Tandem Mass Tag-Based Quantitative Proteome Analysis of Porcine Deltacoronavirus (PDCoV)-Infected LLC Porcine Kidney Cells

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ABSTRACT: Porcine deltacoronavirus (PDCoV) is a newly emerging porcine pathogenic enteric coronavirus that can cause diarrhea, vomiting, dehydration, and a high mortality rate in piglets. At present, the understanding of PDCoV pathogenesis is very limited, which seriously hinders effective prevention and control. In this study, liquid chromatography tandem-mass spectrometry (LC-MS/MS) combined with tandem mass tag (TMT) labeling was performed to compare the differential expression of proteins in PDCoV-infected and mock-infected LLC-PK cells at 18 h post-infection (hpi). In addition, the parallel reaction monitoring (PRM) technique was used to verify the quantitative proteome data. A total of 4624 differentially expressed proteins (DEPs) were quantitated, of which 128 were significantly upregulated, and 147 were significantly downregulated. Bioinformatics analysis revealed that these DEPs were involved mainly in the defense response, apoptosis, and the immune system, and several DEPs may be related to interferon-stimulated genes and the immune system. Based on DEP bioinformatics analysis, we propose that PDCoV infection may utilize the apoptosis pathway of host cells to achieve maximum viral replication. Meanwhile, the host may be able to stimulate the transcription of interferon-stimulated genes (ISGs) through the JAK/STAT signaling pathway to resist the virus. Overall, in this study, we presented the first application of proteomics analysis to determine the protein profile of PDCoV-infected cells, which provides valuable information with respect to better understanding the host response to PDCoV infection and the specific pathogenesis of PDCoV infection.

1. INTRODUCTION
Porcine deltacoronavirus (PDCoV) is a newly emerging virus belonging to the Deltacoronavirus genus of the Coronavirus family, which can cause watery diarrhea, vomiting, dehydration, and high mortality in suckling piglets. The typical symptoms of PDCoV are so similar to those of porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) such that they are clinically indistinguishable, but PDCoV-induced diarrhea is more mild than that induced by PEDV and TGEV. Meanwhile, PDCoV often coinfected with PEDV, porcine rotavirus, or TGEV, which causes more serious disease than single-virus infection. PDCoV is an enveloped, single-stranded, positive-sense RNA virus. The viral genome is approximately 25.4 kb in length, encoding four nonstructural proteins (replicase 1a/b, NS6, and NS7) and four major structural proteins (spike (S), envelope (E), membrane (M), and nucleocapsid (N)). So far, although PDCoV can be accurately and rapidly detected in the laboratory, knowledge of its pathogenesis and an effective vaccine are still insufficient, and PDCoV has caused substantial economic losses to the swine industry worldwide.

In 2012, PDCoV was first reported in swine feces during molecular surveillance of coronavirus in Hong Kong, while the outbreak and first isolation of PDCoV in the United States occurred in 2014. Since then, PDCoV has been reported to have been found in 18 states in the United States, with the mortality rate reaching 40% in suckling piglets. Subsequently, ongoing outbreaks of PDCoV have been documented in China, Canada, South Korea, Thailand, Japan, Vietnam, and Mexico. Despite years of research on PDCoV, studies have focused on virus isolation, diagnosis, and viral protein functions. LLC porcine kidney (LLC-PK) cells are widely used in the isolation and cultivation of PDCoV strains due to their high susceptibility to PDCoV infection. Therefore, LLC-PK cells are suitable because the PDCoV

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infection model provides a new way to provide insight into PDCoV pathogenesis mechanisms.

In recent years, proteomics has been used as an effective tool to detect protein-protein interactions, protein expression profiles, protein kinetics, and complex regulatory mechanisms. Additionally, with the development of bioinformatics, the effectiveness and speed of large-scale data processing in proteomics have been significantly improved, which makes proteomics technology increasingly widely used to investigate virus-host interactions, such as those for influenza virus, foot and mouth disease virus, transmissible gastroenteritis virus, severe acute respiratory syndrome coronavirus, and porcine epidemic diarrhea virus. Tandem mass tags (TMT) are accurate and reliable for quantitative proteomics, representing a technique that has been developed into one of the most powerful proteomics approaches in recent years because of its efficiency, high sensitivity, wide range of use, and high throughput. Parallel reaction monitoring (PRM) is a technique for verifying proteins, which can provide reliable qualitative and quantitative proteomics results. The strategy of combining TMT-based quantitative proteomics with PRM has been widely used in proteome analysis and verification of various pathogenic microorganisms.

To date, little is known about the proteome function in the interaction between PDCoV and the host. In the present study, TMT labeling coupled with liquid chromatography tandem-mass spectrometry (LC-MS/MS) was applied to compare the differences in the protein expression levels of LLC-PK cells before and after infection with PDCoV. Protein bioinformatics analysis predicted that these differential proteins are involved in a variety of signaling pathways. Moreover, the pivotal proteins screened from the differentially expressed protein profiles were verified by PRM to show the accuracy of the proteome data, and a network of proteins related to PDCoV infection was constructed. To our knowledge, this study is the first to analyze the proteome changes in host cells after PDCoV infection. Our findings provide valuable information for understanding the host response to PDCoV infection and the pathogenesis of PDCoV, which may contribute to the development of effective treatments and vaccines for PDCoV.

2. RESULTS

2.1. PDCoV Propagation in LLC-PK Cells. To determine the kinetics of PDCoV proliferation in LLC-PK cells, cytopathic effects (CPEs), viral protein expression, and virus titer monitoring were performed at 6, 12, 18, 24, and 30 hpi. As shown in Figure 1A, distinct CPEs could be observed at 18 hpi, but no significant host cell lysis, shedding, or membrane fusion was observed at that time. At 24 hpi, the cells were obviously enlarged and rounded and underwent shedding, and almost all of them were detached at 30 hpi, while no CPEs were found in the mock-infected cells at 30 hpi. In addition, IFA results indicated that PDCoV began to replicate at 6 hpi, which increased gradually by 24 hpi (Figure 1B). Moreover, a one-step growth curve confirmed that PDCoV production increased steadily during infection and reached its peak at 30 hpi (Figure 1C). According to previous proteomics studies of porcine coronaviruses (such as PEDV), when no significant host cell lysis, shedding, or membrane fusion is observed but a high virus yield is achieved, that point is regarded as the optimal time for proteomics analysis. Therefore, to ensure the effectiveness of the proteome data, considering the cellular integrity and high-infection-rate cells, the time point of 18 hpi was selected for further proteome analysis.

Figure 1. PDCoV infection in LLC-PK cells. (A) Cytopathic effects (CPEs) of LLC-PK cells at 6, 12, 18, 24, and 30 h after PDCoV infection and mock-infected cells at 30 h as a negative control. (B) Infected LLC-PK cells were stained for immunofluorescence at 6, 12, 18, and 24 h after infection, and mock-infected cells at 36 h were used as a control. (C) One-step growth curve of the PDCoV strain CH/XJYN/2016 in LLC-PK cells.
2.2. Analysis of the Differentially Regulated Proteins.

As described in experimental section 2, PDCoV- and mock-infected LLC-PK cell proteins were extracted. In total, 5369 proteins were successfully detected, and 4624 proteins were quantitated. Of these proteins, 128 were significantly upregulated, and 147 were significantly downregulated according to our differentially expressed protein identification criteria.

**Figure 2.** Subcellular location of the differentially expressed proteins in LLC-PK cells infected with PDCoV. (A) Upregulated protein subcellular localization in PDCoV-infected cells. (B) Downregulated protein subcellular localization in PDCoV-infected cells.

**Figure 3.** Functional characterization of upregulated and downregulated proteins. (A) Biological process; (B) cellular component; (C) molecular function.

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The biological process, cellular component, molecular function, and subcellular location of these DEPs were analyzed using GO and the UniProt database. As shown in Figure 2A, the 128 upregulated proteins in infected cells were localized mainly to the cytoplasm, nucleus, extracellular environment, and mitochondria. The 147 downregulated proteins in infected cells were located mainly in the nucleus, plasma membrane, cytoplasm, and mitochondria (Figure 2B).

The identified differentially expressed proteins were submitted to the GO database to gain insight into the functional enrichment of DEPs, including their biological process, cellular component, and molecular function. For biological process annotation, the upregulated proteins in PDCoV-infected cells were involved mainly in the defense response, including the defense response to virus, defense response to other organisms, response to virus, and response to type I interferon, indicating that PDCoV infection stimulates the defense response of the host (Figure 3A). However, the downregulated proteins were related mainly to chromosome regulation, such as DNA conformation change, chromatin assembly or disassembly, DNA packaging, and protein–DNA complex assembly (Figure 3A). For the cellular component annotation, the upregulated proteins were localized mainly in the perinuclear region of the cytoplasm, the extracellular side of the membrane, the cytoplasmic side of the plasma membrane, and the extracellular region (Figure 3B). The downregulated proteins were localized mainly in the nucleusosome, the DNA packaging complex, the integral component of the membrane, and the intrinsic component of the membrane (Figure 3B). Meanwhile, the upregulated proteins, for the molecular function, were involved mainly in glutathione binding, oligopeptide binding, purine-nucleoside phosphorylase activity, and 2'-5'-oligoadenylate synthetase activity (Figure 3C), which might be related to antiviral effects. Furthermore, the downregulated proteins were related mainly to DNA binding, DNA topoisomerase activity, chromatin binding, and lysine-acetylated histone binding (Figure 3C), suggesting that the virus may inhibit the replication of cells.

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### Table 1. Analysis and Comparison of Quantitative Results of Nine Candidate Proteins TMT and PRM

| Protein Description                        | Gene Symbol | Signature Peptides          | PD/N ratio TMT | PD/N ratio PRM | PRM p value |
|-------------------------------------------|-------------|----------------------------|----------------|----------------|-------------|
| Growth factor receptor-bound protein 2    | GRB2        | ESESAPGDFSLSVK              | 1.27           | 1.23           | 2.5 x 10^-3 |
| Alpha-2-macroglobulin receptor-associated | LRPAP1      | SEGLDEGDGEKEYNILLETLSR      | 0.81           | 0.76           | 4.7 x 10^-2 |
| Protein precursor                         |             |                            |                |                |             |
| UDP-glucuronosyl transferase              | UGT1A6      | YTTAFSDR                    | 0.80           | 0.74           | 2.2 x 10^-2 |
| Poly[ADP-ribose] polymerase              | PARP1       | DSEEAEITRTTNGILSGQLR        | 0.76           | 0.60           | 1.7 x 10^-3 |
| Histone H4                                |             | ISGLYEETVRFLYIRDAVYTYTEHAK  | 0.56           | 0.31           | 5.7 x 10^-6 |
| DNA topoisomerase 2                       | TOP2B       | LLFAPVDDNLKSKQDFGNLFSFSYSQK | 0.80           | 0.48           | 1.0 x 10^-4 |
| Histone cluster 1 H1 family member e     | HIST1H1E    | ASGPPVSLEITKSGVSLAALK       | 0.50           | 0.32           | 1.8 x 10^-4 |
| Promyelocytic leukemia                    | PML         | QVVAHLAQEQEQLLEGVNASPEATSTPR| 1.38           | 1.26           | 5.7 x 10^-2 |
| Interferon-induced GTP-binding protein Mx2| MX2         | GPENNLYSPFEK                | 2.18           | 19.29          | 9.7 x 10^-5 |
analysis were involved mainly in apoptosis and related pathways (Figure 4B), indicating that PDCoV may achieve maximum replication by modulating apoptosis. Finally, the DEPs were further searched using the STRING database version 11.0 to display the visual network functional relationship between differentially expressed proteins. The potential network interaction diagram of the DEPs is shown in Figure 4C. Among the proteins present in these network analyses, the upregulated proteins are shown in red shadow, and the downregulated proteins are shown in green. These data reveal that many proteins have different functions and can be connected by modulating the protein interaction. It is worth noting that the network interaction diagram highlights the process of immune response and apoptosis involved in PDCoV CH/XJYN/2016 infection.

2.4. Validation of Candidate Differentially Expressed Proteins by PRM. Based on their annotations and different ratios to select proteins, the differential expression changes of nine candidate proteins (GRB2, LRPA1, UGT1A6, PARP1, Histone H4, TOP2B, HIST1H1E, PML, and MX2) were evaluated by PRM analysis to verify the reliability of TMT-based quantitation results. These candidate proteins are involved mainly in the defense response to virus, chromatin assembly or disassembly, immune system, DNA packaging, virus carcinogenesis, apoptosis, hippo signaling pathway, and JAK–STAT signaling pathway. As shown in Table 1, the results of PRM analysis of eight proteins, except MX2, were consistent with the results of TMT-based quantitation. Although the results of PRM analysis showed that the level of high expression of MX2 was much higher than that in the TMT-based quantitation results, there was a similar trend of change between them. Therefore, our PRM results show that the nine candidate proteins exhibit a similar trend to TMT-based quantitation results, which further confirms the authenticity and reliability of the TMT-based quantitation results.

3. DISCUSSIONS

Virus infection can cause changes in protein expression in host cells, which will affect the normal physiological function of host cells and determine the pathogenicity of the virus. Therefore, the study of differential protein expression profiles after virus infection by the proteomics technique is helpful to further reveal the interaction mechanism between the virus and host and the molecular virus pathogenesis mechanism. TMT-based quantitative analysis is a kind of protein identification and quantification technology based on stable isotope chemical labeling and has a wide range of applications in proteomics research. To date, proteomics analysis of PDCoV-infected host cells has not been reported. Here, we utilized TMT-based quantitative analysis technology to investigate the interaction between PDCoV and LLC-PK cells. A total of 4624 proteins were found, among which the number of significantly differentially expressed upregulated and downregulated proteins was 128 and 147, respectively. Furthermore, PRM quantitative results were also in accordance with the TMT-based quantitative results. These DEPs were analyzed and compared to explore the changes in host proteins after PDCoV infection to provide information further revealing the pathogenic mechanism of PDCoV.

The interaction between the virus and the host cell has been demonstrated to involve a variety of complex biology processes. During the infection, virus invasion can cause the host cell to employ a variety of strategies to inhibit the replication of the virus, resulting in an antiviral state. In the present study, we found involvement of several immune system response proteins, including STAT2, MX1, MX2, GRB2, OA51, OA52, IFIT2, IFIT3, IFIT5, RSAD2, and JUN. In order to successfully proliferate in host cells, many coronaviruses have evolved different strategies to evade the innate cellular immune response. Among these proteins, GRB2, STAT2, MX1, RSAD2, and OA51 were also found to be related to the innate immunity of the host in this study. In addition, it has been reported that porcine RSAD2 and MX2 significantly inhibit PEDV infection and in a dose-dependent manner. Compared with those in mock-infected cells, these DEPs were significantly upregulated in PDCoV-infected cells, suggesting that these DEPs may be involved mainly in the process of combating PDCoV infection.

The GRB2 protein has been reported to be the major adaptor protein for coupling cell surface receptors to intracellular signaling events, which can also promote the entry of viruses and infection of host cells. Inhibition of endogenous GRB2 protein activity by application of GRB2 RNA interference (RNAi) mediated a sustained and significant decrease in viral and cell membrane fusion. In addition, GRB2 has been verified as a marker of adaptive immune deficiency in the process of viral infection. Our proteome data showed that PDCoV infection significantly increased the expression of GRB2, which was confirmed by PRM. However, there are relatively poor reports on the pathogenesis of coronavirus. Whether GRB2 plays an important role in the pathogenesis of coronavirus remains to be confirmed.

There are seven STAT proteins in mammals, which are involved mainly in the JAK/STAT signaling pathway. Among them, STAT1 and STAT2 play a critical role in the IFN signaling pathway. For many viruses, inhibition of STAT-mediated signal transduction can not only prevent STAT phosphorylation but also interfere with the degradation of STAT protein, which is one of the main mechanisms to evade the antiviral response. It has been reported that PDCoV antagonizes IFN-α signaling transduction by cleavage of STAT2, while cleavage of STAT2 is dependent on the activity of PDCoV nonstructural protein 5 (nsp5) protease. Moreover, nsp5 was also proven to effectively inhibit IFN signaling by targeting human STAT2 in Zika virus (ZIKV) infection. Guo et al. confirmed that PEDV infection could target the degradation of STAT1 to inhibit IFN-β production in vitro. However, our proteomics data showed that STAT1 did not significantly change in this study, but notably, PDCoV infection significantly increased STAT2 expression. These data indicate that STAT2 may exert a positive effect on resisting PDCoV infection.

Interferons bind to the corresponding receptors to stimulate JAK/STAT signaling pathways, which in turn drive the transcription of ISGs and induce cells to enter an antiviral state. Li et al. used comparative proteome analysis and found that PEDV infection significantly increased the expression of ISGs, such as OA51, OA52, MX1, MX2, and IFIT3, in host cells. Furthermore, overexpression of OA51 has been verified to significantly inhibit the replication of PRRSV in marc-145 cells. Similarly, in the present study, the expression of OA51, OA52, MX1, MX2, and IFIT3 also increased significantly after PDCoV infection of host cells. Therefore, we speculate that after PDCoV infection, the host may upregulate the expression.
of STAT2 and stimulate the transcription of ISGs to achieve an antiviral effect.

Apoptosis is an important defense mechanism for host cells to resist viral infection. Some viruses utilize apoptosis-inducing mechanisms as a way to release and disseminate progeny viruses. Six apoptosis-related proteins were identified in our proteome quantitative analysis, namely, Caspase-3, Caspase-7, Caspase-8, PARP1, CTSH, and JUN. The caspase family is an important initiator and executor of apoptosis, playing a key role in the induction, transduction, and amplification of apoptosis signaling in the cell. The cleavage of PARP1 by Caspase-3 is seen as a general feature of the death of apoptotic cells. Interestingly, almost every caspase can inhibit PARP1, thus contributing to the development of cell apoptosis. It is well demonstrated that in the extrinsic apoptotic pathway, death receptors and ligands activate Caspase-8, which directly activates the occurrence of apoptosis. Together, GO enrichment, KEGG enrichment, and network pathway analysis provide a comprehensive review of the host response to PDCoV infection, implying that the defense response of the host may achieve maximum viral replication through modulating the apoptosis pathway.

4. CONCLUSIONS

In conclusion, this study is the first attempt to determine the protein expression profile of PDCoV-infected cells by TMT-based quantitative analysis combined with LC–MS/MS techniques. These data revealed that PDCoV infection modulates a variety of biological processes in the host cells to escape viral infection, including the stress response, apoptosis, and the immune system. This study provides an important basis for further understanding the defense and regulation mechanism of host cells in the process of PDCoV infection and may be helpful to improve the pathogenicity and infectivity of PDCoV.

5. MATERIALS AND METHODS

5.1. Cells and Virus. LLC porcine kidney (LLC-PK) cells were purchased from the ATCC (ATCC CL-101) and cultured at 37 °C with 5% CO₂ in the modified Eagle medium (MEM) (Invitrogen, Carlsbad, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% MEM nonessential amino acids (NEAAs) (Gibco, Grand Island, NY, USA), 1% HEPES (Gibco, Grand Island, NY, USA), and 1% antibiotic-antimyocotic (Gibco, Grand Island, NY, USA). The PDCoV strain CH/XJYN/2016 (GenBank accession number: MN064712) was isolated from intestinal samples of a suckling piglet experiencing acute diarrhea using LLC-PK cells in our laboratory.

5.2. Virus Inoculation. When LLC-PK cells grew to 80% confluence in T-25 culture flasks, the cell growth medium was removed, and cells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2) (Gibco, Grand Island, NY, USA). Then, 3 mL of the PDCoV strain CH/XJYN/2016 was inoculated into the flasks at a multiplicity of infection (MOI) of 0.1 with 20 μg/mL trypsin (Gibco, Grand Island, NY, USA). After incubation at 37 °C for 5% CO₂ for 1 h, 1 mL of the maintenance medium (MEN supplemented with 1% NEAAs, 1% HEPES, and 1% antibiotic-antimyocotic) was added to the T-25 culture flasks and cultured at 37 °C for 5% CO₂. Uninfected cells served as the mock-infected group. Subsequently, CPEs were observed every 6 h post-infection (hpi), and the viruses were harvested from the mock-infected group. Viral RNA was extracted using an RNeasy Mini kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer’s protocol, and the viral replication kinetics were determined by real-time PCR (developed in our laboratory).

5.3. Immunofluorescence Assay (IFA). LLC-PK cells cultured in a 60 mm dish until the culture reached 70~80% confluence were inoculated with the PDCoV strain CH/XJYN/2016 at an MOI of 0.1. The medium was removed at 6, 12, 18, 24, 30, and 36 hpi, and the cells were washed with sterile PBS three times. Then, the cells were fixed at 4 °C for 30 min with 4% cold paraformaldehyde, and then, 1 mL of 0.25% Triton X-100 (Solarbio, Beijing, China) was added at room temperature for 10 min. After washing three times with sterile PBS, the cells were blocked with 5% bovine serum albumin (Solarbio, Beijing, China) for 1 h. The PDCoV-infected cells were detected by a mouse monoclonal anti-PDCoV-N protein antibody (prepared and stored in our laboratory) and Alexa Fluor® 488-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK). The cell nuclei were stained with 0.01% 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA) for 10 min. Fluorescent images were captured by using a fluorescence microscope (Olympus, Tokyo, Japan).

5.4. Protein Extraction and Quantitative Analysis. According to the cellular integrity and high-infection-rate cells, we selected 18 h after PDCoV infection to analyze the protein changes of LLC-PK cells. Cultures of PDCoV-infected and mock-infected cells were collected with a cell spatula, centrifuged at 300 g/min for 10 min, and washed twice with ice-cold PBS containing 1 mM sodium fluoride and 1 mM pervaeanade; three independent biological replicates were carried out. A 4-fold sample volume of lysis buffer (8 M urea, 1% protease inhibitor, 3 μM TSA, 50 mM NAM and 2 mM EDTA) was added to each protein sample for further protein solubilization by sonication, and the samples were centrifuged at 4 °C and 12,000 g/min for 10 min to remove cellular debris. The protein supernatants were collected, and the protein concentration was measured using a BCA protein quantitation kit (Thermo, USA) according to the manufacturer’s instructions.

5.5. Protein Digestion, TMT Labeling, and Fractionation. For digestion, the protein solution was reduced with 5 mM dithiothreitol at 56 °C for 30 min and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM tetraethylammonium bromide (TEAB) to a urea concentration less than 2 M. Finally, trypsin was added at a 1:50 trypsin-to-protein mass ratio for the first digestion.
overnight and a 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion. After trypsin digestion, a Strata X C18 SPE column (Phenomenex, Torrance, USA) was used for desalination and vacuum drying. The peptides were recombined in 0.5 M TEAB and processed according to the manufacturer’s TMT kit protocol (Thermo, USA). Briefly, one unit of TMT reagent was thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 h at room temperature, pooled, desalted, and dried by vacuum centrifugation. The tryptic peptides were fractionated into 50 fractions by high-pH reverse-phase high-performance liquid chromatography (HPLC) using a Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). The peptides were first separated with a gradient of 8 to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 6 fractions and dried by vacuum centrifugation. All samples were stored at −80 °C until LC–MS/MS/MS analysis.

5.6. LC–MS/MS Analysis. The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a homemade reverse-phase analytical column (15 cm length, 75 μm i.d.) and then separated using an EASY-nLC 1000 ultra-high-performance liquid chromatography system. The gradient was comprised of an increase from 6 to 24% solvent B (0.1% formic acid in 90% acetonitrile), 24 to 33% in 8 min, climbing to 75% in 3 min, and then holding at 75% for the last 3 min, all at a constant flow rate of 700 nL/min with an EASY-nLC 1000 UPLC system. The peptides were subjected to an NSI source followed by tandem mass spectrometry (MS/MS) in a Q ExactiveTM Plus (Thermo, USA) coupled inline to the UPLC.

The electrospray voltage applied was 2.0 kV, and peptide parent ions and their secondary fragments were detected and analyzed using a high-resolution Orbitrap. The first-level mass spectrum range was set to 350–1800 m/z, the scanning resolution was set to 70,000, the fixed start point of the secondary mass spectrum scanning range was 100 m/z, and the secondary scanning resolution was set to 35,000. The data acquisition mode used a data-dependent scanning (DDA) program; that is, after the first scan, the first 20 peptide parent ions with the highest signal intensity were selected to enter the HCD collision pool in turn, and 28% fragmentation energy was used for fragmentation, and the second-order mass spectrometry analysis was also carried out in turn. To improve the effective utilization of mass spectrometry, automatic gain control (AGC) was set at 2E3, the signal threshold was set to 2000 ions/s, the maximum injection time was set to 50 ms, and the dynamic elimination time of tandem mass spectrometry scanning was set to 20 s to avoid repeated scanning of parent ions.

5.7. Data Analysis. The resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8). Retrieval parameter settings were the following: the database was Sus_scrofa_9823_PM_20180816 (40,708 sequences); an inverse library was added to calculate the false positive rate (FDR), which is caused by random matching; and a common pollution library was added to the database to eliminate the influence of contaminating protein in the identification results. Trypsin/P was specified as a cleavage enzyme, allowing up to 2 cleavages. The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search, and the mass tolerance for fragments was set as 0.02 Da. Carboxymethyl on Cys was specified as a fixed modification, and acetylation modification and oxidation on Met were specified as variable modifications. The quantitative method was set as TMT-plex, and protein identification and the FDR were determined by PSM, and at least two peptides had to have higher than 95% confidence for protein quantification. The statistical significance of the protein expression level difference among the samples was compared using Student’s t-test to correct the multiplicity test. For accurate comparison between samples, protein quantification data with a fold change >1.2 or <0.83 and a p value <0.05 were identified as significantly DEPs.

5.8. Bioinformatics Analysis. These DEPs were analyzed by bioinformatics through Gene Ontology (http://www.ebi.ac.uk/interpro/) and divided into different biological processes, molecular functions, and cellular components. The Kyoto Encyclopedia of Genes and Genomes (KEGG) online service tool (http://www.genome.jp/kaas-bin/kaas_main) was used to annotate the submitted protein, and the annotated proteins were then matched into the corresponding pathways in the databases through the KEGG mapper (http://www.kegg.jp/kegg/mapper.html). KEGG and InterPro were used for the pathway and domain enrichment analysis of DEPs using Fisher’s exact test. After the DEP input, STRING software (version 11.0) was used to make a DEP network diagram, and the DEP interaction relationship was obtained according to a confidence score >0.7 (high confidence).

5.9. Targeted Protein Quantification by PRM. To further verify the original proteomics results obtained by TMT-based quantitative proteomics analysis, nine DEPs with reliable identification were selected for quantitative analysis via LC-PRM/MS. In brief, the peptide segments were from the proteomics residual peptide segment, and the protein extraction and trypsin digestion methods were the same as those in the TMT experiment. The peptides were separated by tandem mass spectrometry (MS/MS) and subject to an NSI ion source for ionization and then analyzed by Q Exactive Plus mass spectrometry. The electrospray voltage applied was 2.0 kV, and both the peptide master ion and its secondary fragments were detected and analyzed using a high-resolution Orbitrap. The full mass scan range was set to 350–1050 m/z, and the scan resolution was set to 70,000; the MS/MS Orbitrap scan resolution was set to 17,500. The data acquisition mode used a data-independent scanning (DIA) program, and the fragmentation energy of the HCD collision pool was set to 27. AGC was set at 3E6 for full MS and 1E5 for MS/MS. The maximum IT was set at 50 ms for full MS and auto for MS/MS. The isolation window for MS/MS was set at 2.0 m/z.

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indole; TEAB, tetrachloroethylene bromide; HPLC, high-performance liquid chromatography; DDA, data-dependent scanning; AGC, automatic gain control; KEGG, the Kyoto Encyclopedia of Genes and Genomes

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