Original Article

P-glycoprotein expression in ulcerative colitis patients and its role in intestinal barrier

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Received December 21, 2016; Accepted February 14, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Aims: Efflux transporters such as P-glycoprotein (Pgp; MDR1/ABCB1) protect the enterocytes from potentially toxic substances. The aim of this study was to evaluate Pgp expression in inflamed mucosa of patients with active Ulcerative Colitis (UC), and to compare the results of Pgp expression with healthy subjects. We also explored the relationship between Pgp expression and the pathogenesis of UC in this study. Methods: Pgp expression was determined with real-time PCR (SYBR GREEN), immunohistochemistry (IHC-SP method) and Western blot in inflamed mucosa of newly diagnosed (n = 102) patients with UC. Expression levels of Pgp expression were compared with control subjects (n = 31). The serum endotoxin and D-lactic acid levels of newly diagnosed UC patients and control subjects were detected by ELISA and ultraviolet spectrophotometer respectively. Results: Compared with control subjects, Pgp expression was significantly reduced in inflamed mucosa of newly diagnosed patients with UC. MDR1 (Multi-Drug Resistance 1) expression depicts a similar pattern. Pgp expression was found elevated in intestinal mucosa of UC patients after two months’ treatment with prednisone. The serum endotoxin and D-lactic acid levels in UC patients before and after prednisone treatment were negatively relevant with Pgp expression level. Conclusions: Compared with healthy controls, the expression of Pgp was reduced in inflamed tissue of patients with active UC. The Pgp may play a role in the pathogenesis of UC.

Keywords: Ulcerative colitis, p-glycoprotein, multidrug resistance gene, intestinal mucosa barrier

Introduction

The ulcerative colitis is a common chronic intestinal nonspecific inflammatory disease with unknown etiology and pathogenesis. It is reported that MDR gene knockout mouse tend to idiopathic colitis. The P-glycoprotein which encoded by human MDR1 (Multidrug resistance 1) gene, is the member of the ATP-binding cassette (ABC) transporter family. Pgp was first detected in multi-drug resistant tumor cells. Pgp has a protective function against potentially toxic xenobiotics and is highly expressed in tissues that are important for uptaking and elimination of toxic substances originated from the intestine, the kidney, the liver, and the blood brain barrier. Pgp is localized at the apical membrane of intestinal cells which limit the absorption of orally administered drugs and ingested toxins. Therefore, Pgp expression was investigated in newly diagnosed untreated patients with UC, patients treated with prednisone and healthy controls. In patients with active UC, special interest was paid to evaluate Pgp expression in inflamed mucosa before and after prednisone treatment. We hypothesized that expression of Pgp may be altered in UC.

Materials and methods

Patients’ characteristics

In this study, 102 patients with newly diagnosed UC and 31 healthy controls were included. The diagnosis of patients with UC was based on a typical clinical history, laboratory findings as well as endoscopic and histological criterias. Tissue biopsies were sampled by experienced gastroenterologists. We obtained biopsies from the inflamed mucosa of the
patients with active UC and all the inflamed mucosa were characterized by macroscopic/endothelial signs of inflammation including ulceration, edema, discoloration, hemorrhagic appearance or mucinous/fibrinous coating. Control subjects had an indication for a gastrointestinal tract endoscopy within a cancer screening program. Biopsy specimens were obtained during routine colonoscopy, then submerged in RNA-later solution (Qiagen, Hilden, Germany) and stored at -80°C until further processing.

Real-time RT-PCR analysis (SYBR green)

Total RNA was isolated from 2 biopsies of each subject. The RNA was extracted with the RNeasy Mini Kit (Qiagen) following the instructions provided by the manufacturer, and then quantified with a GeneQuanPhotometer (Pharmacia, Uppsala, Sweden). After DNase I digestion (Gibco Life Technologies, Basel, Switzerland), 0.5 μg of the total RNA was reverse-transcribed by 5 × Prime Script RT Master Mix (Takara Biotechnology, Dalian) according to the manufacturer's protocol. SYBR Premix Ex Taq™ kit was bought from Takara Biotechnology Company Ltd, Dalian.

SYBR analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). Primers for SYBR analysis were designed according to the guidelines of Applied Biosystems with the help of the Primer 6.0 software. ABCB1/MDR1 (NM-000927) gene primers were as follows, Right primer sequence, ATTCCTCGAGAAACTGCGAA; Left primer sequence, TCACTTCAGGAAGC-AACCAG. All samples were quantified using a standard curve. For each sample the transcript numbers of MDR1 and 18S RNA were determined. By calculating the ratio of MDR1 to 18S RNA, the mRNA expression was normalized. 18S RNA gene primers were as follows, Right primer sequence, TGCGAGTACTCAACACCAACA; Left primer sequence, GCATATCTTCGGCCCACA. This approach was established to account for variations in the enterocyte content of all the biopsies.

Immunohistochemistry

Human colonic tissue were mounted in OCT (Optimal cutting temperature) compound, snap-frozen in liquid nitrogen and stored at -80°C. Frozen sections were air dried overnight and fixed with a periodate-lysine paraformaldehyde solution (3%). After three washes with the washing solution, a perhydrol solution [H₂O₂ (0.3%), sodium azide (0.1%) in PBS] was used to destroy the endogenous peroxidase activity. Then the sections were washed with washing solution (TBS/NaCl, Tween 0.05%) and incubated with normal bovine serum as blocking solution for one hour at room temperature. For Pgp staining, the tissue sections were incubated with a 1:100 dilution of the Pgp polyclonal antibody (Anti-ABCB1 antibody produced in rabbit, SIGMA-ALDRICH) overnight at 4°C. Then the samples were washed three times with washing solution and incubated with the goat anti-rabbit IgG secondary antibody for 1 hour at room temperature. The tissue staining was performed with the 3,3'-diaminobenzidine (DAB) as organic reagent (1:10 dilution) for three minutes.

Semiquantitative analysis of Pgp staining was performed in biopsies of control subjects (n = 31), and inflamed tissues of patients with newly diagnosed UC (n = 102) before and after prednisone treatment. Rating of protein staining on blinded specimens was done by two trained pathologists. Expression levels were rated as follows: 0 = no expression, 1 = low expression, 2 = intermediate expression, 3 = high expression.

Western blot analysis

All protein samples were separated by 15% SDS-PAGE gel. The SDS-separated proteins were equilibrated in transfer buffer (25 mM Tris, pH 8.5, 0.2 M glycine and 20% methanol) and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA).

The membranes were incubated with 5% non-fat milk for one hour in Tris-buffered saline containing 0.1% Tween. These membranes were then washed and incubated with appropriate dilutions of specific antibodies [including anti-ABCB1 antibody (1:1000, WH0005243M1, mouse, SIGMA), GAPDH antibody (1:5000, mouse, 60004-1, Proteintech)] at 4°C overnight. After incubation with anti-mouse fluorescence antibody (1:10000, Odyssey) at room temperature for one hour, the bound secondary antibodies were revealed by Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).
P-glycoprotein expression in ulcerative colitis

Figure 1. Detection of serum endotoxin and D-lactic acid levels in health control, UC patients before treatment and UC patients with prednisone treatment.

Table 1. Correlation analysis of serum endotoxin and D-lactic acid with Pgp

<table>
<thead>
<tr>
<th>Group</th>
<th>Endotoxin and Pgp R value</th>
<th>Endotoxin and Pgp P value</th>
<th>D-lactic acid and Pgp R value</th>
<th>D-lactic acid and Pgp P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health controls</td>
<td>-0.175</td>
<td>0.044</td>
<td>-0.255</td>
<td>0.003</td>
</tr>
<tr>
<td>UC before treatment</td>
<td>-0.212</td>
<td>0.013</td>
<td>-0.219</td>
<td>0.010</td>
</tr>
<tr>
<td>UC after treatment</td>
<td>-0.060</td>
<td>0.023</td>
<td>-0.177</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Figure 2. The expression of MDR1 mRNA level in health control, UC patients before treatment and UC patients with prednisone treatment were detected using quantitative real time PCR assay which were normalized to 18S RNA.

Detection of D-lactic acid

We obtained neutralized-protein-free plasma (NPFP) from 1 milliliter serum. Then we mixed 3.3 ml 2.43 mmol/L nicotinamide-adenine dinucleotide glycine diammonium solution with 1.1 ml NPFP in EP tubes; and dispensed the mixture into four cuvettes with 1.0 ml each. 0.05 ml D-lactic dehydrogenase were put into two cuvettes of the four. The other two control cuvettes were infused with 0.05 ml distilled water. All the cuvettes were incubated at 25°C for ninety minutes. Ultraviolet spectrophotometer was used to read the absorbance value at 340 nm. The 340 nm absorbance value of sample tubes and standard tubes were recorded respectively. The computational formula of serum D-lactic acid concentration is, D-lactic acid concentration = (sample tube absorbance value/standard tube absorbance value) × the concentration of the standard substance.

Statistics

All values were expressed as means ± SD.

Enzyme-linked immunosorbent assay (ELISA) analysis

Blood samples were collected in commercially available EDTA-treated tubes (Gongdong Medical Technology Co., Ltd). Plasma was obtained by centrifugation for fifteen minutes at 2,000 × g using a refrigerated centrifuge at 4°C. The plasma samples were stored at -80°C. Plasma levels of Pgp were analyzed using commercially available ELISA kit (ADL, San Diego CA, USA) according to the manufacturer’s protocol. Specimens and standard solutions were dispensed into appropriate wells and 50 μl of Enzyme Conjugated Reagent was dispensed into each well. The wells were incubated at 37°C for one hour after gently mixing. The incubation mixture was removed and the microtiter wells were rinsed for 5 times. 50 μl of color A and color B Reagents were dispensed into each well. Stop solution was added to each well. A microtiter plate reader was used to read the absorbance value at 450 nm.
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Figure 3. A. Immunohistochemical stain analysis of P-glycoprotein expression in health control (normal), UC patients before treatment; Original magnification, (left) 200 ×, (right) 400 ×; B. Immunohistochemical stain analysis of P-glycoprotein expression in UC patients before treatment, UC patients with prednisone treatment; Original magnification, (left) 200 ×, (right) 400 ×.

Table 2. Results of IHC

<table>
<thead>
<tr>
<th>Group (rate)</th>
<th>Negative</th>
<th>Weakly positive</th>
<th>Positive</th>
<th>Strong positive</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health controls</td>
<td>17 (54.84)</td>
<td>3 (9.68)</td>
<td>11 (35.48)</td>
<td>0 (0)</td>
<td>14 (45.16)</td>
</tr>
<tr>
<td>UC before treatment</td>
<td>83 (81.37)</td>
<td>16 (15.69)</td>
<td>3 (2.94)</td>
<td>0 (0)</td>
<td>19 (18.63)</td>
</tr>
<tr>
<td>UC after treatment</td>
<td>66 (64.71)</td>
<td>19 (18.63)</td>
<td>13 (12.75)</td>
<td>4 (3.92)</td>
<td>36 (35.29)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
<td>&lt;0.01*</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

P value: *means healthy controls (n = 31) VS UC before treatment (n = 102); †means UC before treatment (n = 102) VS UC after treatment (n = 102).

Table 3. Correlations between serum endotoxin and D-lactic acid levels

<table>
<thead>
<tr>
<th>Correlation</th>
<th>ρ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum endotoxin &amp; D-lactic acid</td>
<td>0.56</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum endotoxin &amp; MDR1 mRNA expression</td>
<td>0.63</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

P-glycoprotein expression was analyzed using Pearson correlation analysis. The correlations between serum endotoxin and D-lactic acid levels, and serum endotoxin and MDR1 mRNA expression were significant (Table 3).

Results

Serum endotoxin and D-lactic acid are upregulated in the newly diagnosed UC patients; serum endotoxin and D-lactic acid are down-regulated in UC patients after prednisone treatment compared with these UC patients before prednisone treatment (Figure 1; Table 1).

MDR1 mRNA expression is downregulated in the inflamed mucosa of patients with active UC; MDR1 mRNA expression is upregulated in UC patients with two months’ treatment compared with these patients before treatment (Figure 2).

MDR1 mRNA expression was analyzed in 31 control subjects and compared with patients with newly diagnosed UC (102 biopsy specimens from inflamed mucosa). We also analyzed MDR1 mRNA expression in 102 newly diagnosed UC patients with two months’ prednisone treatment compared with these patients before treatment (Figure 2).
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P-glycoprotein (Pgp) expression in ulcerative colitis (UC) patients has been studied extensively. The expression and function of Pgp are decreased in inflamed mucosa of untreated newly diagnosed UC patients, suggesting a close relationship between intestinal inflammation and Pgp expression. In vitro data indicate that the transcriptional regulation of Pgp is regulated by PXR [4]. Several proinflammatory cytokines have been shown to influence transporter gene expression [5, 6]. The findings further suggest that Pgp is likely involved in the pathogenesis of UC.

Pgp is an important component of the intestinal barrier against bacterial toxins, carcinogens and drugs [7-11]. Altered expression and/or altered function of Pgp have been proposed to contribute to the pathogenesis of inflammatory disorders [12, 13]. The disorders of barrier function in gastrointestinal mucosa are responsible for the pathogenesis of Inflammatory Bowel Diseases. Endotoxin is a component of gram-negative bacterial cell wall and its main component is lipopolysaccharide (LPS).

D-lactic acid is a metabolic product of intestinal flora. Both of the levels of endotoxin and D-lactic acid could increase in serum when intestinal barrier function is damaged. Therefore, the serum endotoxin and D-lactic acid levels which are negatively correlated with intestinal barrier function can be used to reflect the barrier function of colon mucosa.

We found that the expression of Pgp in colon mucosa of UC patients reduced significantly accompanied with the levels of serum endotoxin and D-lactic acid elevated. Pgp is an efflux pump for a variety of toxic substances, so its decreased expression tend to disturb the intestinal barrier and the defense function of the intestine. Furthermore, the decreased expression of Pgp in inflamed intestinal mucosal sites could lead to the abnormal accumulation of exogenous substances and potent carcinogens in enterocytes. These results might somewhat explain the phenomenon that UC patients have a higher risk of developing colorectal carcinomas [14, 15].

We further demonstrated the expression of MDR1 on the mRNA and protein levels of UC patients before treatment and after two months’ prednisone treatment. Patients treated with prednisone showed an increase in Pgp expression in the inflamed mucosa of untreated newly diagnosed UC patients suggests that this reduction is closely related to intestinal inflammation. In vitro data indicate that the transcriptional regulation of Pgp is regulated by PXR [4]. Several proinflammatory cytokines have been shown to influence transporter gene expression [5, 6]. The findings further suggest that Pgp is likely involved in the pathogenesis of UC.

Expression of Pgp was evaluated by immunohistochemistry and western blot in control subjects and newly diagnosed UC patients. In the group of newly diagnosed UC patients, tissue samples were obtained from inflamed mucosa. As expected, Pgp was localized on the apical membrane of the epithelial cells. Pgp was significantly decreased in inflamed mucosa of newly diagnosed UC patients before treatment compared with healthy controls (Figure 3A). Pgp expression was upregulated in UC patients after two months’ treatment with prednisone when compared with these patients before treatment (Figure 3B). We also obtained the same results as above through western blot assay (Figure 4).

Discussion

Pgp encoded by MDR1 gene is a glycoprotein weighting 170 kDa, also known as a member of ATP-dependent transmembrane pumps [1]. It is reported that the expression and activity of a number of efflux transporters such as MRP2, MRP3 and MRP4, are diminished during inflammatory processes [2, 3]. Inflammation is known to suppress the expression and activity of several efflux transporters; also, the expression and function of intestinal MDR1 is reduced in a rat model of intestinal inflammation. The results of this study demonstrate that the expression of Pgp is downregulated in patients with UC on the transcriptional and protein levels in the inflamed mucosa. The reduction in Pgp expression in inflamed mucosa of untreated newly diagnosed UC patients suggests that this reduction is closely related to intestinal inflammation. In vitro data indicate that the transcriptional regulation of Pgp is regulated by PXR [4]. Several proinflammatory cytokines have been shown to influence transporter gene expression [5, 6]. The findings further suggest that Pgp is likely involved in the pathogenesis of UC.

Pgp levels are reduced in the inflamed mucosa in patients with active UC (Figure 3A; Table 2); Pgp levels are upregulated in UC patients with two months’ prednisone treatment compared with these patients before treatment (Figure 3B; Table 2).

Expression of Pgp was evaluated by immunohistochemistry and western blot in control subjects and newly diagnosed UC patients. In the group of newly diagnosed UC patients, tissue samples were obtained from inflamed mucosa. As expected, Pgp was localized on the apical membrane of the epithelial cells. Pgp was significantly reduced in inflamed mucosa of newly diagnosed UC patients before treatment compared with healthy controls (Figure 3A). Pgp expression was upregulated in UC patients after two months’ treatment with prednisone when compared with these patients before treatment (Figure 3B). We also obtained the same results as above through western blot assay (Figure 4).
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expression accompanied with a decrease in serum endotoxin and D-lactic acids.

Although prednisone was identified as a substrate for Pgp [16], it is not known to induce the expression of Pgp. We illustrate that Pgp expression might be induced by prednisone through the above study.

In conclusion, we have shown that patients with newly diagnosed UC exhibit a decreased expression of MDR1, both on the mRNA and protein levels in inflamed sites of the intestinal mucosa. The downregulation of Pgp might play a role in the pathogenesis of UC. The increased expression of MDR1 in UC patients might be induced after prednisone treatment. Through this study, we may provide a new therapeutic target of UC and give help to reveal the pathogenesis of UC.

Acknowledgements

This work is funded by National Natural Science Foundation (Number, 81370554, 81570551), Key research and development program of Shandong Province (2016GSF201018), Jinan science and technology project (201503043).

Disclosure of conflict of interest

None.

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