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Profiling of Ubiquitination Modification Sites in Talin in Colorectal Carcinoma by Mass Spectrometry

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Abstract Talin protein was partially purified from human colorectal carcinoma tissues, which was subject to tryptic digestion. Immunoaffinity precipitation with specific antibodies that recognize diglycyl-lysine(Lys) remnants from tryptic digestion of ubiquitinated peptides was used to enrich ubiquitinated sites in talin. Mass spectrometry coupled with capillary reverse-phase high-performance liquid chromatography was used to analyze the enriched peptides. Specifically, four peptides containing diglycyl-Lys remnants from talin, namely, TAK(ub)VLVEDTK, QQQYK(ub) FLPSELRDEH, K(ub)STVLQQQYNR, and EGILK(ub)TAK can be determined using mass spectrometric data. This study provides an analytical method for further study in the relationship between ubiquitination modification of talin and its biological activity in colorectal cancer tissues with different pathological processes.

Keywords Talin; Ubiquitination; Colorectal carcinoma; Mass spectrometry

1 Introduction

Colorectal carcinoma(CRC) is a common malignant tumor in Western Europe, North America, and other developed countries. In recent years, CRC in China has developed from low to high incidence[1,2]. Metastasis is the main cause of death of malignant CRC[3,4]. Recent studies have shown that talin, a cytoskeletal protein mainly distributed in cell and intercellular matrixes, is closely related to the occurrence, invasion, and metastasis of malignant tumors[5]. For example, the mRNA level of talin in colorectal cancer tissues is significantly reduced, which is closely related to metastasis and invasion of colorectal cancer[6]. During inside-out signaling, talin head domain can bind to the cytoplasmic tail of β-integrin to activate integrin[7]. Talin was recently identified as a substrate for SUMOylation(a kind of ubiquitin-like modification) in migrating cancer cells[8], thereby highlighting the key role of ubiquitin and ubiquitin-like modifications of talin in the CRC metastasis.

Protein ubiquitination is an important post-translational modification of proteins in organisms. Many cellular biological processes, such as signal transduction, gene expression, cell differentiation, apoptosis, and tumorigenesis, can be precisely regulated by ubiquitination and ubiquitination-like modifications[9—11]. Talin can be modified by ubiquitination, thereby affecting its turnover and biological functions. Studies have shown that talin ubiquitination and degradation can be inhibited through phosphorylation by cyclin-dependent kinase 5[12]. To date, knowledge on the types and sites of post-translational modifications of talin remains limited. Therefore, research of talin ubiquitination, especially under tumor and other pathological conditions, is important for further understanding tumor pathogenesis and metastasis and developing new anti-tumor drugs.

Modern mass spectrometry has been extensively used in many research fields considering its high resolution, quality accuracy, sensitivity, and scanning speed[13—15]. In the present work, talin proteins in human colorectal cancer tissues were extracted and purified through salt-induced precipitation and ion exchange chromatography, and their peptides were obtained through trypsin hydrolysis. The peptides containing diglycyl-lysine(Lys) residue that was a remnant from tryptic digestion of ubiquitinated peptides were enriched by immunoprecipitation and then analyzed by capillary reverse high-performance liquid chromatography(HPLC) and mass spectrometry.

2 Experimental

2.1 Materials

Urea, dithiothreitol(DTT), trifluoroacetic acid(TFA), 4-morpholineethanesulfonic acid(MES), idioacetamide, acetonitrile(ACN), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), ethylenediaminetetraacetic acid(EDTA), sodium dodecyl sulfate(SDS), and DEAE ion exchange column were purchased from Sigma-Aldrich(St. Louis, MO, USA). Sequencing grade trypsin was obtained from Promega(Madison, WI, USA). Anti-diglycyl-Lys(Clone GX41) antibody was obtained from Lucerna(Brooklyn, NY, USA). Sep-Pak C_{18} solid

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phase extraction columns were acquired from Waters(Milford, MA, USA). OMIX C_{18} tips were purchased from Agilent Technologies(Sandwich, MA, USA). Immunoprecipitation kit was obtained from Pierce(Carlsbad, CA, USA). All other reagents with analytical grade were obtained from Sinopharm Chemical Reagent Beijing Company, China.

2.2 Purification of Talin from CRC Tissues

Tissue samples were collected from patients with colorectal cancer without chemotherapy. The type of colorectal cancer was Dukes B adenocarcinoma, as confirmed by histopathological examination. The samples were homogenized in a 10-fold volume of lysis buffer containing 0.5 mmol/L protease inhibitor phenylmethanesulfonyl fluoride(PMSF). Subsequently, centrifugation was performed at 10000 r/min for 15 min. The supernatant was discarded, and a buffer(pH=9.0) containing 500 mmol/L NaCl, 20 mmol/L Tris, 1 mmol/L EGTA, 3 mmol/L mercaptoethanol, 0.5 mmol/L PMSF, was added to the precipitate and stirred overnight at 4 °C to extract the proteins from the precipitate. Ammonium sulfate with concentrations between 70 and 280 g/L was used to fractionate the proteins and then full dialysis was performed in a buffer (pH=8.0) containing 20 mmol/L Tris, 3 mmol/L EGTA, 3 mmol/L mercaptoethanol, 0.5 mmol/L PMSF. After verification by centrifugation, the protein solution was loaded onto a DEAE ion exchange column(DE52), which was previously washed with the same buffer. A linear salt gradient of 0—200 mmol/L NaCl was applied to elute the proteins. The eluates between 45 and 70 mmol/L NaCl were collected and fully dialyzed against a buffer containing 2 mol/L urea, 3 mmol/L EDTA, and 50 mmol/L NH_4HCO_3. Then, the protein sample was kept at −80 °C until use.

2.3 Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis

The protein sample was boiled with an equal volume of reducing sample buffer[20 g/L SDS, 20%(volume fraction) glycerol, 20 mmol/L Tris-HCl, pH=6.8, 6 mmol/L mercaptoethanol, and trace bromophenol blue] for 5 min and then separated by vertical electrophoresis on Mini-PROTEAN Tetra Cell. After electrophoresis, the films were stained with Coomassie brilliant blue R250 and scanned.

2.4 Sample Preparation for Immunoprecipitation

A total of 1 mol/L DTT solution was added to the protein sample at the final concentration of 5 mmol/L and kept at 56 °C for 1 h. Then, 1 mol/L iodoacetamide solution was added to the final concentration of 10 mmol/L and kept in the dark at room temperature for 30 min. Subsequently, the sequencing-grade trypsin was added at the mass ratio of 1:100. The protein solution was kept overnight at 37 °C. Formic acid solution[1%(volume ratio) final concentration] was added to satisfy the enzymatic reaction. Afterward, the solution was verified at 12000 r/min and transferred to a new tube. After enzyme digestion, the peptide samples were desalted by Sep-Pak C_{18} solid-phase extraction column and then freeze-dried and stored at −20 °C.

2.5 Immunoprecipitation

Anti-diglycyl-Lys antibody was coupled to the surface of agarose magnetic beads in accordance with the instructions of the immunoprecipitation kit, followed by washing with an immunoprecipitation buffer[50 mmol/L 3-(*N*-morpholino)-propanesulfonic acid(MOPS)/NaOH, pH=7.2, 10 mmol/L Na_2HPO_4, and 50 mmol/L NaCl). The freeze-dried peptides were dissolved in the above mentioned immunoprecipitation buffer, mixed with the magnetic beads, and kept being shaken at room temperature for 2 h. Then, the beads were collected by magnetic force, followed by several times of washing. The peptides on the beads were eluted with an elution buffer then desalted by OMIX C_{18} tips, freeze-dried and stored at −20 °C.

2.6 LC-MS/MS Analysis

The dried peptide samples were dissolved in buffer A(99% water+1% acetonitrile+0.1% formic acid) and loaded onto a homemade reversed-phase capillary column(150 mm, 0.075 mm, and 3 μm) using an Easy-nLC 1000 Nanoliter liquid chromatography system(Thermo Scientific Company, USA). The peptides were eluted by gradient combination with mobile phases A and B(B: 99% acetonitrile +1% water +0.1% formic acid). Then, the eluted peptides were analyzed using Q exactive mass spectrometer(Thermo Scientific Company, USA), under which the scanning mode was data-dependent acquisition (DDA).

2.7 Mass Spectrometry Data Processing

The collected mass spectra were searched against the *Homo sapiens* sub-database of the SwissProt protein database using Maxquant software(version 1.5). The search parameters were set as follows: the protease was trypsin; up to two missed cleavages were allowed; the maximum error for MS1 ion was 0.15; the maximum allowable error of daughter ion was 0.6; and carbamidomethylation at cysteine was set as a fixed modification. Oxidation of methionine and diglycyl adduct of Lys was set as variable modification. The resulting data were filtered with the following criteria: the protein error detection rate was controlled below 1%, and the peptide level threshold exceeded 95%.

3 Results and Discussion

3.1 Isolation and Purification of Talin from CRC Tissues

Colorectal cancer tissue samples were cut into pieces and homogenized in deionized water, followed by centrifugation. The supernatant was discarded, and the protein components in the precipitation were extracted using a buffer. The crude extract of talin was obtained through salt-induced precipitation and ion exchange chromatography. In Fig.1, talin(molecular weight 270000) was effectively separated from other small

molecular weight proteins. After digestion by trypsin, the proteins were converted into low molecular peptides, of which the bands were undetected.

Fig.1 Purification and tryptic digestion of talin
Lane 1: crude extract from CRC tissues; lane 2: purified talin from CRC tissues; lane 3: tryptic digestion of talin samples.

3.2 Mass Spectrometry Analysis of Digestion of Partially Purified Talin

The partially purified protein sample was subject to reduction using DTT, followed by alkylation *via* iodoacetamide. Then, the protein sample was digested by sequencing-grade trypsin, and the resulting peptide samples were desalted and analyzed by mass spectrometry. Database search results indicated that most peptide signals were derived from talin, whereas several peptides, such as ACTA2, TUBB, HSPA5, COL6A1, HBB, and DES, from other proteins that were co-purified with talin, were also identified. Talin was identified with a sequence coverage of 74.84%. As expected, no peptides containing diglycyl-Lys remnants were detected. The identification information of all these proteins is summarized in Table 1.

Table 1 Summary of identified proteins in partially purified sample preparation

Protein	Molecular weight	Isoelectric point	Amino acid count	Coverage(%)
TLN1 Talin-1	269767	5.71	2541	74.84
ACTA2 Actin, aortic smooth muscle	42009	5.12	377	70.62
TUBB Tubulin *β*-chain	49671	4.64	444	30.14
HSPA5 HSPA5 protein	72422	4.92	655	23.16
COL6A1 Collagen alpha-1(VI) chain	108529	5.13	1028	22.86
HBB Hemoglobin subunit β	15998	6.88	147	51.16
DES Desmin	53536	5.07	470	54.04
IGHA2 Ig alpha-2 chain C region	36508	5.67	340	42.88
HSPD1 60000 heat shock protein, mitochondrial	61055	5.59	573	34.50
LAMB2 Laminin subunit *β*-2	195981	6.06	1798	9.23
RPSA 40S ribosomal protein SA	32854	4.64	295	23.46

3.3 Mass Spectrometry Analysis of Ubiquitination Sites in Talin

The detection of low abundant ubiquitinated proteins in a physiological environment has been a considerable challenge in proteomics fields. To date, an effective enrichment by a specific antibody that can recognize diglycyl-Lys remnants prior to mass spectrometric analysis has been frequently utilized. In the present study, the peptide mixture from tryptic digestion was incubated with the anti-diglycyl-Lys antibody, by which the peptides containing diglycyl-Lys remnants were significantly enriched. Then, the enriched peptides were subjected to mass spectrometry analysis and database searches. Database search results showed that 10 unique peptide sequences from talin protein are positively identified, as summarized in Table 2. Among these sequences, four peptides containing diglycyl-Lys remnants from talin were identified; the four peptides were TAK(ub)VLVEDTK, QQQYK(ub)FLPSELRDEH, K(ub)STVLQQQYNR, and EGILK(ub)TAK, as indicated in Fig.2(A—D), respectively.

In addition to the modified peptides, six unmodified peptide sequences, namely, HKAGFLDLKDFLPK, TYGVSFFLVK, GLAGAVSELLR, GVGAAATAVTQALNELLQHVK, TMLESAGGLIQTAR, and TLSHPQQMALLDQTK, were co-purified from the tryptic peptide mixture, as illustrated in Fig.3(A—F), respectively.

Table 2 Peptide sequences of talin identified from affinity enrichment by anti-diglycyl-Lys antibody

Sequence	Modification*	MS, *m/z*		Charge	Error	Start	Stop
		Experimental	Theoretical mass(M+H)				
HKAGFLDLKDFLPK		407.98	1628.91	4	−0.00035	255	268
TYGVSFFLVK		580.82	1160.63	2	−0.00002	307	316
kSTVLQQQYNR	DiGG(+114)	493.59	1478.77	3	−0.00006	428	438
GLAGAVSELLR		543.32	1085.63	2	−0.00010	614	624
GVGAAATAVTQALNELLQHVK		1046.08	2091.16	2	−0.00016	766	786
TMLESAGGLIQTAR		724.38	1447.76	2	−0.00020	1605	1618
TLSHPQQMALLDQTK		570.97	1710.88	3	−0.00033	1752	1766
EGILkTAK	DiGG(+114)	325.19	973.57	3	−0.00004	2017	2024
TAkVLVEDTK	DiGG(+114)	609.34	1217.67	2	−0.00020	2022	2031
QQQYkFLPSELRDEH	DiGG(+114)	508.75	2031.99	4	−0.00023	2527	2541

* Refers to the type of modifications(diglycyl-Lys, DiGG) identified in the peptide sequences.

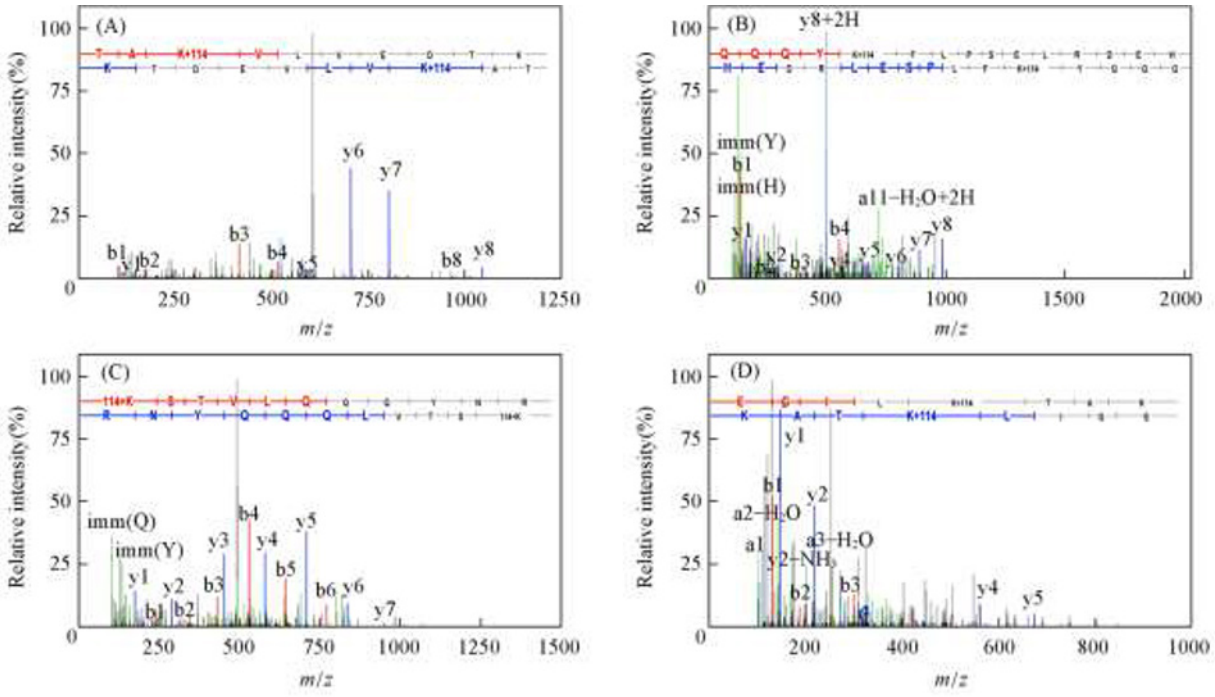

Fig.2 MS/MS spectra of identified peptides with a double-charged peak of TAkVLEDTK(A), a quadruple-charged peak of QQQQYkFLP-SELRDEH(B), a triple-charged peak of kSTVLQQQYNR(C) and triple-charged peak of EGILkTAK(D), respectively

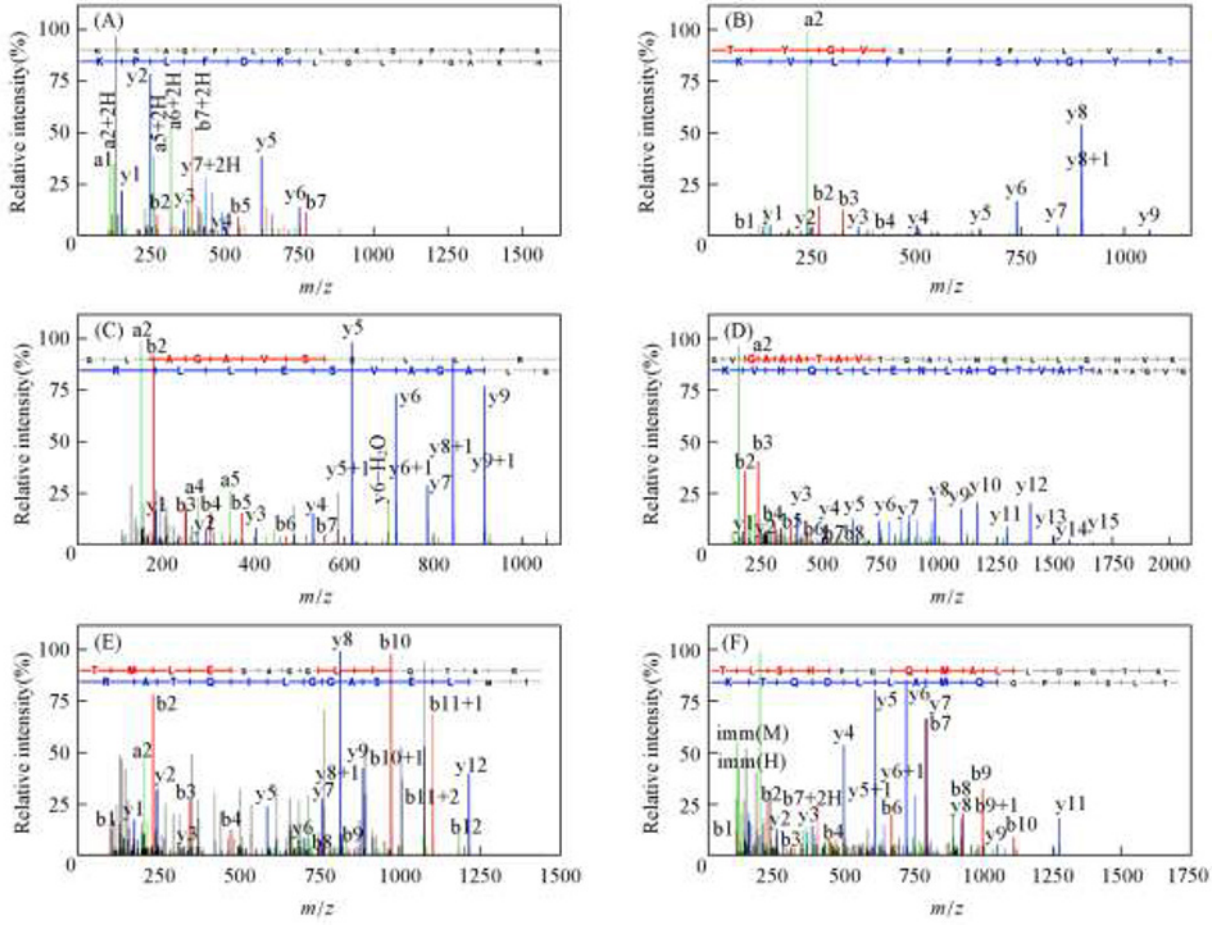

Fig.3 MS/MS spectra of identified peptides with a quadruple-charged peak of HKAGFLDLKDFLPK(A), a double-charged peak of TYGVSFFLVK(B), a double-charged peak of GLAGAVSELLR(C), a double-charged peak of GVGAAATAV-TQALNELLQHVK(D), a double-charged peak of TMLESAGGLIQTAR(E) and a triple-charged peak of TLSHPQQMALLDQTK(F), respectively

In Fig.3, all the six peptide sequences were consistent with the MS/MS spectra. For example, we selected an MS/MS spectrum with a double-charged peak at *m/z* 724.38 to further illustrate the confirmation of the identified sequence (TMLESAGGLIQTAR). In Fig.3(E), nearly all y-series fragment ions, such as y1(*m/z* 175.12), y2(*m/z* 246.18), y3 (*m/z* 346.18), y4(*m/z* 475.27), y5(*m/z* 588.35), y7(*m/z* 758.46), y8(*m/z* 815.47), y9(*m/z* 886.51), y10(*m/z* 973.54), y11 (*m/z* 1102.58), and y12(*m/z* 1215.68), were definitely assigned. Additionally, most b-series fragment ions, including b1 (*m/z* 102.06), b2(*m/z* 233.10), b3(*m/z* 346.18), b4(*m/z* 475.27), and b10(*m/z* 973.54), could be easily assigned. The identified fragment ions displayed *m/z* errors below 0.15, which reflected a high quality of mass data obtained in this experiment, as depicted in Fig.4.

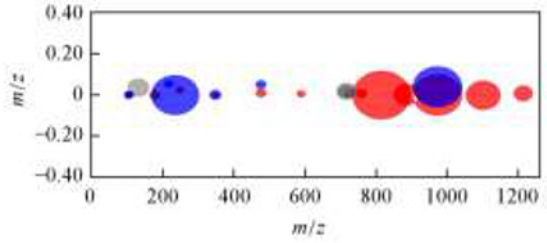

Fig.4 Bubble plot of the *m/z* error of fragment ions of a double-charged peak at *m/z* 724.38 identified as TMLESAGGLIQTAR

The y- and b-series ions were displayed in red and blue, correspondingly. The size(diameter) of the bubbles represented the magnitude of the *m/z* error of each assigned fragment ion.

The application of mass spectrometry can not only qualitatively and quantitatively analyze proteins in various physiological conditions[16—18] but also precisely locate various post-translational modifications of proteins[19—21]. Given the low abundance of ubiquitinated proteins *in vivo*, modified proteins or peptides must be enriched to minimize the influence of high abundance of non-modified peptides and improve the sensitivity of mass spectrometry detection of modified peptides. At present, immunoaffinity precipitation with specific antibodies that recognize the diglycyl-Lys remnants from tryptic digestion of ubiquitinated peptides is a primary method for enriching ubiquitinated peptides. In the present study, we used this approach to profile the ubiquitinated sites in talin partially purified from CRC tissues. Four peptides containing diglycyl-Lysine remnants from talin, such as, namely, TAK(ub)-VLVEDTK, QQQYK(ub)FLPSELRDEH, K(ub)STVLQQQ-YNR, and EGILK(ub)TAK were positively identified.

4 Conclusions

In the present study, tryptic peptides from talin, partially purified from human CRC tissues, were immunoaffinity precipitated with a specific antibody that can recognize the peptides containing diglycyl-Lys remnants. The enriched peptides were analyzed by mass spectrometry coupled with capillary reverse-phase HPLC. Specifically, four peptides, namely, TAK(ub)VLVEDTK, QQQYK(ub)FLPSELRDEH, K(ub)STVLQQQYNR, and EGILK(ub)TAK, containing diglycyl-Lys remnants from talin can be determined using the mass spectrometric data. This study provides an analytical method for further studying the relationship between the ubiquitination modification of talin and its biological activity in colorectal cancer tissues with different pathological processes.

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