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Molecular Markers and Diagnosis

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New Features of Molecular Diagnostics of Ulcerative Colitis

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Abstract

The purpose of this study was to search for new molecular markers for the diagnosis of ulcerative colitis (UC). The study included 65 patients (range from 22 to 35 years, 24 men and 41 women) with left-sided UC (Montréal classification), mild and moderate activity, infrequent (≤1/year) relapses according to the inclusion/exclusion criteria in the research. Criteria of the diagnosis of UC corresponded to ECCO Consensus. The duration of UC was 5.3 years. The control group included 30 healthy individuals. Molecular phenotyping of colon mucosa was processed with methods of proteomics. The data of the molecular interactions were received with STRING 10.0 database. Potentially new molecular markers of the development of UC were identified. **(Int J Biomed. 2016; 6(1):70-73.).**

Keywords: *ulcerative colitis; colon mucosa; proteomics; diagnostic markers.*

Introduction

The worldwide incidence rate of ulcerative colitis (UC) varies between 0.5 and 24.5 cases per 100,000 persons. Currently, the incidence of UC in Russia is 5–30 cases per 100,000 per year. The peak age of onset for UC is most common between 15 and 30 years, although it may occur at any age. About 10% of the cases occur in individuals under the age of 18. UC is slightly more common in males [1,2].

UC is characterized by an even and continuous distribution of the inflammatory infiltrate that only affects the lamina propria. Furthermore, the disruption of normal crypt architecture and the presence of crypt abscesses are the main histological characteristics of UC. UC patients have a well-known risk of colorectal cancer [3].

So far, mechanisms of UC remain to be fully understood and require a detailed multidisciplinary approach [4-6]. In 1990, an international consortium revealed universal genetic sequences which enable a genetic map of UC [7]. According to Medscape, methods of cell and molecular biology have shown the role of thrombocytosis (1966), antibodies to E.coli (1969), key components of the inflammation with UC using granulocytes labeled Indium-111 (1985), interleukin-6 (IL-6), tumor necrosis factor (TNF)-α, pANCA, C-reactive protein (CRP) (1990), thrombocytes, sedimentation rate of erythrocytes in differential diagnosis between UC and infectious diarrhea (1991), IL-12 (1995), fecal lactoferrin (1996), the level of α4β7-integrin in T-lymphocytes (1997), pANCA and ASCA (1998), bacterial antibodies and fecal calprotectin (1999), serum protein S100A12 (2003,2006), and IL-23 in colon mucosa (2004).

Modern achievements of proteomic methods of analysis are ideal for research that is free from hypotheses and allows us to define molecular characteristics of inflammation in colon mucosa of UC patients.

Material and Methods

The study conducted in accordance with WMA Declaration of Helsinki (1964-2013) and the permission of the Ethics Committee of the Rostov-on-Don State Medical University (Rostov-on-Don, Russia).

The study was prospective comparative cohort with parallel design and included 65 patients (range from 22 to 35 years, 24 men and 41 women) with left-sided UC (Montréal classification) [8], mild and moderate activity (Truelove - Witts' criteria, Mayo score) [9,10], infrequent (≤1/year) relapses according to the inclusion/exclusion criteria in the research. Criteria of the diagnosis of UC corresponded to ECCO Consensus [11]. The duration of UC was 5.3 years. The induction of UC remission assumed the acceptance of mesalazine 3 to 4 g/day p.o. and rectally 1 to 2 g/day; the maintenance of UC remission included the acceptance of mesalazine 1.5 g/day p.o. for 3 years by patients with intolerant UC. The duration of therapy was 4.6 years. The control group included 30 healthy individuals.

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At the stage of data collection and screening, we applied standard methods for identification of UC: the assessment of the patient`s symptoms, risk factors, medical history, physical examination, complete blood count, erythrocyte sedimentation rate (ESR) (Advia 120, Bayer Diagnostics, Germany), biochemical analysis of blood and urine, serological markers - perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA) (ELISA, Siemens 2000, Germany), faecal calprotectin (ELISA, Buhlmann, Швейцария), the stool culture (culture-dependent methods, DNA-PCR and FISH analysis), patient's immunization status to various viral diseases and tuberculosis status.

Biosamples of colon mucosa (3-10mg) in patients with UC in the active stage and in healthy persons were received by ileocolonoscopy (Olympus, Japan) with colon mucosa biopsy (Rachmilewitz index). We used endoscopic scoring by the Schroeder classification for UC and Endoscopic Index of Severity (UCEIS) [12,13]. Histological characteristics of colon mucosa in UC in the active stage were performed by light-microscopy (architectural features, epithelial abnormalities, and inflammatory features).

The storage of biosamples before proteomic analysis was carried out at -80ºC. Sample preparation was conducted as follows: biosamples were homogenized and processed by lysis buffer (1mg bioptate/10µl lysis buffer, pH 3–10, GE Healthcare, Sweden), CHAPS (Applichem, Germany), and 1% DTE (Sigma-Aldrich D8255) in water. After the incubation during 2h at room temperature, lysed cells were centrifuged at 10000 RPM for 20 min at 4 ºC.

The separation of individual proteins of colon mucosa was based on technologies of IEF, SDS-PAGE, 2DPAGE, by standard sets (MB-HIC C8 Kit, MB-IMAC Cu, MB-Wax Kit, «Bruker», USA). Automated mass spectrometry imaging was performed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS/MS, Ultraflex II, «Bruker», USA). The partially identified sequences were then submitted to "BLAST protein-protein" and screened against the *Homo sapiens* Swissprot database to check if this identification matched the MASCOT-identification (Matrix Science, UK). The data of the molecular interactions and functional features of proteins were received with STRING 10.0 database.

Based on the data of standard methods of identification of UC and molecular phenotyping of colon mucosa we conducted new prognostic markers, molecular pathways of UC and diagnostic tests in patients with UC.

Statistical analysis of the survey data was performed using the software "Statistica 12.0" (Statsoft, Russia). Baseline characteristics were summarized as frequencies and percentages for categorical variables and as mean±SEM for continuous variables. Student's unpaired paired t-tests were used to compare two groups for data with normal distribution. Group comparisons with respect to categorical variables are performed using chi-square tests with Yates correction or, alternatively, Fisher's exact test when expected cell counts were less than 5. A probability value of $P<0.05$ was considered statistically significant.

Results

Clinical-anamnestic and laboratory characteristics of UC patients are presented in Table 1.

Table 1.

Clinical-anamnestic and laboratory characteristics of UC patients

Variable	Group patients with UC (n=65)			Control group (d)
	Active stage (a)	Remission (1st year) (b)	Remission (3rd year) (c)	
Sex (male/female), n	39/26	39/26	39/26	17/13
Age, yrs	29.2±2.5	30.3±2.7	32.1±2.9	30.1±3.3
Weight, kg	58.3±1.7*	60.4±1.8**	64.3±2.2	69.7±2.3
Height, cm	171.2±1.7	169.4±1.6	170.5±1.6	170.2±1.7
BMI, kg/m^2	19.9±1.3	21.2±1.6	22.3±1.9	25.8±2.1
UC duration,yrs	4.2±1.1	4.0±1.0	4.4±1.1	-
UC activity: mild moderate	 39 26	 52 13	 65 -	-
Mayo score: 1 2	 39 26	 52 13	 65 -	-
Weight loss, n	65	57	43	-
Anemia, n	65	52	33	
Arthralgia, n	29	9	9	-
Erythema nodosum, n	14	9	9	-
Laboratory parameters				
pANCA (>1:40), n	65	57	45	-
ASCA, RU/ml	35.4±3.2	18.9±2.6	19.2±2.7	-
Faecal calprotectin, µg/g	234.5±9.3^	186.8±8.4^	145.3±7.1^	32.4±3.1

** - P = 0.004 between (a) - (d); ** - P = 0.029 between (b) - (d);*
^ - P = 0.000 between (a) - (d), (b) - (d), and (c) - (d).

Results of ileocolonoscopy with biopsy and histological characteristics of UC are shown in Table 2. Parameters of complete blood count, ESR, serum urea, creatinine, electrolytes, liver enzymes, serum iron levels, CRP were changed in active stage of UC: high platelet count (n=32), elevated ESR (n=65), hypokalemia (n=35), hypomagnesemia (n=32), elevated level of CRP (n=65), elevated level of serum urea and creatinine (n=8), alaninaminotrasferase (n=37), a decreased level of serum iron (n=65).

All laboratory tests were in the range of reference values for patients in the stage of induction and the maintenance of UC remission.

We found a dysbiotic relationship between protective and aggressive bacterial species in patients with UC in the active stage and in the stage of induction of UC remission: the increase of *Escherichia coli, lactose-negative strains* (n=57; 10^5/g), *Proteus spp.* (n=45;10^5/g), *Enterococcus spp.* (n=42;10^{4-6}/g), *Staphylococcus spp.* (n=50,10^{5-6}/g), *Streptococcus spp.* (n=42;10^{5-6}/g), *Bacteroides spp.* (n=55;10^{4-6}/g), *Clostridium spp.* (n=38;10^4/g) and the decrease of *Bifidobacterium spp.* (n=63;10^5/g), *Lactobacterium spp.* (n=63;10^{4-6}/g) in stool culture.

Table 2.

Histological characteristics of ulcerative colitis

The characteristic findings at ileocolonoscopy (Truelove - Witts' criteria, Schroeder classification, histological characteristics)		n
Active stage		
Mild activity of UC[2] (grade 1)	Erythema, decreased vascular pattern, mild friability Basal plasmacytosis, the inflammatory infiltrate in the lamina propria, absent crypt architectural distortion	39
Moderate activity of UC (grade 2)	Marked erythema, absent vascular pattern, friability, erosions Basal plasmacytosis or subcryptal, heavy, diffuse transmucosal lamina propria cell increase and widespread crypt architectural distortion	26
Induction of remission		
Mild activity of UC (grade 1)	Erythema, decreased vascular pattern, mild friability Basal plasmacytosis, the inflammatory infiltrate in the lamina propria, absent crypt architectural distortion	52
Moderate activity of UC (grade 2)	Marked erythema, absent vascular pattern, friability, erosions Basal plasmacytosis or subcryptal, heavy, diffuse transmucosal lamina propria cell increase and widespread crypt architectural distortion	13
Maintenance of remission		65
Mild activity of UC (grade 1)	-	-
Moderate activity of UC (grade 2)	-	-

All these changes correlate with different expressions of peptides and proteins in damaged and undamaged colon mucosa in patients with UC in the active stage and in healthy persons (Table 3). Molecules of peptides and proteins were seen to interact among themselves and with other molecules as participants in universal pathways in patients with UC in the active stage: cytokine, oxidative stress, Klotho, STAT-JAK signaling pathway, PPAR γ, TLR, NF-kB, β-defensin, INK4 tumor suppressor proteins pathway, MUC1-mediated signaling pathways. Bioinformatics analysis revealed the presence of molecules that are the participants in the universal pathways of UC in the active stage, and the molecular interactions involved.

Table 3.

Qualitative profile of peptides and proteins in colon mucosa

Protein name	MW (Da)	pI	CG (n=30)	UC (active stage) (n=65)	*P-level*
IL-2	17628	7.6	2	22	*0.005*
RBP4	23010	5.4	1	7	*0.428*
SMAD2	48081	6.1	2	11	*0.096*
HSP47	70052	9.0	2	13	*0.058*
HSP27	27000	6.12	1	9	*0.162*
HSP2	90000	5.1	1	9	*0,162*
TNF-α	25644	6.4	2	24	*0.002*
KNG1	71957	4.8	1	10	*0.164*
APOC3	10852	4.6	1	15	*0.018*
NF-kB	105356	5.5	2	25	*0.001*
RTKs	104000	6.96	1	13	*0.058*
PPARγ	57620	5.78	12	9	*0.007*
IL-6	23718	6.17	1	12	*0.028*
IL-8	11098	9.1	2	13	*0.133*
IL-12A	24874	8.4	1	7	*0.428*
IL-1β	30748	6.1	1	6	*0.426*
CASP8	55391	5.12	2	9	*0.493*
CASP10	58951	5.97	2	10	*0.328*
HβD-1	7420	4.1	16	3	*0.000*
CFTR	168142	8.91	13	1	*0.000*
PHB	29804	9.8	12	2	*0.000*

pI – isoelectric point; P-value between groups based on Fisher's Exact Test

Discussion

We identified following functional groups of peptides and proteins in molecular patterns of bioptates of colon mucosa in UC patients: peptides and proteins regulating the barrier function of colon mucosa; proteins-participants of specific metabolism in epitheliocytes and endocrinocytes; proteins of the fibrosis in colon mucosa; proteins regulating cell cycle, oncogenesis, proteolysis in cell, hormones processing, angiogenesis, coagulation factors; proteins of free radical oxidation and antioxidant system; proteins regulating the receptor activity of epitheliocytes and immune cells; structural proteins of colon mucosa; transcription and translation factors regulating the activity of cell nucleus, regulators of protein folding; transport proteins; proteins-enzyms of detoxification. Below we have provided the functional activity of some of them.

SMAD family member 2 (SMAD2) activates the transcription of TFG1β, which increases the activity of Rho/ROCK signaling pathway in fibroblasts of colon submucosa that leads to specific regulation of the CCN2 gene in cells and the development of fibrosis in colon submucosa in UC patients. The stimulation of the expression of apoC-III in affected colon mucosa in UC is associated with the activation of the FOXO1 signaling pathway that supports inflammatory processes in colon mucosa.

The second small heat shock protein (HSP2) controls the apoptosis of colonocytes and immune response in damaged colon mucosa through expression of Bcl-2 and IL-17; HSP2 is also responsible for the mucosa resistance to therapeutic strategies. Anti-apoptotic functions of HSP27 are possible through the interaction with DAXX7, the activation of Akt and the inhibition of the apoptosis. HSP47 interacts with collagen I, II, III, IV and V types, which contributes to the launch of autoimmune process in UC.

Caspase 8 protects colonpcytes from TNFα-induced cell death through a necroptosis mechanism via the blockade of the RIP3 expression. The expression of prohibitin maintains

optimal activity of the electronic transport chain through the activity of transcription factor STAT3 and the decrease in the TNFα expression.

Significant decrease of the PPARγ expression promotes the activation of STAT and AP-1 signaling pathways, which promotes an increase in the synthesis of IL-2,6,8,12, TNFα, matrix metalloproteinases, the activity of immune and inflamation processes in colon mucosa. A significant increase in the NF-kB expression in colon mucosa is associated with the activation of TNFα and IL-1, which promotes the increase of immune processes in colon mucosa.

The molecular interactions of β-defensin-1 are presented in Figure 1. The reduction of the β-defensin-1 expression in cells of colon mucosa is accompanied by increased expression of CCR6, which promotes the formation of inflammatory infiltrates in coloc submucosa in UC.

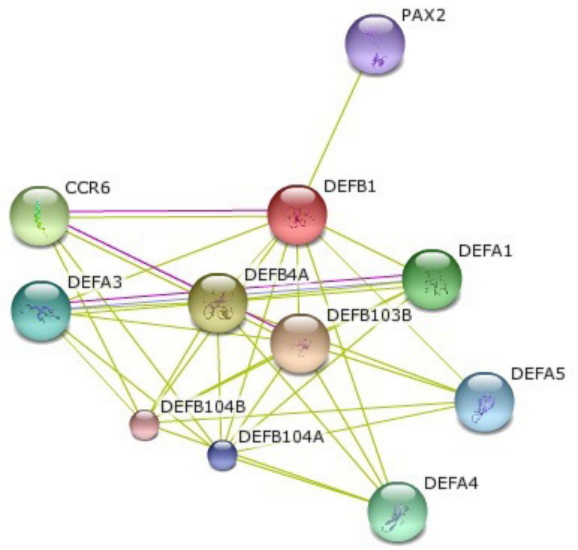

Figure 1. Molecular interactions of β-defensin -1 (STRING 10.0 database)

DEFB1*, defensin beta 1;* ***DEFA4****, defensin alpha 4, corticostatin;* ***DEFB4A****, defensin, beta 4A;* ***DEFA3****, defensin alpha 3, neutrophil-specific;* **DEFA5,** *defensin alpha 5, Paneth cell-specific;* ***DEFA6****, defensin alpha 6, Paneth cell-specific;* ***CCR6,*** *chemokine (C-C motif) receptor 6;* ***ALB****, albumin; MYC, v-myc avian myelocytomatosis viral oncogene homolog;* ***CAMP****, cathelicidin antimicrobial peptide;* ***PAX2****, paired box 2.*

In conclusion, we identified potentially new molecular markers of the development of UC. This information may provide new avenues for the development of novel diagnostic tests for UC.

Competing interests

The authors declare that they have no competing interests.

References

1. Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: up or down? World J Gastroenterol. 2006;12(38):6102-8.
2. Büsch K, Ludvigsson JF, Ekström-Smedby K, Ekbom A, Askling J, Neovius M. Nationwide prevalence of inflammatory bowel disease in Sweden: a population-based register study. Aliment Pharmacol Ther. 2014;39(1):57–68.
3. Garrett WS, Gordon JJ, Glimcher LH. Homeostasis and inflammation in the intestine. Cell. 2010;140(6):859-70.
4. Andersen V, Christensen J, Ernst A, Jacobsen BA, Tjønneland A, Krarup HB, et al. Polymorphisms in NF-kB, PXR, LXR, PPARγ and risk of inflammatory bowel disease. World J. Gastroenterol. 2011;17(2):197–206.
5. Andersen V, Nimmo E, Krarup HB, Drummond H, Christensen J, Ho GT, et al. Cyclooxygenase-2 (COX-2) polymorphisms and risk of inflammatory bowel disease in a Scottish and Danish case–control study. Inflamm Bowel Dis. 2011;17(4):937–46.
6. Comelli EM, Lariani S, Zwahlen MC, Fotopoulos G, Holzwarth JA, Cherbut C, et al. Biomarkers of human gastrointestinal tract regions. Mamm Genome. 2009;20(8):516–27.
7. Li X, Conklin L, Alex P. New serological biomarkers of inflammatory bowel disease. World J. Gastroenterol. 2008;14(33):5115–24.
8. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut. 2006;55(6):749–53.
9. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. Br Med J. 1955;2(4947):1041–8.
10. D'Haens G, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, et al. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. Gastroenterology. 2007;132(2):763–86.
11. Dignass A, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis. Part 1: Definitions and diagnosis. J Crohns Colitis. 2012; 6(10):965–90.
12. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. N Engl J Med. 1987;317(26):1625–9.
13. Travis SP, Schnell D, Krzeski P, Abreu MT, Altman DG, Colombel JF, et al. Developing an instrument to assess the endoscopic severity of ulcerative colitis: the Ulcerative Colitis Endoscopic Index of Severity (UCEIS). Gut. 2012;61(4):535–42.