Up-regulation of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in sentinel lymph nodes of colorectal cancer

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AIM: To investigate the early metastasis-associated proteins in sentinel lymph node micrometastasis (SLNMM) of colorectal cancer (CRC) through comparative proteome.

METHODS: Hydrophobic protein samples were extracted from individual-matched normal lymph nodes (NLN) and SLNMM of CRC. Differentially expressed protein spots were detected by two-dimensional electrophoresis and image analysis, and subsequently identified by matrix assisted laser desorption/ionization-time of flight mass spectrometry-mass spectrometry and Western blotting, respectively.

RESULTS: Forty proteins were differentially expressed in NLN and SLNMM, and 4 metastasis-concerned proteins highly expressed in SLNMM were identified to be hnRNP A1, Ezrin, tubulin β-2C and Annexin A1. Further immunohistochemistry staining of these four proteins showed their clinicopathological characteristics in lymph node metastasis of CRC.

CONCLUSION: Variations of hydrophobic protein expression in NLN and SLNMM of CRC and increased expression of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in SLNMM suggest a significantly elevated early CRC metastasis.

Key words: Colorectal cancer; Micrometastasis; Proteomics; Sentinel lymph node

INTRODUCTION

At present, colorectal cancer (CRC) is the third most common cause of cancer-related death worldwide[1]. Its incidence in China has increased rapidly during the past few decades[2]. Since CRC metastasis has a great effect on the survival of its patients and selection of its treatment mo-
Tissue sample collection

Forty-three cases of moderately differentiated colorectal adenocarcinoma (24 males and 19 females) at the age of 39-80 years (mean ± SD = 51.2 ± 12.6 years), who underwent operation from January 2007 to January 2008, were randomly collected from Department of General Surgery, Second Affiliated Hospital of Nanjing Medical University, China. Endoscopic ultrasonography was carried out 1 d before operation to identify the invasion extent and 0.1% isosulfan blue was injected circumferentially around the neoplasm to mark SLN [8].

A set of lymph nodes were collected during operation and stained with HE and cytokeratin-20 immunohistochemistry (CK-IHC) immediately by two experienced pathologists. Based on HE staining and CK-IHC, the lymph nodes were divided into NLN and SLNMM. All specimens were snap frozen in liquid nitrogen and stored at -80°C until further analysis. All patients recruited in this study received neither chemotherapy nor radiotherapy before surgery. Permission for this study was obtained from the Ethics Committee of Second Affiliated Hospital of Nanjing Medical University. All specimens were anonymous and handled according to the ethical and legal standards.

Protein sample preparation

Protein was extracted from 50 mg of frozen tissue by homogenization in lysis buffer containing 4% CHAPS, 2 mol/L thiourea, 7 mol/L urea, 2% NP-40, 1% Triton X-100, 100 mmol/L DTT, 5 mmol/L PMSF, 0.5 mmol/L EDTA, 2% pharmalyte, 1 mg/mL RNase I, 0.25 mg/mL RNase A, and 40 mmol/L tris-HCl, at pH 8.5, and incubated at room temperature for 2 h. The mixture was centrifuged at 40,000 g for 1 h at 4°C. The supernatant was saved and stored at -70°C. Supernatants from 10 individual specimens corresponding to each group were pooled to minimize the individual variations, and the protein concentration in each mixed sample was measured with the bicinchoninic acid method using PBS as the standard.

Two-dimensional gel electrophoresis and image analysis

Three hundred micrograms protein of each group was loaded onto a 240 mm linear IPG strip (pH 3-10, Amersham Biosciences, Piscataway, NJ) for first-dimensional isoelectric focusing. Protein separation in the second dimension SDS-PAGE (Bio-Rad, Hercules, CA) was carried out on vertical systems, IPG strips were loaded and run on a 125 g/L acrylamide SDS-PAGE gel in electrode buffer (Tris 0.025 mol/L, glycine 0.192 mol/L, SDS 1 g/L, pH 8.3). Electrophoresis was performed with a current of 30 mA/gel for 15 min, followed by 60 mA/gel for 4 h. Each sample was subjected to 2D gel electrophoresis three times to avoid procedural errors. After electrophoresis, the gels were stained with silver nitrate and scanned with an Imagescanner (Amersham Biosciences). The software of PD-Quest 7.3.1 (Bio-Rad) was employed for image analysis, including background abstraction, spot intensity calibration, spot detection, and matching.

Protein identification

Differential protein spots selected were excised from 2-DE gels and cut into small pieces, which were destained, reduced and digested with trypsin overnight. Tryptic digests were extracted and analyzed in a matrix assisted laser desorption/ionization-time of flight mass spectrometry-mass spectrometry (MALDI-TOF-MS) (Bruker, Daltonics, Billerica, MA, USA). The resultant MS data were then searched against NCBI nr and SWISS-PROT databases using the MASCOT search program (Matrix Science, London, UK; http://www.matrixscience.com). Protein identities were assigned if at least 4 peptide masses were matched within a maximum of 100 ppm error spread across the data set and the candidate agreed with the estimated pI and molecular weight from the 2-DE gel.

Western blotting

Tissue samples were lysed following the method for 2-DE described above. Aliquots of protein extracts (50 mg) were separated on a 12.5% SDS-polyacrylamide gel. Sub-
sequently, the protein was electrophoretically transferred onto a PVDF membrane (Bio-Rad). After blocked with TBS-Tween 20 (TBST) containing 10% skim milk, the membranes were incubated with mouse monoclonal antibodies against mouse hnRNP A1 and tubulin β-2C, and rabbit polyclonal antibodies against mouse Annexin A1 and Ezrin for 1 h, respectively, followed by peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:10000 in TBST for 1 h. Finally, blots were developed with chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA). In order to equal protein loading, blots were re-stained using anti-actin antibody (Santa Cruz Biotechnology) as a control.

**Immunohistochemistry analysis**

Formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated using xylene and a series of graded alcohol, respectively. Tissue sections were treated with 3% hydrogen peroxidase for 15 min at room temperature, followed by incubation overnight at 4°C with anti-hnRNP A1 (1:50 Gmbh, Forckenbeckstr, Aachen, Germany), anti-tubulin β-2C (1:50 Saier Biotechnology Inc, Wuhan, China), anti-Annexin A1 (1:100 Saier Biotechnology Inc, Wuhan, China), and anti-Ezrin (1:50 Gmbh, Forckenbeckstr, Aachen, Germany) antibodies, respectively. Finally, the tissue sections were incubated with ready to use peroxidase-conjugated goat anti-rabbit antibody (MaiXin, Fuzhou, China), developed with diaminobenzidine as chromogen, and counterstained with hematoxylin.

**Statistical analysis**

Experimental data were analyzed by Student’s t-test and χ² test using SPSS 10.0. P < 0.05 was considered statistically significant.

**RESULTS**

**Harvesting and identification of SLNMM**

A total of 62 NLN and 126 blue-stained lymph nodes from 43 patients were excised and processed. As a result, 37 and 54 blue-stained lymph nodes were considered to be SLNMM with HE staining (29.36%, Figure 1A) and CK-IHC (42.85%, Figure 1B), respectively, and at least one SLNMM was detected in each case.

**Differential expression of proteome in NLN and SLNMM**

Sixty-three protein spots were differentially expressed in NLN and SLNMM (Figure 2). Among the 63 protein spots, some could not be identified with incomplete poly-peptide fragments, and some were too low in abundance to obtain useful data. Finally, 40 protein entries were identified by MALDI-TOF-MS analyses (Table 1). The expression was up-regulated and down-regulated in 15 and 25 protein entries, respectively. The 15 proteins with their expression up-regulated were then grouped and classified according to their biological functions (http://www.geneontology.org/) into cytoarchitecture reorganization- concerned proteins including VCL, PDZD8, LMNA, RUVBL1, TCP11, CLIC1,
ALB and PFN1, cytometabolism-concerned proteins including SERPINB1, PURH and GLO1, and metastasis-concerned proteins including hnRNP A1, Ezrin, tubulin β-2C and Annexin A1. The expression and distribution of these 4 metastasis-concerned proteins were further studied to assess the role they play in the progress of early CRC metastasis.

Detection of differentially expressed proteins by Western blotting

Western blotting showed that the expression level of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1, was mainly expressed in cell nuclei and cytoplasm, and tubulin β-2C chain precursor was mainly expressed in cell membrane. Ezrin was

| Spot ID | International protein index accession No. | Protein | Score | Sequence coverage (%) | Mr/pl |
|---------|------------------------------------------|---------|-------|------------------------|-------|
| Up      | IPI00307162                              | VCL Isoform 2 of Vinculin | 98    | 11                     | 124292/5.50 |
| 482     | IPI00186098                              | PDZD8 PDZ domain-containing protein 8 | 198   | 15                     | 129681/5.78 |
| 513     | IPI00027834                              | HNRNPI heterogeneous nuclear ribonucleoprotein L isoform a | 119   | 17                     | 64720/8.46 |
| 501     | IPI00216952                              | LMNA Isoform C of Lamin-A/C | 190   | 41                     | 65413/5.93 |
| 530     | IPI00289499                              | ATIC Bifunctional purine biosynthesis protein PURH | 252   | 46                     | 65089/6.27 |
| 741     | IPI00021187                              | RUVBL1 Isoform 1 of Ruv-B-like 1 | 133   | 39                     | 50538/6.02 |
| 1071    | IPI00027444                              | SERPINB1 Leukocyte elastase inhibitor | 94    | 38                     | 42829/5.90 |
| 1266    | IPI00007752                              | TUBB2C Tubulin β-2-chain | 134   | 30                     | 50255/4.79 |
| 1411    | IPI00103433                              | TCP11 Isoform 1 of T-complex protein 11 homolog | 108   | 24                     | 56675/5.08 |
| 1449    | IPI00218918                              | ANXA1 Annexin A1 | 196   | 50                     | 38918/6.57 |
| 1649    | IPI00554767                              | CLC1 18 kDa protein | 96    | 52                     | 17927/4.71 |
| 1650    | IPI00746388                              | EZR Ezrin | 133   | 21                     | 69355/5.86 |
| 1967    | IPI00878282                              | ALB 23 kDa protein | 94    | 36                     | 23414/5.93 |
| 2102    | IPI00207666                              | GLO1 Lactoylglutathione lyase | 107   | 48                     | 20992/5.12 |
| 2410    | IPI00216691                              | PFN1 Profilin-1 | 62    | 40                     | 15216/8.44 |
| Down    | IPI000021405                             | LMNA Isoform A of Lamin-A/C | 204   | 42                     | 74380/6.57 |
| 509     | IPI00022463                              | TP Serotransferrin precursor | 65    | 19                     | 79280/6.81 |
| 701     | IPI00878282                              | ALB 23 kDa protein | 127   | 52                     | 23414/5.93 |
| 719     | IPI00553177                              | SERPIN1A Isoform 1 of α-1-antitrypsin precursor | 247   | 52                     | 46878/5.37 |
| 994     | IPI00027223                              | IDH1 Isocitrate dehydrogenase (NADP) cytoplasmic | 150   | 50                     | 46915/6.53 |
| 1042    | IPI00021926                              | PSMC6 26S protease regulatory subunit S10B | 85    | 25                     | 44430/7.10 |
| 1204    | IPI00298497                              | FGB Fibrinogen β chain precursor | 204   | 46                     | 56577/8.54 |
| 1433    | IPI00455315                              | ANXAX2 Annexin A2 | 130   | 41                     | 58808/7.57 |
| 1442    | IPI00295889                              | SRF19 Signal recognition particle 19 kDa protein | 129   | 38                     | 16374/9.87 |
| 1550    | IPI00847278                              | ANXA4 Annexin A4 | 217   | 60                     | 23500/5.84 |
| 1572    | IPI00745668                              | ANXA3 Uncharacterized protein ANXA3 (Fragment) | 121   | 41                     | 36623/5.53 |
| 1715    | IPI00394878                              | CIQTNF1 Clq and tumor necrosis factor related protein 1 | 273   | 68                     | 22841/8.40 |
| 1856    | IPI00003766                              | ETHE1 ETHE1 protein, mitochondrial precursor | 124   | 45                     | 28368/6.35 |
| 1863    | IPI00465028                              | TPII Isoform 1 of Triosephosphate isomerase | 292   | 80                     | 31057/5.65 |
| 1890    | IPI00025512                              | HPSB1 Heat shock protein β-1 | 86    | 48                     | 22826/5.98 |
| 1919    | IPI00853525                              | APOA1 Apolipoprotein A1 | 191   | 64                     | 28005/5.80 |
| 1954    | IPI00220766                              | GLO1 Lactoylglutathione lyase | 107   | 48                     | 20992/5.12 |
| 1961    | IPI00219022                              | PSMA2 Proteasome subunit β type-2 | 275   | 70                     | 25994/6.92 |
| 1995    | IPI0003815                               | ARHGDIA Rho GDP-dissociation inhibitor 1 | 91    | 43                     | 23500/5.02 |
| 2104    | IPI0014832                               | PDK2 [Pyruvate dehydrogenase kinase isozyme 2, alpha] | 152   | 19                     | 51389/7.67 |
| 2237    | IPI00096066                              | SUCLG2 Succinyl-CoA ligase [GDP-forming] | 184   | 23                     | 46824/6.15 |
| 2245    | IPI00384679                              | RNFI70 20 kDa protein | 184   | 54                     | 20773/6.40 |
| 2349    | IPI00796366                              | MLX16 16 kDa protein | 97    | 51                     | 16451/4.56 |
| 2540    | IPI00295844                              | PRF1-429EE11.3 Novel protein | 153   | 65                     | 15089/5.49 |
| 2564    | IPI00796606                              | HBB Hemoglobin (Fragment) | 98    | 64                     | 11554/5.90 |

1Over-expressed proteins in sentinel lymph nodes and their function-related metastasis of cancer cells.

Immunohistochemical analysis of proteins in NLN and SLNMM

The representative immunohistochemistry staining of different proteins in each group is shown in Figure 4. The positive expression rate was 54.8% and 69.8% respectively for hnRNP A1, 81.1% and 87.3% respectively for Ezrin, 19.3% and 74.6% respectively for tubulin β-2C, and 14.5% and 53.9% respectively for Annexin A1, in NLN and SLNMM. Statistical analysis demonstrated that the positive expression rate for the 4 proteins was significantly higher in SLNMM than in NLN (Table 2).

Furthermore, these four proteins were negatively or weakly expressed in NLN, but strongly expressed in SLNMM (Figure 4). Annexin A1 and hnRNP A1 were mainly expressed in cell nuclei and cytoplasm, and tubulin β-2C was mainly expressed in cell membrane. Ezrin was
enriched on cell membrane surface of SLNMM, but its distribution in cytoplasm of NLN was uniform.

**DISCUSSION**

The proteome approach, applied in this study is of clinical importance to identify the differentially expressed proteins in NLN and SLNMM of CRC, since these proteins can be potentially used as tumor markers and anticancer targets.

We marked SLN using isosulfan blue and identified micrometastasis of CRC with HE staining and CK-IHC. The total positive rate was 42.85%, which is consistent with the reported data[^20]. A total of 40 proteins were differentially expressed in NLN and SLNMM. Of these 40 proteins, 15 were up-regulated and 25 were down-regulated. The 15 proteins with their expression up-regulation were then divided into 3 groups according to their functions. Western blotting and immunohistochemistry analysis showed that the expression and distribution of 4 metastasis-concerned proteins in NLN and SLNMM were significantly different.

The Annexins are a family of calcium-regulated phospholipid-binding proteins with a diverse role in cell biol-
to 12 Annexins have been found in higher vertebrates. Although no exact physiological function of Annexins has been described, there is evidence that they are differentially expressed in various carcinomas. For example, expression of Annexins at mRNA and protein level is sharply up-regulated in many cancers, while some data indicate that declined expression of Annexins may play a significant role in tumorigenesis and metastasis. So, the precise role of Annexin expression in pathogenesis of tumors is still unknown. In this study, Western blotting and IHC showed that the expression level of Annexin A1 was significantly higher in SLNMM than in NLN, suggesting that up-regulated expression of Annexin A1 may contribute to early CRC metastasis.

Ezrin, a membrane-cytoskeleton anchor, can affect cell adhesion and regulate tumor cell invasion and metastasis. Wang et al. reported that Ezrin expression level is obviously higher in CRC tissue than in normal colorectal mucosa tissue, which is closely related to CRC invasion and metastasis. Elzagheid et al. found that intense Ezrin immunoreactivity in cytoplasm can predict poor survival of CRC patients, thus providing clinically valuable information on the biological behavior of CRC. In this study, Ezrin was expressed on cell membrane surface or in cytoplasm, but not uniformly expressed in cytoplasm, which is consistent with the reported findings in pancreatic cancer, indicating that membrane translocation of Ezrin may also play an important role in early CRC metastasis.

Cell locomotion, including cancer cell invasion, is closely associated with dynamics of cytoskeletal structures. Tubulin isotype composition may affect polymerization properties and dynamics of microtubules. Portyanko et al. showed that tubulin β-2C is associated with tumor budding grade, and changes in tubulin isotypes can modulate the invading activity of CRC cells. In our study, the expression of tubulin β-2C was about 2-fold higher in SLNMM than in NLN, and IHC showed that the staining of tubulin β-2C was weak and mostly gathered around nuclei of NLN but stronger and diffused in cytoplasm of SLNMM, suggesting that the expression and distribution of tubulin β-2C are different in NLN and SLNMM of CRC, and the increased expression of tubulin β-2C is associated with early lymph node micrometastasis, thus leading to poor prognosis of CRC.

HnRNP is most abundantly expressed in nuclear protein of mammalian cells, which is associated with pre-mRNA processing and other aspects of mRNA metabolism and transport. As a class of protein family, many of its subtypes are related to the occurrence of different tumors, and hnRNP A2/B1 subtype is now used as an indicator in early diagnosis of lung cancer. In our study, Western blotting and IHC showed the expression level of hnRNP A1 was higher in SLNMM than in NLN, indicating that hnRNP A1 plays an important role in the occurrence and development of CRC and can thus be considered a potential molecular indicator/biomarker of tumorigenesis in CRC.

In summary, comparative proteomics technologies can be used in study of protein profiles in NLN and SLNMM and in identification of early CRC metastasis-related proteins. Increased expression of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in SLN suggests a significantly elevated incidence of early CRC metastasis. However, further study is needed to verify their role in therapeutic target of CRC.

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