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The regulatory role of Nrf2 in antioxidants phase2 enzymes and IL-17A expression in patients with ulcerative colitis

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ABSTRACT

Background: Reactive oxygen species (ROS) is one of the pathogenic factors responsible for intestinal injury in Ulcerative colitis (UC). Nuclear factor erythroid-2 related factor 2 (Nrf2) plays a critical role against ROS factors to conserve epithelial integrity. This study aimed to localize Nrf2 and IL-17A protein in the inflamed mucosa of patients with ulcerative colitis. The gene expression of Nrf2 was also correlated with GST-A4 and PRDX1.

Materials and methods: A total of 20 patients and 20 healthy controls with definite UC based on the clinical criteria were enrolled for this study. The expression pattern of Nrf2 and IL-17A protein was compared in inflamed and non-inflamed colonic biopsies by immunohistochemical staining. *Nrf2*, *GST-A4* and *PRDX1* gene expression were determined by real-time polymerase chain reaction (RT-PCR).

Results: In inflamed colonic biopsies, an increased level of Nrf2 protein factor was detected in epithelial cells. Conversely, IL-17A protein was presented more in mononuclear cells in mucosa and lamina propria regions. A significant increase of *Nrf2*, *GST-A4* gene expression was observed in both mild and severe patients with ulcerative colitis. *GST-A4* gene expression indicated a high exponential rate in logistic regression.

Conclusion: Oxidative stress in inflamed colonic tissue can induce *Nrf2* gene expression. The performance of Nrf2 transcription factor may lead to the induction of GST-A4 and PRDX1. IL-17A is less detected in intestinal inflammation, presenting Nrf2 factor. The present findings suggest that Nrf2 function in the gut plays a role in arresting both inflammatory response and oxidative damages of UC.

1. Introduction

The inflammatory bowel diseases (IBDs) has a wide range of associated disorders like ulcerative colitis (UC), characterised by recurrent chronic and incurable inflammatory conditions in the colon and rectum [1,2]. The interaction between genetic, nutritional and environmental agents initially presents more of the inflammatory conditions in UC [3]. Potentially, Inflammation damages the mucosal barrier function and amplify inflammatory response in the gastrointestinal tract [4]. Recent investigations have also indicated an association to exist between the degree of inflammation and the enhanced production of reactive oxygen species (ROS) such as nitric oxide and superoxide [5]. Under oxidative stress, nuclear factor erythroid 2 related factor 2 (Nrf2) can be released from Keap1 and translocates into the nucleus of cells [6]. The Nrf2 is mainly expressed in the nucleus of epithelial cells to conserve the intestinal integrity. The protective effects of Nrf2 in a chronic colitis mice caused lower expression of inflammatory cytokines [7]. Activated Nrf2 has the potential to suppress interleukin (IL)-17 expression in autoimmune disease [8]. In murine dextran sodium sulfate (DSS)-induced colitis, IL-17 augments inflamed intestinal by pro-inflammatory

Abbreviations: ROS, reactive oxygen species; UC, ulcerative colitis; Nrf2, nuclear factor erythroid 2 related factor 2; IBD, inflammatory bowel diseases; DSS, dextran sodium sulfate; IL-17, interleukin-17; APE1, apurinic/apyrimidinic endonuclease; PRDX1, peroxiredoxin-1; GST-A4, glutathione *S*-transferase-alpha4; 4-HNE, trans-4-hydroxy-2-nonenal; IEC, intestinal epithelial cells; COPD, chronic obstructive pulmonary disease; Hmox-1, heme oxygenase-1; RORct, retinoic orphan receptor gamma(t); MAPK, mitogen-activated protein kinase

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effects on epithelium [9]. In addition to high level of IL-17 in serum and tissue, the existence of T helper-17 illustrates the pathogenesis role of this cytokine in the UC patients (Graphical abstract) [10,11].

The Nrf2 factor controls the expression of phase2 antioxidant enzymes by binding to the Antioxidant Response Element (ARE) region [12]. Peroxiredoxin-1 (PRDX1), a cytoprotective gene, is induced by Nrf2 activation under oxidative stress condition to protect the cell particularly against superoxide. PRDX1 can act as chaperone, protecting protein factors through thiol-specific groups in the cytoplasm [13]. PRDX1 enzyme engages in several redox factors like apurinic/apyrimidinic endonuclease1 (APE1), consequently causing a reduction in inflammatory cytokines [14]. Nrf2 can also target ARE region to induce Glutathione S-transferase alpha 4 (GST-A4) against oxidative damage. In response to accumulation of lipid peroxidation, GST-A4 attenuates toxic lipid aldehydes by conjugating to glutathione [15]. GST-A4 is dominantly capable of detoxifying trans-4-hydroxy-2-nonenal (4-HNE) which is considered as an endogenous mutagen in inflammation-associated colorectal cancer [16].

In this study, the *Nrf2* gene expression and its relation with mRNA level of PRDX1 and GST-A4 in control colonic biopsy compared to UC patient, will be examined. The expression pattern of the Nrf2 and IL-17A protein in histological tissues will be assessed. A negative protein expression between Nrf2 and IL-17A will be identified. Moreover, Nrf2 factor is more presented in mucosa layer of biopsies. These data suggest that GST-A4 gene expression appears to be dependent on Nrf2 transcription factors.

2. Materials and methods

2.1. Sample collection and preparation

The study group comprised of 20 patients with UC and 21 healthy individuals as control (Table 1). Over the period of 6 months in 2017, all cases were attended in Gastroenterology Unit of Hajar hospital, Shahrekord, Iran, from January to May 2017. Prior to the colonoscopy procedure, clinical information of UC patients such as age, gender, food habits, stress, medicine consumption and history of abdominal surgery were collected by opt query and medical files. The disease activity index (DAI) of ulcerative colitis was marked in regard to the weight loss, blood in the stool and stool consistency [17]. In addition to the clinical criteria, inflammation extension in colonoscopy and histological manifests were analyzed based on the Montreal Classification for the confirmation of UC [18]. 14 active UC patients had a mild proctosigmoiditis (inflammation was limited from the colorectum distal to the splenic flexure/erythema is detected in rectum and sigmoid, decreased friability and vascular pattern/Montreal class: E2). Six severe patients had consumed corticosteroid six months before sampling date (Involvement extends proximal to the splenic flexure/Montreal class: E3). The Control group was selected among healthy individuals without immune-mediated diseases. These subjects were undergone colonoscopy due to screening for colorectal cancer or polyp surveillance without inflammatory disease or any medication. This project was approved by Shahrekord University of Medical Sciences Ethics Committee, and all informed participants complemented a written consent.

Table 1
demographic information of the patients.

	Control	Mild colitis	Severe colitis	P value
Age	33.81 ± 8.85	35.43 ± 9.55	32.00 ± 9.06	0.729
Gender (male/female)	12/9	7/7	3/3	0.913
Smoking	9.5%	21.4%	46.7%	**0.019**[a]
Habitat (rural/urban)	2/19	4/10	3/3	0.078
History of abdominal surgery	19%	64.3%	50%	0.295

[a] Number of smoking people were statistically significance.

2.2. RNA isolation and quantitative RT-PCR

The total RNA was extracted from nitrogen frozen colon biopsies with TRIzol® reagent (Invitrogen/Thermo Fisher Scientific, Inc, catalog number. 15596026). The concentration of each RNA samples was determined by Thermo Scientific™ NanoDrop 2000. The 260/280 - 260/230 ratios for samples were measured, and cDNAs were synthesized using the Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, K1622) with 1.5 µg of RNA in a reaction volume of 20 µL. To eliminate possible DNA contamination, DNaseI (Fermentas EN0521) enzyme was used. The real-time RT-PCR to indicate the quantification of mRNA was performed on a Rotor-Gene RG-300 (Corbett Research, Sydney, AU) and SYBR Green Real-time PCR Master Mix Kit (TAKARA, Japan, catalog number. RR820Q) according to the protocol provided by the manufacturer. Then, three pairs primers were exploited for the specific amplification of Nrf2, PRDX1 and GST-A4 (Supplement File 1). The primers were designed by Primer3.0 (http://bioinfo.ut.ee/primer3-0.4.0) web-based server. We checked out the lack of SNPs in the genomic region corresponding to the 3′ ends of primers by looking through the dbSNP database. The primers specificity was checked by the in-silico-PCR tool in UCSC genome browser and Primer blast of NCBI genome browser. Thermal cycling for running a process started the denaturation step at 95 °C for 5 min first denaturation step at 95 C for 5 min and followed by 38 cycles of 95 °C for 15 s, 61 °C for 20 and 72 for 25 s. Melting curve confirmed the amplification specificity of three gene expression of Nrf2, PRDX1 and GST-A4 were normalised versus GAPDH as an internal control and relative quantification ($2-\Delta\Delta Cq$) showed the fold changes of each mRNA.

2.3. Immunohistochemistry

Immunohistochemical staining was implemented with the streptavidin biotin peroxidase-complex method according to the Abcam protocol [[19]]. Briefly, sections of biopsy specimens were cut into 4-µm-thick sections and stuck on poly-L-lysine slides. The sections were deparaffinized and rehydrated after placed in xylene and a series of alcohols (100%, 100%, 80% and 70%). In antigen retrieving stage, sections immersed in citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) were exposed to pressure for 20 min. Sections were then incubated with protein block (Abcam, England) containing albumin for 120 min to prevent nonspecific background staining. Rabbit anti-human Nrf2 antibody (ab31163, Abcam, UK) at a 1:700 dilution and anti-IL17A antibody (ab79056) were incubated overnight 4 C. It is followed by adding 0.3% H_2O_2 solved in TBS to inhibit endogenous peroxidase activity. After incubating biotinylated IgG antibody (ab93697, Abcam, UK) and Streptavidin-Peroxidase Plus at room temperature, 3-diaminobenzidine tetrahydrochloride DAB (ab94665, Abcam, UK) was used to visualise specific antigen. Sections were counterstained with hematoxylin and washed with cool water. The number of nuclei with positive reactivity for Nrf2 and IL-17A were counted in the 3 areas of mucosal epithelium and lamina propria. The expression level of Nrf2 and IL-17A were assessed using a 6-score system (0 = negative, 0.5 = 0–5% positive, 1 = 5–15% positive, 2 = 16%–40% positive, 3 = 41%–90% positive, and 5 more than 90% positive).

2.4. Statistical analysis

All data were presented as mean ± SD and were evaluated by SPSS19.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). The distribution of data was normal and the relationship between age of subjects and groups was addressed with the Fischer exact test. Unpaired *t*-test was