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Histopathology of melanosis coli and determination of its associated genes by comparative analysis of expression microarrays

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Abstract. Melanosis coli (MC) refers to the condition characterized by abnormal brown or black pigmentation deposits on the colonic mucosa. However, the histopathological findings and genes associated with the pathogenesis of melanosis coli remain to be fully elucidated. The present study aimed to examine the histopathological features and differentially expressed genes of MC. This involved performing hematoxylin and eosin staining, specific staining and immunohistochemistry on tissues sections, which were isolated from patients diagnosed with MC. DNA expression microarray analysis, western blotting and immunofluorescence assays were performed to analyze the differentially expressed genes of melanosis coli. The results demonstrated that the pigment deposits in MC consisted of lipofuscin. A TUNEL assay revealed that a substantial number of apoptotic cells were present within the macrophages and superficial lamina propria of the colonic epithelium. Expression microarray analysis revealed that the significantly downregulated genes were CYP3A4, CYP3A7, UGT2B11 and UGT2B15 in melanosis coli. Western blotting and immunofluorescence assays indicated that the expression of CYP3A4 in the normal tissue was higher than in the MC tissue. The results of the present study provided a comprehensive description of the histopathological characteristics and pathogenesis of MC and for the first time, to the best of our knowledge, demonstrated that the cytochrome P450-associated genes were significantly downregulated in melanosis coli. This novel information can be used to assist in further investigations of melanosis coli.

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Introduction

Melanosis coli (MC) is a condition, in which the mucous membrane of the colon and rectum appear darker than usual, with the depth of color varying between pale grey and brown or black (1). Billiard first described the occurrence of colonic mucosal hyperpigmentation in 1825, which Virchow termed melanosis coli in 1857. In 1928, Bartle indicated that MC was associated with long time use of laxatives, and subsequent studies investigated this association (2-4). Investigations on animal models of melanosis have indicated that anthraquinone laxatives, including aloe, senna and rhubarb cause MC (5,6), however, their role in the etiology and pathogenesis of MC remains to be elucidated.

Several hypotheses have been suggested to explain the pigmentation of MC. The majority suggest that the formation of pigment granules is associated with purgative-induced apoptosis of colonic mucous membrane epithelial cells. The laxative effect of anthranoid laxatives induces damage on the epithelial cells; which causes alterations in absorption, secretion and motility. The outcome is harmful to the cells in the lining of the intestine and leads to apoptosis. These apoptotic cells are subsequently phagocytized by adjacent macrophages, which form a substance that appears as dark pigmentation granules (7). The distinctive pigmentation of the bowel wall develops when a sufficient number of cells have been damaged. It has also been suggested that improvements in standards of living and lack of proper exercise contribute to decreases in bowel movements and leads to chronic constipation. This, in turn, leads to an increased quantity of protein-rich foods remaining in the intestinal tract. The intestine absorbs the protein degradation products and converts them into melanin or lipofuscin by fermentation within the connective tissue cells. When melanin or lipofuscin is phagocytized by macrophages in the lamina propria, conditions are favorable for the

development of MC (8,9). Therefore, determination of whether there apoptotic cells are present in the colonic mucosa of MC patients is required.

The pigment bodies in the intestine may be composed of lipofuscin, melanin, hemosiderin or bile pigment, however, no clear experimental evidence has confirmed the type of pigment present in MC (10). Lipofuscin granules are residual bodies containing oxidized and/or undigested lipids. These granules are considered to result from the residue of cellular organelles within lysosomes (11). Melanin is synthesized through oxidation of tyrosine to dopamine and eventually melanin in the melanosome (12). Due to macrophage phagocytosis of erythrocytes and/or their breakdown products, hemosiderin develops within residual bodies (13). Each granule type is distinctive and can be visualized using specific staining. Confirmation of the type of pigment granules present in MC is required.

The presence of MC may indicate an increased risk for the development of colorectal cancer. High doses of anthraquinone cause tumor development in animals, and colorectal adenomas occur more frequently in patients with MC (14,15). Therefore, MC has clinical significance, and further analysis of its clinical features and pathogenesis is necessary. In previous years, several studies have been performed to investigate MC, however, the requirement for comprehensive investigation remains, and comparative analysis of gene expression differences in MC have not been determined. Therefore the present study aimed to investigate MC in terms of its endoscopic features, histopathological characteristic and gene expression differences, and provide a novel framework for understanding the pathogenesis of MC.

Table I. Clinical features of patients with melanosis coli.

Clinical feature	Number	Rate (%)
Gender		
Male	14	53.85
Female	12	46.15
Age (years)		
30-50	6	23.07
51-69	12	46.15
≥70	8	30.76
Obstipation		
Laxative use	20	76.92
Bloody stools	3	11.53
Abdominal pain	8	30.76
Abdominal distension	5	19.23
Constipation	12	46.15
Dry stool	12	46.15
Loose stools	3	11.53
Colonoscopic findings		
Brown	18	69.23
Red	8	30.76
Mucosal edema	6	23.07
Snake-skin appearance	7	26.92
Neoplasm	10	38.46
Adenocarcinoma	9	34.62

Materials and methods

Tissue and patients. A total of 26 patients with MC were recruited in the present study (Table. I), and tissue specimens were collected from the First Affiliated Hospital of Chengdu Medical College (Chengdu, China). Colonoscopy and biopsy were performed for chronic constipation, abdominal pains, distention or occasional bloody mucinous diarrhea. The tissue specimens were surgically removed under endoscopic monitoring. The Institutional Ethics Committee of Chengdu Medical College approved the present study. All patients provided informed consent prior to commencement.

Hematoxylin and eosin (H&E) staining. The histopathological characteristics of the MC tissue specimens were evaluated using H&E staining (Beyotime Institute of Biotechnology, Inc., Shanghai, China). The tissues were fixed in 10% formalin and embedded in paraffin, and then sectioned into 4 μm slices prior to staining with H&E.

Pathology-specific staining of MC. All grain sizes of the tissue blocks were prepared for specific staining by deparaffinization in xylol and rehydration in serial dilutions of ethanol and distilled water. All the chemical reagents used for specific staining were obtained from Chengdu Changzheng Glass Co., Ltd. (Chengdu, China). The periodic acid Schiff reaction (PAS) was used to detect lipofuscin. Following incubation with 0.5% periodic acid for 5 min at room temperature, sections were washed with distilled water for 15 min. Sections were incubated with Schiff's reagent (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature, followed by washing under running tap water for 5 min. All sections were counterstained with hematoxylin for 3 min. Masson-Fontana ammoniacal silver staining was used for melanin analysis. The sections were incubated with ammoniacal silver solution (a few drops of ammonia were added into 5% silver nitrate solution until the precipitation disappeared) in the dark for 15 min at room temperature and then washed twice with distilled water. The sections were then incubated with 0.2% gold chloride for 2 min at room temperature and subsequently washed with distilled water. Sections were then fixed in 2% sodium thiosulfate and finally counterstained in neutral red for 1 min. Bilirubin is oxidized to biliverdin in an acid medium, and this oxidation reaction occurs rapidly by ferric chloride in trichloroacetic acid solution (16). Sections were incubated with freshly prepared Fouchet's solution (1% $FeCl_3$, 25% CCl3COOH) for 5 min at room temperature. Sections were washed with distilled water and stained with Van Gieson's solution [1% fuchsin acid:1.22% picric acid (1:9)] for 5 min at room temperature. Tissue hemosiderin was detected using Prussian blue staining for ferric ion. Sections were incubated with a freshly prepared solution of a 1:1 mixture of 2% potassium ferrocyanide and 2% hydrochloric acid for 20 min at 60˚C. After washing with distilled water, the sections were counterstained in neutral red solution.

Immunohistochemistry. Staining was performed using an Histostain Plus kit (Zhongshan Golden Bridge, Co., Ltd.,

Beijing, China). The specimens were stained with mouse monoclonal immunoglobulin G (IgG) anti-melanoma antibody (cat. no. ZM0187; Zhongshan Golden Bridge Co., Ltd.). The antibody was diluted at 1:200 and incubated for 30 min at room temperature.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Cell apoptosis in the MC tissues was detected using a TUNEL assay kit (KeyGen Biotech Co., Ltd., Nanjing, China) following the manufacturer's instructions. Briefly, slides containing the tissue sections were incubated with 20 μg/ml proteinase K solution (Sigma-Aldrich) for 30 min at room temperature. Endogenous peroxidases were inactivated by immersing the slides in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4 and 2 mmol/l KH_2PO_4). A reaction mixture of rTdT (2% Biotin-11-dUTP and 5% TdT enzyme in PBS) was added to the slides, and the sections were incubated at 37˚C for 60 min to allow the end-labeling reaction to occur. The sections were then incubated with streptavidin-fluorescein isothiocyanate (FITC) solution (1:20 dilution) for 30 min at room temperature. After washing with PBS three times, the sections were incubated with peroxidase-conjugated anti-FITC solution (1:10 dilution) for 30 min at room temperature. Diaminobenzidine was then added for chromogenesis, for detecting the appearance of a light brown background.

Expression microarray analysis. Total RNA was extracted from the colon MC and normal colon tissues using TRIzol reagent (Gibco Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quantity and quality were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed using standard denaturing agarose gel electrophoresis. Sample labeling and array hybridization were performed, according to the Agilent One-Color Microarray-based gene expression analysis protocol (Agilent Technologies, Inc., Santa Clara, CA, USA). Briefly, total RNA from each sample was linearly amplified and labeled with Cy3-UTP. The Labeled cRNAs were purified by RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using the NanoDrop ND-1000. 1 μg each labeled cRNA was fragmented by adding 11 μl 10X blocking agent (Takara Bio, Inc., Shiga, Japan and 2.2 μl 25X fragmentation buffer (Takara Bio, Inc.), then heated at 60˚C for 30 min. Finally, 55 μl 2X gene expression hybridization buffer (Takara Bio, Inc.) was added to dilute the labeled cRNA. Hybridization solution (100 μl) was dispensed into the gasket slide and assembled to the gene expression microarray slide, and the slides were incubated for 17 h at 65˚C. The hybridized arrays were washed with gene expression wash buffer, fixed and scanned using the G2505C Agilent DNA Microarray Scanner (Agilent Technologies, Inc.). Agilent feature extraction software (version 11.0.1.1; Agilent Technologies, Inc.) was used to analyze the acquired array images. Differentially expressed genes with statistical significance were identified through volcano plot filtering. Hierarchical clustering was performed using Agilent Genespring GX software (version 11.5.1; Agilent Technologies, Inc.).

Gene ontology (GO) and pathway analysis were performed according to the standard enrichment computation method (17). The GO project provides a controlled vocabulary to describe gene and gene product attributes in any organism. The ontology covers three domains: i) Biological process; ii) cellular components; and iii) molecular function. Fisher's exact test establishes whether there is more overlap between the differentially expressed list and the GO annotation list, than would be expected by chance. The P-value denotes the significance of GO term's enrichment in the differentially expressed genes. The lower the P-value, the more significant the GO term (P<0.05). Pathway analysis is a type of functional analysis that maps genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/pathway.html). The P-value denotes the significance of the pathway correlated with the conditions. When the P-value is lower the pathway is more significant is (P<0.05).

Western blotting. The samples (60 μg) were electrophoresed on 10% SDS-PAGE gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The rabbit anti-human CYP3A4 polyclonal antibody (cat. no. bs-1472R; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) was diluted at 1:500, added to the membranes and incubated for 2 h at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated IgG (Zhongshan Golden Bridge Co., Ltd.) and analyzed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunofluorescence assay. Briefly, the blocked tissue sections were incubated overnight at 4˚C with rabbit anti-human CYP3A4 polyclonal antibody (cat. no. bs-1472R; Beijing Biosynthesis Biotechnology Co., Ltd.). The sections were then incubated with Dylight 649-conjugated secondary antibodies (GeneTex, Inc., San Antonio, TX, USA) for 30 min. The slides were visualized under a fluorescence microscope (TI-S; Nikon Corporation, Tokyo, Japan).

Statistical analysis. Statistical analysis of the results in each experiment was determined using one- or two-way analysis of variance analysis using SPSS 16.0 analysis software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Endoscopic and histopathological characteristics of MC. Examining the characteristics of MC revealed colonoscopy characteristics in three representative cases. Severe MC, in which a marked black-brownish pigmentation was apparent in the mucosa of the whole colon; moderate MC, in which diffuse and brown pigmentation was observed throughout the colon; and mild MC, in which the mucosa had a diffusely brownish, snake-skin appearance (Fig. 1A). H&E staining revealed that the yellow-brown granular pigment was confined to the tunica propria of the mucosa of large mononuclear histiocytes (Fig. 1B). Of the 26 patients who underwent endoscopy,

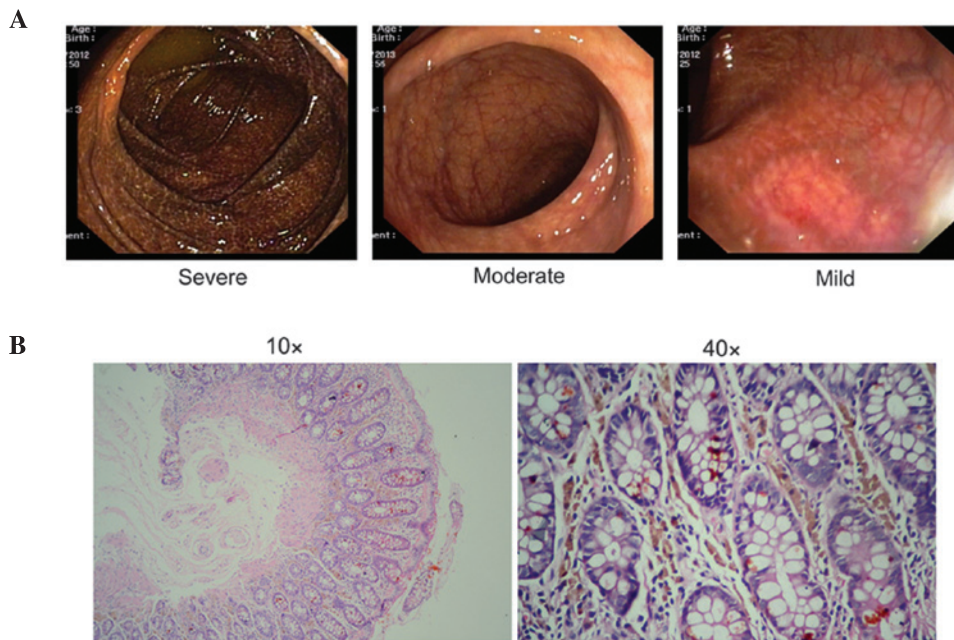

Figure 1. Pigmentation characteristics of melanosis coli. (A) Representative endoscopic images of the three characterized severities. Severe, black-brownish pigmentation of the mucosa of the entire colon. Moderate, diffusely brownish pigmentation. Mild, edematous, snake-skin appearance and pigmentation of the mucosa. (B) Hematoxylin and eosin staining revealing typical pigments in granule-laden macrophages in the lamina propria.

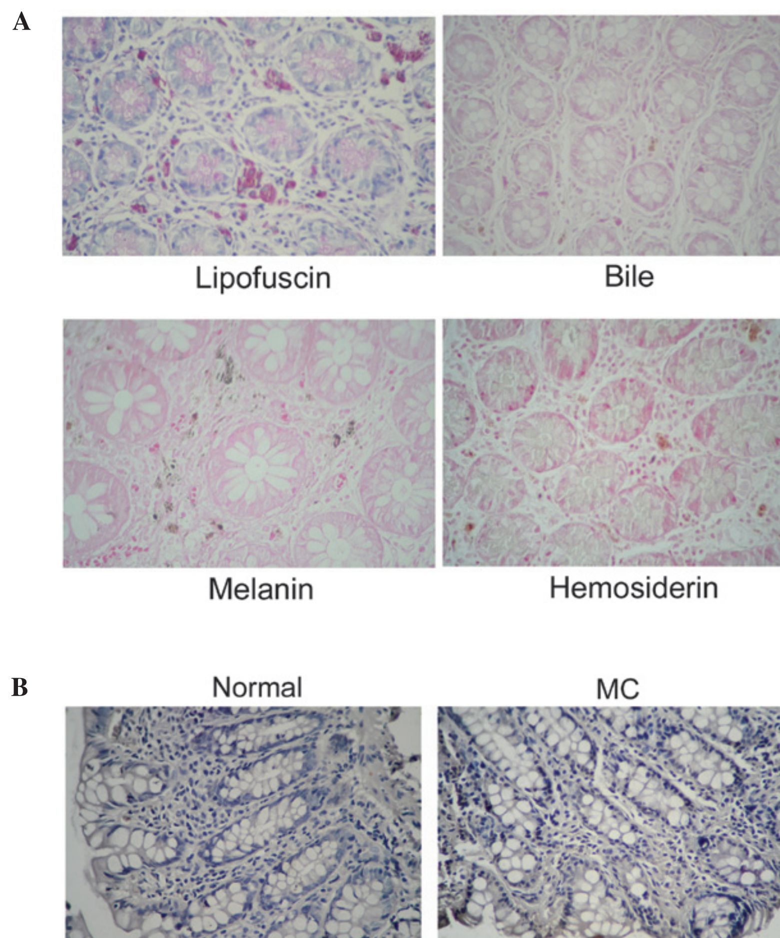

Figure 2. Specific staining and immunohistochemistry for pigment detection in MC tissues. (A) Lipofuscin, periodic acid Schiff reaction demonstrated the presence of lipofuscin, which appears as purple particles. Bile, ferric chloride in trichloroacetic acid medium analysis revealed the absence of bile. Melanin, no obvious black particles were observed following Masson-Fontana ammoniacal silver staining. Hemosiderin, prussian blue staining for ferric ions revealed a negative result for hemosiderin in tissue. Magnification, x40. (B) Paraffin wax-embedded tissues stained using a melanin-antibody. No melanin expression was observed in the MC tissues. Magnification, x40. MC, melanosis coli.

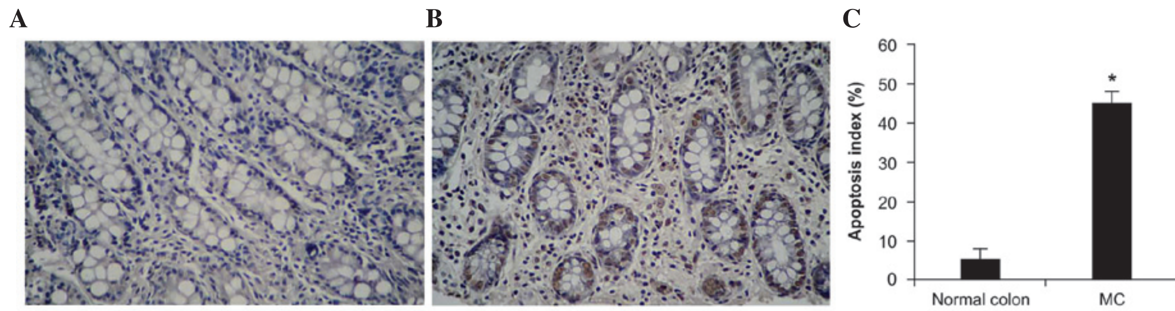

Figure 3. Apoptosis of the colonic cells in MC, determined using a TUNEL assay. (A and B) Sections detected using a TUNEL assay (magnification, x40). (C) Apparent increase in number of apoptotic cells and apoptotic index within the MC tissues, compared with the normal colon tissues. The bar graph indicates the mean ± standard deviation. *P<0.05, compared with the normal colon tissues. MC, melanosis coli. TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

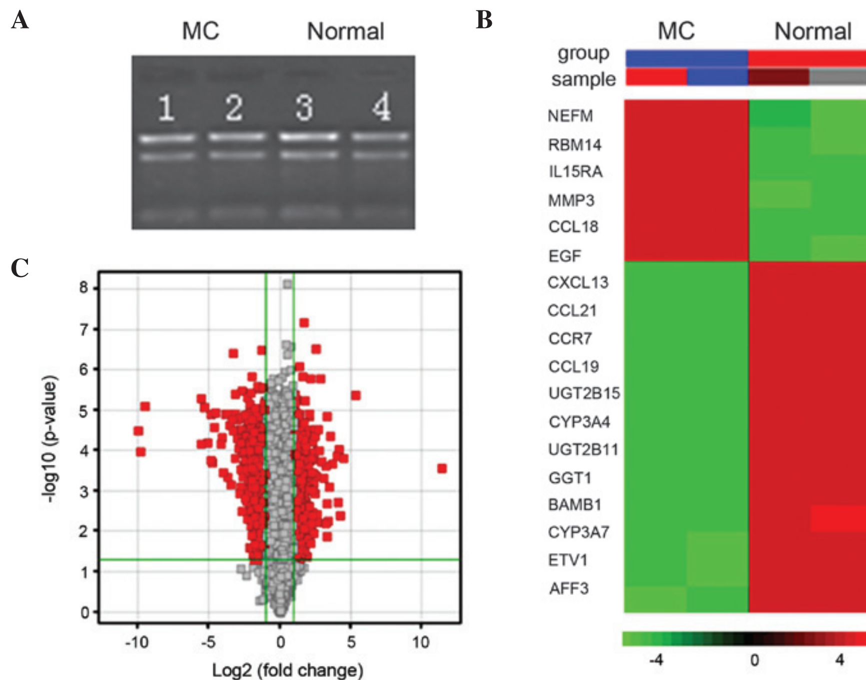

Figure 4. Differentially expressed genes in MC, determined using expression microarray analysis. (A) Total RNA of the samples: Lane 1-2, donors where endoscopic examination revealed MC; lane 3-4, donors where endoscopic examination demonstrated no abnormalities. (B) Heat map for signature genes in each group. Color scale, log2-transformed expression (red, high; green, low) for each gene (row) normalized by the mean of all samples. (C) Volcano plot representation of the differentially expressed genes. The vertical green lines on the left and right correspond to 2.0-fold downregulation and upregulation, respectively, and the horizontal line indicates a P-value of 0.05. The red points in the plot indicate the differentially expressed genes between the normal and MC with statistical significance. MC, melanosis coli.

10 (38.46%) had neoplasia and 10 (34.62%) had adenocarcinoma. It total, 76.92% of the patients with MC had a history of long-term laxative use (Table I). This suggested that the use of laxatives was associated with MC.

Pigment type in MC. Specific staining and immunohistochemical analyses of the MC tissues indicated that the pigment granules in the lamina propria indicated lipofuscin, but not melanin, bile pigment or hemosiderin. PAS is used to detect the presence of lipofuscin. Light microscopy of the stained sections revealed blue nuclei, a pale red gland cavity and several uniform purple particles in the lamina propria, and there were higher numbers of purple particles in the MC sections, compared with the normal sections. In addition, no green or blue staining was identified to indicate the presence of bile pigment and hemosiderin, and no obvious black particles were observed in the specimens (Fig. 2A). To confirm the type of pigmentation, 26 MC tissues and 10 normal colon tissues were analyzed using immunohistochemistry. The results indicated that the expression of melanin was absent in the MC and normal colon tissues (Fig. 2B). These results confirmed that the pigment deposits in MC were lipofuscin, not melanin.

Apoptosis in MC tissues. Apoptosis of the colonic cells in MC tissues was evaluated using a TUNEL assay, with which the 26 MC tissues and 10 normal colon specimens were analyzed. Numerous apoptotic cells were observed in the MC tissue sections, and the apoptotic rate was higher than that observed in the normal colon tissue sections. Apoptotic bodies were observed within the macrophages and superficial lamina

Table II. GO analysis of differentially expressed genes in Melanosis coli.

GO.ID	Term	Ontology	Count	Pop Hits	List Total	Pop Total	Fold Enrichment	P-value	Enrichment Score	Representative gene
GO:0006955	Immune response	Biological process	61	1023	463	14742	1.8985	8.8294E-07	6.0540	C3/FCER1A/IL7R/ELK1/ TIRAP/NOS2/CCR7/CD37
GO:0045321	Leukocyte activation	Biological process	37	505	463	14742	2.3328	1.5604E-06	5.8067	CCR7/CCL19/CCL21/ITK/ CX3CR1/CD1C/GPR183/
GO:0006959	Humoral immune response	Biological process	14	113	463	14742	3.9448	1.1939E-05	4.9229	C3/CD37/CXCL13/CCR7/ CLU/C8G/CR2/CR1/CD28
GO:0060326	Cell chemotaxis	Biological process	15	129	463	14742	3.7023	1.2787E-05	4.8932	CALCA/CCL23/TNFSF11/ GREM1/IL16/CX3CR1/LEF1
GO:0001637	GPC chemoattractant receptor activity	Molecular function	5	25	474	15325	6.4662	0.0008	3.0545	CX3CR1/CCR9/CCR7/ CXCR5/CXCR4
GO:0048020	CCR chemokine receptor binding	Molecular function	4	15	474	15325	8.6216	0.0009	3.0267	CCL23/CCL19/CCL21/ CXCL13
GO:0017144	Drug metabolic process	Biological process	4	36	463	14742	3.5377	0.0255	1.5919	AKR1C1/CYP3A4/CYP2B6/ BCHE
GO:0042379	Chemokine receptor binding	Molecular function	9	55	395	15325	6.3486	1.0206E-05	4.9911	CXCL1/CXCL2/CXCL3/PF4/ CCL3/CCL18/CXCL11
GO:0004857	Enzyme inhibitor activity	Molecular function	15	306	395	15325	1.9018	0.0133	1.8753	APOC1/PHACTR1/SERPINC1/ SERPINB13/SERPINB4
GO:0016878	Acid-thiol ligase activity	Molecular function	3	20	395	15325	5.8196	0.0139	1.8542	ACSM5/C10ORF129/ACSF3
GO:0015125	Bile acid transmembrane transporter activity	Molecular function	2	11	395	15325	7.0540	0.0312	1.5051	SLC10A2/ZAR1
GO:0071241	Cellular response to inorganic substance	Biological process	8	83	373	14742	3.8094	0.0011	2.9292	SLC18A2/MT1F/MT1G/ MT1H/MT1X/FOS/SLC18A1
GO:0097006	Regulation of plasma lipoprotein particle levels	Biological process	6	45	373	14742	5.2697	0.0008	3.0498	APOC2/APOC1/PCSK9/MPO/ PLA2G7/MSR1

GO.ID, ID of gene ontology term; Count, number of DE genes associated with the listed GOID; Pop.Hits, number of background population genes associated with the listed GOID; List.Total, number of DE genes; Pop.Total, total number of background population genes; Fold.Enrichment, Fold Enrichment value of the GOID (Count/Pop.Hits)/(List.Total/Pop.Total); Pvalue, significance testing value of the GOID; Enrichment.Score, Enrichment Score value of the GOID (-log10(Pvalue); GPC, G-protein coupled.

Table III. Top KEGG pathways enriched with downregulated expressed genes and their corresponding Fisher's exact test P-values.

KEGG pathway name (entry ID)	P-value	Differentially expressed genes	Number of genes	Ratio
Intestinal immune network for IgA production (hsa04672)	3.3E-05	9	50	0.180
NF-κB signaling pathway (hsa04064)	4.3E-05	12	91	0.132
Primary immunodeficiency (hsa05340)	0.0001	7	36	0.194
Cytokine-cytokine receptor interaction (hsa04060)	0.0002	21	271	0.077
Hematopoietic cell lineage (hsa04640)	0.0006	10	88	0.114
Vascular smooth muscle contraction (hsa04270)	0.0013	12	131	0.092
Steroid hormone biosynthesis (hsa00140)	0.0026	7	57	0.123
Retinol metabolism (hsa00830)	0.0051	7	64	0.109
Starch and sucrose metabolisms (hsa00500)	0.0103	6	56	0.107
Metabolism of xenobiotics by cytochrome P450 (hsa00980)	0.0112	7	74	0.095
Arachidonic acid metabolism (hsa00590)	0.0191	6	64	0.094

KEGG, Kyoto Encyclopedia of Genes and Genomes. The ratio represents the percentage of differentially expressed genes in the indicated pathway.

Table IV. Top KEGG pathways enriched with upregulated expressed genes and their corresponding Fisher's exact test P-values.

KEGG pathway name (entry ID)	P-value	Differentially expressed genes	Number of genes	Ratio
Cytokine-cytokine receptor interaction (hsa04060)	0.0011	17	271	0.063
Chemokine signaling pathway (hsa04062)	0.0019	13	189	0.069
Salmonella infection (hsa05132)	0.0022	8	86	0.093
Mineral absorption (hsa04978)	0.0024	6	51	0.118
Rheumatoid arthritis (hsa05323)	0.0034	8	92	0.087
Butirosin and neomycin biosynthesis (hsa00524)	0.0069	2	5	0.400
Bile secretion (hsa04976)	0.0133	6	72	0.083
Glycine, serine and threonine metabolism (hsa00260)	0.0191	4	38	0.105
Serotonergic synapse (hsa04726)	0.0355	7	114	0.061
Collecting duct acid secretion (hsa04966)	0.0361	3	27	0.111
Melanoma (hsa05218)	0.0436	5	71	0.070

KEGG, Kyoto Encyclopedia of Genes and Genomes. The ratio represents the percentage of differentially expressed genes in the indicated pathway.

propria of the colonic epithelium (Fig. 3). These results indicates that pigment storage is a consequence of apoptosis in colonic epithelial cells.

Analysis of the gene chip expression profile. Data from three independent samples demonstrated that 1,718 genes were differentially expressed between the MC and control samples (Fig. 4). Of these, 879 genes were downregulated and 739 genes were upregulated, as shown by the Volcano plot representation in Fig. 4C. The most significantly upregulated genes were CCL18, NEFM, EGF and IL15RA, and the most significantly downregulated genes were CYP3A4, CYP3A7, UGT2B11 and UGT2B15 (Fig. 4B). The GO functional class scoring of the differentially expressed genes demonstrated that the most affected categories were as follows: Immune response, lymphocyte activation, humoral immune response, cell chemotaxis, G-protein-coupled chemoattractant receptor activity, CCR chemokine receptor binding and drug catabolic process for the downregulated genes and chemokine receptor binding, enzyme inhibitor activity, acid/thiol ligase activity, bile acid transmembrane transporter activity, cellular response to inorganic substance and lipoprotein particle for the upregulated genes (Table II). Accordingly, the most affected pathways for the downregulated genes were as follows: Immune network, NF-κB signaling pathway, metabolism of xenobiotics by cytochrome P450, vitamin digestion and absorption (Table III) the most affected pathways for the upregulated genes were as follows: Salmonella infection, mineral absorption, bile secretion, collecting duct acid secretion and melanoma (Table IV).

Detection of CYP3A4. P450 families of CYP1, CYP2 and CYP3 are the predominant contributors to the oxidative metabolism of >90% of clinical drugs. CYP3A4 is one of

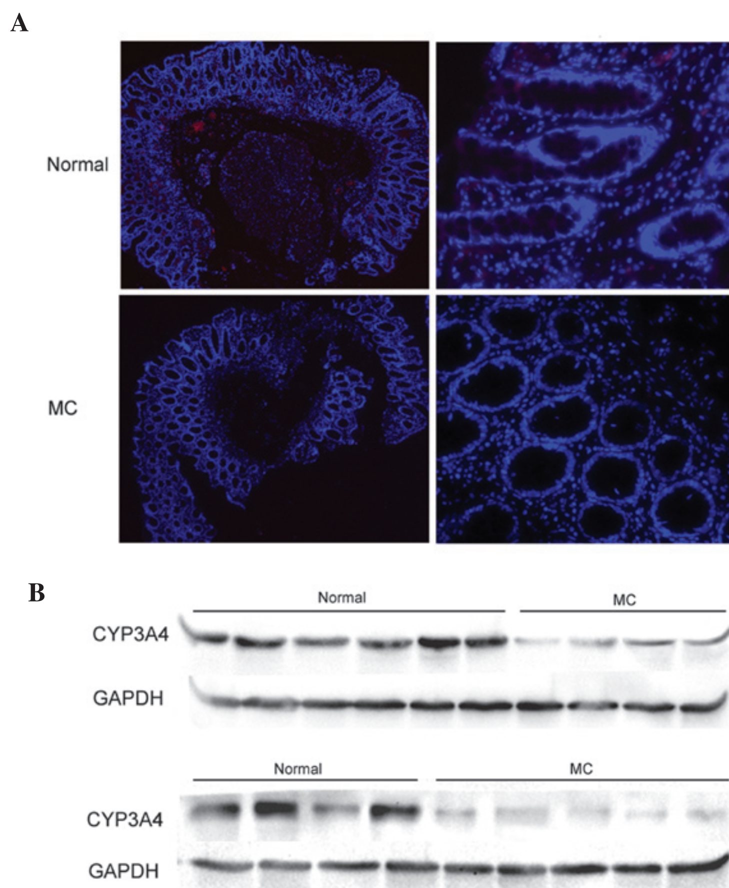

Figure 5. Elevated expression of CYP3A4 in MC and normal tissues. (A) Immunofluorescence assay. Red staining indicates protein expression of CYP3A4, while blue staining indicates the nuclei of the cells. Magnification: Left column, x4; right column, x20. (B) Immunoblot analysis of the expression of CYP3A4. Results are representative of 19 independent sample tissues per group. Expression of CYP3A4 was standardized to that of GAPDH. MC, melanosis coli.

these, and is predominantly present in the intestine. The chip expression data revealed that CYP3A4 was downregulated in MC by 11.0-fold, compared with normal tissue. To further verify the results of the gene chip screening, the expression levels of CYP3A4 were assayed using western blotting and an immunofluorescence assay in the present study. The results indicated that the expression of CYP3A4 in MC was higher than in normal tissue (Fig 5) and were, therefore, in accordance with the results of the gene chip screening.

Discussion

MC refers to an abnormality in which brown or black pigmentation is deposited in the colonic mucosa. It is a relatively common finding in colonic biopsies and resected specimens, however, the histopathology and pathogenesis of MC remain to be fully elucidated. In the present study, the type of pigment in MC was investigated by performing specific staining and immunohistochemical analyses in 26 MC specimens. The pigment deposits in MC were observed to contain lipofuscin and not melanin, bile pigments or hemosiderin. This condition, in which pigment deposits consist of lipofuscin rather than melanin is also referred to as pseudo-MC. In addition, there were a higher number of apoptotic cells in MC, compared with normal tissues. Expression microarray analysis demonstrated that the significantly downregulated genes were CYP3A4, CYP3A7, UGT2B11 and UGT2B15 in MC tissue, and western blotting and immunofluorescence analyses indicated that the expression of CYP3A4 in normal tissue was higher than that in MC.

The pathogenesis of MC has not been investigated in previous studies at depth. Several hypotheses have been suggested to explain pigment formation in MC. For example, it has been suggested that the formation of pigment granules is associated with apoptosis in colonic mucous membrane epithelial cells induced by purgatives (18). It has also been suggested that constipation leads to the retention of protein-rich foods in the intestinal tract, and that the protein degradation products are converted into melanin or lipofuscin, which are phagocytized by macrophages in the lamina propria (8). Despite these hypotheses, there is no clear experimental evidence to support any single pathogenesis for MC. In the present study, TUNEL apoptosis analysis revealed numerous apoptotic bodies in the epithelium and superficial lamina propria in the colonic mucosal biopsies from patients with MC. Pigment storage is a consequence of colonic epithelial cells apoptosis, in which the apoptotic cells are swallowed by macrophages, which migrate in the lamina propria and the conversion into lipofuscin pigment occurs by lysosomal enzymes (19).

The gene chip technique has been widely used to detect gene expression differences using comparative analysis. In the present study, the Agilent gene chip to analyze the gene

expression profile of human MC and normal colon tissues. As shown in Fig. 4, significant changes were observed in the expression of several genes in MC. These genes included those involved in the intestinal immune network, NF-κ B signaling pathway and metabolism of xenobiotics and drug by cytochrome P450 and melanoma. The most significantly downregulation genes were CYP3A4, CYP3A7, UGT2B11 and UGT2B15. These genes belong to the cytochrome P450 superfamily, which is involved in the metabolism of xenobiotics and drugs (20). The human CYP superfamily contains 57 functional genes and 58 pseudogenes. Among these, the members from the CYP1, CYP2 and CYP3 families are the predominant contributors to the oxidative metabolism of >90% of clinical drugs (21,22), and CYP3A4 is one of these, which is predominantly present in the intestine.

Aloe and emodin are anthraquinones known to be metabolized by P450s. It has been reported that aloe vera juice inhibits CYP3A4 and CYP2D6 irreversibly *in vitro*, having significantly different half maximal inhibitory concentration values (23), and emodin inhibits P450 with an antimutagenic effect (24,25). These biological effects of emodin prompted the present study to investigate anthraquinones as potential P450 inhibitors. The chip expression data in the present study demonstrated that CYP3A4 was downregulated in the MC tissues by 11.0-fold, compared with normal tissues. Western blotting and immunofluorescence assays also indicated that the expression of CYP3A4 in the MC tissue was lower than in the normal tissue (Fig. 5).

The data of the present study demonstrated that the pigment deposits in MC contain lipofuscin, and do not contain melanin, bile pigment or hemosiderin, and numerous apoptotic bodies were observed in the epithelium and superficial lamina propria in the colonic mucosal biopsies. Expression microarray analysis revealed that the P450-associated genes were significantly downregulated in MC tissues, and further experiments confirmed that the expression of CYP3A4 in the normal tissue was higher than in the MC tissue. To the best of our knowledge, this is the first time to demonstrate that, for MC patients, long time use of anthraquinone laxatives may inhibit P450, particularly CYP3A4, in the intestine. These findings increase understanding for assistance in further investigations of MC.

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References

1. Li D, Browne LW and Ladabaum U: Melanosis coli. Clin Gastroenterol Hepatol 7: A20, 2009.
2. Kunkel J, Schmidt S, Loddenkemper C, Zeitz M and Schulzke JD: Chronic diarrhea and melanosis coli caused by wellness drink. Int J Colorectal Dis 24: 595-596, 2009.
3. Samenius B: The clinical importance of melanosis coli. Proc R Soc Med 52 (Suppl): 105-106, 1959.
4. Pardi DS, Tremaine WJ, Rothenberg HJ and Batts KP: Melanosis coli in inflammatory bowel disease. J Clin Gastroenterol 26: 167-170, 1998.
5. Chen JY, Pan F, Zhang T, Xia J and Li YJ: Experimental study on the molecular mechanism of anthraquinone cathartics in inducing melanosis coli. Chin J Integr Med 17: 525-530, 2011.
6. Chen JY, Pan F and Zhang T: Rhubarb induced change of tumor necrosis factor-alpha level in guinea pig model of melanosis coli and its significance. Zhongguo Zhong Xi Yi Jie He Za Zhi 29: 140-142, 2009 (In Chinese).
7. Wang T, Chen ZW and Streutker CJ: Melanosis coli sparing adenomatous polyps: Novel findings using cleaved caspase-3 immunohistochemistry. Histopathology 62: 819-821, 2013.
8. Pearce CB, Martin H, Duncan HD, Goggin PM and Poller DN: Colonic lymphoid hyperplasia in melanosis coli. Arch Pathol Lab Med 125: 1110-1112, 2001.
9. Loveday RL, Hughes MA, Lovel JA, and Duthie GS: Melanosis coli in the absence of anthranoid laxative use harbouring adenoma. Colorectal Dis 15: 1044-1045, 2013.
10. Franken FH and Wiechers B: [Melanosis coli(author's transl)]. Leber Magen Darm 5: 269-271, 1975 (In German).
11. Park C, Cho NH and Jeong HJ: Melanosis coli - histochemical and immunohistochemical comparison of the pigments of melanosis coli and Dubin-Johnson syndrome. Yonsei Med J 31: 27-32, 1990.
12. Benavides SH, Morgante PE, Monserrat AJ, Zárate J and Porta EA: The pigment of melanosis coli: A lectin histochemical study. Gastrointest Endosc 46: 131-138, 1997.
13. Allen EA, Ali SZ and Erozan YS: Pigment-laden macrophages in ascitic fluid associated with melanosis coli. Acta Cytol 41: 1249-1251, 1997.
14. Tsobanidou Ch: Melanosis coli in two patients with colorectal neoplasia. J BUON 10: 131-133, 2005.
15. Puppa G and Colombari R: Brown colon (melanosis coli) harbouring pale tumors (adenocarcinoma and an adenomatous polyp). J Gastrointestin Liver Dis 18: 509-511, 2009.
16. Onks DL, Robertson AF and Brodersen R: The effect of chloral hydrate and its metabolites, trichloroethanol and trichloroacetic acid, on bilirubin-albumin binding. Pharmacol Toxicol 71: 196-197, 1992.
17. Tian L, Greenberg SA, Kong SW, Altschuler J, Kohane IS and Park PJ: Discovering statistically significant pathways in expression profiling studies. Proc Natl Acad Sci USA 102: 13544-13549, 2005.
18. Walker NI, Bennett RE and Axelsen RA: Melanosis coli. A consequence of anthraquinone-induced apoptosis of colonic epithelial cells. Am J Pathol 131: 465-476, 1988.
19. Chen JY, Pan F, Zhang T, Xia J and Li YJ: Experimental study on the molecular mechanism of anthraquinone cathartics in inducing melanosis coli. Chin J Integr Med 17: 525-530, 2011.
20. Pandey AV and Flück CE: NADPH P450 oxidoreductase: structure, function, and pathology of diseases. Pharmacol Ther 138: 229-254, 2013.
21. Nebert DW and Russell DW: Clinical importance of the cytochromes P450. Lancet 360: 1155-1162, 2002.
22. Guengerich FP: Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS J 8: E101-E111, 2006.
23. Djuv A: Co-use of drugs and herbal remedies in general practice and *in vitro* inhibition of CYP3A4, CYP2D6 and P-glycoprotein by the common herb aloe vera (Doctoral thesis). Department of Cancer and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, 333: 1503-8181, 2013 (In Norwegian).
24. Sridhar J, Liu J, Foroozesh M and Klein Stevens CL: Inhibition of cytochrome p450 enzymes by quinones and anthraquinones. Chem Res Toxicol 25: 357-365, 2012.
25. Takahashi E, Fujita K, Kamataki T, Arimoto-Kobayashi S, Okamoto K and Negishi T: Inhibition of human cytochrome P450 1B1, 1A1 and 1A2 by antigenotoxic compounds, purpurin and alizarin. Mutat Res 508: 147-156, 2002.