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Review Article

Differential proteomics mass spectrometry of melanosis coli

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Abstract: This study aims to reveal the biological relevancy between melanosis coli (MC) with colon cancer by analyzing the proteomics differences of tissues of melanosis coli, colon cancer, and normal ones to probe into the causes and development mechanisms of MC from the perspective of biomolecules. Fourteen differential protein spots were found in the study after using two-dimensional gel electrophoresis (2-DE) and bio-mass spectrometry (MALDI-TOF/TOF-MS). Specifically, six and eight differential protein spots in the melanosis coli tissues were detected, respectively, compared with the normal tissues and colon cancer tissues. Eight kinds of proteins, including keratin 8 (KRT8), keratin 18 (KRT18), fibrinogen beta chain isoform 2 preproprotein (FGB), catalase (CAT), 26s protease regulatory subunit 10b (PSMC6), isoform 1 of tropomyosin alpha-4 chain (TPM4), carbonic anhydrase 1 (CA1), isoform of prelammin-A/C (LMNA), were retrieved through the mass spectral database, which could be deemed as associated proteins of MC and colon cancer. The different expressions in the disease tissues indicate that these proteins may be connected with the carcinogenesis of MC as well as the malignant proliferation, development, differentiation, and diffusion of cancer cells.

Keywords: Melanosis coli (MC), colon cancer, proteomics, two-dimensional gel electrophoresis (2-DE), bio-mass spectrometry (MALDI-TOF/TOF-MS)

Introduction

Melanosis coli (MC) is a rare non-inflammatory, benign, and reversible intestinal disease. MC can involve the esophagus, stomach, duodenum, lymph nodes and appendix around the colon, etc. The pathology is manifested by macrophage lipid brown pigment-like substances in the lamina propria of the colonic mucosa causing mucosal pigmentation [1]. The incidence of MC in China is lower than that in Europe and America. However, with the improvement of people's living standards, the change of diet structure and living habits, and the development of electronic colonoscopy technology, the incidence of MC in China showed an increasing trend [2]. MC for the current study mainly focused on the etiology and pathogenesis. For long-term constipation or long-term application of stimulant laxatives, especially anthraquinones drugs, can cause the MC has basically reached a consensus, but the exact cause and pathogenesis of MC is still unclear. Some researchers in China believe that although many studies have confirmed that anthraquinones can cause apoptosis of mucosal epithelial cells, some MC patients have neither constipation nor history of taking anthraquinones [3-6]. Therefore, it can be inferred that constipation and the history of oral laxatives cannot be used as the sole cause of MC, and it is still necessary to further explore the causes and mechanisms of the MC.

In the current development of the MC study, the relationship between MC and colon cancer has been controversial. One study suggested that multiple factors such as constipation in MC patients repeatedly stimulated the formation of adenomatous polyps in the intestinal mucosa, which may eventually develop into cancer [7]. Du Chun et al. believed that MC was associated

with colon tumors to some extent [8]. However, many studies have concluded that MC is not necessarily related to colon cancer. For example, Kassim study suggested that MC is related to proliferative polyps, but has no clear relationship with the occurrence of adenocarcinoma [9]. A single-center retrospective study considered that the MC was not associated with the diagnosis of colorectal cancer [10]. There is still no definitive answer as to whether MC may become cancerous.

In recent years, the research concepts and technologies of proteomics have continuously developed, and they interact with genomics to explain life phenomena at a deep level. Get a deeper understanding of the occurrence, development, and transformation of diseases, to discover the targets of diagnosis and treatment, and use them to guide clinical trials [11]. Proteomics technologies mature and widely used in various fields, provided favorable conditions for the further study of MC and its relationship with colon cancer. Our team zhou xin et al. analyzed the association between MC and colon cancer using proteomics technology and obtained some preliminary results, which still need to be further explored [12, 13].

In this study, we used 2-DE technology to obtain colonic mucosal differential protein spots of normal tissues, MC tissues, and colon cancer tissues. The mass spectrometry database was used to find and analyze the types and functions of these proteins, and to study the causes and development mechanisms of MC at the biomolecular level, and to reveal the biological association with colon cancer.

Materials and methods

Patients and clinical samples

Patients admitted to The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology from January 2013 to June 2014 were selected. The MC group included 15 patients, including 6 males and 9 females. The age ranged from 30 to 65 years old, with an average age of 45.31±10.36 years old. The colon cancer group included 15 patients, including 5 males and 10 females. The age ranged from 32 to 70 years old, with an average age of 46.28±11.23 years old. The normal control group included 15 patients, including 8 males and 7 females. The age ranged from 36 to 72 years old, with an average age of 48.18±11.23 years old. One-way ANOVA excluded the influence of age. Chi-square test excluded the impact of gender on this experiment.

Two experienced pathologists blindly confirmed all samples. Four pieces of tissue were taken from the diseased or normal parts, rinsed with precooled phosphate buffer saline (PBS), and stored in liquid nitrogen for later use. MC inclusion criteria: microscopically visible brown, shallow brown, or black changes of colonic mucosa. The intestinal lumen is obviously darkened, and the patch shape and snakeskin changes can be intermittent or continuously distributed. Pathological examination found that the other structural layers of the intestinal wall were basically normal, and there were a large number of mononuclear macrophages that engulfed pigment particles in the lamina propria. Exclusion criteria: merge to other intestinal disorders, patients with a history of intestinal surgery; recently treated patients; with severe heart, brain, lung, liver, kidney, and other patients with serious diseases; Patients with disorders of human protein metabolism. The normal control group was confirmed by histopathology without MC, colon cancer, intestinal polyps and inflammatory or non-inflammatory bowel disease. Patients with history of abdominal surgery, combined with digestive system diseases, severe heart, brain, lung, kidney and other diseases were excluded. The patient approved all specimens included in the experiment and approved by the Ethics Committee of Henan University of Science and Technology.

Specimen protein acquisition

All experiments were performed in specialized laboratories provided by SCI-LONGS BIOTECH INC (Beijing). Take out the samples from liquid nitrogen, weigh about 0.1 g respectively, rinse with PBS solution (4°C) and add liquid nitrogen to grind into powder. Add 1 mL RIPA solution and use ice ultrasound cell crushing apparatus for 4-5 cycles, 1 min each. In the 4°C environments for 2 hours, then placed in a high-speed centrifuge (Shimadzu, Japan) (program setting: > 12000 g, centrifuged for 5 min). Let stand

after collecting the centrifuged supernatant on to determine protein concentration.

Protein quantification with the Bradford method

Prepare standard liquid. Respectively 0, 2, 4, 6, 8, 10 μL standard bovine albumin (BSA) solution (2 mg/mL) was added to the 96 well plates and diluted to the scale with water. Aspirated sample 2 μL of the original solution, added 98 μL of H_2O to the 96-well plate, added Bradford working solution and mix well. After 5 minutes of standing, test the absorbance of A595 nm of each well, and substitute the formula to calculate the protein concentration. The data was input into Excel and a standard curve was drawn. After obtaining the slope equation, the protein concentration of the sample was calculated. The protein concentrations of the MC group, colon cancer group, and normal control group were measured as: 6.65 g/L, 6.03 g/L, and 2.90 g/L. The specimens were quantitatively separated and stored at -80°C placed.

Isoelectric focused electrophoresis with first solid pH gradient (IPG-IEF)

Calculate the volume of the sample loaded according to the length of the rubber strip, and operate 854 ug for each group of experimental samples. Add DTT 9 μL (containing 152.4 mg DTT and 0.85 mL H_2O) into the final volume concentration of 50 mmol/L required, IPG buffer added to the volume of 0.5% of the final concentration, according to the length of the strip, add rehydration buffer to strip the hydration fluid volume is 450 μL. The impurities were removed by centrifugation (high-speed centrifugation for 5 min) and stored at -80°C for later use.

IPG strips prefabricated thawed (length: 24 cm pH: 3-10) 10 min, removing the protective layer, place the strip face down on the focus plate sample solution, and cover the surface of the strip with 2-3 mL mineral oil, at room temperature stand for 12-16 h. Filter paper shall be applied at the place where electrodes are put on both sides of the rubber strip (add 80 mL of H_2O to each side). After the electrode is dried, electrophoresis is carried out, set the time and program parameters (current 50 μA/strip, time 23-24 h, temperature 20°C). The optimal time was established according to the sample size, pH range, and length of the strip.

Second vertical plate SDS-PAGE electrophoresis

Different molecular weight proteins by SDS-PAGE separation of protein mixture can be separated based on two-dimensional planes, preparation for SDS-PAGE gel. 0.16 L volume of 13% gel solution was prepared and injected into the prepared gel model along the glass wall. Natural solidification occurs at 25°C after filling. 8 test tubes were selected, and 8 mL balanced buffer mother liquor was added. Added 0.16 mL of DTT wherein 4 test tubes, was manufactured into strip balanced I, the remaining 4 tubes were added 148 mg iodoacetamide, made-manufactured strips equilibrium liquid II. The strip was successively placed in equilibrium liquid I and II, and the horizontal shaker was slowly shaken for 15 min. After two equilibrations, the strip was immersed in SDS-PAGE electrophoresis buffer for the 30 s. The dissolved agarose was dropped onto the upper surface of SDS-PAGE gel, and the subsequent electrophoresis operation was performed after the agarose was completely condensed. The electrophoretic solution was precooled, and the gel was placed in the electrophoresis tank for sealant treatment. Set temperature 20°C, and switching on the the cooled electrode buffer. The initial setting was 4 W/gel for 1 h. And then constant power 10 W/gel, 6-7 h. The gel was placed in the fixator, placed on the shaker for 2 h, and the protein was fixed.

Coomassie brilliant blue stain

The fixed solution was extracted with a vacuum pump, cleaned repeatedly with ultrapure water, and added with coomassie brilliant blue staining solution. The staining time was about 12-22 h. The decolorization time after finishing the staining is about 2 h. The decolorization process is performed twice, and DD H_2O is used for washing for 2-3 times after completion.

Image comparison and analysis

Image scanner III (GE, USA) and compare Software image master 7.0 (GE Healthcare, USA) was used to shear and modify the gel images. After adjusting the parameters, differential protein spots were screened and manu-

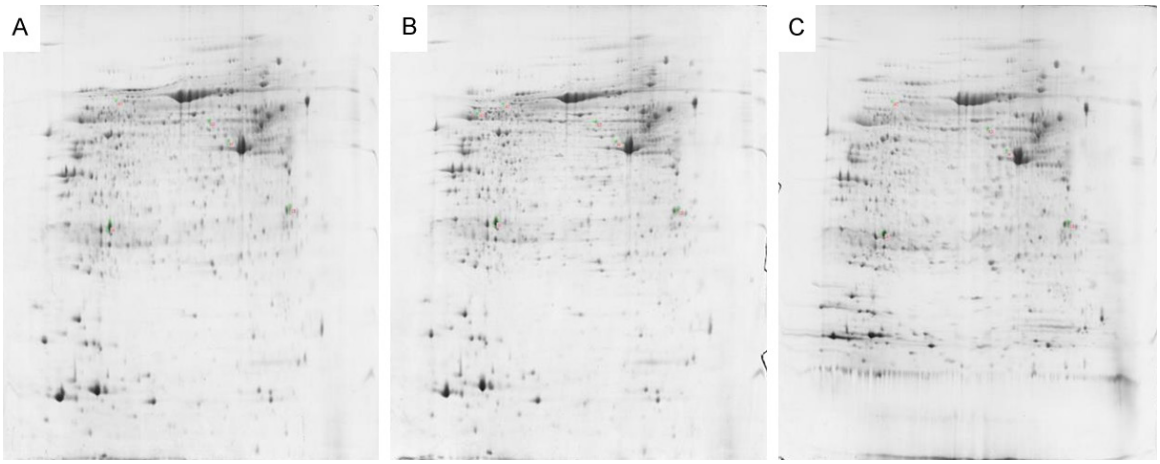


Figure 1. MC tissues three times electrophoresis image contrast. Comparison of images obtained by the first (A), second (B), third (C) two-dimensional gel electrophoresis of MC tissues showed a clear background and matching rate > 80%, indicating good repeatability of the experiment. After analysis and processing, the relative protein content of each protein strip could be generated for further experiments.

ally corrected. Chosen the protein spots with a difference of expression greater than 1.5 times as the study objects for mass spectrometry identification and analysis of protein function.

Statistical analysis

Statistical analysis was performed using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation (SD). Chi-square test was used for comparison data classification variables between the groups, one-way ANOVA was used for the comparison of continuous variables, and T-test was used for the comparison between the two groups. $P<0.05$ was considered statistically significant.

Proteolysis

Significantly differential protein spots on the gel were cut off and put into a centrifugal tube containing 0.5 mL DI. We used ultrapure water rinsing twice. Add the decolorizing liquid and shake it repeatedly until the decolorization is complete. Dry with filter paper for 5 min. Each tube was added with 10 μL of enzymatic hydrolysate solution for bloating for about 30 min. The filter paper adsorbed the remaining enzymatic hydrolysate, supplemented with 10 μL covering solution (25 mmol/L NH_4HCO_3, 5% ACN), sealed, and kept warm at 37°C water bath for 10 h. The supernatant was placed in a clean centrifuge tube, and the remaining gel blocks were added with an extraction solution containing 0.1% TFA and 33% ACN, followed by ultrasound for 30 min. Absorb the extract, add an aqueous solution of 0.1% trifluoroacetic acid and 100% acetonitrile, and sonicate for 10 min. Clear liquid on the merger, vacuum freezing centrifugal drying 5-6 μL mass spectrometry detection.

Biomass spectrum manipulation and database retrieval

Mass spectrometry analysis and identification of 0.5-1 μL samples were performed after 2-3 times of spot target treatment and drying treatment with spot matrix. The API4800 tandem time-of-flight mass spectrometer (MALDI-TOF/TOF ABI, USA) was used to select the appropriate scanning mode, laser energy, and scanning range. Integrate primary and secondary mass spectrometry data. We used the retrieval software Mascot2.1 (Matrix Science, UK) and GPS Explorer software3.6 (ABI, USA), the results of our analysis of the mass spectrum.

Results

Two-dimensional gel image has good reproducibility

The gel image of MC tissues protein gel samples obtained after three experiments (**Figure 1**). After image contrast, the background of the three images visible, less texture, high resolu-

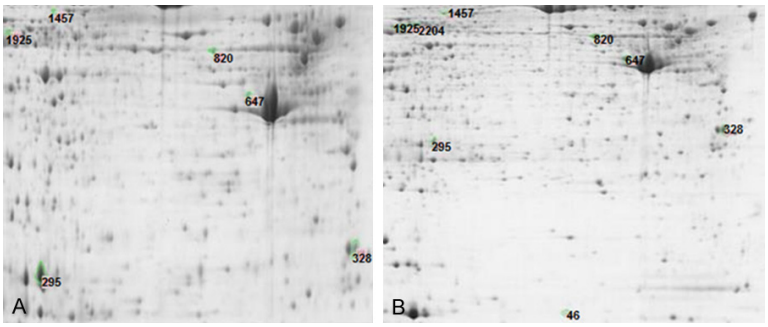


Figure 2. The MC group and colon cancer group images the distribution of differences in protein spots. A. There were six differential protein spots in the MC group, including 295, 328, 647, 820, 1457, and 1925. B. In addition to the presence of 295, 328, 647, 820, 1457, and 1925, the colon cancer group had the presence spots of 46 and 2204.

tion, image spots were a clear separation between the matching rate > 80%, illustrating the experimental repeatability is excellent, in the process of sample preparation of organization to eliminate the impurity in the group effect is good. After the gel image analysis, we can obtain the relative protein content of each protein band. Two-dimensional gel image analysis of colon cancer tissues and normal colon tissues can be made to ensure the accuracy and reliability of experimental data.

Image comparison and data analysis between the MC group and the colon cancer group

The average protein spots of the three groups were 1970±138 (MC group), 2150±240 (colon cancer group), and 2028±176 (normal tissue group). The MC images were compared with colon cancer and normal tissues respectively, and fourteen protein spots with a difference in expression level of more than 1.5 times were found. Comparison between the MC and colon cancer image: after sufficient comparative analysis, a total of eight protein spots with expression differences more than 1.5 times were found, encoding: 46, 295, 328, 647, 820, 1457, 1925, 2204. There were six common differential protein spots, including 295, 328, 647, 820, 1457, and 1925 (**Figure 2A** and **2B**); spots 46 and 2204 appeared only in the colon cancer group (**Figure 2B**).

Compare the content of eight differentially expressed protein spots between the MC group and the colon cancer group on the gel image. The upper part of each image is the colon cancer group, and the lower part is the MC group. It can be seen that the shade and area of the different spot staining are different. The expression levels of spots 295 were higher than those of the colon cancer group (**Figure 3B**). In contrast, those of spots 328, 647, 820, 1457, and 1925 were lower than those of the colon cancer group (**Figure 3C-G**), among which spots 46 and 2204 appeared only in the colon cancer group (**Figure 3A** and **3H**). The difference of spot protein content between MC and colon cancer tissues was analyzed (**Table 1**), and the difference was statistically significant by t-test (P<0.01).

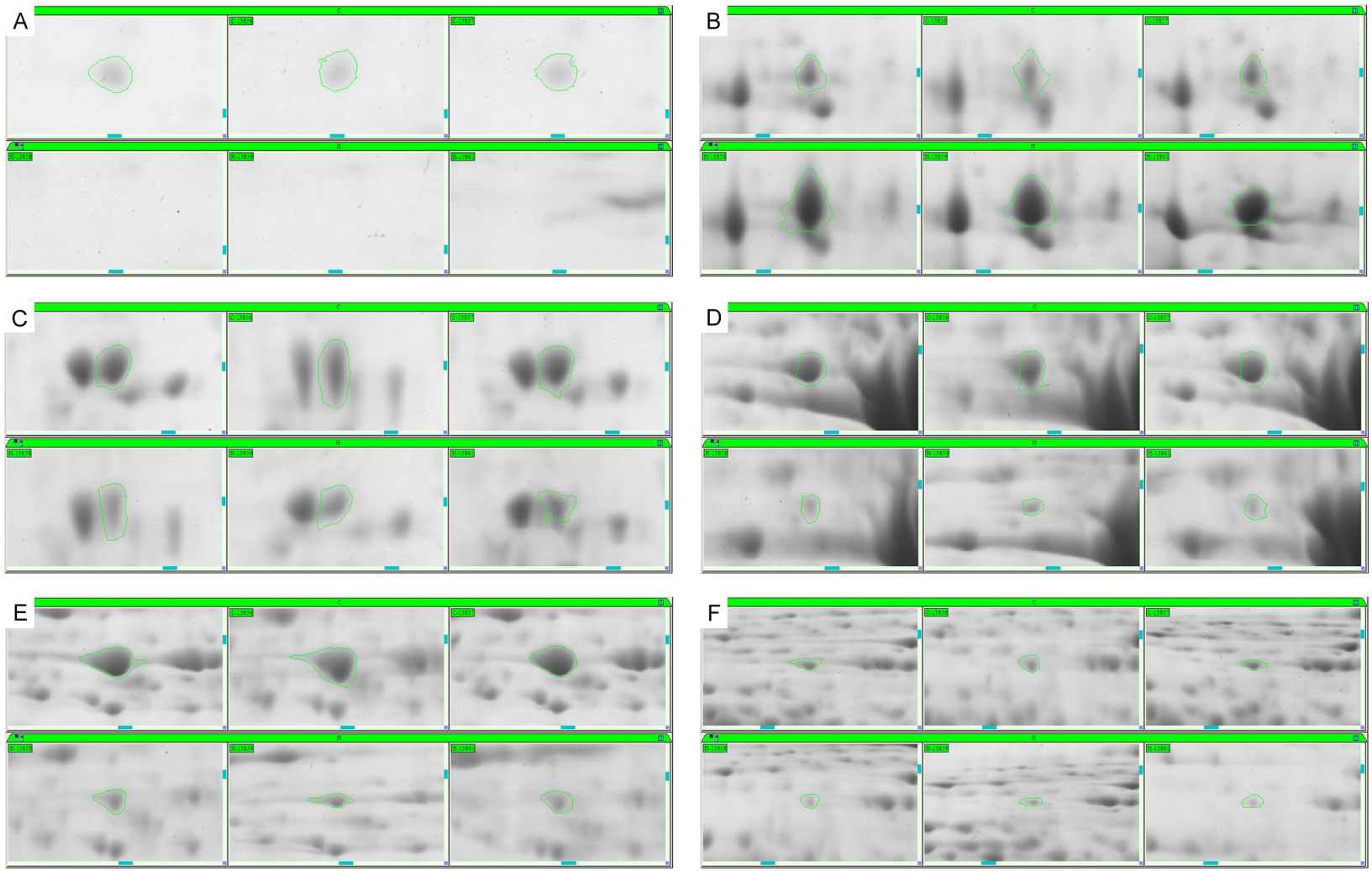
Image comparison and data analysis between the MC group and the normal group

We were comparing the MC and the normal group image: after sufficient comparative analysis. We found a total of six protein spots with expression differences more than 1.5 times, encoding: 602, 647, 817, 820, 938, 1783. There were five common differential protein spots, including 602, 647, 817, 820, 938 (**Figure 4A** and **4B**), spot 1783 appeared only in the MC group (**Figure 4A**).

Compare the content of six differentially expressed protein spots between the MC group and the normal group on the gel image. The upper part of each image is the MC group, and the lower part is the normal group. It can be seen that the shade and area of the difference spot staining are different. The expression levels of spot 938 were higher than those of the normal group (**Figure 5E**), while those of spots 602, 647, 817 and 820 were lower than those of the normal group (**Figure 5A-D**), spot 1783 appeared only in the MC group (**Figure 5F**). The difference of spot protein content between MC and normal tissues was analyzed (**Table 2**), and the difference was statistically significant by t-test (P<0.05).

Protein identification results and analysis

Using Mascot software (Matrix Science, UK) to search the NCBInr database, a matching score


A
B
C
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E
F

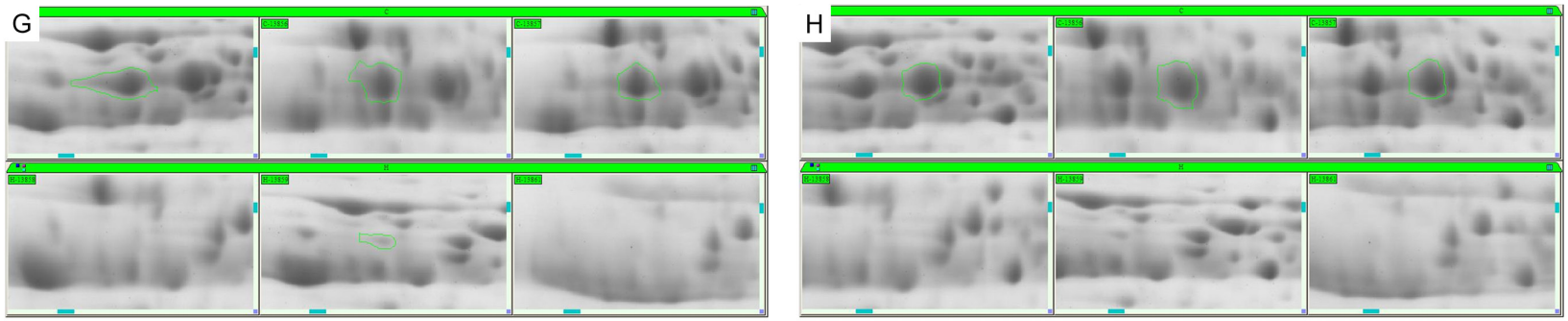


Figure 3. The MC and the colon cancer group differences in protein spots content. (Deep shade and a large area of the image indicate high content, the upper part of each image is the colon cancer group, and the lower part is the MC group). A. Display spot 46 appeared only in the colon cancer group. B. Display spot 295 expressed in MC group content is higher than that of the colon cancer group. C. Display spot 328 expressed in MC group content is lower than that of the colon cancer group. D. Display spot 647 expressed in MC group content is lower than that of the colon cancer group. E. Display spot 820 expressed in MC group content is lower than that of the colon cancer group. F. Display spot 1457 expressed in MC group content is lower than that of colon cancer group. G. Display spot 1925 expressed in MC group content is lower than that of the colon cancer group. H. Display spot 2204 appeared only in the colon cancer group.

Table 1. MC and colon cancer tissue differences in spot protein content

Differential speckle coding	Average protein content (%VOL)		P
	MC	Colon Cancer Tissue	
46	NO	0.078	P<0.01
295	0.640	0.158	P<0.01
328	0.183	0.330	P<0.01
647	0.041	0.251	P<0.01
820	0.065	0.370	P<0.01
1457	0.016	0.038	P<0.01
1925	0.010	0.231	P<0.01
2204	NO	0.278	P<0.01

MC, melanosis coli.

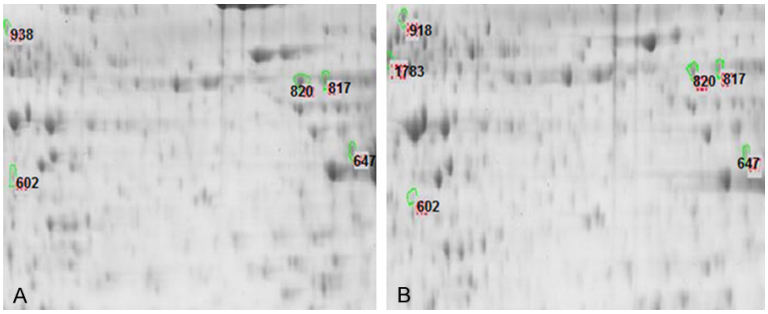


Figure 4. The MC group and the normal group images the distribution of differences in protein points. A. There were six differential protein spots in the MC group, including 602, 647, 817, 820, 938, and 1783. B. In the normal group, there were five spots with points 602, 647, 817, 820, 938.

of more than 62 was considered a successful match. The results showed that ten protein spots were identified (among which two groups had the same identification results; spots 46, 1925 matching score less than 62 points determine failure), a total of eight proteins (**Tables 3** and **4**). Including keratin 18 (KRT18), carbonic anhydrase 1 (CA1), isoform c of prelamin-A/C (LMNA), catalase (CAT), fibrinogen beta chain (FGB), 26s protease regulatory subunit 10b (PSMC6), keratin 8 (KRT8), tropomyosin 4 (TPM4) (**Figure 6A-H**). Combined with the comparison of the content of differentially expressed protein spots, the comprehensive analysis showed that KRT8 and KRT18 were down-regulated in MC and up-regulated in colon cancer. FGB expression was up-regulated in MC and colon cancer, but no expression in normal tissues. CAT expression in MC was higher than that in normal tissues. PSMC6 expression in MC is lower than normal tissue. The expression of TPM4 and LMNA in MC was lower than that in colon cancer tissues. CA1 expression was higher in MC than in colon cancer tissues.

Discussion

MC is a metabolic lesion in the lamina propria of colonic mucosa, in which macrophages contain lipid-brown-like substances. It is characterized by mucosal pigmentation and is also a non-inflammatory, benign and reversible lesion. The diagnosis mainly depends on endoscopic examination [14]. In recent years, a large number of experimental and retrospective studies have shown that, although MC is a reversible and non-inflammatory disease, it may be associated with common intestinal diseases such as intestinal polyps, colorectal cancer, and inflammatory bowel disease. With the improvement of living standards, the detection rate of MC increases year by year, and people's research on MC has gradually entered a more in-depth level [15]. The development of biomass spectrometry and proteomics technologies such as 2-DE has provided a perfect technical guarantee for our experiment, and has been widely applied in various neighborhoods in [16]. We used proteomics techniques to explore the causes and mechanisms of MC development and to uncover the biological link with colon cancer.

In this study, to guarantee the accuracy and reliability of the test and reduce systematic errors, we performed three repeated gel electrophoresis on each tissue sample and calculated the average electron value of the gel images. In the present study, we selected 24 cm long nonlinear IPG bands with pH between 3-10, since the protein spots displayed by these bands were more distinct and independent, and the separation area was larger. Protein Coomassie brilliant blue staining is relatively simple, easy to control, and more repetitive than silver staining. Therefore, we used coomassie brilliant blue in this study.

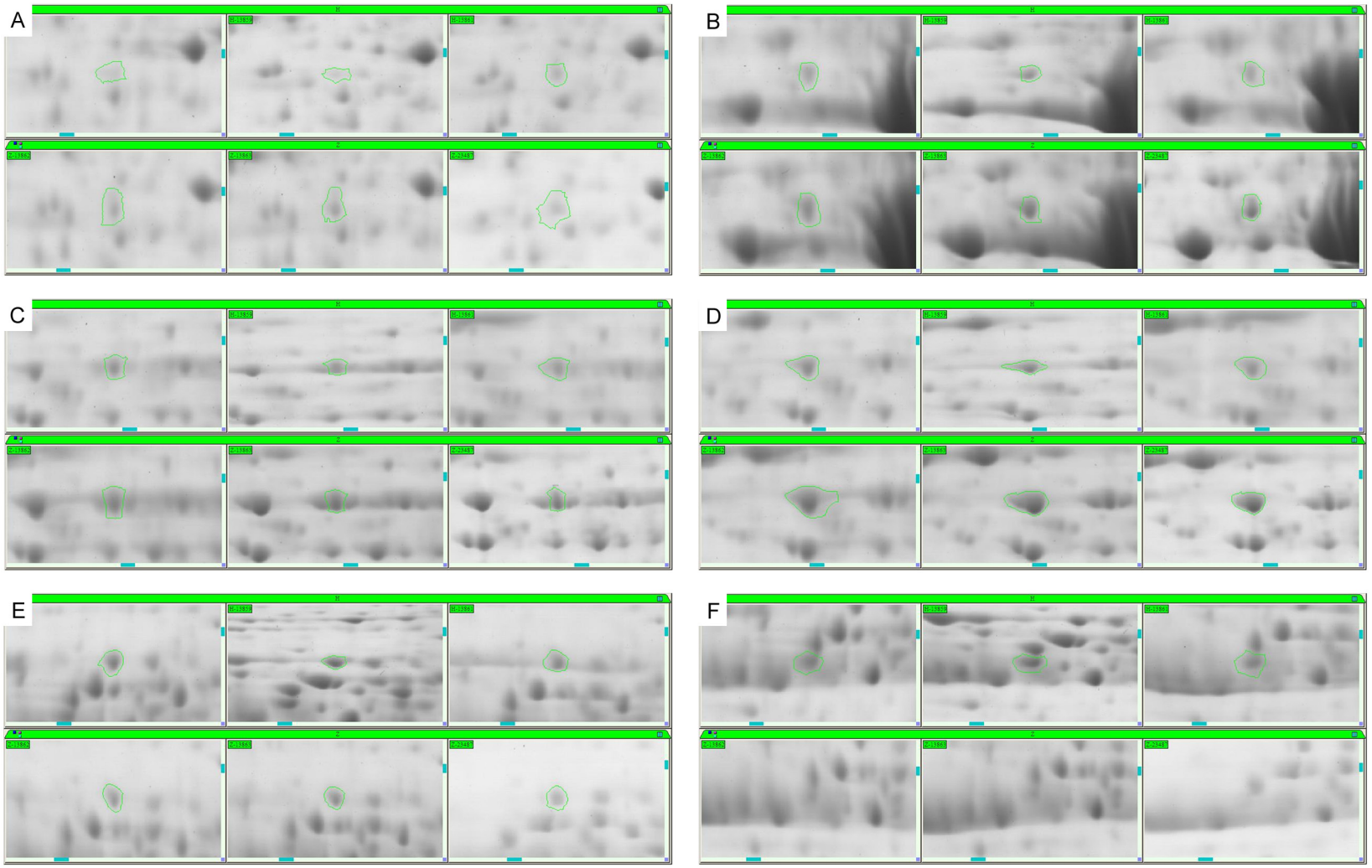


Figure 5. The MC and the normal group differences in protein spots content. (Deep shade and a large area of the image indicate high content, the upper part of each image is the MC group, and the lower part is the normal group). A. Display spot 602 expressed in MC group content is lower than that of the normal group. B. Display spot 647 expressed in MC group content is lower than that of the normal group. C. Display spot 817 expressed in MC group content is lower than that of the normal group. D. Display spot 820 expressed in MC group content is lower than that of the normal group. E. Display spot 938 expressed in MC group content is higher than that of the normal group. F. Display spot 1783 appeared only in the MC group.

Table 2. Normal tissue and MC tissue differences in spot protein content

Differential speckle coding	Average protein content (%VOL)		P
	MC	Normal Tissue	
602	0.022	0.047	P<0.01
647	0.041	0.084	P<0.05
817	0.051	0.088	P<0.01
820	0.065	0.185	P<0.05
938	0.064	0.044	P<0.05
1783	0.078	NO	P<0.05

MC, melanosis coli.

The two-dimensional gel electrophoresis technology (2-DE) platform for colon cancer, MC, and normal tissues of proteomics established in this study provides technical support for subsequent comparative proteomics studies in colon cancer. Through detailed research and comparison, we found 8 proteins, including keratin 8, keratin 18, fibrinogen beta chain, catalase, 26s protease regulatory subunit 10b, tropomyosin 4, carbonic anhydrase 1, isoform c of prelammin-A/C, which could be deemed as associated proteins of MC and colon cancer.

This study showed that keratin 8/18 (KRT8/18) was down-regulated in MC and up-regulated in colon cancer. Keratin is one of the cytoskeletal components of cells, providing mechanical stability and integrity to epithelial tissues by forming structural scaffolds that protect them from various cellular stresses. KRT8/18 mainly in multiple abnormal expression in squamous cell carcinoma, which is associated with increased invasiveness and poor prognosis. Also, to using as a diagnostic marker for tumors, KRT8/18 has also been reported to regulate different signaling pathways involved in tumor progression [17]. Analysis by Jian Fang's team proved that KRT8 is related to the overall survival rate of gastric cancer patients. Patients with high expression of KRT8 often have a poor prognosis. KRT8 can regulate the occurrence of gastric cancer and may become a potential target for anti-tumor therapy. Plasminogen activation promoted the infiltration of tumor cells [18]. These studies suggest that KRT8 may be involved in the development of MC and colon cancer, which may be a potential molecular marker for the diagnosis of MC and colon cancer.

In this study, the expression of fibrinogen beta chain (FGB) was up-regulated in MC and colon cancer, but not in normal tissues. Fibrin is part of the major components of a blood clot, whose primary function is blood clotting, in addition to fibrin deposition, which is associated with infection and promotes antimicrobial immune responses through innate and T-cell mediated pathways. In recent years, the role of FGB in tumors has been gradually revealed, and the endogenous fibrinogen can promote the growth of lung cancer and prostate cancer cells. FGB is one of the key effectors of epithelial-mesenchymal transformation associated with cell adhesion and cell communication in lung cancer. FGB can be used as a biomarker for the diagnosis of bladder cancer. Besides, FGB is also highly expressed in renal cell carcinoma [19-22]. Yang's team finally detected a significant correlation between plasma FGB and colorectal cancer by tandem mass spectrometry and western blot, which could be utilized as a potential biomarker for early diagnosis of colorectal cancer [23]. This study showed that FGB was up-regulated in MC and colon cancer tissues, which may indicate that MC is related to the growth and development of colon cancer.

This study showed that catalase (CAT) in colon cancer tissues was significantly less than that in the other two groups, and CAT expression in MC was higher than that in normal tissues. CAT is widely found in living organisms. Its principal function is to catalyze the decomposition of hydrogen peroxide into oxygen and water. The CAT may signal by reducing hydrogen peroxide and prevent apoptosis, to prolong life. It has been confirmed that the prevalence of overexpression of mitochondrial CAT was decreased in mice [24]. Many studies have confirmed that the expression of CAT in cancer cells is decreased [25], which is consistent with our results. The significant reduction of CAT in colon cancer tissues may indicate that the free radical scavenging system in tumor tissues is damaged in the process of tumor occurrence. In recent years, many scholars have studied the CAT in the tissues and fluid-level relationships with tumor diagnosis and systemic treatment. CAT can be used as a tumor marker to assist diagnosis, guide treatment, and determine the prognosis of malignant tumors.

Table 3. Results of MC and colon cancer tissue differential protein

Difference spot number	Acession number	Protein name	score	Mr	pI	Sequence coverage
295	IPI00215983	CA1 (Carbonic anhydrase 1)	253	28909	6.59	60%
328	IPI00010779	TPM4 (Isoform 1 of Tropomyosin alpha-4 chain)	149	28619	4.67	44%
647	IPI00554788	KRT18 (Keratin)	457	48029	5.34	72%
820/817	IPI00554648	KRT8 (Keratin)	295	53671	5.52	52%
1457	IPI00216952	LMNA (Isoform C of Prelamin-A/C)	132	65153	6.40	38%
2204/1783	IPI00965713	FGB (fibrinogen beta chain isoform 2 preproprotein)	66	50436	8.22	21%

MC, melanosis coli.

Table 4. Results of differential proteins between MC and normal colon tissue

Difference spot number	Acession number	Protein name	score	Mr	pI	Sequence coverage
602	IPI00926977	PSMC6 (26S protease regulatory subunit 10B)	105	46053	7.64	34%
647	IPI00554788	KRT18 (Keratin)	457	48029	5.34	72%
817/820	IPI00554648	KRT8 (Keratin)	295	53671	5.52	52%
938	IPI00465436	CAT (Catalase)	95	59947	6.90	18%
1783/2204	IPI00965713	FGB (fibrinogen beta chain isoform 2 preproprotein)	66	50436	8.22	21%

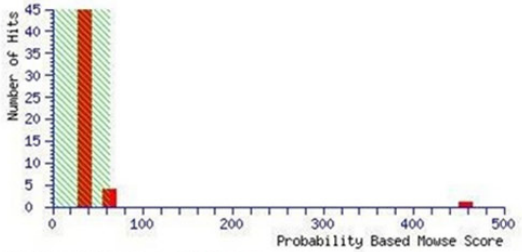
MC, melanosis coli.

In this study, we found that 26s protease regulatory subunit 10b (PSMC6) was down-regulated in MC tissues and was not found in colon cancer. PSMC6 is a core protease for ATP and protein degradation in the eukaryotic cytoplasm and nucleus [26]. A study confirmed that PSMC6 was abnormal in MC patients and was a candidate biomarker related to MC apoptosis. This could provide a theoretical basis for the early development of MC and the prevention of complications. PSMC6 is also involved in cell surface receptor signaling pathways and proteasome pathways activated by innate immune responses. Lack of PSMC6 expression in human multiple myeloma cells may induce resistance to apoptosis [27, 28]. The results of our study the function and mechanism of PSMC6 can be further studied, which is helpful to reveal further the physiological processes such as protein degradation, regulation of cell cycle, inhibition of tumor genes, expression of tumor antigen, activation or degradation of transcription factors.

This study indicated that tropomyosin 4 (TPM4) was expressed more than MC in colon cancer tissues. At present, TPM4 is abnormally regulated in human tumors such as ovarian cancer, breast cancer, colon cancer, and esophageal squamous cell carcinoma. It acts as an oncogene or a tumor suppressor gene [29]. One study showed a decrease in TPM4 in colon cancer tissues and cell lines. Low TPM4 expression was significantly correlated with clinical stage, infiltration depth, lymph node metastasis, distant metastasis, and differentiation. Survival analysis showed that low TPM4 expression was an independent prognostic factor for patients with colon cancer. In vitro experiments indicated that the up-regulation of TPM4 expression could inhibit the expression of genes related to migration, invasion, and metastasis of colon cancer cells. In summary, TPM4 is associated with the clinical progression of colon cancer patients and has a role in cancer inhibition in colon cancer cells [30]. This study showed that the expression of TPM4 was up-regulated in colon cancer patients, which may indicate that these patients get a better prognosis.

In this study, the expression of carbonic anhydrase 1 (CA1) was highly expressed in MC tissues and down-regulated in colon cancer tissues. Studies in recent years have shown that CA can regulate the pH of body fluids and is widely present in most tumor tissues. Its expression level is closely related to the invasion and spread of tumor cells and is involved in the generation and proliferation of most tumors. CA1 can be used as a potential biomarker for the detection of prostate cancer [31]. Therefore, it is speculated that the CA1 expression observed in colorectal cancer may predict better surgical outcomes and overall

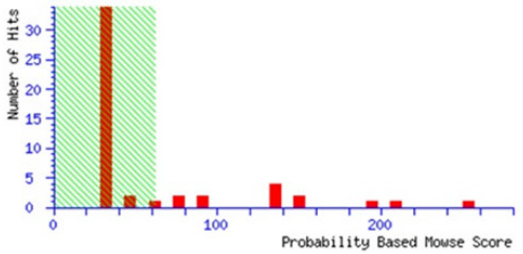
A



Matched peptides shown in **Bold Red**

```
  1 MSFITRSTFS TNYRSLGSVQ APSYGARPVS SAASVYAGAG GSGSRISVSR
 51 STSFRGGMGS GGLATGIAGG LAGMGGIQNE KETMQSLNDR LASYLDRVRS
101 LETENKKLES KIKEHLEKEG PQVKDWSHTF KIIEDLKAQI FANTVDNAKI
151 VLQIDNAKLA ADDFKVKYET ELAMRQSVEN DIHGLKEVID DTNITRLQLE
201 TEIEALKEEL LFMKKNHEEE VKGLQAQIAS SGLTVEVDAP KSQDLAKIMA
251 DIRAQYDELA RKNREELDKY WSQQIEESTI VVTTQSAEVG AAETTLTELR
301 RTVQSLEIDL DSMRNLKASL ENSLREVEAR YALQMEQLNG ILLHLESELA
351 QTRAEGQRQA QEYEALLNIK VKLEAEIATY RKLLEDGEDF NLGDALDSSN
401 SMQTIQKITI RRIVDGKVVS EINDTKVLRH
```

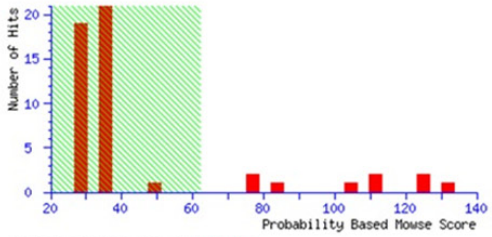
B



Matched peptides shown in **Bold Red**

```
  1 MASPDWGYDD KNGPEQWSKL YPIANGNNQS PVDIKTSETK HDTSLKPISV
 51 SYNPATAKEI INVGHSFHVN FEDNDNRSVL KGGPFSDSYR LFQFHFHWGS
101 TNEHGSEHTV DGVKYSAELH VAHWNSAKYS SLAEAASKAD GLAVIGVLMK
151 VGEANPKLQK VLDALQAIKT KGKRAPFTNF DPSTLLPSSL DFWTYPGSLT
201 HPPLYESVTW IICKESISVS SEQLAQFRSL LSNVEGDNAV PMQHNNRPTQ
251 PLKGRTVRAS F
```

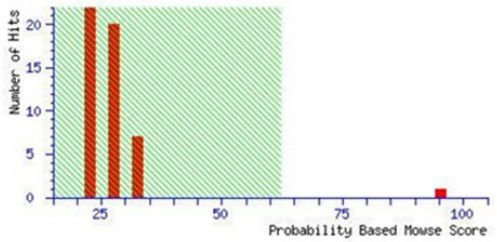
C



Matched peptides shown in **Bold Red**

```
  1 MEIPSQRRAT RSGAQASSTP LSPTRITRLQ EKEDLQELND RLAVYIDRVR
 51 SLETENAGLR LRITESEEVV SREVSGIKAA YEAELGDARK TLDSVAKERA
101 RLQLELSKVR EEFKELKARN TKKEGDLIAA QARLKDLEAL LNSKEAALST
151 ALSEKRTLEG ELHDLRGQVA KLEAALGEAK KQLQDEMLRR VDAENRLQTM
201 KEELDFQKNI YSEELRETKR RHETRLVEID NGKQREFESR LADALQELRA
251 QHEDQVEQYK KELEKTYSAK LDNARQSAER NSNLVGAAHE ELQQSRIRID
301 SLSAQLSQLQ KQLAAKEAKL RDLEDSLARE RDTSNRLLAE KEREMAEMRA
351 RMQQQLDEYQ ELLDIKLALD MEIHAYRKLL EGEEERLKLS PSPTSQRSRG
401 RASSHSSQTQ GGGSVTKKRK LESTESRSSF SQHARTSGRV AVEEVDEEGK
451 FVKLRNKSNE DQSMGNWQIK RQNGDDPLLT YRFPPKFTLK AGQVVTIWAA
501 GAGATHSPPT DLVVKAQNTV GCGNSLRTAL INSTGEEVAN RKLVRSVTVV
551 EDDEDEDGDD LLHHHHVSGS RR
```

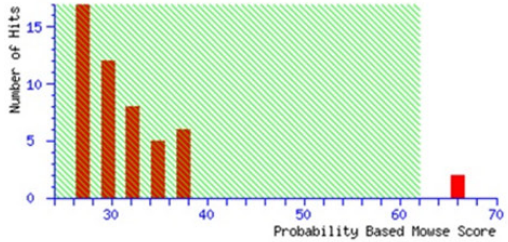
D



Matched peptides shown in **Bold Red**

```
  1 MADSRDPASD QMQHWKEQRA AQKADVLTTG AGNPVGDKLN VITVGPRGPL
 51 LVQDVVFTDE MAHFDRERIP ERVVHAKGAG APGYFEVTHD ITKYSKAKVF
101 EHIGKKIPIA VRFSTVAGES GSADTVRDPR GFAVKFYTED GNWDLVGNNT
151 PIFFIRDPIL FPSFIHSQKR NPQTHLKDPD MVWDFWSLRP ESLHQVSFLF
201 SDRGIPDGHR HMNGYGSHTF KLVNANGEAV YCKFHYKTDQ GIKNLSVEDA
251 ARLSQEDPDY GIKDLFNAIA TGKYPSWTFY IQVMTFNQAE TFPFNPFDLT
301 KVWPHKDYPL IPVGKLVLNR NPVNYFAEVE QIAFDPSNMP PGIEASPDKM
351 LQGRLFAYPD THRHRLGPNY LHIPVNCPYR ARVANYQRDG PMCMQDNQGG
401 APNYYPNSFG APEQQPSALE HSIQYSGEVR RFNTANDDNV TQVRAFYVNV
451 LNEEQKKRLC ENIAGHLKDA QIFIQKEAVK NFTEVHPDYG SHIQALLDKY
501 NAEKPKNAIH TFVQSGSHLA AREKANL
```

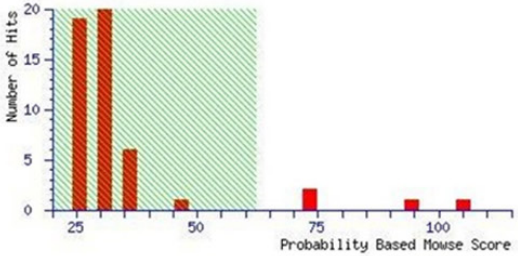
E



Matched peptides shown in **Bold Red**

```
  1 MKRMVSWSFH KLKTMKHLLL LLLCVFLVKS QGVNDNEEGF FSARGHRPLD
 51 KKREEALLQQ ERPIRNSVDE LNNNVEAVSQ TSSSSFQYMY LLKDLWQKRQ
101 KQVKDNENVV NEYSSELEKH QLYIDETVNS NIPTNLRVLR SILENLRSKI
151 QKLESDVSAQ MEYCRTPCTV SCNIPVVSGK BCEEIIRKGG ETSEMYLIQP
201 DSSVKPYRVY CDMNTENGGW TVIQNRQDGS VDFGRKWDPY KQGFGNVATN
251 TDGKNYCGLP GEYWLGNDKI SQLTRMGPTE LLIEMEDWKG DKVKAHYGGF
301 TVQMEANKYQ ISVNKYRGTA GNALMDGASQ LMGENRTMTI HNGMFFSTYD
351 RDNDGWLTSD PRKQCSKEDG GGWWYNRCHA ANPNGRYYWG GQYTWDMAKH
401 GTDDGVVWMN WKGSWYSMRK MSMKIRPFFP QQ
```

F



Matched peptides shown in **Bold Red**

```
  1 MAIPGIPYER RLLIMADPRD KALQDYRKKL LEHKEIDGRL KELREQLKEL
 51 TKQYEKSEND LKALQSVGQI VGEVLKQLTE EKFIVKATNG PRYVVGCRRQ
101 LDKSKLKPGT RVALDNTTLT IMRYLPREVD PLVYNMSHED PGNVSYSEIG
151 GLSEQIRELR EVIELPLTNP ELFQRVGIIP PKGCLLYGPP GTGKTLLARA
201 VASQLDCNFL KVVSSSIVDK YIGESARLIK EMFNYARDHQ PCIIFMDEID
251 AIGGRRFSEG TSADREIQRT LMELLNQMDG FDTLHRVKMI MATNRPDTLD
301 PALLRPGRLD RKIHIDLPNE QARLDILKIH AGPITKHGEI DYEAIVKLSD
351 GFNGADLRNV CTEAGMFAIR ADHDFVVQED FMKAVRKVAD SKKLESKLDY
401 KPV
```

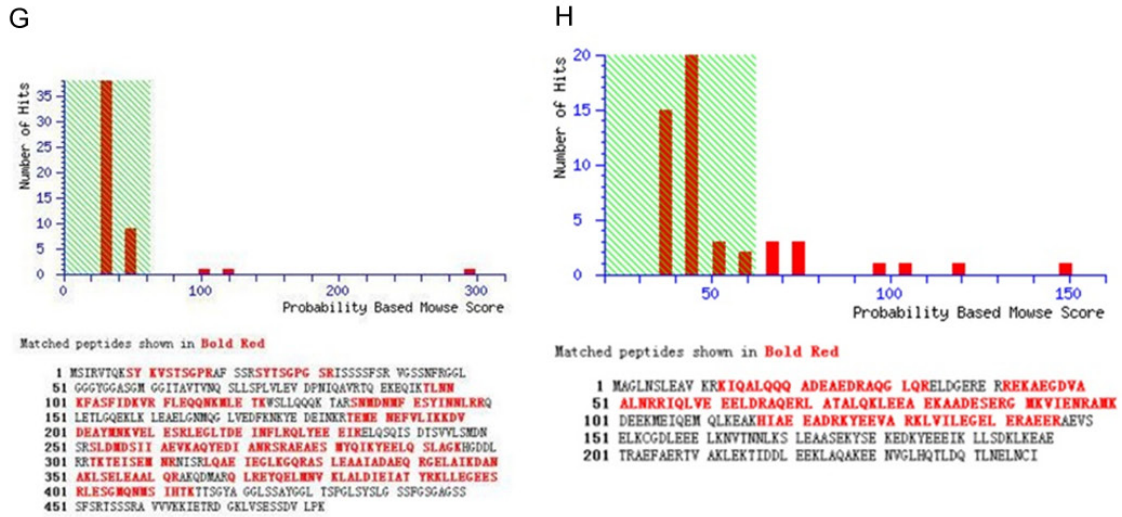


Figure 6. Results of protein mass spectrometry. (Red part of the matching sequence). A. KRT18 Mascot score histogram and protein amino acid sequence. B. CA1 Mascot score histogram and protein amino acid sequence. C. LMNA Mascot score histogram and protein amino acid sequence. D. CAT Mascot score histogram and protein amino acid sequence. E. FGB Mascot score histogram and protein amino acid sequence. F. PSMC6 Mascot score histogram and protein amino acid sequence. G. KRT8 Mascot score histogram and protein amino acid sequence. H. TPM4 Mascot score histogram and protein amino acid sequence.

survival in cancer patients. For tumor patients, increasing the expressive content of related CA in vivo has a good effect on improving patients' quality of life, surgical prognosis, and inhibiting cancer cell metastasis [32]. Further research on the relationship between tumors and CA has important theoretical significance and clinical value for exploring the pathogenesis of the tumor, carrying out gene-targeted chemotherapy, and improving the anticancer effect.

This study showed that lamin A/C expression in MC was lower than in colon cancer tissues. Lamin A is part of a nuclear membrane protein, plays a key role in the stability of the core, and many other biochemical processes, which is derived from a precursor protein, called prelamin A [33]. At the systemic level, prelamin A isoforms cause changes in the growth hormone axis, changes in glucose and lipid metabolism, and induction of chronic inflammatory responses. A cancer-induced mouse experiment showed that prelamin A did not affect the occurrence of tumors, but prevented the invasion of cancers, and was confirmed in human oral cancer, lung cancer and breast cancer cells [34]. Currently, there is a hypothesis that the diseases caused by mutations in different components of the lamin family are various; that is, they have different effects on various body tissues. For example, the expression level of lamin A in patients with skin cancer and ovarian cancer is significantly increased. In contrast, the expression level of lamin A/C in tumors of the blood system and the digestive system is significantly decreased considerably. Therefore, in combination with our experimental results, a detailed study on the lamin family may improve the cognition of the proliferation, differentiation, and metastasis of most tumors.

In conclusion, this study identified eight proteins differentially expressed in MC, colon cancer and normal colon tissues by two-dimensional gel electrophoresis, gel image contrast, and bio-mass spectrometry analysis software. These proteins could be deemed as associated proteins of MC and colon cancer, which provides a new direction for the follow-up study. Further study of the function of these proteins may provide new targeted molecular markers for the diagnosis and treatment of MC and colon cancer to achieve early detection and effective treatment and improve the overall therapeutic effect.

Acknowledgements

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Disclosure of conflict of interest

None.

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