Original Article

Identification of Ubiquitinated Proteins from Human Multiple Myeloma U266 Cells by Proteomics*

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Abstract

Objective To identify ubiquitinated proteins from complex human multiple myeloma (MM) U266 cells, a malignant disorder of differentiated human B cells.

Methods Employing a globally proteomic strategy combining of immunoprecipitation, LC-MS/MS and SCX-LC-MS analysis to identified ubiquitination sites, which were identified by detecting signature peptides containing a GG-tag (114.1 Da) and an LRGG-tag (383.2 Da).

Results In total, 52 ubiquitinated proteins containing 73 ubiquitination sites of which 14 and 59 sites contained LRGG-tag and GG-tag were identified, respectively.

Conclusion Classification analysis by of the proteins identified in the study based on the PANTHER showed that they were associated with multiple functional groups. This suggested the involvement of many endogenous proteins in the ubiquitination in MM.

Key words: Ubiquitination; Multiple myeloma; Immunoprecipitation; Ubiquitinated proteins

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INTRODUCTION

Ubiquitin (Ub) is an 8.5 kDa protein that exists in all eukaryotes. The protein is involved in critical cellular functions, mediating selective degradation of regulatory proteins[1]. Ub is extremely well conserved and can be transiently attached to thousands of different proteins[2-3]. Ubiquitin-mediated degradation plays a crucial role in cellular growth, immune function, cell-cycle regulation, membrane-protein endocytosis, intracellular trafficking, chromatin-mediated transcriptional regulation, and the assembly of signaling complexes[4-5]. A better understanding of the ubiquitin system is important for elucidating therapeutic strategies for diseases including cancers, some severe types of mental retardation and neurodegenerative disorders[6-7].

Proteins in eukaryotic cells may be subjected to a variety of post-translational modifications that greatly extend the functional diversity and dynamics of the cellular proteome. Ubiquitination is one such post-translational modification in which proteins are modified by attaching an Ub group. The identification of substrates that undergo ubiquitination is essential to unveil the mechanisms

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corresponding to different cellular functions and in the pathogenesis of disease. Thus, several proteomic investigations using different methods, including affinity purification, trypsin digestion, and mass spectroscopy (MS) analysis have been performed to identify ubiquitinated proteins in different species, tissues and cells[8].

Multiple myeloma is a hematologic malignancy characterized by the clonal proliferation of plasma cells in the bone marrow with similar clinical phenotypes characterized by over-production of monoclonal immunoglobulins[9-11]. Multiple Myeloma cells are extremely refractory to chemotherapy because of the multiplicity of anti-apoptotic signaling mechanisms[12-14]. In addition, the cytokine IL-6 as an MM growth, survival, and drug-resistance factor plays an important role in the pathogenesis and malignant growth of MM cells[15-16]. MM is the second most common adult hematologic malignancy, and the most common cancer with skeleton as its primary site. Existing treatments including conventional chemotherapy, hematopoietic stem cell transplantation, immune therapy, and target site treatment can prolong survival and improve the quality of life of patients. However, finding a complete cure for MM related cancers is a long-term prospect. Each year, 19 900 new cases are reported in the United States, which accounts for 10% of hematologic malignancies and 1% of all regional cancer deaths. In China, annual incidence of MM is approximately one case per 100 000 persons[17].

To date, a few reports on the application of proteomic technologies towards the study of MM have been published[18-20], but a proteomic study has been lacking to specifically identify ubiquitinated proteins in MM cells. In the present work, we employed a proteomic strategy combining immunoprecipitation with LC-MS/MS and SCX-LC-MS methodologies to globally identify ubiquitinated proteins from MM U266 cell lysate. The ubiquitination sites were identified by detecting signature peptides containing a GG-tag (114.1 Da) and an LRGG-tag (383.2 Da) located internally in lysine residues as well as a GG-tag found on the C-terminus of ubiquitinated peptides[21]. In total, 52 ubiquitinated proteins were identified, including nucleic acid binding proteins, cytoskeletal proteins, Ub-ligating enzymes, transcription factor proteins, and proteins involved in cell cycle regulation, apoptosis and signal transduction. This work represents the first proteomic analysis of native ubiquitinated proteins from genetically unmodified human MM cells.

MATERIALS AND METHODS

Cell Culture

Human MM U266 cells were cultured at 37 °C in 5% CO_2 in DMEM medium(Gibco products, Inc) supplemented with 10% fetal calf serum. After reaching 80% confluence, cells were harvested, washed three times with ice-cold phosphate buffered saline (PBS) and transferred to a clean 1.5 mL Eppendorf tube. Cells were lysed with RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L PMSF, 100 mmol/L leupeptin, and 2 mg/mL aprotinin, pH 8.0). Cellular debris was removed by centrifugation for 30 min at 13 200 g at 4 °C and supernatants were collected. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad) and confirmed by SDS-PAGE.

Purification of the Ubiquitin-related Proteome

The supernatant was collected and 10 mg of the supernatant was pre-cleared with 50 µL equal protein A-Sepharose and G-Sepharose mixture for 1 h at 4 °C and immunoprecipitated with 10 µg of mouse mAb (FK2 clone) (Biomol, Plymouth Meeting, PA) on a rocker at 4 °C overnight. Twenty microliters of equal protein A-Sepharose and G-Sepharose mixture was used to isolate the pellet. After washing the pellet three times in lysis buffer, the protein A- and G-Sepharose-bound immune complexes were resuspended in SDS loading buffer and separated with 10% SDS-PAGE.

Trypsin Digestion

Protein bands were excised from SDS-PAGE gels and subjected to in-gel tryptic digestion as previously described[22]. Digestions were performed for 12 h at 37 °C with MS-grade trypsin (Promega, USA). The supernatants were transferred into a 200 µL microcentrifuge tube and the gels were extracted once with extraction buffer (67% acetonitrile [ACN] containing 2.5% trifluoroacetic acid). The peptide extract and the supernatant of the gel slice were combined and dried completely in a SpeedVac centrifuge.

Peptide Analysis by LC-MS/MS

Peptide Analysis by 1D-LC-MS/MS Dried peptides were reconstituted in 5% ACN/0.1% formic

acid and analyzed with a Finnigan Surveyor HPLC system coupled to a LTQ-Orbitrap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanospray source. The peptide mixture was loaded on a C18 reverse phase column (100 μm i.d, 10 cm long, 5 μm resin; Michrom Bioresources, Auburn, CA) using an autosampler. Peptides were eluted with an increasing 0-35% gradient buffer solution (Buffer A, 0.1% formic acid and 5% ACN; Buffer B, 0.1% formic acid and 95% ACN) over 90 min and monitored by MS. The general MS conditions were: spray voltage, 1.80 kV; without sheath and auxiliary gas flow; ion transfer tube was heated to 200 °C. Ion selection thresholds were: 1 000 counts for MS^2 and 500 counts for MS^3. An activation q=0.25 and activation time of 30 ms were applied to MS^2 acquisitions. The mass spectrometers were operated in positive ion mode using a data-dependent automatic switch between MS and MS^2 acquisition modes. For each cycle, one full MS scan in the LTQ Orbitrap at 1×10^6 AGC target was followed by five MS^2 in the LTQ at 5×10^3 AGC target on the five most intense ions. Selected ions were excluded from further analysis for 90 s. Maximum ion accumulation time was set at 500 ms for full MS scans and 100 ms for MS^2 scans. All MS/MS spectra were collected with normalized collision energy (set at 35%), an isolation window of 3 m/z, and 1 micro-scan. The application of mass spectrometer scan functions and HPLC solvent gradients were controlled by XCalibur data system (Thermo Fisher Scientific, Waltham, MA).

Peptide Analysis by SCX-LC-MS (online 2D-LC-MS/MS) The peptides were firstly loaded on a strong cation exchange (SCX) column using an autosampler, and the peptides were eluted by NH_4Cl with different concentrations (1 mmol/L, 10 mmol/L, 100 mmol/L, 200 mmol/L, 1 mol/L). Each fraction peptide was loaded onto a C18 column (as described earlier) using an autosampler. Peptides were eluted using a 0-35% gradient (using the same composition for buffer A and buffer B as described earlier) over 90 min and online detected in LTQ-Orbitrap. LC-MS/MS analysis was performed as described earlier (Peptide analysis by 1D-LC-MS/MS) .

Protein Identification

The acquired raw files were processed with BioWorks 3.3.1 (Thermo Finnigan, San Jose, CA) and the MS/MS spectra were searched using the Mascot 2.2.04 search engine (Matrix Science, London, UK) against a real and reverse ipi.HUMAN.3.49.fasta database respectively (each database included 74 017 protein entries). The following search criteria were employed: full tryptic specificity was required and two missed cleavages were allowed; parent ion mass tolerance 10 ppm; Cys (+57.0215 Da, Carbamidomethylation) was set as fixed modification and ubiquitination (GG and LRGG remnant tags) was considered as variable modifications. The false positive rate for peptide identification was kept below 1%. MS/MS spectra of ubiquitinated proteins were manually verified to confirm and map ubiquitination sites.

Protein Categorization

The identified proteins were classified based on the PANTHER (Protein ANalysis THrough Evolutionary Relationships) system (http://www.pantherdb.org), a unique resource that classifies genes and proteins by their functions. The PANTHER ontology, a highly controlled vocabulary(ontology terms) by biological process, molecular function and molecular pathway was used to categorize proteins into families and subfamilies with shared functions.

RESULTS

SDS-PAGE Map of Ubiquitinated Proteins from Human Multiple Myeloma Cells

Ubiquitinated proteins from human MM U266 cells were separated by SDS-PAGE gel electrophoresis and silver staining (Figure 1). Anti-ubiquitin antibodies

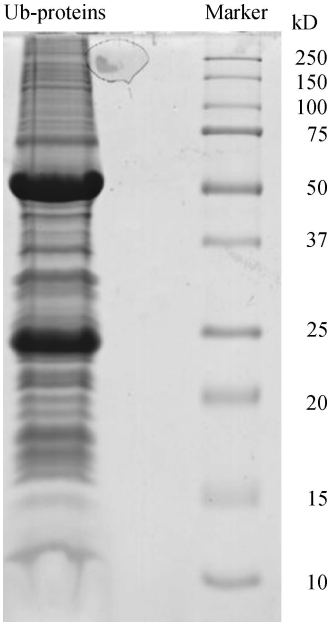

Figure 1. Purification of ubiquitinated proteins from human multiple myeloma U266 cells.

were used to enrich the yield of ubiquitinated proteins by immunoprecipitation. A series of immunoprecipitations were performed using the anti-Ub antibodies to confirm the efficiency of the purification of ubiquitinated proteins from human MM U266 cells and thus to optimize the experimental conditions. The optimization factors that were considered included the ratio of anti-Ub beads and sample material, incubation time to ensure strong antibody-antigen interactions, the frequency of washing steps to eliminate non-specific background proteins and the optimal of elution buffer[1]. The typical gel pattern shown in Figure 1 demonstrated the efficient immunoprecipitation under the optimized conditions.

Identification of Ubiquitinated Proteins and Sites

Analysis by LC-MS/MS and SCX-LC-MS of gel bands subjected to tryptic digestion identified a total of 52 ubiquitinated proteins. These proteins embody 73 ubiquitination sites including 59 ubiquitinated sites with the GG-tag and 14 ubiquitinated sites with the LRGG-tag (Table 1). The proportion of the ubiquitination sites identified as internal GG or LRGG tags and the C-terminal GG tag is shown in Figure 2.

Functional Analysis of Identified Ubiquitinated Proteins

We categorized the ubiquitinated proteins identified in accordance with their molecular function in the biological process using the PANTHER classification system. The ubiquitinated proteins may be classified into 14 groups with different functions (Figure 3), and thus providing an overview for the ubiquitinated proteome in MS in humans.

About 45% of the ubiquitinated proteins were nucleic acid binding proteins, representing the largest proportion of all the proteins identified. The second largest category of ubiquitinated proteins identified were cytoskeletal proteins (~13%). The other ubiquitinated proteins were further categorized into other functional groups including

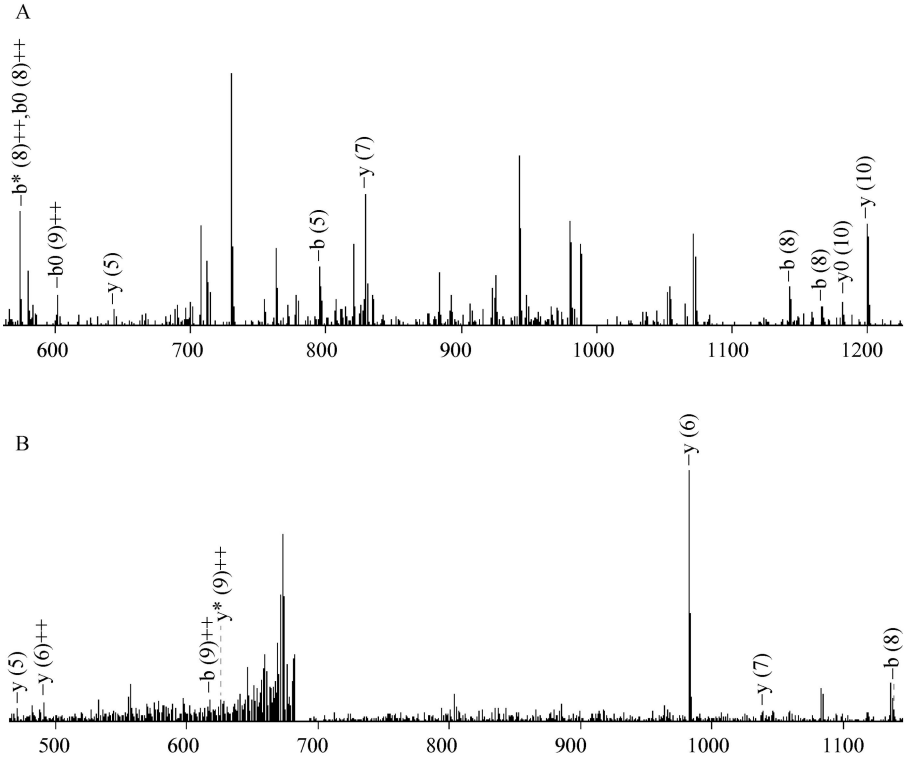

Figure 2. Identification of ubiquitination sites by SCX-LC-MS and LC-MS/MS. (A) Single tag detection for the ubiquitinated peptide (IVAPGK$_{28}$GILAADESTGSIAK). A single GG tag is located at K28. (B) Dual tag detection for the ubiquitinated peptide (EKIAAK$_{541}$NALESYA FNMK$_{552}$). Both GG and LRGG tags are detected at positions K541 and K552, respectively. In the MS/MS analysis, the matched b- and y-ions for the given peptide sequence are labeled.

Table 1. Proteins with Ubiquitination Sites Identified from Human Multiple Myeloma U266 Cells

Acc No	Protein ID	Prot_ Score	GG tag Position	GG tag Count	LRGG tag Position	LRGG tag Count	Total_ Count	MS Method for Detection
IPI00304596	Non-POU domain-containing octamer-binding protein	5 232	96; 99	2	0	0	2	SCX-LC-MS
IPI00218343	Tubulin alpha-1C chain	4 993	326	1	0	0	1	SCX-LC-MS
IPI00792677	cDNA FLJ60097, highly similar to Tubulin alpha-ubiquitous chain	4 832	291	1	0	0	1	SCX-LC-MS
IPI00011654	Tubulin beta chain	4 463	58	1	0	0	1	SCX-LC-MS
IPI00013475	Tubulin beta-2A chain	3 431	336	1	0	0	1	SCX-LC-MS
IPI00007752	Tubulin beta-2C chain	3 391	58	1	0	0	1	SCX-LC-MS
IPI00012726	Isoform 1 of polyadenylate-binding protein 4	1 832	240	1	0	0	1	SCX-LC-MS
IPI00016249	Isoform 1 of fragile X mental retardation syndrome-related protein 1	872	416	1	0	0	1	SCX-LC-MS
IPI00554715	Isoform 2 of fragile X mental retardation syndrome-related protein 1	872	416	1	0	0	1	SCX-LC-MS
IPI00007702	Heat shock-related 70 kDa protein 2	806	129; 139	2	0	0	2	SCX-LC-MS
IPI00903278	Heterogeneous nuclear ribonucleoprotein D	474	144	1	149	1	2	SCX-LC-MS
IPI00028888	Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	474	165	1	170	1	2	SCX-LC-MS
IPI00844578	ATP-dependent RNA helicase A	321	733;735;737	3	0	0	3	SCX-LC-MS
IPI00470658	Heterogeneous nuclear ribonucleoprotein A3 pseudogene 1	294	0	0	199	1	1	SCX-LC-MS
IPI00384463	Eukaryotic translation initiation factor 4 gamma, 1 isoform 2	264	777	1	0	0	1	SCX-LC-MS
IPI00011603	26S proteasome non-ATPase regulatory subunit 3	259	194	1	0	0	1	SCX-LC-MS
IPI00029012	Eukaryotic translation initiation factor 3 subunit A	234	38	1	0	0	1	SCX-LC-MS
IPI00013881	Heterogeneous nuclear ribonucleoprotein H	208	14	1	0	0	1	SCX-LC-MS
IPI00384992	Myosin light chain 4	132	9	1	0	0	1	SCX-LC-MS
IPI00008530	60S acidic ribosomal protein P0	124	50; 57	2	0	0	2	SCX-LC-MS
IPI00647650	cDNA FLJ35809 fis, clone TESTI2006016, highly similar to eukaryotic translation initiation factor 3 subunit 3	94	38	1	0	0	1	SCX-LC-MS
IPI00005614	Isoform long of spectrin beta chain, brain 1	79	968	1	0	0	1	SCX-LC-MS

(Continued)

Acc No	Protein ID	Prot_ Score	GG tag Position	GG tag Count	LRGG tag Position	LRGG tag Count	Total_ Count	MS Method for Detection
IPI00021926	Proteasome 26S ATPase subunit 6	58	0	0	336	1	1	SCX-LC-MS
IPI00031627	DNA-directed RNA polymerase II subunit rpb1	55	874	1	0	0	1	SCX-LC-MS
IPI00291755	Isoform 1 of nuclear pore membrane glycoprotein 210	54	212; 217	2	0	0	2	SCX-LC-MS
IPI00889791	Putative uncharacterized protein	37	427	1	0	0	1	SCX-LC-MS
IPI00746059	HECT, UBA and WWE domain containing 1	458	16; 20	2	0	0	2	SCX-LC-MS
IPI00299904	Isoform 1 of DNA replication licensing factor MCM7	67	641	1	0	0	1	SCX-LC-MS
IPI00376143	Minichromosome maintenance complex component 7 isoform 2	67	465	1	0	0	1	SCX-LC-MS
IPI00552897	Isoform 1 of mediator of DNA damage checkpoint protein 1	59	1010	1	0	0	1	SCX-LC-MS
IPI00465439	Fructose-bisphosphate aldolase A	46	28	1	0	0	1	SCX-LC-MS
IPI00179330	Ubiquitin and ribosomal protein S27a precursor	18764	48	1	0	0	1	LC-MS/MS
IPI00015953	Isoform 1 of nucleolar RNA helicase 2	4928	75	1	0	0	1	LC-MS/MS
IPI00083708	Isoform 7 of BAT2 domain-containing protein 1	777	1630	1	0	0	1	LC-MS/MS
IPI00024067	Isoform 1 of clathrin heavy chain 1	415	1113; 1118; 1122	3	0	0	3	LC-MS/MS
IPI00301277	Heat shock 70 kDa protein 1L	333	541	1	552	1	2	LC-MS/MS
IPI00165434	Ylp motif containing 1	301	0	0	318	1	1	LC-MS/MS
IPI00384463	Eukaryotic translation initiation factor 4 gamma, 1 isoform 2	293	794	1	0	0	1	LC-MS/MS
IPI00001159	Translational activator GCN1	288	0	0	1134	1	1	LC-MS/MS
IPI00400922	Protein rrp5 homolog	257	553;560;571	3	0	0	3	LC-MS/MS
IPI00010700	Isoform 1 of large proline-rich protein BAT2	214	0	0	990	1	1	LC-MS/MS
IPI00215743	Isoform 3 of ribosome-binding protein 1	172	51	1	47	1	2	LC-MS/MS
IPI00384456	Isoform GTBP-N of DNA mismatch repair protein Msh6	126	334	1	0	0	1	LC-MS/MS
IPI00420014	Isoform 1 of U5 small nuclear ribonucleoprotein 200 kDa helicase	109	1142; 1145	2	0	0	2	LC-MS/MS
IPI00022774	Transitional endoplasmic reticulum ATPase	83	0	0	8	1	1	LC-MS/MS
IPI00025447	Elongation factor 1-alpha	80	429	1	432	1	2	LC-MS/MS

(Continued)

Acc No	Protein ID	Prot_ Score	GG tag Position	GG tag Count	LRGG tag Position	LRGG tag Count	Total_ Count	MS Method for Detection
IPI00299254	Eukaryotic translation initiation factor 5B	69	406	1	410	1	2	LC-MS/MS
IPI00456969	Cytoplasmic dynein 1 heavy chain 1	52	1498	1	0	0	1	LC-MS/MS
IPI00004290	Digestive organ expansion factor homolog	40	680	1	0	0	1	LC-MS/MS
IPI00178352	Isoform 1 of Filamin-C	39	517	1	514	1	2	LC-MS/MS
IPI00004970	Small subunit processome component 20 homolog	35	2510; 2519	2	2509	1	3	LC-MS/MS
IPI00797574	Protein Fam44A	33	2921	1	0	0	1	LC-MS/MS

hydrolases (~7%), transcription factors (~5%), lyases (~4%), chaperones(~2%), select regulatory molecules (~2%), membrane traffic proteins (~2%), select calcium binding proteins (~2%), receptors (~2%), extracellular matrix proteins (~2%), ligases (~1%), and transferases (~1%). The remaining proteins with their subfamily members that could not be assigned to a particular protein class were categorized as "molecular function unclassified" (~9%) (Figure 3).

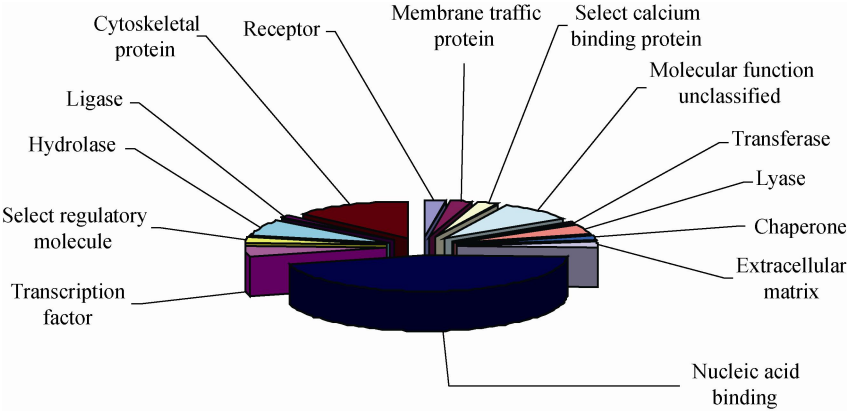

Figure 3. Functional classification of ubiquitinated proteins found in human multiple myeloma U266 cells. The pie chart represents the distribution of identified ubiquitinated proteins according to their functions. Categorization was based on information obtained from the online resource PANTHER classification system.

DISCUSSION

Ubiquitination is an important protein posttranslational modification that plays a pivotal role in the dynamic balance and functional regulation of proteins. Although many substrates of the Ub-proteasome pathway have been individually characterized by biochemical and genetic approaches, only several large-scale researches on ubiquitinated proteins have been reported primarily due to the difficulties encountered in isolating these low-abundance proteins using conventional purification methods[1]. In 2003, Peng et al. identified 1 075 potential ubiquitination substrates from a yeast strain stably expressing 6x His-tagged Ub[23]. Excellent work was also performed by Hitchcock et al. in 2003 in the identification of membrane-associated ubiquitinated proteins in response to mutations in the endoplasmic reticulum degradation machinery[24]. Apart from these studies, our current work represents the first global identification of ubiquitinated proteins in MM U266 cells. We found that the identification of ubiquitination sites should not solely be limited to the analysis of GG tags on internal lysine residues and more ubiquitination sites may be identified if additional mass tags are

considered in analysis[21].

Nucleic acid binding proteins play an important role in transcription, replication, repair and translation of DNA and RNA[25]. Proteins such as HUWE1 (HECT, UBA, and WWE domain containing 1) and UBC (ubiquitin and ribosomal protein S27a precursor) identified in this study, suggests their involvement in the ubiquitination process in myeloma cells.

E3 ubiquitin ligases catalyze the final step of ubiquitin conjugation and regulate numerous cellular processes. The HUWE1 class of E3 ubiquitin ligases directly transfer Ub from the bound E2 enzyme to a myriad of substrates. The catalytic domain of Ub ligases HUWE1 has a bilobal architecture that separates the E2 binding region and catalytic site. The function of HUWE1 is to control Ub ligase activity and specificity[26]. E3 Ubiquitin assists in ribosome biogenesis and also functions as a posttranslational modifier after its release from S27a where Ub is expressed[27]. In this context, HUWE1 and E3 Ub ligases may play a role in the formation and expression of ubiquitination in myeloma.

Cytoskeletal proteins mainly involve in cell movement, mitosis, particle intake and discharge, cell adhesion, axonal transport, and the regulation of receptors in signal transduction[28]. The present experiment identified 11 cytoskeletal proteins, which can be divided into five groups, namely myosin, tubulin, spectrin, dynein, and actin proteins.

The five identified tubulin proteins were TUBA1C (tubulin alpha-1C chain), TUBA1B (cDNA FLJ60097, highly similar to the tubulin alpha-ubiquitous chain), TUBB (tubulin beta chain), TUBB2A (tubulin beta-2A chain), and TUBB2C (tubulin beta-2C chain). The tubulin proteins represent a large number of cytoskeletal proteins with a crucial role in the regulation of microfilaments which may be implicated in many pathologies. For example, the correct folding of tubulin monomers and the formation of functional heterodimers regulated by a series of cellular events involving of chaperonins and cofactors[29]. Research results also showed that microtubules and tubulin alpha/beta heterodimers are bound strongly by ubiquitinated proteins and enhance ubiquitination and degradation of alpha and beta-tubulin. In this regard, E3 ligase activity may play an important role in regulating misfolded tubulins produced during the complex and reversible folding processes[30].

A large proportion of proteins identified in this study belong to the Ub-proteasome system, including protein VCP (transitional endoplasmic reticulum ATPase), HSPA2 (heat shock-related 70 kDa protein 2), and RPS27A (ubiquitin and ribosomal protein S27a precursor). Those proteins can regulate many different cellular functions including transcription, antigen processing, signal transduction and cell cycle.

The proteasome is a multisubunit complex responsible for the degradation of ubiquitinated substrates, which plays a critical role in regulating a variety of biological processes[31]. Alterations of in the ubiquitination pathway contribute to the pathogenesis of diseases including cancer, neurodegenerative disorders and viral infections[32]. In this study, the identification of ubiquitination sites may help in elucidating the involvement of ubiquitination associated pathways in the molecular mechanism of human multiple myeloma[33].

It becomes increasingly evident that ubiquitination is critically involved not only in the degradation of proteins needlessly, but also in a plethora of fundamental eukaryotic cellular processes including cell cycle progression, DNA repair, signal transduction, and membrane trafficking[34-35]. As a systematic approach, proteomics has demonstrated its advantage in the comprehensive identification of ubiquitinated proteins[36-37]. The current study is a preliminary application of proteomics to analyze the ubiquitination in genetically unmodified/untransfected in human MM U266 cells, allowing the identification of 52 ubiquitination proteins or substrates and 73 ubiquitination sites. Analyses of these Ub proteins and sites may aid our understanding of the ubiquitination of endogenous proteins under physiologic and pathologic conditions[38] Further work is required to characterize the proteins and sites identified in the study and thus to demonstrate their involvement in ubiquitination pathways in human myeloma**.**

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