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.

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

Correspondence to: Dr Xiao-An Li, The Gastroenterology Tumor and Microenvironment Laboratory, Department of Gastroenterology, The First Affiliated Hospital of Chengdu Medical College, Chengdu Medical College, 4 Baoguang Road, Xindu, Chengdu, Sichuan 610041, P.R. China
E-mail: zqzy1983@163.com

*Contributed equally

Key words: melanosis coli, pigmentation, CYP3A4, laxatives, expression microarray
development of MC (8,9). Therefore, determination of whether
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 oxida-
tion of tyrosine to dopamine and eventually melanin in the
melanosomes (12). Due to macrophage phagocytosis of erythro-
cytes 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 anthra-
quinone 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 differ-
cences 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.

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 depara-
fination 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%
CCl$_3$COOH) 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% potas-
sium 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.,
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    |
| Obstruction              |        |          |
| 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    |
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 protease 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 Na2HPO4 and 2 mmol/l KH2PO4). 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 spectrophotometer. 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 (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 Biotec 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 horse-radish 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 Biotec 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.
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/ C3/CD37/CXCL13/CCR7/ CLU/C8G/CR2/CR1/CD28 |
| 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/ CX3CR1/CCR5/CXCR4 |
| GO:0048020 | CCR chemokine receptor binding            | Molecular function | 4     | 15       | 474        | 15325     | 8.6216         | 0.0009    | 3.0267           | CCL23/CCL19/CCL21/ CX3CR1/CCR9/CCR7 |
| 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/SERPINC4 |
| 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/PCS9/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, 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); P-value, significance testing value of the GOID; Enrichment.Score, Enrichment Score value of the GOID (-log10(P-value); GPC, G-protein coupled.
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 catalytic 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

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 |

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.
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 tissue 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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