to the red marked node in the left diagram. ID number is the Swiss-Prot accession number. Ratio (T/N)=Ratio of tumors to controls. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

invasion and metastasis (Fig. 2C). Mitophagy involves the engulfment of any material in a double-membrane enclosed autophagosome, which subsequently fuses with lysosomes. In autophagosomes, mitochondrion, proteins or peroxisome were usually observed. Autophagosomes fuse with lysosomes and emit high-energy substances, including fatty acid and amino acid (Zimmermann & Reichert 2017). In this progress, mitophagy is dependent on the general autophagy machinery and relies on a growing cadre of 'mitophagy adaptors' and regulatory molecules, such as FUNDC1, BNIP3L(NIX), PGAM5, CK, OPA1, prohibitin2, OPTN, TBK1, p62 and Bcl2-L13 (Drake *et al.* 2017). This current study identified those proteins too, even though few of them appeared to have changing expression patterns (Table 2). The results were consistent with previous studies, for those proteins activated downstream mitophagy by post-translational modifications (Zimmermann & Reichert 2017).

A peroxisome is a kind of organelle in nearly all eukaryotic cells. It is involved in metabolism of fatty acids, amino acids and polyamines, and reduction of reactive oxygen, and also contains two enzymes in the pentose phosphate pathway, so it is important for

energy metabolism (Wanders & Waterham 2006). The peroxisome-related proteins were notably increased in EOCs relative to controls (fold change >1.5), including NUDT19 (fold change=1.54, $P=0.006$), PECR (fold change=1.56, $P=0.0004$), PEX13 (fold change=1.61, $P=0.003$), ABCD3 (fold change=1.72, $P=0.003$), PEX14 (fold change=1.98, $P=0.0006$), IDH2 (fold change=2.01, $P=0.002$), GSTK1 (fold change=2.13, $P=0.0000004$) and ACAA1 (fold change=2.70, $P=0.01$) (Fig. 3 and Table 3), which indicated peroxisome was metabolically active in EOC tissues. Mitophagy is the selective degradation of mitochondria by autophagy. Defective mitochondria following stress or damage were swallowed by phagosomes (Lemasters 2005). The phagosome-related proteins were significantly increased in EOCs relative to controls (fold change >1.5), including TAP (fold change=1.60, $P=0.008$), MSR1 (fold change=1.61, $P=0.02$), FcyR (fold change=1.64, $P=0.005$), SEC22 (fold change=1.69, $P=0.01$) and p22phox (fold change=2.10, $P=0.0002$) (Fig. 4 and Table 4), which indicated phagosome was metabolically active in EOC tissues.

Autophagosomes fuse with lysosomes to form autolysosome, and 'goods' in autophagy has also been

Table 4 Phagosome pathways involved identified proteins operated in ovarian cancer biological system.

Pathway code	Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
ATPase	P27449	V-type proton ATPase 16 kDa proteolipid subunit	ATP6V0C	11.61	1	18	15.73	8.44	1.63	0.074595
ATPase	P38606	V-type proton ATPase catalytic subunit A	ATP6V1A	23.5	13	19	68.26	5.52	1.12	0.948809
ATPase	P21281	V-type proton ATPase subunit B, brain isoform	ATP6V1B2	11.55	2	6	56.46	5.81	0.99	0.037427
ATPase	P21283	V-type proton ATPase subunit C 1	ATP6V1C1	15.18	6	7	43.91	7.46	1.186	0.000291
ATPase	P36543	V-type proton ATPase subunit E 1	ATP6V1E1	10.18	3	6	26.13	8.00	1.41	0.494573
ATPase	Q16864	V-type proton ATPase subunit F	ATP6V1F	7.56	1	1	13.36	5.52	1.09	0.00215
C3b	P01024	Complement C3	C3	44.02	59	144	187.03	6.40	0.89	0.001276
CANX	P27824	Calnexin	CANX	38.51	15	167	67.53	4.60	1.41	0.011842
calnexin	D6RB85	Calnexin (Fragment)	CANX	50.69	1	58	15.99	4.74	1.38	0.007655
collectins	Q9BWV8	Collectin-11	COLEC11	4.06	1	1	28.65	5.41	0.53	0.018448
coronin	P31146	Coronin-1A	CORO1A	29.07	8	11	50.99	6.68	1.16	0.561446
cathepsin	Q9HBQ7	Cathepsin L, isoform CRA_b	CTSL	9.27	1	1	16.83	4.58	1.09	0.000904
p22phox	P13498	Cytochrome b-245 light chain	CYBA	11.28	2	3	21.00	9.54	2.10	0.000256
gp91	P04839	Cytochrome b-245 heavy chain	CYBB	12.98	8	15	65.29	8.63	1.41	0.031874
Dynein	Q9Y6G9	Cytoplasmic dynein 1 light intermediate chain 1	DYNC1LI1	10.52	5	7	56.54	6.42	0.83	0.006861
Dynein	O43237	Cytoplasmic dynein 1 light intermediate chain 2	DYNC1LI2	10.16	4	7	54.07	6.38	0.80	0.009882
EEA1	Q15075	Early endosome antigen 1	EEA1	6.95	8	10	162.37	5.68	0.81	0.005954
FcγR	P12318	Low affinity immunoglobulin gamma Fc region receptor II-a	FCGR2A	3.15	1	1	34.98	6.68	1.64	0.005709
αvβ3	P05556	Integrin beta-1	ITGB1	34.46	23	67	88.36	5.39	0.85	0.009677
αvβ5	P18084	Integrin beta-5	ITGB5	9.14	5	7	88.00	6.06	1.46	0.222117
M6PR	P20645	Cation-dependent mannose-6-phosphate receptor	M6PR	20.94	5	10	30.97	5.83	0.93	0.01956
MPO	P05164	Myeloperoxidase	MPO	26.17	16	33	83.81	8.99	1.40	0.002417
MRC2	Q9UBG0	C-type mannose receptor 2	MRC2	9.06	12	16	166.57	5.83	0.61	0.00081
MSR1	P21757	Macrophage scavenger receptor types I and II	MSR1	1.55	1	1	49.73	5.91	1.61	0.020868
Rab5	P20339	Ras-related protein Rab-5 ^a	RAB5A	40	3	14	23.64	8.15	1.27	0.005865
Rab7	P51149	Ras-related protein Rab-7a	RAB7A	76.33	13	29	23.47	6.70	1.20	0.001324
SEC22	O75396	Vesicle trafficking protein SEC22b	SEC22B	29.3	6	19	24.58	6.91	1.69	0.012313
SEC22	I1VE18	SEC22 vesicle trafficking protein B (Fragment)	SEC22B	37.74	2	3	6.12	4.94	1.37	0.026863
SEC61A1	P61619	Protein transport protein Sec61 subunit alpha isoform 1	SEC61A1	15.55	7	19	52.23	8.06	1.40	0.333448
STX7	O15400	Syntaxin-7	STX7	15.71	3	3	29.80	5.55	0.97	0.001201
TAP	X5CMH5	Antigen peptide transporter 2	TAP2	28.31	17	36	77.65	7.85	1.60	0.008156
TfR	P02786	Transferrin receptor protein 1	TFR	27.63	18	39	84.82	6.61	1.45	0.244793
α2β1	F5H4Z8	Thrombospondin-3	THBS3	8.13	5	7	103.19	4.64	1.06	0.019908
TUBB	P07437	Tubulin beta chain	TUBB	70.95	4	215	49.64	4.89	1.30	0.029027
TUBB	Q13885	Tubulin beta-2A chain	TUBB2A	62.47	3	151	49.87	4.89	0.86	0.879846
TUBB	Q9BUE5	Tubulin beta-6 chain	TUBB6	46.19	5	66	49.83	4.88	0.99	0.012313

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N), Ratio of tumors to normal controls.

Figure 5

Lysosome pathway altered in an ovarian cancer. Green rectangle with red mark means the identified proteins. Green rectangle without red mark means species-specific enzymes. White rectangle means reference pathway. The solid line means molecular interaction. The dot line means indirect effect. The circle means mostly chemical complex. The pathway node in the right panel corresponds to the red marked node in the left diagram. ID number is the Swiss-Prot accession number. Ratio (T/N)=Ratio of tumors to controls. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

degraded into products (fatty acids and amino acids). Those kind of high-energy substances were transported to the cytoplasm of cells for reuse (Lemasters 2005). The lysosome pathway-related proteins were significantly increased in EOCs relative to controls (fold change >1.5), including CD63 (fold change=1.76, $P=0.007948$), ATP6V0C (fold change=1.63, $P=0.000424$), GNPTG (fold change=1.57, $P=0.018055$) and ACP2 (fold change=1.64, $P=0.006068$), AP1M2 (fold change=1.603138, $P=0.003299$), CTSG AP1M2 (fold change=1.62, $P=0.029695$) (Fig. 5 and Table 5), which indicated lysosome pathway was metabolically active in EOC tissues. The fatty acid degradation-related proteins were significantly increased in EOCs relative to controls (fold change >1.5), including ECHS1 (fold change=1.52, $P=0.003$), EHHADH (fold change=1.62, $P=0.002$), ECI1 (fold change=1.64, $P=0.001$) and CPT2 (fold change=2.05, $P=0.019$) (Fig. 6 and Table 6), which indicated fatty acid degradation pathway was metabolically active in EOC tissues.

The valine, leucine and isoleucine degradation-related proteins were significantly increased in EOCs relative to controls (fold change >1.5), including ECHS1 (fold change=1.52, $P=0.003$), HIBCH (fold change=1.58, $P=0.002$), EHHADH (fold change=1.61, $P=0.002$),

IL4I1 (fold change=1.82, $P=0.008$), HMGCS2 (fold change=2.17, $P=0.002$) and ACAA1 (fold change=2.70, $P=0.0100$) (Fig. 7 and Table 7), which indicated valine, leucine and isoleucine degradation pathway was metabolically active in EOC tissues.

qRT-PCR and Western blotting validated the consistency of iTRAQ quantitative mitochondrial proteomics

To validate the DEPs identified by iTRAQ quantitative mitochondrial proteomics, we examined the protein expressions of the identified DEPs, including GLDC, PCK2, IDH2, CPT2 and HMGCS2 in the mitochondrial protein samples prepared from EOC and control tissues, and the mRNA and protein expressions of those five DEPs in the cultured EOC cells TOV21G and control cells IOSE80. Except for HMGCS2, a significant increase in the mRNA and protein expression levels of GLDC, PCK2, IDH2 and CPT2 was observed in the cultured cells by q-PCR and Western blot, respectively (Fig. 8A and B). The results of Western blot in the prepared mitochondrial samples had a good consistency with the results of iTRAQ quantitative mitochondrial proteomics (Fig. 8C).

Table 5 Lysosome pathways involved identified proteins operated in ovarian cancer biological system.

Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
F8VV56	CD63 antigen	CD63	20.69	3	10	16.00	7.88	1.76	0.007948
P27449	V-type proton ATPase 16 kDa proteolipid subunit	ATP6V0C	11.61	1	18	15.72	8.44	1.63	0.000424
Q8TBM3	V-type proton ATPase subunit a	ATP6V0A2	6.18	2	2	43.14	5.82	1.40	0.002528
P17900	Ganglioside GM2 activator	GM2A	13.47	3	3	20.82	5.31	1.23	0.00379
A2VDJ4	GNPTG protein (Fragment)	GNPTG	3.42	1	1	32.93	6.79	1.57	0.018055
A0A1B0GTP7	Acid ceramidase	ASAHI	30.21	1	16	32.55	7.62	0.77	0.007232
I3NI22	N-sulphoglucosamine sulphohydrolase (Fragment)	SGSH	6.7	1	1	21.69	6.19	0.68	0.00013
P11117	Lysosomal acid phosphatase	ACP2	14.66	6	12	48.31	6.74	1.64	0.006068
K7EJJ1	AP-1 complex subunit mu-2 (Fragment)	AP1M2	6.94	1	1	27.87	5.58	1.60	0.003299
Q13367	AP-3 complex subunit beta-2	AP3B2	3.51	1	5	118.98	5.59	1.28	0.01856
Q6PK82	AP3D1 protein (Fragment)	AP3D1	7.75	5	6	98.47	9.13	0.80	0.009833
Q9BR63	FARSB protein (Fragment)	FARSB	28.72	15	19	65.65	6.84	0.70	0.004887
A0A1B0GW44	Cathepsin D	CTSD	39.01	13	32	43.66	6.54	0.78	0.004092
P08311	Cathepsin G	CTSG	35.69	7	13	28.81	11.19	1.62	0.029695
Q00610	Clathrin heavy chain 1	CLTC	40.12	57	128	191.49	5.69	0.81	0.000287
P09496	Clathrin light chain A	CLTA	14.92	5	7	27.06	4.51	0.87	0.050676
P16278	Beta-galactosidase	GLB1	14.33	8	12	76.02	6.57	1.24	0.003044
A0A024R8Q1	Glucosidase, alpha acid (Pompe disease, glycogen storage disease type II), isoform CRA_a	GAA	11.34	2	8	105.27	5.99	1.41	0.003841
P08236	Beta-glucuronidase	GUSB	13.52	9	16	74.68	7.02	1.39	0.036837
E5RH11	Heparan-alpha-glucosaminide N-acetyltransferase	HGSNAT	8.25	1	1	10.65	8.94	0.42	0.004235
Q9BVJ8	HEXA protein (Fragment)	HEXA	11.98	5	9	47.06	5.00	1.42	0.009748
P07686	Beta-hexosaminidase subunit beta	HEXB	20.68	10	19	63.07	6.76	1.37	0.008104
P11717	Cation-independent mannose-6-phosphate receptor	IGF2R	10.36	18	24	274.20	5.94	1.24	0.014874
M0QXC5	Napsin-A (Fragment)	NAPSA	2.61	1	1	29.09	6.39	1.84	0.000808
P07602	Prosaposin	PSAP	34.35	17	58	58.07	5.17	1.40	0.013245
Q14108	Lysosome membrane protein 2	SCARB2	23.43	10	30	54.26	5.14	1.35	0.012884
B3KY44	Solute carrier family 11 (Proton-coupled divalent metal ion transporters), member 2, isoform CRA_c	SLC11A2	2.68	1	1	44.86	7.65	2.67	0.048925
E9LUE7	Sphingomyelin phosphodiesterase 1 isoform 5	SMPD1	5.28	3	3	67.21	7.20	1.24	0.023889
Q8NBK3	Sulfatase-modifying factor 1	SUMF1	12.3	4	5	40.53	6.65	1.94	0.002598

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N); Ratio of tumors to normal controls.

Figure 6

Fatty acid degradation pathway altered in an ovarian cancer. Green rectangle with red mark means the identified proteins. Green rectangle without red mark means species-specific enzymes. White rectangle means reference pathway. The solid line means molecular interaction. The dot line means indirect effect. The circle means mostly chemical complex. The pathway node in the upper panel corresponds to the red marked node in the lower diagram.

ID number is the Swiss-Prot accession number. Ratio (T/N)=Ratio of tumors to controls. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

Discussion

EOC is the leading cause of death from gynecologic cancer and the concealed characteristics caused difficulty to be diagnosed in the early stage (Chan *et al.* 2006). The symptoms index plus CA125 screening may be the best way to identify women who may have an EOC (Karabudak *et al.* 2013). However, the 5-year overall survival rate for patients diagnosed with stage III–IV EOC remains still poor (about 30%) (Miller *et al.* 2016). Joint detection of different tumor biomarkers is necessary to

increase the specificity of the immunoassay. Proteomics provides a feasible approach for large-scale screening of EOC-related proteins to enhance the understanding of EOC pathogenesis. There are few reports on proteomics analysis of EOCs (Hiramatsu *et al.* 2016). Until now, it is the first time to use iTRAQ-based quantitative proteomics to identify EOC-related proteins in mitochondria. Mitochondria play a central role in the regulation of cellular signals, metabolism and apoptosis in cancer cells. In this study, one aimed at identifying the potential mitochondrial biomarkers for the prediction, prevention,

Table 6 Fatty acid degradation pathway involved the identified proteins in ovarian cancer biological system.

Pathway code	Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
1.3.99	D4QEZ8	Short-chain acyl-CoA dehydrogenase	ACADS	27.67	8	19	44.33	7.72	1.33	6.42809E-06
1.3.8.9	P49748	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADVL	49.62	12	154	70.35	8.75	1.20	0.0057736
2.3.1.9	P24752	Acetyl-CoA acetyltransferase, mitochondrial	ACAT1	42.86	19	81	45.17	8.85	0.90	0.0055291
6.2.1.3	O60488	Long-chain-fatty-acid--CoA ligase 4	ACSL4	18.71	7	14	79.14	8.38	1.10	0.0571799
6.2.1.3	Q9ULC5	Long-chain-fatty-acid--CoA ligase 5	ACSL5	7.47	3	5	75.94	6.92	1.20	0.0335853
1.1.1.1	P11766	Alcohol dehydrogenase class-3	ADH5	30.75	11	28	39.70	7.49	0.52	0.000616293
1.2.1.3	P30837	Aldehyde dehydrogenase X, mitochondrial	ALDH1B1	43.52	18	76	57.17	6.80	1.11	0.0450192
1.2.1.3	P05091	Aldehyde dehydrogenase, mitochondrial	ALDH2	55.9	20	90	56.35	7.05	0.76	0.00533149
1.2.1.3	P49189	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	37.04	18	48	53.77	5.87	0.99	0.75121
CPT2	P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2	42.4	5	77	73.73	8.18	2.05	0.0199416
4.2.1.17	P30084	Enoyl-CoA hydratase, mitochondrial	ECHS1	75.86	19	156	31.37	8.07	1.52	0.00356432
5.3.3.8	P42126	Enoyl-CoA delta isomerase 1, mitochondrial	ECI1	34.44	8	50	32.80	8.54	1.64	0.00199072
4.2.1.17	Q08426	Peroxisomal bifunctional enzyme	EHHADH	33.47	22	34	79.44	9.14	1.62	0.00225176
1.3.8.6	Q92947	Glutaryl-CoA dehydrogenase, mitochondrial	GCDH	23.29	8	16	48.10	8.06	1.21	0.0654308
1.1.1.35	Q16836	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	HADH	64.01	16	69	34.27	8.85	1.34	0.0210962
1.1.1.211	P40939	Trifunctional enzyme subunit alpha, mitochondrial	HADHA	69.07	42	379	82.94	9.04	1.18	0.0130629
2.3.1.16	P55084	Trifunctional enzyme subunit beta, mitochondrial	HADHB	64.77	5	251	51.26	9.40	1.07	0.0654946

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N), Ratio of tumors to normal controls.

Figure 7

Valine, leucine and isoleucine degradation pathway altered in an ovarian cancer. Green rectangle with red mark means the identified proteins. Green rectangle without red mark means species-specific enzymes. White rectangle means reference pathway. The solid line means molecular interaction. The dot line means indirect effect. The circle means mostly chemical complex. The pathway node in the right panel corresponds to the red marked node in the left diagram. ID number is the Swiss-Prot accession number. Ratio (T/N)=Ratio of tumors to controls. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

diagnosis and treatment of EOCs. Mitochondrial purification was progressed by Nycodenz density gradient approach, followed by an 6-plex iTRAQ proteomics to identify mtEPs in EOCs relative to controls. Among 5115 iTRAQ-identified proteins, 262 proteins were significantly related to overall survival in EOC patients. The iTRAQ and TCGA data were integrated, and GO analysis, protein-protein interaction and gene coexpression were analyzed. Moreover, 63 proteins were identified as potential markers for the development of an EOC, such that ERBB2, PTBP1 and H2AFX as reported were significantly related to overall survival. To some degree, these results indicate that our findings were consistent with previous studies and also make new discoveries. Moreover, we searched

CoolGen database with key words of 'marker or biomarker' and 'cancer' to understand the situation of our findings in the research of other cancers, not only EOCs. One found that 17 biomarkers had been reported in other cancers, including ANXA2, TACSTD2, TNFSF10, HNRNP2B1, RBP4, PPP1R13L, UGT8, VTN, BCAT1, PTPN2, PDIA6, MFAP4, GSTZ1, NUCB2, P4HB and PTBP1, which indicated our new found biomarkers were reliable. Numerous reported EOC protein biomarkers such as MUC16, MSLN, ERBB2, CHI3L1, MUC1, CD44, VTCN1 and CRP (Simon *et al.* 2007, Chiang *et al.* 2015, Stewart *et al.* 2015, Jiang *et al.* 2017, Bartakova *et al.* 2018, Fortner *et al.* 2018) were also identified in this iTRAQ proteomics study. It demonstrated that iTRAQ proteomic strategy is

Table 7 Valine, leucine and isoleucine degradation pathways involved the identified proteins in ovarian cancer biological system.

Pathway code	Accession number	Protein name	Gene name	Coverage (%)	Unique peptides	PSMs	MW (kDa)	Calc. pI	Ratio (T/N)	t-test P value
6.2.1.16	Q86V21	Acetoacetyl-CoA synthetase	AACS	2.68	2	2	75.10	6.24	1.35	0.00053
2.6.1.22	P80404	4-aminobutyrate aminotransferase, mitochondrial	ABAT	50.8	18	24	56.40	7.96	1.05	0.032216
2.3.1.16	P09110	3-ketoacyl-CoA thiolase, peroxisomal	ACAA1	46.23	4	50	44.26	8.44	2.70	0.010033
2.3.1.16	H7C131	3-ketoacyl-CoA thiolase, peroxisomal (Fragment)	ACAA1	56.21	1	38	30.31	8.63	0.92	0.363631
1.3.99	Q9UKU7	Isobutyryl-CoA dehydrogenase, mitochondrial	ACAD8	43.13	14	23	45.04	7.85	1.42	0.011082
1.399.12	P45954	Short/branched-chain-specific acyl-CoA dehydrogenase, mitochondrial	ACADS8	25.93	8	15	47.46	6.99	1.29	0.010581
2.3.1.9	P24752	Acetyl-CoA acetyltransferase, mitochondrial	ACAT1	42.86	19	81	45.17	8.85	0.90	0.005529
6.2.1	Q4G176	Acyl-CoA synthetase family member 3, mitochondrial	ACSF3	23.26	11	15	64.09	8.37	1.27	0.039637
1.2.1.3	P30837	Aldehyde dehydrogenase X, mitochondrial	ALDH1B1	43.52	18	76	57.17	6.80	1.11	0.045019
1.2.1.3	P05091	Aldehyde dehydrogenase, mitochondrial	ALDH2	55.9	20	90	56.35	7.05	0.76	0.005331
1.2.1.3	Q02252	Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial	ALDH6A1	56.26	25	96	57.80	8.50	0.88	4.4E-05
1.2.1.3	P49189	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	37.04	18	48	53.77	5.87	0.99	0.75121
2.6.1.42	B3KS13	Branched-chain amino acid aminotransferase	BCAT2	37.78	11	20	39.89	8.24	1.03	0.224773
2.3.1.168	P11182	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	DBT	38.38	18	67	53.45	8.51	1.18	0.001486
4.2.1.17	P30084	Enoyl-CoA hydratase, mitochondrial	ECHS1	75.86	19	156	31.37	8.07	1.52	0.003564
4.2.1.17	Q08426	Peroxisomal bifunctional enzyme	EHHADH	33.47	22	34	79.44	9.14	1.62	0.002252
4.2.1.17	Q16836	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	HADH	64.01	16	69	34.27	8.85	1.34	0.021096
1.1.1.35	P40939	Trifunctional enzyme subunit alpha, mitochondrial	HADHA	69.07	42	379	82.95	9.04	1.18	0.013063
1.1.1.31	P31937	3-hydroxyisobutyrate dehydrogenase, mitochondrial	HIBADH	31.85	9	31	35.31	8.13	1.12	0.007616
3.1.2.4	Q6NVY1	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	HIBCH	34.46	14	29	43.45	8.19	1.58	0.002504
2.3.3.10	P54868	Hydroxymethylglutaryl-CoA synthase, mitochondrial	HMGCS2	12.8	7	11	56.60	8.16	2.17	0.002099
1.1.1.178	Q99714	3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	73.95	15	66	26.91	7.78	1.35	0.00185
1.4.3.2	Q96RQ9	L-amino-acid oxidase	IL4I1	13.76	6	13	62.84	8.68	1.82	0.008397
1.3.8.4	P26440	Isovaleryl-CoA dehydrogenase, mitochondrial	IVD	37.35	15	51	46.29	8.19	1.28	0.001903
6.4.1.4	Q96RQ3	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	MCCC1	44.55	21	66	80.42	7.78	1.25	0.073636
6.4.1.4	Q9HCC0	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	MCCC2	45.29	21	64	61.29	7.68	1.45	0.000881
5.1.99.1	Q96PE7	Methylmalonyl-CoA epimerase, mitochondrial	MCEE	38.07	5	8	18.74	9.08	1.22	0.048685
5.4.99.2	P22033	OS=Homo sapiens GN= PE=1 SV=1 - [MCEE_HUMAN]	MUT	33.73	19	43	83.08	6.93	1.13	0.01005
6.4.1.3	P05165	Methylmalonyl-CoA mutase, mitochondrial	PCCA	34.62	20	48	80.01	7.52	1.03	0.226927
2.8.3.5	P55809	Propionyl-CoA carboxylase alpha chain, mitochondrial	OXCT1	32.12	14	34	56.12	7.46	0.98	0.379129

MW, Molecular weight; PSMs, Peptide sequence matches; Ratio (T/N), Ratio of tumors to normal controls.

Figure 8

qRT-PCR and Western blot analyses to validate results of iTRAQ quantitative mitochondrial proteomics. (A) qTR-PCR analysis to quantify the expression levels of GLDC, PCK2, IDH2, CPT2 and HMGCS2 between EOC cells TOV21G and control cells IOSE80. (B) Protein expression levels of GLDC, PCK2, IDH2, CPT2 and HMGCS2 in EOC cells TOV21G and control cells IOSE80. (C) Mitochondrial proteins of EOC and control tissues were analyzed by Western blot using antibodies against GLDC, PCK2, IDH2, CPT2 and HMGCS2. The levels of GLDC, PCK2, IDH2, CPT2 and HMGCS2 were normalized relative to β -actin. Data represent mean $\pm$ s.d. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0243>.

a reliable tool for identity of EOC biomarker. Compared to previous studies, 992 proteins of 5115 identified proteins have been reported to associate ovary checked by CoolGeN database. Among 992 proteins, there were 33 proteins upregulated more than two-fold; and 12 of those 33 identified proteins have not been reported in previous EOC biomarker studies, including IDH2, RAB43, SYNGR2, RDH10, CYBA, HMGCS2, PCK2, RIDA, KIF23, UGT8, SC11A2 and AIG1, which indicates that those proteins need further studies to testify them as novel EOC biomarkers. In the documented study, most of them endeavor on the effect of single factor or gene mutation on the development of cancer. However, some studies found that only one single molecular event does not lead to the occurrence of cancer. A typical cancer occurrence model needs the mutation of two to eight driver genes (van der Wal *et al.* 2018). In this study, a recognized biomarker pattern should be put forward, and it means that the use of a set of biomarkers builds a data model to improve the accuracy and specificity of prediction, diagnosis, prognosis and therapy of an EOC. Despite its common use in cancer treatment, biomarkers have not really entered the era of precision medicine to predict, prevent and personalize the treatment of an EOC, and there have been no approaches to adjust personalized medicine based on biological differences between or within tumors. A genome-based model for adjusting radiotherapy dose (GARD), which was reported in 2017, has attracted much attention (Scott *et al.* 2017). One is going to use the gene expression-based drug-reaction index and the linear quadratic model to derive the drug dose, which one speculates would relate to clinical

outcome. In the future, it is necessary for one to collect prospective clinical data to validate the model.

Mitophagy involves the engulfment of any material in a double-membrane enclosed autophagosome, which subsequently fuses with lysosomes. In autophagosomes, mitochondria, proteins or peroxisomes were usually observed. Autophagosomes fuse with lysosomes and emit high-energy substances, including fatty acids and amino acids (Zimmermann & Reichert 2017). Pathway network analysis mapped the identified proteins to 70 signaling pathways. Interestingly, the results demonstrated that cancer cells exhibited an increased dependence on mitophagy, such as peroxisome, phagosome, valine, leucine and isoleucine degradation, fatty acid degradation pathway, which might play an important role in EOC invasion and metastasis. In this progress, mitophagy is dependent on the general autophagy machinery and relies on a growing cadre of 'mitophagy adaptors' and regulatory molecules, such as *FUNDC1*, *BNIP3L(NIX)*, *PGAM5*, *CK*, *OPA1*, *prohibitin 2*, *OPTN*, *TBK1*, *p62* and *Bcl2-L13* (Drake *et al.* 2017). This study identified those proteins too, even though few of them appeared expression changing. The results were consistent with previous studies, because those proteins activated downstream mitophagy by post-translational modifications (Wu *et al.* 2016). Two mitophagy mechanisms in mammalian cells had been reported. Firstly, receptor-mediated mitophagy is activated by phosphorylation, increasing its binding proteins for Atg8-like and recruiting them to mitochondria. Secondly, the highly ubiquitylated mitochondrial outer membrane proteins recruit adapter proteins, which in turn recruits Atg8-like proteins. For NIX (BNIP3L), BNIP3 and

BCL2L13, only the activated phosphorylation mechanism and the modified residues are known but the kinases and phosphatases have not been identified; yet, for FUNDC1, both the activated and deactivated phosphorylation mechanisms, the modified residues and participated enzymes are known (Zimmermann & Reichert 2017). In this regard, one could not ignore those identified proteins without significant change. In the future, more and more proteins experienced post-translational modifications might also be biomarker, even become more effective (Peng *et al.* 2015).

In conclusion, this study used iTRAQ-SCX-LC-MS/MS to identify and quantify 5115 mtEPs in EOCs relative to controls. The first quantitative reference map of a human EOC tissue mitochondrial proteome has been achieved here. These findings enriched human mitochondrial database and a series of identified biomarkers could be useful for clinical practice. These results demonstrated that iTRAQ-SCX-LC-MS/MS is a reliable method to identify and quantify mitochondrial protein profiles and could assist in exploiting potential biomarkers and the novel mechanisms of EOC carcinogenesis.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0243>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N L analyzed data, prepared figures and tables, designed and wrote the manuscript. H L collected samples and prepared mitochondrial samples. L C collected tumor tissue samples and performed clinical diagnosis. X Z conceived the concept, designed experiments and manuscript, instructed experiments and data analysis, supervised results, coordinated, critically revised/wrote manuscript and was responsible for its financial supports and the corresponding works. All authors approved the final manuscript.

References

- Bartakova A, Michalova K, Presl J, Vlasak P, Kostun J & Bouda J 2018 CD44 as a cancer stem cell marker and its prognostic value in patients with ovarian carcinoma. *Journal of Obstetrics and Gynaecology* **38** 110–114. (<https://doi.org/10.1080/01443615.2017.1336753>)
- Borgfeldt C, Bendahl PO, Ferno M & Casslen B 2003 High preoperative plasma concentration of tissue plasminogen activator (tPA) is an independent marker for shorter overall survival in patients with ovarian cancer. *Gynecologic Oncology* **91** 112–117. ([https://doi.org/10.1016/S0090-8258\(03\)00493-1](https://doi.org/10.1016/S0090-8258(03)00493-1))
- Chan JK, Cheung MK, Husain A, Teng NN, West D, Whittemore AS, Berek JS & Osann K 2006 Patterns and progress in ovarian cancer over 14 years. *Obstetrics and Gynecology* **108** 521–528. (<https://doi.org/10.1097/01.AOG.0000231680.58221.a7>)
- Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ & He J 2016 Cancer statistics in China, 2015. *CA: A Cancer Journal for Clinicians* **66** 115–132. (<https://doi.org/10.3322/caac.21338>)
- Cheng T & Zhan X 2017 Pattern recognition for predictive, preventive, and personalized medicine in cancer. *EPMA Journal* **8** 51–60. (<https://doi.org/10.1007/s13167-017-0083-9>)
- Chiang YC, Lin HW, Chang CF, Chang MC, Fu CF, Chen TC, Hsieh SF, Chen CA & Cheng WF 2015 Overexpression of CHI3L1 is associated with chemoresistance and poor outcome of epithelial ovarian carcinoma. *Oncotarget* **6** 39740–39755. (<https://doi.org/10.18632/oncotarget.5469>)
- Cleland TP 2018 Solid digestion of demineralized bone as a method to access potentially insoluble proteins and post-translational modifications. *Journal of Proteome Research* **17** 536–542. (<https://doi.org/10.1021/acs.jproteome.7b00670>)
- Cymbaluk-Ploska A, Chudecka-Glaz A, Pius-Sadowska E, Machalinski B, Menkiszak J & Sompolska-Rzechula A 2018 Suitability assessment of baseline concentration of MMP3, TIMP3, HE4 and CA125 in the serum of patients with ovarian cancer. *Journal of Ovarian Research* **11** 1. (<https://doi.org/10.1186/s13048-017-0373-9>)
- Drake LE, Springer MZ, Poole LP, Kim CJ & Macleod KF 2017 Expanding perspectives on the significance of mitophagy in cancer. *Seminars in Cancer Biology* **47** 110–124. (<https://doi.org/10.1016/j.semcancer.2017.04.008>)
- Fortner RT, Schock H, Le Cornet C, Husing A, Vitonis AF, Johnson TS, Fichorova RN, Fashemi T, Yamamoto HS, Tjønneland A, *et al.* 2018 Ovarian cancer early detection by circulating CA125 in the context of anti-CA125 autoantibody levels: results from the EPIC cohort. *International Journal of Cancer* **142** 1355–1360. (<https://doi.org/10.1002/ijc.31164>)
- Gadducci A, Cosio S, Zola P, Landoni F, Maggino T & Sartori E 2007 Surveillance procedures for patients treated for epithelial ovarian cancer: a review of the literature. *International Journal of Gynecological Cancer* **17** 21–31. (<https://doi.org/10.1111/j.1525-1438.2007.00826.x>)
- Gonzalez-Angulo AM, Iwamoto T, Liu S, Chen H, Do KA, Hortobagyi GN, Mills GB, Meric-Bernstam F, Symmms WF & Pusztai L 2012 Gene expression, molecular class changes, and pathway analysis after neoadjuvant systemic therapy for breast cancer. *Clinical Cancer Research* **18** 1109–1119. (<https://doi.org/10.1158/1078-0432.CCR-11-2762>)
- Hanaoka T, Hasegawa K, Kato T, Sato S, Kurosaki A, Miyara A, Nagao S, Seki H, Yasuda M & Fujiwara K 2017 Correlation between tumor mesothelin expression and serum mesothelin in patients with epithelial ovarian carcinoma: a potential noninvasive biomarker for mesothelin-targeted therapy. *Molecular Diagnosis and Therapy* **21** 187–198. (<https://doi.org/10.1007/s40291-017-0255-2>)
- Hiramatsu K, Yoshino K, Serada S, Yoshihara K, Hori Y, Fujimoto M, Matsuzaki S, Egawa-Takata T, Kobayashi E, Ueda Y, *et al.* 2016 Similar protein expression profiles of ovarian and endometrial high-grade

- serous carcinomas. *British Journal of Cancer* **114** 554–561. (<https://doi.org/10.1038/bjc.2016.27>)
- Hu R, Wang X & Zhan X 2013 Multi-parameter systematic strategies for predictive, preventive and personalised medicine in cancer. *EPMA Journal* **4** 2. (<https://doi.org/10.1186/1878-5085-4-2>)
- Huhta H, Helminen O, Palomaki S, Kaupila JH, Saarnio J, Lehenkari PP & Karttunen TJ 2017 Intratumoral lactate metabolism in Barrett's esophagus and adenocarcinoma. *Oncotarget* **8** 22894–22902. (<https://doi.org/10.18632/oncotarget.15284>)
- Jiang XS, Zhou H, Zhang L, Sheng QH, Li SJ, Li L, Hao P, Li YX, Xia QC, Wu JR, *et al.* 2004 A high-throughput approach for subcellular proteome: identification of rat liver proteins using subcellular fractionation coupled with two-dimensional liquid chromatography tandem mass spectrometry and bioinformatic analysis. *Molecular and Cellular Proteomics* **3** 441–455. (<https://doi.org/10.1074/mcp.M300117-MCP200>)
- Jiang Y, Yan F, Liang L, Wan Y, Liu J & Cheng W 2017 Meta-analysis demonstrates no association between p16 (ink4a) promoter methylation and epithelial ovarian cancer. *Archives of Gynecology and Obstetrics* **295** 697–704. (<https://doi.org/10.1007/s00404-016-4264-x>)
- Karabudak AA, Hafner J, Shetty V, Chen S, Secord AA, Morse MA & Philip R 2013 Autoantibody biomarkers identified by proteomics methods distinguish ovarian cancer from non-ovarian cancer with various CA-125 levels. *Journal of Cancer Research and Clinical Oncology* **139** 1757–1770. (<https://doi.org/10.1007/s00432-013-1501-6>)
- Khan A, Eikani CK, Khan H, Iavarone AT & Pesavento JJ 2018 Characterization of *Chlamydomonas reinhardtii* core histones by top-down mass spectrometry reveals unique algae-specific variants and post-translational modifications. *Journal of Proteome Research* **17** 23–32. (<https://doi.org/10.1021/acs.jproteome.7b00780>)
- Kim TW, Kim B, Kim JH, Kang S, Park SB, Jeong G, Kang HS & Kim SJ 2013 Nuclear-encoded mitochondrial MTO1 and MRPL41 are regulated in an opposite epigenetic mode based on estrogen receptor status in breast cancer. *BMC Cancer* **13** 502. (<https://doi.org/10.1186/1471-2407-13-502>)
- Kim HK, Noh YH, Nilius B, Ko KS, Rhee BD, Kim N & Han J 2017 Current and upcoming mitochondrial targets for cancer therapy. *Seminars in Cancer Biology* **47** 154–167. (<https://doi.org/10.1016/j.semcancer.2017.06.006>)
- Ksiezakowska-Lakoma K, Kulczycka-Wojdala D, Kulig A, Baum M & Wilczynski JR 2017 The presence of A5935G, G5949A, G6081A, G6267A, T9540C mutations in MT-CO1 and MT-CO3 genes and other variants of MT-CO1 and MT-CO3 gene fragments in the study population diagnosed with endometrial cancer. *Ginekologia Polska* **88** 343–348. (<https://doi.org/10.5603/GP.a2017.0065>)
- Lemasters JJ 2005 Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Research* **8** 3–5. (<https://doi.org/10.1089/rej.2005.8.3>)
- Michalak S, Rybacka-Mossakowska J, Gazdulska J, Golda-Gocka I & Ramlau R 2016 The effect on cognition of mitochondrial respiratory system proteins in peripheral blood mononuclear cells in the course of lung cancer. *Advances in Experimental Medicine and Biology* **911** 45–52. (https://doi.org/10.1007/5584_2016_221)
- Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R & Jemal A 2016 Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians* **66** 271–289. (<https://doi.org/10.3322/caac.21349>)
- Pasing Y, Schniers A & Hansen T 2017 Straightforward protocol for gel-free proteomic analysis of adipose tissue. *Methods in Molecular Biology* [epub]. (https://doi.org/10.1007/7651_2017_82)
- Peng F, Li J, Guo T, Yang H, Li M, Sang S, Li X, Desiderio DM & Zhan X 2015 Nitroproteins in human astrocytomas discovered by gel electrophoresis and tandem mass spectrometry. *Journal of the American Society for Mass Spectrometry* **26** 2062–2076. (<https://doi.org/10.1007/s13361-015-1270-3>)
- Pu M, Wang J, Huang Q, Zhao G, Xia C, Shang R, Zhang Z, Bian Z, Yang X & Tao K 2017 High MRPS23 expression contributes to hepatocellular carcinoma proliferation and indicates poor survival outcomes. *Tumor Biology* **39** 1393380537. (<https://doi.org/10.1177/1010428317709127>)
- Qi Y, Xu F, Chen L, Li Y, Xu Z, Zhang Y, Wei W, Su N, Zhang T, Fan F, *et al.* 2016 Quantitative proteomics reveals FLNC as a potential progression marker for the development of hepatocellular carcinoma. *Oncotarget* **7** 68242–68252. (<https://doi.org/10.18632/oncotarget.11921>)
- Ray S, Reddy PJ, Jain R, Gollapalli K, Moiyadi A & Srivastava S 2011 Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. *Proteomics* **11** 2139–2161. (<https://doi.org/10.1002/pmic.201000460>)
- Riley NM & Coon JJ 2018 The role of electron transfer dissociation in modern proteomics. *Analytical Chemistry* **90** 40–64. (<https://doi.org/10.1021/acs.analchem.7b04810>)
- Sakhujia S, Yun H, Pisu M & Akinyemiju T 2017 Availability of healthcare resources and epithelial ovarian cancer stage of diagnosis and mortality among Blacks and Whites. *Journal of Ovarian Research* **10** 57. (<https://doi.org/10.1186/s13048-017-0352-1>)
- Schonenberger MJ & Kovacs WJ 2017 Isolation of peroxisomes from mouse brain using a continuous nycodenz gradient: a comparison to the isolation of liver and kidney peroxisomes. *Methods in Molecular Biology* **1595** 13–26. (https://doi.org/10.1007/978-1-4939-6937-1_2)
- Simon I, Katsaros D, Rigault DLLI, Massobrio M, Scorilas A, Kim NW, Sarno MJ, Wolfert RL & Diamandis EP 2007 B7-H4 is over-expressed in early-stage ovarian cancer and is independent of CA125 expression. *Gynecologic Oncology* **106** 334–341. (<https://doi.org/10.1016/j.ygyno.2007.03.035>)
- Sloutsky R & Naegle KM 2018 Proteome-level analysis indicates global mechanisms for post-translational regulation of RRM domains. *Journal of Molecular Biology* **430** 41–44. (<https://doi.org/10.1016/j.jmb.2017.11.001>)
- Sotgia F, Whitaker-Menezes D, Martinez-Outschoorn UE, Salem AF, Tsigiris A, Lamb R, Sneddon S, Hulit J, Howell A & Lisanti MP 2012 Mitochondria “fuel” breast cancer metabolism: fifteen markers of mitochondrial biogenesis label epithelial cancer cells, but are excluded from adjacent stromal cells. *Cell Cycle* **11** 4390–4401. (<https://doi.org/10.4161/cc.22777>)
- Stewart ML, Tamayo P, Wilson AJ, Wang S, Chang YM, Kim JW, Khabele D, Shamji AF & Schreiber SL 2015 KRAS genomic status predicts the sensitivity of ovarian cancer cells to decitabine. *Cancer Research* **75** 2897–2906. (<https://doi.org/10.1158/0008-5472.CAN-14-2860>)
- Scott JG, Berglund A, Schell MJ, Mihaylov I, Fulp WJ, Yue B, Welsh E, Caudell JJ, Ahmed K, Strom TS, *et al.* 2017 A genome-based model for adjusting radiotherapy dose (GARD): a retrospective, cohort-based study. *Lancet Oncology* **18** 202–211. ([https://doi.org/10.1016/S1470-2045\(16\)30648-9](https://doi.org/10.1016/S1470-2045(16)30648-9))
- van der Wal L, Bezstarosti K, Sap KA, Dekkers D, Rijkers E, Mientjes E, Elgersma Y & Demmers JAA 2018 Improvement of ubiquitylation site detection by Orbitrap mass spectrometry. *Journal of Proteomics* **172** 49–56. (<https://doi.org/10.1016/j.jprot.2017.10.014>)
- Wanders RJ & Waterham HR 2006 Biochemistry of mammalian peroxisomes revisited. *Annual Review of Biochemistry* **75** 295–332. (<https://doi.org/10.1146/annurev.biochem.74.082803.133329>)
- Wang X, Guo T, Peng F, Long Y, Mu Y, Yang H, Ye N, Li X & Zhan X 2015 Proteomic and functional profiles of a follicle-stimulating hormone positive human nonfunctional pituitary adenoma. *Electrophoresis* **36** 1289–1304. (<https://doi.org/10.1002/elps.201500006>)
- Wu H, Wei H, Sehgal SA, Liu L & Chen Q 2016 Mitophagy receptors sense stress signals and couple mitochondrial dynamic machinery for mitochondrial quality control. *Free Radical Biology and Medicine*

- 100** 199–209. (<https://doi.org/10.1016/j.freeradbiomed.2016.03.030>)
- Zhan X, Long Y, Zhan X & Mu Y 2017a Consideration of statistical vs. biological significances for omics data-based pathway network analysis. *Med One* **2** e170002. (<https://doi.org/10.20900/mo.20170002>)
- Zhan X, Long Y & Lu M 2017b Exploration of variations in proteome and metabolome for predictive diagnostics and personalized treatment algorithms: innovative approach and examples for potential clinical application. *Journal of Proteomics* [epub]. (<https://doi.org/10.1016/j.jprot.2017.08.020>)
- Zhan X, Qian S & Huang Y 2017c The untapped potential of nitroproteomics for medicine. *Med One* **2** e170027. (<https://doi.org/10.20900/mo.20170027>)
- Zhan X, Yang H, Peng F, Li J, Mu Y, Long Y, Cheng T, Huang Y, Li Z, Lu M, *et al.* 2018 How many proteins can be identified in a 2-DE gel spot within an analysis of a complex human cancer tissue proteome? *Electrophoresis* **39** 965–980. (<https://doi.org/10.1002/elps.201700330>)
- Zhang LL, Shao SL & Wu Y 2010 Expressions of osteopontin and B7-H4 in epithelial ovarian neoplasm and their significance. *Chinese Journal of Cancer* **29** 25–29. ([https://doi.org/1000-467X\(2010\)01-0025-05](https://doi.org/1000-467X(2010)01-0025-05))
- Zhang H, Meltzer P & Davis S 2013 RCircos: an R package for Circos 2D track plots. *BMC Bioinformatics* **14** 244. (<https://doi.org/10.1186/1471-2105-14-244>)
- Zhang H, Liu T, Zhang Z, Payne SH, Zhang B, McDermott JE, Zhou JY, Petyuk VA, Chen L, Ray D, *et al.* 2016 Integrated proteogenomic characterization of human high-grade serous ovarian cancer. *Cell* **166** 755–765. (<https://doi.org/10.1016/j.cell.2016.05.069>)
- Zhou JY, Chen L, Zhang B, Tian Y, Liu T, Thomas SN, Chen L, Schnaubelt M, Boja E, Hiltke T, *et al.* 2017 Quality assessments of long-term quantitative proteomic analysis of breast cancer xenograft tissues. *Journal of Proteome Research* **16** 4523–4530. (<https://doi.org/10.1021/acs.jproteome.7b00362>)
- Zimmermann M & Reichert AS 2017 How to get rid of mitochondria: crosstalk and regulation of multiple mitophagy pathways. *Biological Chemistry* **399** 29–45. (<https://doi.org/10.1515/hsz-2017-0206>)

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