Down-regulation of Vinculin in Human Colorectal Carcinoma Identified by Proteomics Analysis

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Abstract In the present study, we aimed to globally profile the proteins involved in colorectal carcinoma(CRC), in order to find clues to the pathological process of CRC. Pairs of malignant tissues and their adjacent healthy tissues from patients with colorectal cancer were subject to differential proteomics analysis. Two dimensional electrophoresis coupled with mass spectrometry(2-DE/MS) was used to identify differentially expressed proteins between pairs of tissue samples. A list of proteins relevant to the progression of colorectal tumor was identified by two dimensional gel electrophoresis(2-DE)-based proteomics approach. Among the identified proteins, vinculin was found to be remarkably down-regulated in colorectal carcinoma tissues. In addition, three phosphorylation modifications within the isolated vinculin were identified by in-depth liquid chromatography-tandem mass spectrometry(LC-MS/MS) analysis. Our results provide a basis for further understanding the pathological significance of vinculin in the regulation of carcinogenesis, invasion and metastasis of colorectal tumors.

Keywords Mass spectrometry; Vinculin; Colorectal carcinoma; Phosphorylation
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1 Introduction

Colorectal carcinoma(CRC) remains one of the major public health problems, especially in western countries[1]. Despite the existence of a variety of screening and preventive strategies, the underlying mechanisms of the carcinogensis, invasion and metastasis of CRC is still unclear. Moreover, most of CRCs undergo hepatic metastasis which leads to the high mortality of this disease[2,3]. Until now, efforts have been made to identify genes involved in the development of CRC, some of which may be further evaluated as candidate molecules for diagnostic and prognostic purpose. For instance, Ashktorab *et al*.[4] reported that SEL1L might be a potential CRC biomarker, whose expression is significantly higher in adenoma cells than that in normal mucosa. Sarkar *et al*.[5] found that cyclin D2 expression at the invasive margin of CRC means the metastasis of CRC to liver. Several large-scale screening approaches, including high-throughput tissue microarray, have been applied to identifying the proteins that are associated with poor prognosis of CRC[6,7]. Accumulated evidence suggests that the process of CRC is much complicated and hard to be exclusively attributed to certain gene or protein. Therefore, there is an urgent need for global approaches to identify genes or proteins involved in pathological process of CRC.

Mass spectrometry-based proteomics has become the powerful tool to perform a large scale analysis of complex protein mixtures[8—10]. Specifically, mass spectrometry has been extensively used to characterize post-translational modifications (PTMs) of proteins, such as identifying PTM'ed proteins[11], localizing PTM sites of proteins[12], as well as determining functional PTMs by site-specific quantitation[13], which play pivotal roles in a variety of biological processes[14—17]. To explore the proteins relevant to the progression of colorectal tumor, we adopted two dimensional electrophoresis coupled with mass spectrometry(2-DE/MS) to examine differential expression of proteins between colorectal carcinoma tissue and its adjacent normal colon tissue from 18 patients who received no chemo- or radio-therapeutic treatment prior to surgery.

2 Materials and Methods

2.1 Materials

All chemicals used in this work were purchased from Sigma-Aldrich(St. Louis, USA). Precast immobile pH gradient(IPG) dry strips(pH=4—7) were purchased from Bio-Rad(Hercules, CA, USA). Sequencing grade trypsin was obtained from Promega(Madison, WI, USA). Supel-Tips Ti Pipette Tips were purchased from Supelco.

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2.2 Tissue Samples

A total of 18 pairs of malignant tissues and their adjacent healthy tissues were obtained from patients with CRC diagnosed at dukes B stage colorectal cancer in the Third Hospital of Jilin University(China). Informed consents were obtained from these patients prior to this study. Ethical approval was obtained from the Clinical Research Ethics Committee of the Third Hospital of Jilin University(China). None of the patients received neoadjuvant therapy prior to operation. Tissue samples were carefully isolated, followed by washing with phosphate-buffered saline in sterile Petri dishes. Then, the tissue samples were frozen in liquid nitrogen immediately.

2.3 Protein Extraction and Quantification

Frozen tissues were ground into fine powders under the protection of liquid nitrogen, followed by homogenized in the nine-fold volume of lysis buffer{8 mol/L urea, 40 g/L 3-[(3-cholanidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mmol/L Tris, 50 mmol/L *D*, *L*-dithiothreitol(DTT), 1 mmol/L phenylmethylsulphonyl fluoride and 1 μg/mL protease inhibitors cocktail}.

The homogenate was centrifuged at 40000*g* for 60 min to remove any residue materials. The protein concentration of each diluted sample was determined *via* Bio-Rad protein assay following the instructions of the supplier's manual. The samples were then aliquoted and stored at −80 °C until used for 2-DE.

2.4 2-DE Separation

Samples containing 0.5 mg of protein were diluted to 300 μL with rehydration solution[6 mol/L urea, 20 g/L CHAPS, 65 mmol/L DTT, 0.5%(volume fraction) pH=3—10 Bio-lyte, trace bromophenol blue]. Protein samples were passively absorbed into IPG gel strips(pH=4—7, 17 cm, Bio-Rad) for 16 h. Isoelectric focusing was performed for 80 kVh at 20 ^{o}C on Protean IEF Cell(Bio-Rad, IEF=isoelectric focusing electrophoresis). After equilibrated in equilibration solution, gel strips were subjected to the second dimensional polyacrylamide gel electrophoresis(PAGE) with 12% polyacrylamide. Separation was then carried out on a Protean II xi electrophoresis system (Bio-Rad) at a current of 15 mA/gel until the bromophenol blue reached the bottom of the gel. The gels were stained with silver and then scanned with a high-resolution scanner(GS800, Bio-Rad).

The obtained gel images were analyzed *via* PDQuest software(Version 7.1.1, Bio-Rad) according to the instructions provided by the manufacturer.

2.5 Matrix-assisted Laser Desorption/Ionization Time of Fight(MALDI-TOF) MS Analysis

The spots of interest were excised from the gels and then destained by Farmer's reagent(10 mmol/L potassium ferricyanide and 50 mmol/L sodium thiosulfate) until the silver turned invisible. Having been washed with 200 mL of 25 mmol/L ammonium bicarbonate three times, the gel pieces were dehydrated with acrylonitrile(ACN). The dried gel pieces were then incubated in the digestion solution containing 50 mmol/L NH_4HCO_3 and 0.1 g/L *L*-(tosylamido-2-phenyl)ethyl chloromethyl ketone(TPCK)-trypsin for 12 h at 37 °C. The resulting peptides were extracted three times with 50 μL of aliquots of 5% trifluoroacetic acid in 60% acetonitrile. The extracts were then pooled and concentrated in a vacuum centrifuge and redissolved in 0.5% trifluoroacetic acid(TFA) and 5% ACN for MS analysis. The tryptic peptide sample was mixed with saturated acyano-4-hydroxycinnamic acid(CHCA) matrix solution and vibrated gently. A volume(1 μL) of the mixture containing CHCA matrix was loaded on a 96×2 well hydrophobic plastic surface sample plate(Applied Biosystems) and air-dried. The samples were analyzed with a Voyager DE STR MALDI TOF Mass Spectrometer(Applied Biosystems). Monoisotopic peak masses were used to search against the Swiss-Prot database using MASCOT search engine with the following parameters: one missing cleavage, peptide tolerance of 1 μg/g, variable methionine oxidation and fixed cysteine carbamidomethylation.

2.6 TiO_2 Enrichment of Phosphorylated Peptides

Tips embedded with TiO_2 were successively washed with ACN and 200 mmol/L PBS(pH=7), followed by equilibration with 50% ACN containing 0.1% formic acid. The dried tryptic peptide sample was dissolved in 0.1% formic acid and then loaded onto the fully equilibrated TiO_2 tip. After successively washed with 0.1% formic acid, 100 mmol/L KCl and 50% ACN six times, the peptides were eluted with 1% aqueous ammonia and then lyophilized.

2.7 Nano-LC-MS/MS Analysis

The lyophilized tryptic peptides were redissolved in high performance liquid chromatography(HPLC) buffer A(0.1% formic acid) and then separated on a C_{18} column(100 mm×180 μm i.d.). The elution gradient was from 5% to 80% buffer B(0.1% formic acid, 99% ACN, flow rate: 0.2 μL/min) for 2 h.

Elution peptide ions were detected in a survey scan from 400 to 2000 atomic mass unit(amu, 1 μscans), which was followed by ten data-dependent MS/MS scans(10 μscans each, isolation width 3 amu, 35% normalized collision energy, dynamic exclusion for 1.5 min) in a completely automated fashion on an LTQ XL electrospray ion trap mass spectrometer.

SEQuest was used to search MS/MS spectra against an international protein index(IPI) human protein database. Carbamidomethylation of cysteins was specified as static modification, whereas the phosphorylations of serine, tyrosine and thronine were chosen as variable modifications. The mass tolerance was set to 2.0 and 1.0 for precursor and product ion masses, respectively. Spectral matches were filtered with the following criteria: (1) Rsp=1; (2) DeltaCn$\geqslant$0.19; (3) XCorr$\geqslant$2.2, 2.5, 2.9 for charge states +1, +2 and +3, respectively.

3 Results

3.1 Proteomics Analysis of Vinculin in Human Colorectal Carcinoma

Tissue samples from patients with colorectal cancer were pairwise analyzed by 2-DE. Patients who have received either chemo- or radio-therapeutic treatment prior to surgery were excluded from our study to reduce the interference of chemical remedies. Proteins were extracted from tissue samples and then separated by large format 2-DE. The protein expression pattern of the colorectal carcinoma was compared to that of the adjacent normal colon by means of PDQuest software(Version 7.1.1, Bio-Rad). Fig.1 shows a pair of representative images of the 2-DE. Protein spots with quantitative difference between the pair of samples greater than 2-fold in magnitude were subject to further identification by MALDI-TOF MS. The analysis revealed the changes of 12 reproducible protein spots, including 4 down-regulated and 8 up-regulated protein spots in colorectal carcinoma.

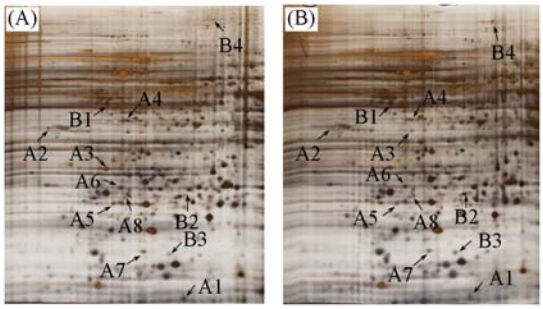

Fig.1 Representative 2-DE maps of colorectal carcinoma(A) and its adjacent normal tissure(B)

IPG strips of pH=4—7(from left to right) were used in the first dimensional IEF separation. Gels were silver-stained. A1. Hemoglobin lepore-baltimore; A2. calreticulin; A3. serpin B8; A4. heterogeneous nuclear ribonucleoprotein K; A5. isoform 3 of tropomyosin alpha-1 chain; A6. vimentin; A7. 6-phosphogluconolactonase; A8. isoform 1 of nucleophosmin; B1. desmin; B2. phosphoglycerate mutase 1; B3. heat shock protein beta-1; B4. vinculin.

The 12 differentially expressed protein spots were identified by MALDI-TOF MS as listed in Fig.2. The expressions of calreticulin(A2), vimentin(A6), isoform 1 of nucleophosmin (A8), serpin B8(A3), 6-phosphogluconolactonase(A7), hemoglobin lepore-baltimore(A1), heterogeneous nuclear ribonucleoprotein K(A4) and isoform 3 of tropomyosin alpha-1 chain(A5) are significantly increased, whereas the expressions of desmin(B1), vinculin(B4), heat shock protein beta-1(B3) and phosphoglycerate mutase 1(B2) are decreased, to some extent, greater than 2-fold.

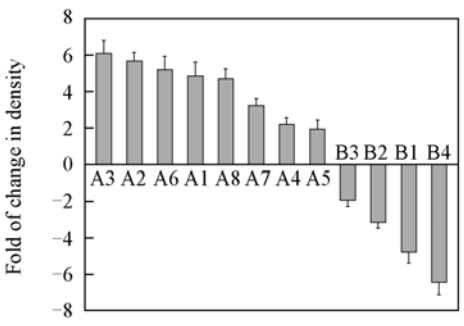

Fig.2 Differentially expressed proteins between colorectal carcinoma and its adjacent normal tissue as determined by 2DE and MS analysis

The up-regulated proteins in the colorectal carcinoma are shown above the *x* axis and the down-regulated proteins in colorectal carcinoma are shown below the *x* axis. Numbers of A1—A8 and B1—B4 are corresponding to those in Fig.1.

3.2 Identification of Phosphorylation Modifications in Vinculin by LC-MS/MS Analysis

To further explore the post translational modifications such as phosphorylation within the identified vinculin, we used TiO_2 embedded in pipette tip to enrich the phosphorylated peptides from tryptic digestion of the isolated vinculin. For the analysis of the enriched peptides, LC-MS/MS was utilized to give an in-depth investigation of phosphorylation modifications in vinculin. In contrast to two-dimensional gel electrophoresis that separates proteins, the non-gel-based LC-MS/MS separates peptides, allowing excellent sensitivity to be achieved due to the direct introduction of peptide samples into the ion source of mass spectrometer[18,19]. Overall, three phosphorylated peptides from the enrichment of the digestion of vinculin were that is, identified, ALASQLQDpSLKDLK[571—584, Fig.3(A)], pSLLDASEEAIKK[721—732, Fig.3(B)], and SFLDSGpYR

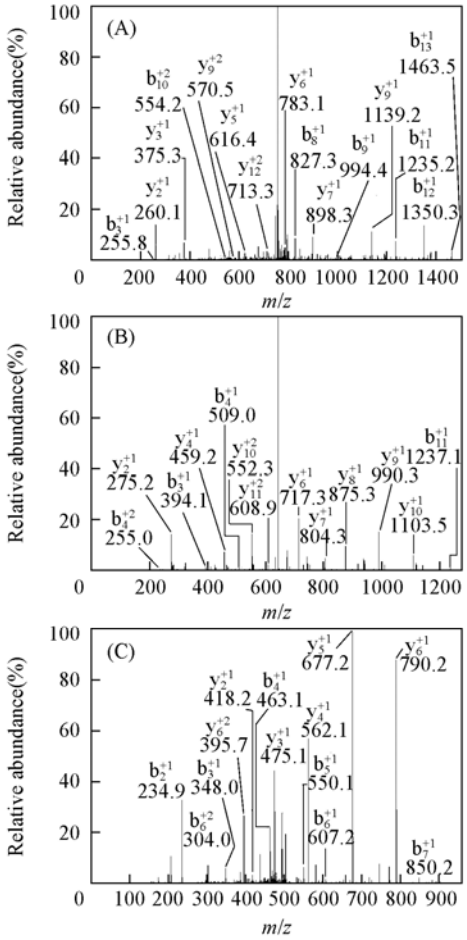

Fig.3 Identification of three phosphorylated peptides from the enrichment of the digestion of vinculin

(A) ALASQLQDpSLKDLK(571—584); (B) pSLLDASEEAIKK(721—732); (C) SFLDSGpYR(816—823).

[816—823, Fig.3(C)].

4 Discussion

A list of proteins showing altered patterns of expression associated with the progression of colorectal carcinoma was identified. Specifically, vinculin was found to be remarkably down-regulated in colorectal carcinoma tissues, compared with that in their normal counterparts. Additionally, in-depth analysis of the isolated vinculin reveals three phosphorylation modifications in this protein, implying a promising role of vinculin in the regulation of the progress of human colorectal carcinoma. It has been well acknowledged that the overexpression of vinculin suppresses cell migration[20,21], whereas the down-regulation of vinculin promotes cell motility[22]. In the present study, down-regulated vinculin in colorectal tumors may reflect a higher invasive potential of the tumor and may be a useful predictive marker for elevated motility of tumor cells in CRC patients.

In conclusion, the current study provides a proteomics analysis on malignant tissues and their adjacent healthy tissues from patients with CRC. Down-regulation of vinculin was identified in malignant tissue, suggesting the elevated tumor cell mobility. This notable feature of vinculin in CRC tissue is relevant to the pathological environment around the tumor cells, which has prognostic significance in the regulation of carcinogenesis, invasion and metastasis of colorectal tumors.

References

[1] Siegel R., Naishadham D., Jemal A., *CA Cancer J. Clin.*, **2012**, *62*, 10

[2] Ishizuka M., Kita J., Shimoda M., Kato M., Sawada T., Kubota K., *Hepatogastroenterology*, **2012**, *59*, 54

[3] Barugel M. E., Vargas C., Krygier W. G., *Expert. Rev. Anticancer Ther.*, **2009**, 9, 1829

[4] Ashktorab H., Green W., Finzi G., Sessa F., Nouraie M., Lee E. L., Morgano A., Moschetta A., Cattaneo M., Mariani-Costantini R., Brim H., Biunno I., *Dig. Dis. Sci.*, **2012**, *57*, 905

[5] Sarkar R., Hunter I. A., Rajaganeshan R., Perry S. L., Guillou P., Jayne D. G., *Colorectal Dis.*, **2010**, *12*, 316

[6] Duncan T. J., Watson N. F., Al-Attar A. H., Scholefield J. H., Durrant L. G., *World J. Surg. Oncol.*, **2007**, *5*, 31

[7] Larsson A., Johansson M. E., Wangefjord S., Gaber A., Nodin B., Kucharzewska P., Welinder C., Belting M., Eberhard J., Johnsson A., Uhlén M., Jirström K., *Br. J. Cancer*, **2011**, *105*, 666

[8] Shi C., Zhao H., Hou N., Chen J., Shi Q., Xue X., Wang J., Zheng C., Zhao L., Yang Y., Wang W., *Chem. Res. Chinese Universities*, **2011**, *27*(1), 87

[9] Wang Z. W., Sun W. C., Zhou Y., Cong Z. Y., Zhou Q. W., Liu N., *Chinese Journal of Analytical Chemistry*, **2012**, *40*, 376

[10] Zhao H., Yang F., Wang W., Wang H., Wei K., *Chem. J. Chinese Universities*, **2010**, *31*(2), 285

[11] Liu N., Song W., Wang P., Lee K. C., Cai Z., Chen H., *Proteomics*, **2010**, *10*, 1875

[12] Baek J. H., Cerda O., Trimmer J. S., *Semin. Cell. Dev. Biol.*, **2011**, *22*, 153

[13] Lu Z., Cheng Z., Zhao Y., Volchenboum S. L., *PLoS One*, **2011**, *6*, e28228

[14] Liu N., Gong K. Z., Cai Y. B., Li Z., *Biochemistry(Moscow)*, **2011**, *76*, 1140

[15] Akimov V., Rigbolt K. T., Nielsen M. M., Blagoev B., *Mol. Biosyst.*, **2011**, *7*, 3223

[16] Liu N., Wang G., Lee K. C., Guan Y., Chen H., Cai Z., *FASEB J.*, **2009**, *23*, 3377

[17] Li Z., Liu N., Zhang L. S., Gong K., Cai Y., Gao W., Liu Z., Liu S., Han Q., Zhang Y., *Proteomics Clin. Appl.*, **2009**, *3*, 1407

[18] Wang H., Wong C. H., Chin A., Kennedy J., Zhang Q., Hanash S., *J. Proteome Res.*, **2009**, *8*, 5412

[19] Liu N., Zhao L., He C., Cai Z., *Current Analytical Chemistry,* **2012**, *8*, 22

[20] Rodríguez Fernández J. L., Geiger B., Salomon D., Ben-Ze'ev A., *Cell Motil. Cytoskeleton*, **1992**, *22*, 127

[21] Rodríguez Fernández J. L., Geiger B., Salomon D., Sabanay I., Zöller M., Ben-Ze'ev A., *J. Cell Biol.*, **1992**, *119*, 427

[22] Rodríguez Fernández J. L., Geiger B., Salomon D, Ben-Ze'ev A., *J. Cell Biol.*, **1993**, *122*, 1285