Chapter

Abnormal Ubiquitination of Ubiquitin-Proteasome System in Lung Squamous Cell Carcinomas

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Abstract

Ubiquitination is an important post-translational modification. Abnormal ubiquitination is extensively associated with cancers. Lung squamous cell carcinoma (LUSC) is the most common pathological type of lung cancer, with unclear molecular mechanism and the poor overall prognosis of LUSC patient. To uncover the existence and potential roles of ubiquitination in LUSC, label-free quantitative ubiquitomics was performed in human LUSC vs. control tissues. In total, 627 ubiquitinated proteins (UPs) with 1209 ubiquitination sites were identified, including 1133 (93.7%) sites with quantitative information and 76 (6.3%) sites with qualitative information. KEGG pathway enrichment analysis found that UPs were significantly enriched in ubiquitin-mediated proteolysis pathway (hsa04120) and proteasome complex (hsa03050). Further analysis of 400 differentially ubiquitinated proteins (DUPs) revealed that 11 subunits of the proteasome complex were differentially ubiquitinated. These findings clearly demonstrated that ubiquitination was widely present in the ubiquitin-proteasome pathway in LUSCs. At the same time, abnormal ubiquitination might affect the function of the proteasome to promote tumorigenesis and development. This book chapter discussed the status of protein ubiquitination in the ubiquitin-proteasome system (UPS) in human LUSC tissues, which offered the scientific data to elucidate the specific molecular mechanisms of abnormal ubiquitination during canceration and the development of anti-tumor drugs targeting UPS.

Keywords: lung squamous cell carcinoma, ubiquitination, ubiquitinated protein (UP), differentially ubiquitinated protein (DUP), ubiquitin-proteasome system (UPS)

1. Introduction

Ubiquitination is one of the important protein post-translational modifications (PTMs) in human body, in which ubiquitin, a 76-amino-acid protein with a molecular weight of 8.5 KDa, is covalently attached its C-terminus to the ε-amino group of the substrate protein lysine residue through a multi-step enzymatic reaction cascade catalyzed by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [1]. As substrate proteins commonly contain multiple lysine residues, there are a variety of ubiquitination forms such as monoubiquitination (only one ubiquitin attached to a protein), multiubiquitination

(several lysine residues of substrate proteins were tagged with single ubiquitin), and polyubiquitination (a polyubiquitin chain is derived from subsequent ubiquitin covalently attached to lysine residues or N-terminus of the former ubiquitin) [2]. It is worth noting that the ubiquitin itself also has seven lysine residues to greatly complicate the topology of the polyubiquitin chain. Different ubiquitination forms perform different functions, such as monoubiquitination or multiubiquitination has been shown to be required for the entry of certain cargo proteins into vesicles at different stages of the secretory/endocytic pathway, while lysine-48 ubiquitin chain is mainly related to proteasome [3]. Like other PTMs, ubiquitination is a reversible reaction, and there are over 100 deubiquitination enzymes that regulate this process [4]. Ubiquitination coordinates with deubiquitination to regulate a broad host of cellular processes, including DNA repair, cell differentiation, signal transduction, enzymatic activity regulation, assembly of multiprotein complexes, protein trafficking, and autophagy [5]. Therefore, abnormal ubiquitination is associated with many diseases, including cancer, neurodegenerative disease, infection, and immune disorders [6]. Considering the importance of ubiquitination in tumorigenesis, different components of ubiquitin-proteasome system could be regarded as targets for discovery of anti-tumor drugs. With the application of first and second therapeutic proteasome inhibitors, such as Bortezomib (FDA has approved it for multiple myeloma and mantle cell lymphoma) [7] and Carfilzomib (FDA has approved it for relapsed and refractory multiple myeloma) [8], more and more anti-tumor drugs targeting UPS have been developed and approved by FDA, such as thalidomide, lenalidomide, and pomalidomide for treatment of multiple myeloma [9, 10].

Lung squamous cell carcinoma (LUSC) is a common type of lung cancer without a clear molecular mechanism. Currently, surgery, radiation, and chemotherapy have made significant advances in lung cancer treatment, especially targeted drug therapy; for example, epidermal growth factor receptor (EGFR) mutation or EML4-ALK fusion-based targeted therapies have improved the survival time of patients with lung adenocarcinoma (LUAD). However, targeted therapy and early-stage diagnosis are still a big clinical challenge in LUSC patients [11]. Although FGFR1 amplification and DDR2 mutation have been nominated as "druggable" targets in LUSC patients, the clinical efficacies of the corresponding drugs are still under clinical trials [12, 13]. Considering that abnormal ubiquitination will lead to the occurrence of a variety of tumors and the widespread clinical applications of anti-tumor drugs for the ubiquitin-proteasome pathway in recent years, the study of quantitative ubiquitinomics in LUSC tissues may provide the direction for the development of biomarkers and new targeted drugs.

High-resolution liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) has been used as a power tool for large-scale identification of various PTMs such as ubiquitination, phosphorylation, acetylation, and N-glycosylation [14]. The challenge of the use of LC-MS/MS to identify endogenous ubiquitination sites on a large scale is the fact that ubiquitination is a low abundance event *in vivo* and the size of the modification itself. The common strategy to identify low-abundance ubiquitinations in a proteome is that the extracted protein sample is firstly digested with trypsin to form tryptic peptide mixture, then the commercially specific anti-K-ε-GG antibodies are used to preferentially enrich ubiquitinated peptides from tryptic peptide mixture before MS/MS analysis in recent years [15]. Anti-ubiquitin antibody (specific anti-K-ε-GG group)-based label-free quantification coupled with LC-MS/MS has been used as an effective method to detect, identify, and quantify ubiquitinated proteins and ubiquitination sites, and more than 10,000 ubiquitination sites have been identified and quantified [16]. For lung cancer, ubiquitinomics is mainly carried out in lung cancer cells [17, 18], while the ubiquitinomics of fresh LUSC tissues is only reported recently in our research

group with label-free quantitative proteomics method and bioinformatics analysis to reveal the functions of ubiquitinome in predictive, preventive, and personalized medicine (PPPM) of LUSC [19].

This book chapter mainly reviewed ubiquitinated proteins (UPs) and differentially ubiquitinated proteins (DUPs) in ubiquitin-proteasome-system (UPS) in LUSC, and emphasized the potential regulatory role of ubiquitination in UPS, which offers scientific data for further research on the regulatory mechanism of ubiquitination on UPS, the molecular mechanism of UPS abnormality in tumor development, and the development of anti-tumor drugs targeting UPS.

2. Materials and methods

2.1 Lung cancer tissues and protein extraction

Human LUSC tissues (n = 5) and tumor-adjacent control lung tissues (n = 5) were surgically removed from patients, immediately stored in liquid nitrogen (−196°C), and then stored in freezer (−80°C). Clinical characteristics of each sample were described previously [19]. LUSC tissues (750 mg, equally mixed 5 tumor tissues) and control tissues (750 mg, equally mixed 5 control tissues) were washed 5 times with 3 mL 0.9% NaCl to clean blood on the surface of the tissues. The washed tissues (LUSC; or controls) were homogenized with urea lysis buffer [2 M thiourea, 7 M urea, 1 mM protein inhibitor PMSF, and 100 mM dithiothreitol(DTT)], sonicated, and centrifuged (15,000 g, 20 min, and 4°C). The supernatant was the extracted protein sample. The protein content was tested with Brandford method. The detailed procedure of protein extraction was described previously [19].

2.2 Protein digestion and enrichment of ubiquitinated peptides

An amount of DTT (final concentration = 10 mM) was added to each extracted protein sample, which was mixed (600 rpm, 1.5 h, and room temperature). An amount of iodoacetamide (final concentration = 50 mM) was added to the DTT-treated protein sample, which was incubated (dark, 30 min). The uranyl acetate (UA) was diluted to 2 M with 50 mM Tris HCl buffer (pH 8.0) and added to each protein sample. An amount of trypsin was added to each protein sample (trypsin:protein = 1:50 at wt:wt), and then incubated (37°C, 15–18 h). A volume of 10% trifluoroacetic acid (TFA; final concentration = 0.1%) was added, and pH was adjusted to ≤ 3 to stop digestion. Each tryptic peptide sample was purified with C18 cartridges and then lyophilized. The lyophilized tryptic peptides were resolved with 1.4 mL immunoaffinity purification (IAP) buffer that contained 50 mM NaCl, 10 mM Na_2HPO_4, 50 mM MOPS/NaOH, and pH 7.2. The anti-K-ε-GG antibodies against ubiquitin remnant motif (K-ε-GG) (Cell Signal Technology) were used to enrich the ubiquitinated peptides, followed by purification with C18 STAGE Tips. The purified ubiquitinated peptide sample was used for MS/MS analysis. The detailed experimental procedure was described previously [19].

2.3 LC-MS/MS

The prepared ubiquitinated peptide sample was analyzed with LC-MS/MS in the Easy nLC and Q Exactive mass spectrometer (Thermo Scientific). The MS/MS data for each sample were used to search protein database using MaxQuant 1.5.3.17 software to identify ubiquitinated proteins and ubiquitination sites and quantify the abundance of ubiquitination. The detailed procedure was described previously [19].

2.4 Bioinformatics

For UPs and DUPs, DAVID software (version 6.8, https://david.ncifcrf.gov/) was used to carry out the gene ontology (GO) enrichment analysis, including cellular components (CCs), molecular functions (MFs), and biological processes (BPs), and group those proteins into different functional clusters [20]. The statistically significant pathways were mined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The KEGG online service tool KOBAS (http://kobas.cbi.pku.cn) was used to annotate the KEGG database description of each protein [21].

3. Results and discussion

3.1 Proteomics analysis of lysine-ubiquitinated profile in LUSC

To identify protein lysine-ubiquitinated sites and quantify the level of ubiquitination in human LUSC tissues, proteins were extracted and digested into peptides with trypsin. Lysine-ubiquitinated peptides were immunoaffinity-enriched with commercially specific anti-K-ε-GG antibodies and analyzed with high-resolution LC-MS/MS. In total, 1209 lysine-ubiquitinated sites in 627 unique proteins were identified. These proteins containing ubiquitinated lysine residues were defined as UPs. **Figure 1** showed two representative MS/MS spectra of the ubiquitinated peptides ^{425}ETNLDSLPLVDTHSK*R^{440} from vimentin (P08670; K* = ubiquitinated lysine residue) (**Figure 1A**), and ^{633}RPVK*DGGGTNSITVR^{647} from multidrug resistance-associated protein 1 (P33527; K* = ubiquitinated lysine residue) (**Figure 1B**). All other ubiquitinated sites and ubiquitinated proteins were identified with the same MS/MS method. The differentially ubiquitinated peptides were determined with amino acid sequences, ratio(tumor/control) > 2.0 or < 0.5, and p-value < 0.05. Proteins containing this type of ubiquitinated peptides were defined as DUPs. Totally, 400 DUPs with 654 ubiquitinated sites were identified in LUSC tissues vs. tumor-adjacent control lung tissues [19].

3.2 UPs and DUPs were significantly enriched in UPS-related biological processes and molecular functions in LUSC

GO functional enrichment analyses of 627 UPs and 400 DUPs were carried out according to BPs, MFs, and CCs. GO enrichment result-based cluster analysis grouped those UPs into seven clusters (**Table 1**), and DUPs into 10 clusters (**Table 2**).

Among GO enrichment results of 627 UPs, many biological processes, molecular functions, and cellular components related to UPS were significantly enriched, including negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle, and positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition in cluster 2, and proteasome-activating ATPase activity, positive regulation of proteasomal protein catabolic process, cytosolic proteasome complex, nuclear proteasome complex, and proteasome regulatory base complex in cluster 3 (**Table 1**). Interestingly, DUPs were also significantly enriched in the similar biological processes, molecular functions, and cellular components, including proteasome accessory complex, negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle, positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition, and protein polyubiquitination in cluster 2, and proteasome-activating ATPase activity, positive regulation of proteasomal protein catabolic process, cytosolic proteasome complex, nuclear proteasome complex, and proteasome regulatory

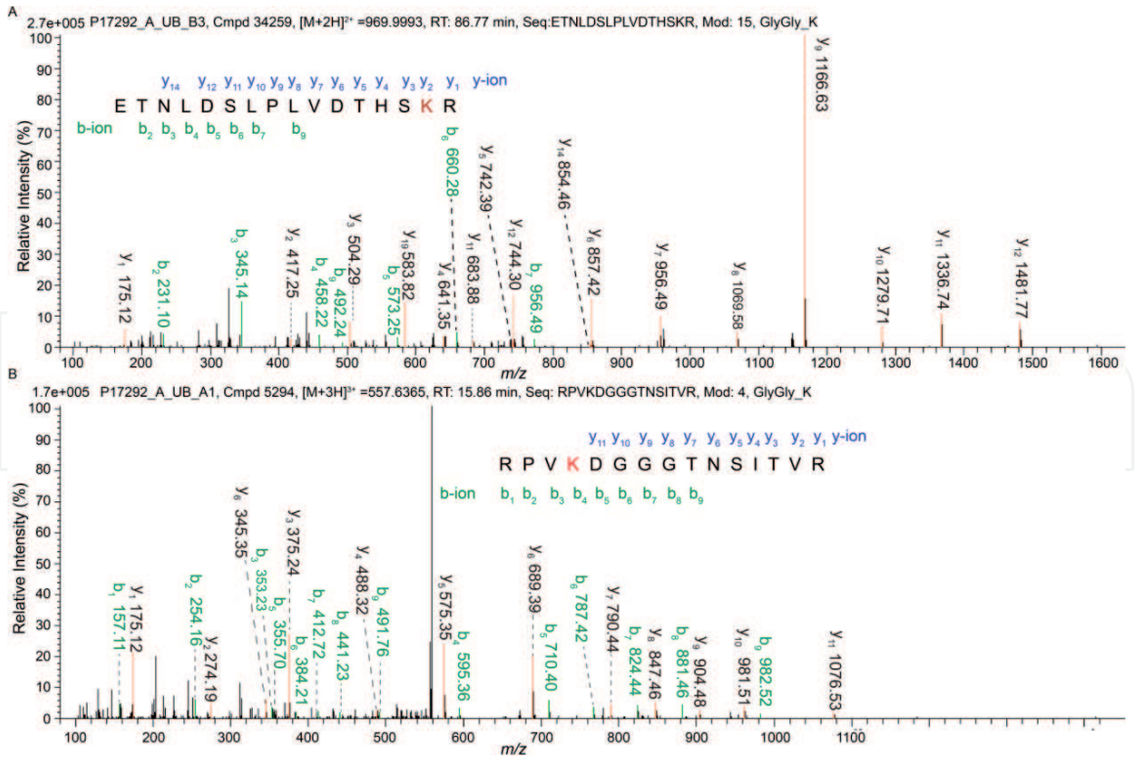


Figure 1.
*Representative MS/MS spectra of ubiquitinated peptides ^{425}ETNLDSLPLVDTHSK*R^{440} from vimentin (P08670) (A) and ^{633}RPVK*DGGGTNSITVR^{647} from multidrug resistance-associated protein 1 (MRP1) (P33527) (B). K* = ubiquitinated lysine residue. Reproduced from Lu et al. [19], with permission from Springer publisher open access article, copyright 2020.*

base complex in cluster 3 (**Table 2**). These findings clearly demonstrated that many ubiquitinated proteins were involved in UPS system, and differential ubiquitination occurred in UPS system in LUSC, implying that ubiquitination participated in the regulation of UPS, and abnormal ubiquitination might play an important role in the development of LUSC.

3.3 UPs involved in UPS-related molecular network alternations in LUSC

KEGG pathway network analysis of 627 UPs revealed 47 statistically significant ubiquitination-mediated signaling pathway alterations ($P < 0.05$ and FDR < 0.05) (**Figure 2**), among which were included two UPS-related pathways—ubiquitin-mediated proteolysis pathway (hsa04120) and proteasome complex (hsa03050).

Ubiquitin-mediated proteolysis pathway showed the detailed process of protein ubiquitination, which involved multiple types of E1s, E2s, and E3s. This study found that one E1 (UBE1), two E2s (UBE2N, and UBE2O), and six E3s (ITCH, HUWE1, UBE4B, PML, CUL4A, and CUL5) were ubiquitinated in LUSC (**Figure 3**). These six E3s belonged to different subfamilies, in which ITCH and HUWE1 belonged to HECT type E3, UBE4B belonged to U-box type E3, PML belonged to single RING-finger type E3, and both CUL4A and CUL5 belonged to multi subunit RING-finger type E3. E1s, E2s, and E3s are the important enzymes to catalyze the occurrence of ubiquitination in a protein. The ubiquitination of these enzymes definitely affects the quitination process of a protein. Currently, studies on these enzymes have focused on their roles in the ubiquitination process, and the effects of PTMs on these enzymes are poorly understood. There are relatively few studies on the ubiquitination of these nine enzymes. For example, ubiquitinated PML (P29590, identified in this study) was mediated by multiple E3s, leading to subsequent proteasomal degradation [22, 23]. Self-ubiquitination of ITCH (Q96J02, identified in this study)

Category	GO term	p -value
Annotation cluster 1		
GOTERM_MF_DIRECT	Cadherin binding involved in cell-cell adhesion	1.43E-29
GOTERM_CC_DIRECT	Cell-cell adherens junction	3.72E-29
GOTERM_BP_DIRECT	Cell-cell adhesion	1.13E-23
Annotation cluster 2		
GOTERM_BP_DIRECT	Wnt signaling pathway, planar cell polarity pathway	5.78E-11
GOTERM_BP_DIRECT	Regulation of cellular amino acid metabolic process	4.14E-09
GOTERM_BP_DIRECT	Stimulatory C-type lectin receptor signaling pathway	4.43E-09
GOTERM_BP_DIRECT	NIK/NF-kappa B signaling	1.37E-08
GOTERM_BP_DIRECT	Tumor necrosis factor-mediated signaling pathway	2.96E-08
GOTERM_BP_DIRECT	Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	3.70E-08
GOTERM_BP_DIRECT	Positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	9.13E-08
GOTERM_BP_DIRECT	Anaphase-promoting complex-dependent catabolic process	1.52E-07
GOTERM_BP_DIRECT	T cell receptor signaling pathway	9.74E-07
GOTERM_BP_DIRECT	Positive regulation of canonical Wnt signaling pathway	1.13E-06
GOTERM_BP_DIRECT	Negative regulation of canonical Wnt signaling pathway	1.57E-05
Annotation cluster 3		
GOTERM_CC_DIRECT	Proteasome regulatory particle, base subcomplex	1.29E-08
GOTERM_CC_DIRECT	Nuclear proteasome complex	1.70E-08
GOTERM_CC_DIRECT	Cytosolic proteasome complex	1.21E-07
GOTERM_MF_DIRECT	Proteasome-activating ATPase activity	1.70E-07
GOTERM_BP_DIRECT	Positive regulation of RNA polymerase II transcriptional preinitiation complex assembly	3.45E-06
GOTERM_MF_DIRECT	TBP-class protein binding	3.27E-05
GOTERM_BP_DIRECT	Positive regulation of proteasomal protein catabolic process	9.97E-05
Annotation cluster 4		
GOTERM_BP_DIRECT	SRP-dependent cotranslational protein targeting to membrane	3.56E-08
GOTERM_BP_DIRECT	Viral transcription	7.74E-08
GOTERM_BP_DIRECT	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	1.92E-07
GOTERM_BP_DIRECT	Translational initiation	6.60E-06
GOTERM_CC_DIRECT	Ribosome	3.32E-05
GOTERM_MF_DIRECT	Structural constituent of ribosome	1.68E-03
Annotation cluster 5		
GOTERM_CC_DIRECT	Haptoglobin-hemoglobin complex	9.95E-05
Annotation cluster 6		
GOTERM_BP_DIRECT	Nucleotide-excision repair, DNA damage recognition	6.40E-04
Annotation cluster 7		
GOTERM_CC_DIRECT	Hemoglobin complex	4.59E-03

Table 1.
The functional categories of 627 ups, identified with GO enrichment analysis.

Category	GO term	p-value
Annotation cluster 1		
GOTERM_MF_DIRECT	Cadherin binding involved in cell-cell adhesion	3.11E-18
GOTERM_CC_DIRECT	Cell-cell adherens junction	2.24E-17
GOTERM_BP_DIRECT	Cell-cell adhesion	1.23E-13
Annotation cluster 2		
GOTERM_CC_DIRECT	Proteasome accessory complex	4.60E-12
GOTERM_BP_DIRECT	Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	1.59E-11
GOTERM_BP_DIRECT	Regulation of cellular amino acid metabolic process	3.49E-09
GOTERM_BP_DIRECT	NIK/NF-kappaB signaling	5.35E-09
GOTERM_BP_DIRECT	Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	1.27E-08
GOTERM_BP_DIRECT	Stimulatory C-type lectin receptor signaling pathway	1.85E-08
GOTERM_BP_DIRECT	Positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	2.83E-08
GOTERM_BP_DIRECT	Anaphase-promoting complex-dependent catabolic process	4.43E-08
GOTERM_BP_DIRECT	Tumor necrosis factor-mediated signaling pathway	8.37E-08
GOTERM_BP_DIRECT	Positive regulation of canonical Wnt signaling pathway	1.04E-07
GOTERM_BP_DIRECT	T cell receptor signaling pathway	2.37E-07
GOTERM_BP_DIRECT	Fc-epsilon receptor signaling pathway	4.90E-07
GOTERM_BP_DIRECT	Protein polyubiquitination	7.65E-07
GOTERM_BP_DIRECT	Negative regulation of canonical Wnt signaling pathway	4.36E-06
Annotation cluster 3		
GOTERM_CC_DIRECT	Proteasome regulatory particle, base subcomplex	4.88E-10
GOTERM_CC_DIRECT	Nuclear proteasome complex	1.00E-09
GOTERM_CC_DIRECT	Cytosolic proteasome complex	7.28E-09
GOTERM_MF_DIRECT	Proteasome-activating ATPase activity	1.54E-08
GOTERM_BP_DIRECT	Positive regulation of RNA polymerase II transcriptional preinitiation complex assembly	3.41E-07
GOTERM_MF_DIRECT	TBP-class protein binding	2.10E-06
GOTERM_BP_DIRECT	Positive regulation of proteasomal protein catabolic process	1.05E-05
GOTERM_BP_DIRECT	Protein catabolic process	1.41E-04
Annotation cluster 4		
GOTERM_BP_DIRECT	Regulation of ventricular cardiac muscle cell action potential	1.11E-03
GOTERM_MF_DIRECT	Cell adhesive protein binding involved in bundle of His cell-Purkinje myocyte communication	5.30E-03
Annotation cluster 5		
GOTERM_CC_DIRECT	Haptoglobin-hemoglobin complex	2.43E-05
GOTERM_CC_DIRECT	Endocytic vesicle lumen	1.71E-04
GOTERM_MF_DIRECT	Haptoglobin binding	1.10E-03
GOTERM_BP_DIRECT	Positive regulation of cell death	1.90E-02

Category	GO term	p-value
GOTERM_CC_DIRECT	Hemoglobin complex	1.97E-02
GOTERM_MF_DIRECT	Oxygen transporter activity	2.90E-02
GOTERM_BP_DIRECT	Oxygen transport	3.43E-02
Annotation cluster 6		
GOTERM_BP_DIRECT	SRP-dependent cotranslational protein targeting to membrane	9.85E-05
GOTERM_BP_DIRECT	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	5.83E-04
GOTERM_BP_DIRECT	Viral transcription	1.69E-03
GOTERM_BP_DIRECT	Translational initiation	5.82E-03
GOTERM_CC_DIRECT	Ribosome	3.40E-02
Annotation cluster 7		
GOTERM_MF_DIRECT	Voltage-gated anion channel activity	5.30E-03
GOTERM_MF_DIRECT	Porin activity	5.30E-03
GOTERM_CC_DIRECT	Pore complex	1.11E-02
GOTERM_BP_DIRECT	Anion transport	3.02E-02
GOTERM_BP_DIRECT	Regulation of anion transmembrane transport	4.34E-02
Annotation cluster 8		
GOTERM_BP_DIRECT	Daunorubicin metabolic process	1.00E-02
GOTERM_BP_DIRECT	Doxorubicin metabolic process	1.00E-02
Annotation cluster 9		
GOTERM_BP_DIRECT	Nucleotide-excision repair, DNA damage recognition	1.00E-02
GOTERM_BP_DIRECT	Global genome nucleotide-excision repair	2.47E-02
Annotation cluster 10		
GOTERM_MF_DIRECT	Neutral amino acid transmembrane transporter activity	1.82E-02
GOTERM_BP_DIRECT	Neutral amino acid transport	2.62E-02

Table 2.
The functional categories of 400 DUPs, identified with GO enrichment analysis. Modified from Lu et al. [19], with permission from Springer publisher open access article, copyright 2020.

through lysine-63 linkages showed an auto-regulatory mechanism controlling ITCH cytoplasmic-nuclear shuffling [24]. Therefore, the effects of the currently known ubiquitination on these enzymes are only the tip of the iceberg. However, one should also realize that this study found ubiquitination of cullin proteins such as Cul4A and Cul5, while cullin proteins can also be modified by NEDD8 to form NEDDylation. It is well known that the use of the K-ε-GG antibody cannot discriminate between proteins modified with ubiquitin and the related proteins NEDD8 and ISG15. Therefore, for deep investigation of this identified ubiquitination of E3s Cul4A and Cul5 in LUSC in the future, additional experiments are needed to discriminate E3s Cul4A and Cul5 that were modified by ubiquitin, NEDD8, or ISG15 [22].

Proteasome was a pivotal component for ubiquitin-mediated proteolysis. The 26S proteasome was a complex including two 19S regulatory particles (PA700) and one 20S core particle. The 20S degradation complex contained two α rings (7 subunits, α1-α7) and two β rings (7 subunits, β1-β7). These α rings and β rings together formed a hollow ground circle. The tube-like structure was highly conserved

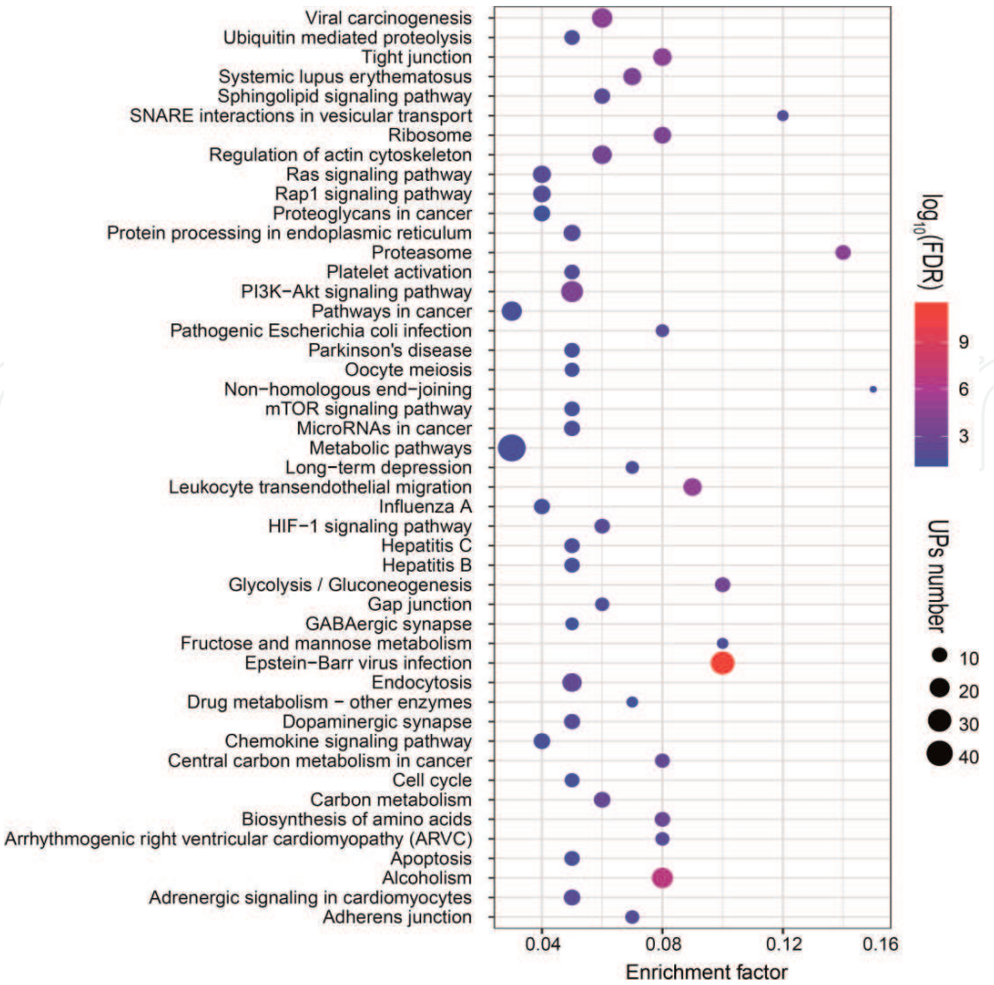
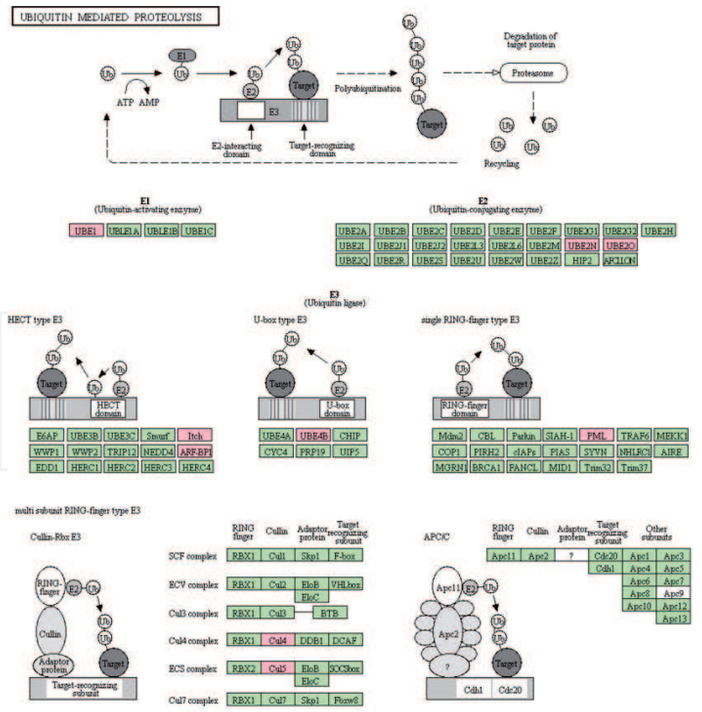


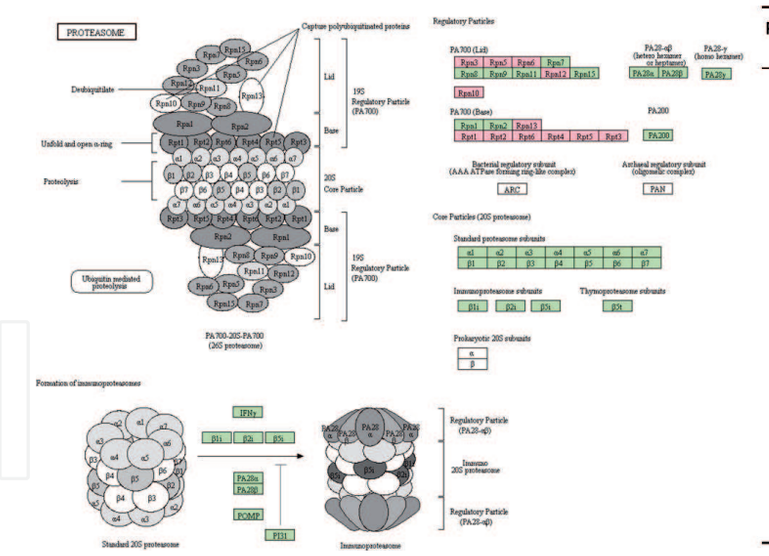
Figure 2.
Statistically significant KEGG pathways enriched from 627 UPs. Those UPs were significantly enriched in 47 KEGG pathways ($p < 0.05$ and FDR < 0.05), among which were included two UPS-related pathways—ubiquitin-mediated proteolysis pathway (hsa04120) and proteasome complex (hsa03050). The darker dot means the more significant enrichment, and the size of the dot represents the number of UPs enriched in the pathway.

from archaea to mammals [25]. Among them, the α ring was located in the outer layer of the cylinder-like structure, which mainly acted on the recognition of the substrate; the β ring was located in the inner layer of the cylinder-like structure, and was mainly responsible for catalyzing the degradation of the substrate [26, 27]. Three subunits that played a catalytic role were located on the inner surface of the β-ring molecule, exhibiting cysteine protease-like activity, trypsin-like activity, and chymotrypsin-like activity [26, 27]. The 19S regulatory complex contained 19 different subunits, which were divided into two parts: "base" and "lid" [28]. Among them, the base part formed the proximal part of the 19S regulatory complex, which was connected to the alpha ring of the 20S degradation complex, and the lip part formed the distal end. The base section contained 6 ATPase-dependent subunits (Rpt1-Rpt6) and 2 ATPase-independent subunits (Rpn1 and Rpn2) [28]. Usually, Rpn10 and Rpn13 were also classified as the base [26]. The lid part consisted of Rpn3, Rpn5-Rpn9, and Rpn11-Rpn12 subunits [28]. The 19S regulatory complex recognized ubiquitin-labeled target proteins (Rpn10 and Rpn13) and before the target protein entered the 20S degradation complex, deubiquitinated the target protein (Rpn11) and opened the folded structure of the target protein [28]. This study discovered five UPs (Rpn3, Rpn5, Rpn6, Rpn10, and Rpn12) in PA700 (Lid), and seven UPs (Rpn13, Rpt1, Rpt2, Rpt3, Rpt4, Rpt5, and Rpt6) in PA700 (Base) in LUSC. No UPs were identified in 20S core particle in LUSC (**Figure 4**).



Pathway nodes	Protein access number	Modified sites	Ratio (T/N)
UBE1	A0A024RDB0	K544	2.90
UBE2N	P61088	K94	0.09
UBE2N	P61088	K92	0.26
UBE2N	P61088	K82	0.55
UBE2N	P61088	K74	NA*
UBE2O	Q9C0C9	K136	0.81
UBE2O	Q9C0C9	K132	0.93
Itch	Q96J02	K446	1.81
Itch	Q96J02	K478	1.83
Itch	Q96J02	K432	3.05
Itch	Q96J02	K350	NA*
ARF-BP1	A0A024R9W5	K3452	2.28
ARF-BP1	A0A024R9W5	K4295	NA*
UBE4B	O95155	K794	NA*
PML	P29590	K394	1.33
Cul4	Q13619	K265	NA*
Cul4	Q13619	K465	NA*
Cul4	Q13619	K635	NA*
Cul5	Q93034	K724	0.96

Figure 3.
UPs identified in ubiquitin-mediated proteolysis pathway (hsa04120). Pink rectangle means the ubiquitinated proteins, and green rectangle means nonubiquitinated proteins. The pathway node in the right panel corresponds to the pink rectangle in the left diagram. Protein access number is the Swiss-Prot accession number. Modified sites refer to ubiquitinated lysine (K). Ratio (T/N) = ratio of tumors to controls. Asterisk (*) represents there is no quantitative intensity on this modification site in both the tumor and the control group.



Pathway nodes	Protein access number	Modified sites	Ratio (T/N)
Rpn13	Q16186	K34	2.27
Rpt2	P62191	K293	T+/N-
Rpt1	A0A140VK70	K64	4.96
Rpt1	A0A140VK70	K415	6.57
Rpt5	A0A140VK42	K53	0.22
Rpt5	A0A140VK42	K276	4.90
Rpt5	A0A140VK42	K79	0.99
Rpt5	A0A140VK42	K372	T+/N-
Rpt3	A8K2M0	K271	T+/N-
Rpt6	A0A140VJS3	K346	T+/N-
Rpt6	A0A140VJS3	K290	T+/N-
Rpt6	A0A140VJS3	K330	T+/N-
Rpt4	A0A087X2I1	K62	2.37
Rpt4	A0A087X2I1	K194	T+/N-
Rpt4	A0A087X2I1	K328	T+/N-
Rpt4	A0A087X2I1	K211	NA*
Rpn3	O43242	K273	T+/N-
Rpn12	P48556	K111	NA*
Rpn6	O00231	K32	T+/N-
Rpn5	A0A0S2Z489	K147	T+/N-
Rpn10	P55036	K40	33.80
Rpn10	P55036	K74	5.16

Figure 4.
UPs identified in proteasome complex (hsa03050). Pink rectangle means the ubiquitinated subunits, and green rectangle means non-ubiquitinated subunits. The pathway node in the right panel corresponds to the pink rectangle in the left diagram. Protein access number is the Swiss-Prot accession number. Modified sites refer to ubiquitinated lysine (K). Ratio (T/N) = ratio of tumors to controls. T+/N– indicates that this modification site has quantitative intensity only in tumor group, while T–/N+ means this modification site has quantitative intensity only in control group. Asterisk (*) represents there is no quantitative intensity on this modification site in both the tumor and the control group.

3.4 DUPs involved in UPS-related molecular network alternations in LUSC

KEGG pathway network analysis of 400 DUPs revealed 39 statistically significant ubiquitination-mediated signaling pathway alterations ($P < 0.05$ and $FDR < 0.05$) (**Figure 5**), including one UPS-related pathway – proteasome complex (hsa03050).

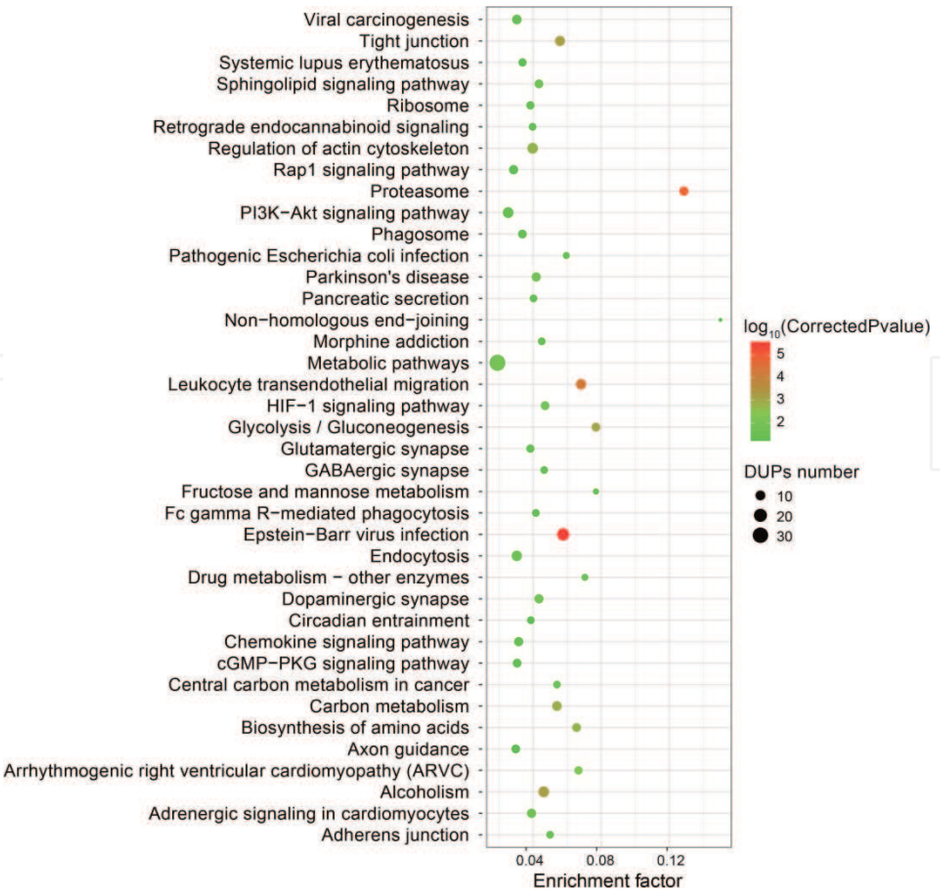
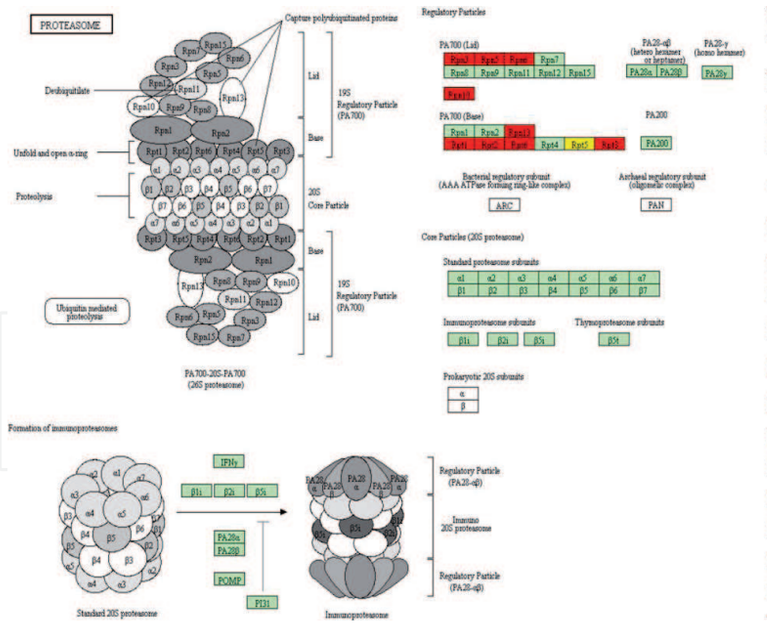


Figure 5.
Statistically significant KEGG pathways enriched from 400 DUPs. Those DUPs were significantly enriched in 39 KEGG pathways ($p < 0.05$ and FDR < 0.05), including one UPS-related pathway – proteasome complex. The darker dot means the more significant enrichment, and the size of the dot represents the number of DUPs enriched in the pathway. Reproduced from Lu et al. [19], with permission from Springer publisher open access article, copyright 2020.

In proteasome complex, this study discovered 4 DUPs (Rpn3, Rpn5, Rpn10, and Rpn6) in PA700 (Lid), and 7 DUPs (Rpn13, and Rpt1-Rpt6) in PA700 (Base). Their ubiquitination levels were significantly increased at residues K_{74} (Ratio = 5.16) in Rpn10, K_{34} (Ratio = 2.27) in Rpn13, K_{293} (T+/N−) in Rpt2, K_{46} (Ratio = 4.96) in Rpt1, K_{372} (T+/N−) in Rpt5, K_{273} (T+/N−) in Rpt4, K_{346} (T+/N−), K_{330} (T+/N−) and K_{290} (T+/N−) in Rpt6, K_{194} (T+/N−), K_{328} (T+/N−) and K_{62} (Ratio = 2.37) in Rpt4, K_{273} (T+/N−) in Rpn3, K_{32} (T+/N−) in Rpn6, and K_{147} (T+/N−) in Rpn5. The ubiquitination level was decreased at residue K_{53} (Ratio = 0.33) in Rpt5 (**Figure 6**).

The proteasome was a pivotal component of UPS to degrade the short-lived regulatory proteins and remove the damaged soluble proteins [29]. Consequently, dysfunction of proteasome might decrease the capability of protein degradation, thus resulting in the increased level of misfolded and damaged proteins, which was closely related to tumorigenesis [30]. The 26S proteasome had one 20S subunit and two 19S regulatory caps. Two 19S caps were necessary to maintain the normal functions of 20S subunit. For example, Rpn 13 in 19S base cap and Rpn 10 in 19S head cap were the recognition-receptors of the ubiquitinated proteins [31, 32]. Further, PTMs (such as phosphorylation, acetylation, myristoylation, and ubiquitination) had been detected in those subunits to greatly complicate the mechanisms of the modulation of proteasome activity. For example, Rpn 10 was mono-ubiquitinated to recruit substrate protein and interact with the shuttle factor of proteasome in drosophila [33, 34]. The multiple ubiquitinations in 19S cap of proteasome such as Rpn 1, Rpn 10, and Rpn 13 were necessary to autophage proteasome [35]. Our study [19] discovered three non-ATPase subunits



Pathway nodes	Protein access number	Modified site	Ratio (T/N)
Rpn13	Q16186	K34	2.27
Rpt2	P62191	K293	T+/N-
Rpt1	A0A140VK70	K46	4.96
Rpt5	A0A140VK42	K372	T+/N-
Rpt5	A0A140VK42	K53	0.33
Rpt4	A8K2M0	K273	T+/N-
Rpt6	A0A140VJS3	K346	T+/N-
Rpt6	A0A140VJS3	K330	T+/N-
Rpt6	A0A140VJS3	K290	T+/N-
Rpt4	A0A087X2I1	K194	T+/N-
Rpt4	A0A087X2I1	K328	T+/N-
Rpt4	A0A087X2I1	K62	2.37
Rpn3	O43242	K273	T+/N-
Rpn6	O00231	K32	T+/N-
Rpn5	A0A0S2Z489	K147	T+/N-
Rpn10	Q5VWC4	K74	5.16

Figure 6.
DUPs identified in proteasome complex (hsa03050). The red rectangle means the intensities of all identified ubiquitination sites in one protein were increased, and yellow rectangle means at least two ubiquitination sites in a protein with inconsistent ubiquitination intensities. The pathway node in the right panel corresponds to the red and yellow rectangle in the left diagram. Protein access number is the Swiss-Prot accession number. Modified sites refer to ubiquitinated lysine (K). Ratio (T/N) = ratio of tumors to controls. T+/N− indicates that this modification site has quantitative intensity only in tumor group, while T−/N+ means this modification site has quantitative intensity only in control group. Reproduced from Lu et al. [19], with permission from Springer publisher open access article, copyright 2020.

(PSMD3, PSMD11, and PSMD12), and three ATPase subunits (PSMC1, PSMC4, and PSMC6) were differentially ubiquitinated in 19S regulatory cap of proteasome in LUSC tissues. It clearly demonstrated that these ubiquitinations in 19S regulatory caps might influence the structure and functions of the proteasome complex. Some studies found that PSMD11 was necessary to assemble proteasome complex and elevate the activity of proteasome in embryonic stem cells [36]. Acetylation [37], phosphorylation [38], and SUMO [39] had been reported to occur in PSMD11, and our study first discovered that PSMD11 was ubiquitinated at residue K_{32} in LUSC tissues but not in control lung tissues [19]. Currently, few literature studies are found regarding the study on the relationship of ubiquitination and function of proteasome subunits. However, the abnormal ubiquitination of proteasome subunits might cause the functional abnormalities of proteasome complex in LUSC tissues and further lead to the imbalance of synthesis and degradation of intracellular proteins. These findings offer the new clues to deeply study and understand the regulation of UPS functions in LUSC.

4. Conclusions

Label-free quantitative ubiquitinomics was an effective approach to identify ubiquitinated proteins and ubiquitination sites and quantifies the levels of ubiquitination in human LUSC tissues. In total, 627 UPs and 400 DUPs were identified, providing the first (differential) ubiquitinome profile based on fresh human LUSC tissues. GO and KEGG analyses of UPs and DUPs revealed the statistically significant ubiquitination-mediated molecular network alternations, among which several proteins in two UPS-related pathways (ubiquitin-mediated proteolysis pathway, and proteasome complex) underwent ubiquitination in LUSC. Furthermore, 11 subunits

of proteasome complex were differentially ubiquitinated in LUSC. These findings demonstrated that ubiquitination was widely present in UPS in LUSC. At the same time, abnormal ubiquitination might affect the functions of the proteasome to promote tumorigenesis and development. This book chapter focused on the status of protein ubiquitination in UPS-related pathways in human LUSC tissues, and provided the scientific data for the elucidation of the specific molecular mechanisms of abnormal ubiquitination during canceration and the development of anti-tumor drugs targeting UPS for lung cancer.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Author's contributions

X.Z. conceived the concept, designed the book chapter, wrote and critically revised the book chapter, coordinated, and was responsible for the correspondence work and financial support. M.L. designed and wrote the book chapter.

Abbreviations

DUPs	differentially ubiquitinated proteins
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
GO	gene ontology
KEGG	kyoto encyclopedia of genes and genomes
LC	liquid chromatography
LUSC	lung squamous cell carcinoma
LUAD	lung adenocarcinoma
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PTM	post-translational modification
PPPM	predictive, preventive and personalized medicine
UPs	ubiquitinated proteins
UPS	ubiquitin-proteasome system

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