Comparative proteomic analysis of melanosis coli with colon cancer

XIN ZHOU[1], PING WANG[2], YING-JIAN ZHANG[1], JIN-JIN XU[1], LA-MEI ZHANG[1], LIN ZHU[1], LI-PING XU[1], XIAO-MIN LIU[1] and HUAN-HUAN SU[1]

[1]Department of Gastroenterology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, Henan 471003; [2]Department of Public Health, Faculty of Medicine, Henan University of Science and Technology, Luoyang, Henan 471003, P.R. China

Received May 3, 2016; Accepted June 21, 2016

DOI: 10.3892/or.2016.5178

Abstract. The present study aimed to investigate the proteomic difference between melanosis coli (MC) alone and melanosis coli with colon cancer (MCCC). Protein expression in patients with different diseases was analyzed using two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS). A total of 14 protein differences with a confidence level of >95% were found. There were six differences between MC and normal tissues, in which two proteins exhibited upregulated expression levels and four proteins exhibited downregulated expression levels in MC. Furthermore, one protein was expressed only in MC ($P<0.05$). In addition, there were differences in the expression of eight proteins between MC and MCCC tissues, in which one protein had an upregulated expression in MC tissues and seven proteins had an upregulated expression in MCCC tissues. Furthermore, two proteins were only expressed in MCCC tissues ($P<0.05$). Eight proteins were identified using mass spectrometry and database search. In conclusion, comparative proteomics accurately displayed the expression differences in eight proteins between MC, MCCC and normal colon tissues.

Introduction

Colon melanosis coli (MC) refers to colonic mucosa pigmentation lesions, in which macrophages in the colonic lamina propria contain lipofuscin-like substances that are rare in non-inflammatory diseases (1-3). At present, most studies have focused on the risk factors of MC (4), or the relationship between MC and proliferative diseases such as colorectal adenoma and colon cancer.

The relationship between MC and tumors is one issue that has received increased research attention. In a retrospective study on 14 study reports, Sonnenberg *et al* (5) proposed that the administration of anthraquinone and glycosides was associated with a higher incidence of colorectal cancer, compared with other laxatives; and the difference was statistically significant. However, Zhang *et al* (6) provided a different conclusion through a prospective study. Various scholars have found through the application of gene chip technology that the expression of protein metallopanstimulin-1 (MPS-1) in colonic MC tissues is 3.9 times higher than that of normal colon tissues, and thioredoxin expression was increased by 3.4 times; which indicates that MC may be associated with the occurrence of colon cancer (7,8). A study conducted by Wang *et al* (9) on the expression of the Hedgehog (Hh) signaling pathway suggested that MC may be associated with colon cancer from one side. Although a variety of clinical studies on the relationship between MC and colon cancer have been carried out domestically and internationally, these results remain inconsistent. In recent years, comparative proteomics has achieved rapid development, and has been widely used in cancer research. Thus, a branch of 'cancer proteomics', has been formed which provides more opportunities or concepts for the diagnosis of tumors (10). The maturation of proteomic technology and its extensive application in various fields provide favorable conditions for the use of comparative proteomics in research concerning the relationship between MC and colon cancer. In the present study, protein expression in MC tissues and its biological function were investigated at the proteomic level. Related protein molecules were identified, which provide favorable conditions for the development of the biological study of MC.

Materials and methods

General information. A total of 45 subjects who received medical service at our hospital from January 2013 to June 2014 were enrolled in the present study. Among these patients, 15 patients were diagnosed with MC, 15 patients were diagnosed with melanosis coli with colon cancer (MCCC), and the

Correspondence to: Dr Ying-Jian Zhang, Department of Gastroenterology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, 24 King Road, Jianxi, Luoyang, Henan 471003, P.R. China
E-mail: yingjianzhangdoc@163.com

Key words: melanosis coli, melanosis coli with colon cancer, 2-DE, proteomics, protein profiling

remaining 15 subjects had normal colon tissues. Four pieces of tissues from diseased or normal sites were obtained from each subject. After washing with phosphate-buffered saline (PBS), the samples were stored in liquid nitrogen. Differences in age and gender across these three groups were not statistically significant.

Inclusion criteria included black, brown or dark gray colonic mucosa under colonoscopy; yellow or pink edges and early pathological tissues that presented tiger skin or snake-skin stripes; were penang section- or patch-shaped. Under microscopy, infiltration of large mononuclear cells containing a great amount of melanin and melanin pigmentation could be observed in the lamina propria of colon mucosa, while other layers of the intestinal wall were normal. From normal subjects, colon tissues without MC, MCCC, polyps and inflammatory or non-inflammatory bowel diseases were obtained. Colon tissue samples were confirmed by a pathologist as MC, MCCC or normal colon tissues. Exclusion criteria included patients with other bowel diseases, patients who underwent treatment, patients who could not tolerate an endoscopic examination and patients with diseases that influence protein metabolism.

Consent was provided by the subjects for the acquisition of the experimental samples, and the study protocol was approved by the Ethics Committee of Henan University of Science and Technology.

Therapeutic methods

Preparation of colon tissue samples. Approximately 0.1 g of sample was obtained and placed in a pre-frozen PBS solution at 4˚C for processing. After the separation of other tissues, the sample was dried by filter paper. After the addition of reagents, the sample was placed in an ice bath, underwent cell disruption using an ultrasonic cell crusher, and was quantitatively analyzed.

Quantitation of protein samples. Protein concentrations in the tissue samples were determined by Bradford protein assay. Protein concentration was 6.03 g/l in the colon cancer tissue, 6.65 g/l in the MC tissue and 2.90 g/l in the normal colon tissue.

Two-dimensional gel electrophoresis. The first dimension included immobilized pH gradient isoelectric focusing [IPG-IEF (pH3-10NL)] for electrophoresis as follows. i) The prepared protein samples were preserved in a refrigerator at -80˚C. ii) Re-swelling solution was added to the groove of the re-swelling tray, in which the surface was covered with an oily DryStrip cover fluid to prevent moisture from evaporating and affect the IEF (pH3-10NL). Then, the sample was maintained overnight (12-16 h at room temperature). iii) Isoelectric focusing electrophoresis was carried out.

The second dimension consisted of vertical plate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Table I): i) SDS polyacrylamide gel with T=13% was prepared. ii) Gel strip equilibration. iii) Vertical plate SDS-PAGE (Table II).

Dyeing and preservation of SDS polyacrylamide gel. This stage consisted of i) Coomassie brilliant blue staining and ii) preservation of the SDS polyacrylamide gel. The gel, which displayed the protein spots after staining, was placed in 7% glacial acetic acid; and image scanning was immediately performed. If the protein spots were not intended to be extracted, the gel would be prepared into a dry strip for long-term preservation.

Table I. Parameter settings for the IEF program.

Step	Voltage (V)	Time (h/Vh)	Mode
S1	500	0.1	Step-on-hold
S2	40	6	Step-on-hold
S3	500	1	Step-on-hold
S4	1,000	2	Gradient
S5	3,000	2	Gradient
S6	5,000	2	Gradient
S7	10,000	3	Gradient
S8	10,000	63,200	Step-on-hold
S9	500	10	Step-on-hold

When IEF was over, the strip was taken out and then all DryStrip cover fluid was absorbed with filter paper. Consequently, the second dimension, vertical plate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted (if not timely, the strip was kept at -80˚C). IEF, isoelectric focusing.

Table II. The gel formulation of the vertical plate SDS-PAGE.

Agent	Volume
H_2O	47.4 ml
Acr/Bis (30/0.8%)	69.2 ml
Tris (1.5 M pH 8.8)	40.0 ml
10% SDS	1,600 μl
10% APS	1,600 μl
TEMED	64 μl
Total volume	160 ml

SDS-PAGE, sulfate-polyacrylamide gel electrophoresis.

Image contrast. For disparate spots acquired by contrast of two-dimensional gel electrophoresis, the UMAX image scanner III (setting, optical resolution at 300 dpi, pixel standard at 16 bits and white light scanning was selected) was used for image acquisition. Image save mode was set as a 16-bit TIF. Then, images were analyzed using ImageMaster 7.0. With an ANOVA value of <0.05 and multiple proportions >1.5, ImageMaster 7.0 was used for image contrast. The spot parameter 'smooth' was set at 3, 'Min area' was set at 65, and 'salience' was set at 250. The image analysis process included the detection, quantification and matching of protein spots.

Analysis software SPSS 19.0 was used for data processing. The two groups of gels were compared by t-test, and $P<0.05$ was considered statistically significant. Spots with multiple proportions >1.5 times (that is, the difference in expression

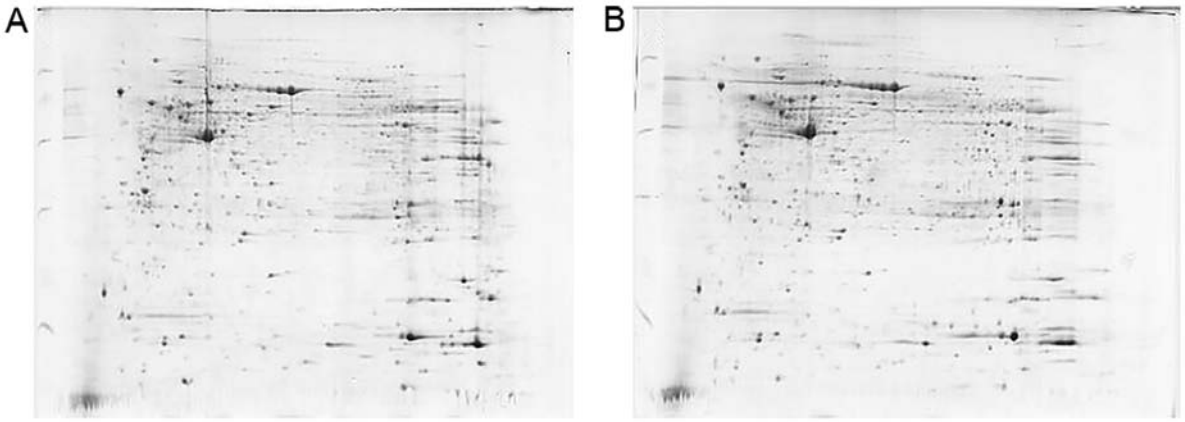

Figure 1. Preliminary experimental results of MC and MCCC tissues. (A) Gel images obtained by the preliminary experiment of colon cancer tissue. (B) Gel images acquired of melanosis coli tissue in the preliminary experiment.

amount was >1.5 times) were selected as protein spots that were sought for in this experiment, and as candidate protein spots for the subsequent mass spectrometry identification.

Mass spectrogram acquisition and analysis. Approximately 0.5-1 μl of sample was obtained, target-dripped and dried. Dripping of the matrix (HCCA; 2.5 mg/ml) was repeated 2-3 times (0.3-0.4 μl at a time). After drying, these were used for shooting on the machine. Mass spectrometer was ABI MALDI TOF 4800 (Applied Biosystems, Inc., Foster City, CA, USA); mass spectrum scanning mode, reflection; scan range, 900-4,000 Da; laser energy, MS4200 and MSMS4800. In each first order mass spectrum, 10 parent ions were selected for the secondary mass spectrum. Search in the NCBI gene sequence database was performed using the software, Mascot ($P<0.05$).

Preliminary experimental results. First, MC and MCCC samples were obtained for the preliminary experiment, in accordance with the above operations; in order to test the feasibility of the operation (Fig. 1).

Results

Repeatability analysis of two-dimensional gel electrophoresis figure. The three images of the two-dimension electrophoresis gel of the same sample were compared using the gel image contrast software, ImageMaster 7.0. This was performed to test the repeatability of the experiment. The three gel images in each group appeared to be basically similar. Each image had a clear separation between the spots, and the matching rate was >80%. These findings indicate that the position of each protein spot on the gel images had good reproducibility. Therefore, gel image analysis could give the relative protein content of each protein band; and the results of the image analyses were also relative reliable.

Contrast analysis of two-dimensional gel electrophoresis figure

Image analysis. This process included the detection of protein spots, quantification of protein spots, removal of the background, and protein spot matching. Before the automatic detection of protein spots, parameters were first set; and the largest, weakest and smallest protein spots were determined. After the automatic detection of protein spots, each protein spot was assigned to a specific SSP code. Then, these protein spots were edited, and some impurities were removed from the protein spots. In comparing between MC and MCCC, one piece of two-dimensional electrophoresis gel with a clear image was assigned as a reference; and the image synthesized from a few pieces of gel was also assigned as a reference. The same method was used to compare the MC and normal tissues. In addition, some corresponding matching spots were set-up, and these matching spots were automatically used to match other protein spots. Protein spots that could not be matched with the spots in the reference gel were automatically added to the reference gel. The sum of the intensity values of all pixels that constitute a protein spot was defined as the amount of the protein spot. The amount of each protein spot was expressed in percentage (% vol); that is, the percentage of one single protein spot accounting for the sum of the total protein spots in the gel. Two groups of gel were compared and analyzed using SPSS 19.0 software. These spots with multiple proportions >1.5 times (that is, the difference of the expression amount was >1.5 times) were selected as protein spots that were sought for in this experiment, and as a candidate protein spot for the subsequent mass spectrometry identification.

Analysis and comparison of the results. i) The average protein spot number of the sample gels of MCCC, MC and normal tissues were 2,150±240, 1,970±138 and 2,028±176, respectively. In comparison, among the nine pieces of gel images, a total of 14 differentially expressed protein spots with a confidence level of >95% were found.

ii) Comparison between the MC and normal tissues was carried out. a) There were six differently expressed protein spots (Fig. 2). Among these, five spots existed in both gel images of the MC and normal tissues; and the number of spots that were expressed only in MC was one, namely spot 1783 ($P<0.05$). b) Spot 602, 647, 817 and 820 revealed a down-regulated expression in the MC tissues; and two spots, 938 and 1783, displayed upregulated expression in the MC tissues. Spot 1783 was expressed only in MC ($P<0.05$), which had an average relative protein content of 0.078 (Table III).

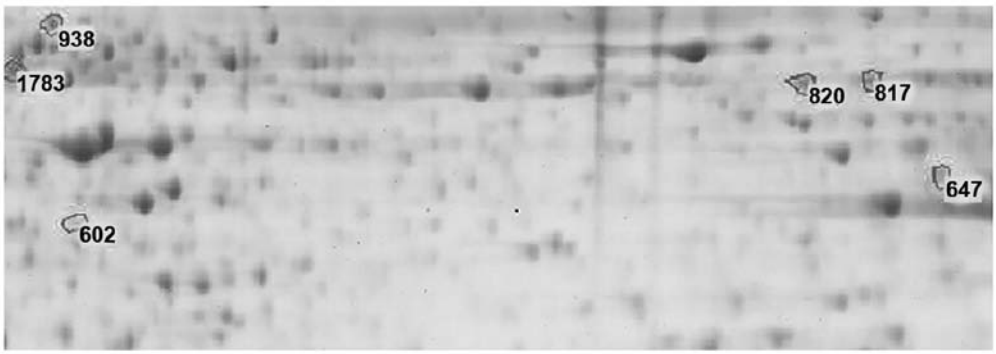

Figure 2. Relative protein content of spot 602, 647, 817, 820, 938 and 1738 in both gel images of MC and normal tissues.

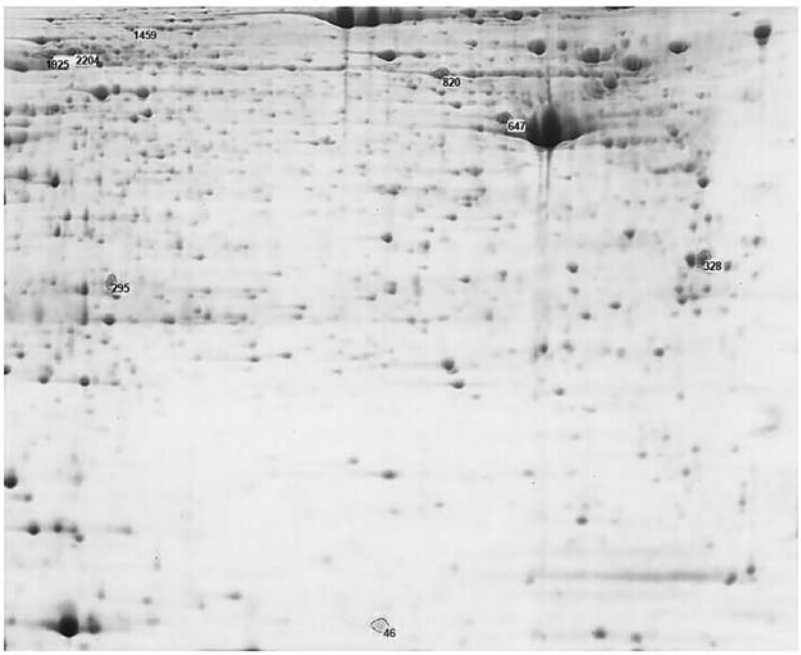

Figure 3. Relative protein content of spot 46, 295, 328, 647, 820, 1457, 1925, 2204 in both gel images of MC and MCCC tissues.

Table III. The protein spots diplaying differential expression between MC and normal tissues.

	Average relative protein content (% vol)		
Protein spot	MC	Normal tissue	P-value
602	0.021774	0.047104	0.01
647	0.041156	0.08436	0.02
817	0.051121	0.08809	0.01
820	0.064543	0.184723	0.00
938	0.064177	0.04425	0.00
1783	0.077631		0.00

MC, melanosis coli.

Fig. 2 shows that the size and dyeing shades of the corresponding differentially expressed protein spots in the gel images of the MC and normal tissues were different. The larger area or deeper staining spot suggests a large relative content that accounts for the total protein spots.

iii) Comparison between MC and MCCC tissues was carried out (Fig. 3). There were eight differentially expressed protein spots, in which six spots existed in the MC and MCCC tissues, and two protein spots were only expressed in the MCCC tissues; and these were spots 46 and 2204. Spot 295 displayed an upregulated expression in the MC tissues, and seven protein spots, namely spots 46, 328, 647, 820, 1457, 1925 and 2204, exhibited upregulated expression in the MCCC tissues. Two proteins were only expressed in the MCCC tissues, namely spots 46 and 2204 ($P<0.05$). Among these, average relative protein contents were 0.0778 and 0.278 (Table IV).

Results of protein identification. Protein identification was performed through comparison between the peptide mass fingerprint spectrum of the protein spots and protein data in the NCBI-nr database using the software, Mascot. A total of 10 protein spots had matching scores >62 (protein identification

Table IV. The protein spots diplaying differential expression between MC and colon cancer tissues.

	Average relative protein content (% vol)		
Protein spot	MC	MC with colon cancer	P-value
46		0.077844	0.00
295	0.639534	0.158345	0.00
328	0.182761	0.329592	0.00
647	0.041156	0.250868	0.00
820	0.064543	0.369613	0.00
1457	0.016213	0.03767	0.00
1925	0.010432	0.230579	0.00
2204		0.278172	0.00

MC, melanosis coli.

results were the same in two groups, spots 46 and 1925 failed to be identified; Table V).

Discussion

In order to ensure the accuracy and reliability of the experiment and reduce system error, three repeats of gel electrophoresis were performed for each tissue sample; and the electronic average of the gel image was calculated. Strip length and pH range are important factors that affect the resolution of the 2-DE map. In most current studies, pH 3-10 L solid phase adhesive strips were used for protein isolation (11-13).

Finally, 24-cm long non-linear IPG strips with pH that ranged from 3-10 were chosen for the present study, since the protein spots that these could display were clearer and independent, and the separation area was larger. Gel dyeing is important. Coomassie brilliant blue staining for proteins is relatively simple and easy to control, and its repeatability was higher than that of silver staining. Hence, Coomassie brilliant blue staining was used in the experiment. However, since it is the same with silver staining, decoloring is necessary before mass spectrum identification. In this experiment, a two-dimensional gel electrophoresis technique platform for the proteomic determination of MCCC, MC and normal tissues was established. This provides technical support for subsequent studies on comparative proteomics of MCCC (14).

Although we took various measures to improve the repeatability and resolution of the gel image, we realize that the 2-DE experimental process is long and has many steps. Furthermore, many processes are needed in its manual operation, not to mention artificial errors and system errors. All these factors result in a high possibility of the occurrence of 'false-negative' or 'false-positive' differences in protein expression. For this experiment, to a certain extent, some differentially expressed protein spots may have been lost or some non-differentially expressed protein may have been mis-displayed in the gel

Table V. MC organization and melanosis disease with colon cancer and normal colon tissue between differentially expressed protein identification results.

Difference point no.	Accession no.	Protein name	Score	M_r	pI	Sequence coverage (%)
MC organization and melanosis disease with colon cancer						
295	IPI00215983	CA1 carbonic anhydrase 1	253	28,909	6.59	60
328	IPI00010779	TPM4 isoform 1 of tropomyosin α-4 chain	149	28,619	4.67	44
647	IPI00554788	KRT18 keratin	457	48,029	5.34	72
820 (817)	IPI00554648	KRT8 keratin	295	53,671	5.52	52
938	IPI00465436	CAT catalase	95	59,947	6.90	18
1457	IPI00216952	LMNA isoform C of prelamin-A/C	132	65,153	6.40	38
2204 (1783)	IPI00965713	FGB fibrinogen β chain isoform 2 preproprotein	66	50,436	8.22	21
MC organization and normal colon tissue						
602	IPI00926977	PSMC6 26S protease regulatory subunit 10B	105	46,053	7.64	34
647	IPI00554788	KRT18 keratin	457	48,029	5.34	72
817 (820)	IPI00554648	KRT8 keratin	295	53,671	5.52	52
938	IPI00465436	CAT catalase	95	59,947	6.90	18
1783 (2204)	IPI00965713	FGB fibrinogen β chain isoform 2 preproprotein	66	50,436	8.22	21

MC, melanosis coli.

images. These factors have been taken into account in the analysis of these results.

Through bioinformatic analysis, eight proteins were finally identified in the present study; which were considered to be related proteins of MC and MCCC.

The identification of spots of differentially expressed proteins between MC and MCCC tissues, and between MC and normal colon tissues, revealed that there were differences in cytokeratins. The main functions of cytokeratins are maintaining cell shape, performing cell movement and resisting external mechanical stress (15,16). In the results of the experiment, it was found that the skeleton protein expression was downregulated in MC tissues and upregulated in MCCC tissues, such as KRT8 and KRT18. It was revealed by recent studies that KRT8 is associated with lung, breast, esophageal and colon cancer infiltration; and these are involved in the multi-drug resistance of colon cancer (17-19). One study revealed that KRT8 may act as a potential receptor of plasminogen, and can activate plasmin through plasminogen activator located on the tumor cell surface. In addition, other studies revealed that the activation of plasminogen promoted tumor cell infiltration. These studies suggest that KRT8 may be involved in the progression of MC and MCCC, which may be used as a potential molecular marker for the diagnosis of MC and MCCC. The specific biological functions in MC and MCCC require further studies.

The identification of spots of differentially expressed proteins between MC and MCCC tissues, and between MC and normal colon tissues, revealed that there were differences in fibrinogen. A present study revealed that fibrinogen levels are abnormally increased in patients with gastric, lung and oral cancer, and many other malignant tumors (20). Another study revealed that fibrinogen can cause an increase in fibroblast growth factor-2 (FGF-2) in prostate and lung cancer cells (21), while FGF-2 is an important growth factor in normal tissues and tumor tissue proliferation, which is closely related to blood vessel hyperplasia in tumor growth. The formation of new blood vessels plays an important role in malignant tumor growth and metastasis. Fibrin in the extracellular matrix of cancer cells forms a stable scaffold, and plasmin promotes the dissolution of fibrin and causes the degradation of the matrix; promoting the spread of cancer cells in the process of angiogenesis (22). This revealed that fibrinogen exhibits upregulated expression in MC and MCCC tissues. Therefore, fibrinogen may be associated with the growth of MC and MCCC, as well as in tumor metastasis.

The identification of spots of differentially expressed proteins between MC and MCCC tissues, and between MC and normal colon tissues, revealed that there were differences in hydrogen peroxidase [also known as catalase (CAT)]. It has been confirmed that increased levels of oxygen-free radicals and changes in antioxidant enzymes can lead to tumorigenesis. Furthermore, cancer patients usually also show oxidation-reduction imbalance in the body, and an interaction between the tumor and antioxidant system. Through comparison of the MCCC, MC and normal tissues, it was found that the CAT was significantly reduced in MCCC tissues. These findings suggest that the radical scavenging system in tumor tissues is damaged during tumorigenesis. In recent years, many scholars have studied the relationship between the CAT levels in tissues, body fluid, and the diagnosis and treatment of systemic tumors. It has been recognized that CAT can be used as a tumor marker for assisting diagnosis, treatment guidance and the prognosis of malignant tumors.

There were differences in the 26s proteasome between MC and normal colon tissues. In the cytoplasm and nucleus of the eukaryote, 26s proteasome is the core protease for protein degradation. Studies have found that ~80-90% of cell protein degradation occurs under the action of the proteasome. The results of the present study showed that 26s proteasome expression was decreased in the MC tissues, but was not found in the MCCC tissues. Therefore, studies on 26s proteasome can promote the understanding of normal or damaged protein degradation, cell cycle regulation, the expression of tumors and antigens, as well as the activation and degradation of transcription factors.

A difference was noted in tropomyosin (TPM) in the MC and MCCC tissues. In recent years, studies have revealed that TPM may abnormally be expressed in a variety of malignant tumors. This experimental study also revealed that TMP expression was significantly higher in MCCC tissues than that noted in the MC and normal tissues. Therefore, TPM may be involved in tumor metastasis. However, the specific signaling pathway involved in its biological function and proteins involved in its interactions require further in-depth studies.

A recent study found that in most tumors, carbonic anhydrase (CA) expression exists. Various tumor tissues have different CA isozyme expression levels (23). The present study revealed that there was a difference in CA between MC and MCCC tissues. CA is involved in the occurrence and progression of most cancers; and its expression is closely related to the invasion and metastatic ability of cancer cells (24). These enzymes that regulate pH balance appear to be able to regulate the behavior of tumor cells. The regulation of the expression level of CA1 proteins is one of the mechanisms of vegetable resisting colorectal cancer. High expression levels of CA1 protein exists in normal colorectal mucosa; but in most colorectal cancer tissues, no CA1 expression appears. In the present study, CA1 displayed a high expression in MC; and exhibited upregulated expression in the MCCC tissues. Therefore, expression of CA1 in colorectal cancer may suggest a good prognosis and extended overall survival rate. The expression of related carbonic anhydrase in cancers may inhibit the metastasis of cancer cells, and improve the prognosis of patients undergoing cancer surgery. Further studies regarding the relationship between tumor and carbonic anhydrase have important theoretical significance and clinical value in the exploration of tumor pathogenesis, the development of gene-targeted chemotherapy, and the improvement in anticancer effects. These may help in further understanding the mechanism of tumors, and help in achieving breakthroughs for tumor-targeted therapies.

A difference was observed in lamin A/C in the MC and MCCC tissues. Lamin A/C has many important physiological functions such as positioning of the nuclear pore complex, nuclear membrane protein binding, peripheral heterochromatin anchoring, gene replication, transcription and DNA damage repair, maintenance of chromosome structure and genome stability (25). It is only a hypothesis that the affected site of laminopathies is selective. Furthermore, it has been

hypothesized that lamin mutations can cause the dysfunction of adult stem cells, leading to abnormal cell differentiation. This may explain the different effects of lamin mutations in different tissues (26). A recent study indicated that the expression of lamin is potentially associated with various types of cancers.

In the present study, using two proteomic methods (2-DE technology and mass spectrometry analysis), eight proteins, which were differentially expressed in MC, MCCC and normal colon tissues, were identified. Further studies of the functions of these proteins may provide a new targeted molecular marker for the diagnosis and treatment of MC and MCCC, in order to achieve the purpose of early detection and effective treatment; and improve overall outcome. For the validation of related proteins and their specific functions, we will conduct further studies as the next step using PCR and immunohistochemical methods based on the present study.

References

1. Liu J, Tian DA, Wang JP, Zhang SZ, Feng J, Zhao ZZ, Hao YX and Liu P: Expression of aquaporin 8 and its relationship with melanosis coli. Chin Med J 124: 3061-3065, 2011.
2. Kapila A, Patel P, Khan O, Murthy R and Young MF: The classic melanosis coli. Indian J Gastroenterol 33: 582-583, 2014.
3. Li D, Browne LW and Ladabaum U: Melanosis coli. Clin Gastroenterol Hepatol 7: A20, 2009.
4. Zhang L and Gao F: New development in melanosis coli. Chin J Gastroenterol Hepatol 24: 257-259, 2015.
5. Sonnenberg A and Müller AD: Constipation and cathartics as risk factors of colorectal cancer: A meta-analysis. Pharmacology 47 (Suppl 1): S224-S233, 1993.
6. Zhang X, Wu K, Cho E, Ma J, Chan AT, Gao X, Willett WC, Fuchs CS and Giovannucci EL: Prospective cohort studies of bowel movement frequency and laxative use and colorectal cancer incidence in US women and men. Cancer Causes Control 24: 1015-1024, 2013.
7. Yang ZY, Jiang H, Qu Y, Wei M, Yan M, Zhu ZG, Liu BY, Chen GQ, Wu YL and Gu QL: Metallopanstimulin-1 regulates invasion and migration of gastric cancer cells partially through integrin β4. Carcinogenesis 34: 2851-2860, 2013.
8. Dai Y, Pierson S, Dudney C, Zeng Y, Macleod V, Shaughnessy JD and Stack BC Jr: Ribosomal protein metallopanstimulin-1 impairs multiple myeloma CAG cells growth and inhibits fibroblast growth factor receptor 3. Clin Lymphoma Myeloma Leuk 11: 490-497, 2011.
9. Wang ZC, Gao J, Zi SM, Yang M, Du P and Cui L: Aberrant expression of sonic hedgehog pathway in colon cancer and melanosis coli. J Dig Dis 14: 417-424, 2013.
10. Zolg W: The proteomic search for diagnostic biomarkers: Lost in translation? Mol Cell Proteomics 5: 1720-1726, 2006.
11. Li DJ, Deng G, Xiao ZQ, Yao HX, Li C, Peng F, Li MY, Zhang PF, Chen YH and Chen ZC: Identificating 14-3-3 sigma as a lymph node metastasis-related protein in human lung squamous carcinoma. Cancer Lett 279: 65-73, 2009.
12. Cheng AL, Huang WG, Chen ZC, Peng F, Zhang PF, Li MY, Li F, Li JL, Li C, Yi H, *et al*: Identification of novel nasopharyngeal carcinoma biomarkers by laser capture microdissection and proteomic analysis. Clin Cancer Res 14: 435-445, 2008.
13. Tian T, Hao J, Xu A, Hao J, Luo C, Liu C, Huang L, Xiao X and He D: Determination of metastasis-associated proteins in non-small cell lung cancer by comparative proteomic analysis. Cancer Sci 98: 1265-1274, 2007.
14. Azad NS, Rasool N, Annunziata CM, Minasian L, Whiteley G and Kohn EC: Proteomics in clinical trials and practice: Present uses and future promise. Mol Cell Proteomics 5: 1819-1829, 2006.
15. Magin TM, Vijayaraj P and Leube RE: Structural and regulatory functions of keratins. Exp Cell Res 313: 2021-2032, 2007.
16. Omary MB, Ku NO, Strnad P and Hanada S: Toward unraveling the complexity of simple epithelial keratins in human disease. J Clin Invest 119: 1794-1805, 2009.
17. Liu F, Chen Z, Wang J, Shao X, Cui Z, Yang C, Zhu Z and Xiong D: Overexpression of cell surface cytokeratin 8 in multidrug-resistant MCF-7/MX cells enhances cell adhesion to the extracellular matrix. Neoplasia 10: 1275-1284, 2008.
18. Bartkowiak K, Wieczorek M, Buck F, Harder S, Moldenhauer J, Effenberger KE, Pantel K, Peter-Katalinic J and Brandt BH: Two-dimensional differential gel electrophoresis of a cell line derived from a breast cancer micrometastasis revealed a stem/progenitor cell protein profile. J Proteome Res 8: 2004-2014, 2009.
19. Makino T, Yamasaki M, Takeno A, Shirakawa M, Miyata H, Takiguchi S, Nakajima K, Fujiwara Y, Nishida T, Matsuura N, *et al*: Cytokeratins 18 and 8 are poor prognostic markers in patients with squamous cell carcinoma of the oesophagus. Br J Cancer 101: 1298-1306, 2009.
20. Seebacher V, Polterauer S, Grimm C, Husslein H, Leipold H, Hefler-Frischmuth K, Tempfer C, Reinthaller A and Hefler L: The prognostic value of plasma fibrinogen levels in patients with endometrial cancer: A multi-centre trial. Br J Cancer 102: 952-956, 2010.
21. Sahni A, Simpson-Haidaris PJ, Sahni SK, Vaday GG and Francis CW: Fibrinogen synthesized by cancer cells augments the proliferative effect of fibroblast growth factor-2 (FGF-2). J Thromb Haemost 6: 176-183, 2008.
22. Wang JF, Guo Z, Tang L, Guo JS, Hu J and Liu JZ: Prognostic associations of preoperative plasma levels of fibrinogen and D-dimer after curative resection in patients with colorectal cancer. Zhonghua Yi Xue Za Zhi 93: 906-909, 2013 (In Chinese).
23. Li Y, Wang H, Tu C, Shiverick KT, Silverman DN and Frost SC: Role of hypoxia and EGF on expression, activity, localization and phosphorylation of carbonic anhydrase IX in MDA-MB-231 breast cancer cells. Biochim Biophys Acta 1813: 159-167, 2011.
24. Dunqwa JV, Hunt LP and Ramanni P: Overexpression of carbonic anhydrase and HIF-1α in Wilms tumours. BMC Cancer 12: 390, 2011.
25. Ostlund C and Worman HJ: Nuclear envelope proteins and neuromuscular diseases. Muscle Nerve 27: 393-406, 2003.
26. Meshorer E and Gruenbaum Y: Gone with the Wnt/Notch: Stem cells in laminopathies, progeria, and aging. J Cell Biol 181: 9-13, 2008.