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An analysis and survey of interleukin-10 receptor mutation in inflammatory bowel disease (IBD) in the first Iranian IBD cohort

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Abstract

Background: Early-onset inflammatory bowel disease (IBD) is classified into Crohn's disease (CD), ulcerative colitis (UC) and unclassified disorders, which has a chronic, relapsing course and can result in substantial long-term morbidity. IBD is a multifactorial disorder with genetic susceptibility, immunological predisposition and environmental triggers. The objective of this study was to generally determine the prevalence of *IL10R* mutation in IBD patients in Isfahan, Iran. We performed sequencing of all exons in *IL10RA* and *IL10RB* in a cohort of IBD patients and healthy controls.

Methods: Total DNA contents of 76 patients and 50 healthy controls were extracted from whole blood and polymerase chain reaction (PCR) amplifications and sequencing of whole exons in *IL10R* were performed.

Results: Overall, we determined 13 single nucleotide polymorphisms (SNPs) in all *IL10R* genes. Of them, rs3135932

and rs2229113 of the *IL10RA1* gene, in exons 4 and 7, respectively, were significantly associated with IBD occurrence in patients.

Conclusions: Our results also confirmed that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding *IL10* receptors. These variants, alone, could only give rise to a sub-clinical manifestation of IBD.

Keywords: immunodeficiency; inflammatory bowel disease; interleukin-10 receptor; monogenic; mutation; single nucleotide polymorphism.

Introduction

Inflammatory bowel disease (IBD) is classified into Crohn's disease (CD), ulcerative colitis (UC) and unclassified disorders, which has a chronic, relapsing period and may result in substantial long-term morbidity [1]. IBD is a multi-factorial disorder with genetic susceptibility, immunological predisposition and environmental triggers [2]. To date, more than 210 genetic loci have been associated with IBD [3, 4]. Genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, barrier function, epithelial restitution, microbial defense, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum (ER) stress and metabolic pathways associated with cellular homeostasis [2, 5].

In IBD, monogenic causes need special consideration. In order to identify high risk patients, specific surveillance (e.g. for malignancy or infection) or specific treatments (e.g. bone marrow transplant) are required [1].

Causative variants of monogenic conditions in interleukin-10 (*IL10*), IL-10 receptor (*IL10R*), X-linked inhibitor of apoptosis protein (*XIAP*), ADAM metalloproteinase domain 17 (*ADAM17*), neutrophil cytosolic factor 4 (*NCF4*) and tetratricopeptide repeat domain 7A (*TTC7A*) were

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identified in very early-onset IBD (VEOIBD) patients; the data presenting the hypothesis that severe infantile colitis frequently start immediately after birth might represent a group of heterogeneous monogenetic diseases [6]. Among these candidate genes, *IL-10* and *IL-10R* gene mutations have been extensively investigated [7]. *IL-10* is an anti-inflammatory cytokine secreted by a variety of cell types and is necessary for maintaining immune homeostasis in the gastrointestinal (GI) tract.

IL-10 has a role in restricting T cell proliferation, downregulating costimulatory protein expression in antigen-presenting cells and limiting pro-inflammatory cytokine production [8].

It is well known that substantial geographical and ethnical variations in IBD incidence exist worldwide. The incidence of IBD is steadily rising in many regions of the world. In Iran, however, the incidence and prevalence rates of IBD are not available. So, we established the first IBD bio-bank in Iran [8] and the registration process of IBD patients is ongoing.

Single nucleotide polymorphisms (SNPs) that affect IL-10 production have been associated with CD and/or UC. As far as we know, there are no data available for the evaluation of IL10R mutation in IBD patients in Iran. Hence, the focus was placed on patient's candidate gene study by sequencing of IL10RA and IL10RB in IBD patients.

Materials and methods

Patient information

We enrolled a total of 76 IBD patients in this study with informed consent. They were referred to the Immunodeficiency Research Center and Al-Zahra University Hospital at the Isfahan University of Medical Sciences. Diagnosis of IBD was based on standard tools including clinical features, endoscopy with biopsies and adequate imaging of the small bowel. We collected their clinical characteristics, including disease type, sex, age of onset and disease behavior. In addition, information pertaining to family history, medical treatment and surgical intervention including the patient's age at operation and history of infections were also obtained. Furthermore, history of immunodeficiency and autoimmunity in the patients was considered.

DNA extraction and PCR amplification

Total DNA content of each patient was extracted from whole blood using the QIAamp DNA Blood Mini Kit

(#51104, Qiagen, Hilden, Germany). A 20- μ L polymerase chain reaction (PCR) reaction mixture contained 100 ng of genomic DNA, 2.5 pmol of each of specific primers for each exon, 100 μ mol/L deoxyribonucleotide triphosphate (dNTP), 1.5 mmol/L $MgCl_2$ and 1 U of AmpliTaq polymerase (PR901643, Cinnagene, Iran).

Amplifications were performed for all primers using a Bio-RADT100™ thermal cycler (Hercules, CA, USA) according to the following parameters: initial denaturation was done at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and elongation at 72 °C for 1 min and ending with a final elongation cycle at 72 °C for 10 min. These conditions were used for all primer pairs.

The resulting PCR product was visualized on a 1% agarose-ethidium bromide gel under ultraviolet (UV) illumination. Five microliters of each reaction was sent for Sanger sequencing and the results were evaluated using the code codon aligner software. Primer set used for PCR reaction was used according to Kotlarz et al.'s paper [2] (Table 1).

Statistical analysis

We used StataCorp software (version 11, StataCorp, College Station, TX, USA) for data analysis and described variables as frequency, percentage, mean and standard deviation. Comparative analysis was done via T-test and χ^2 test in order to explore the effects of variables on IBD incidence. The incidence of SNPs and consanguinity percentage in patients and healthy controls were compared using χ^2 -test. We considered a significant level of 5% for all analyses.

Written informed consent

DNA Sanger sequencing of genes encoding for IL-10R1, IL-10R2 and IL-10 was performed upon written informed consent.

Results

We sequenced the genes encoding for all exons *IL10RA* and *IL10RB* in 76 patients with IBD and 50 healthy controls in Isfahan, Iran, using the candidate gene sequencing approach. Patients were stratified according to gender, age at diagnosis, therapy response, presence of immunodeficiency and autoimmunity and consanguinity condition. Diagnosis of CD and UC was based on standard clinical, endoscopic and histological criteria [9] and the phenotype

Table 1: Primer set used for PCR.

Name of primer	5'→3'
IL10RA 1-F	GACAGTGGTCCCCGTCC
IL10RA 1-R	CACTGGATGGAGAACTTTAATGG
IL10RA 2-F	GAACCTCCCTTTCTTCTTGG
IL10RA 2-R	AGGCAGGTATCTTCCCATGC
IL10RA 3-F	GGCCTCTGCGTCTCCC
IL10RA 3-R	GCAGACATGGTGAGCTATGG
IL10RA 4-F	TCCGTGGACTAATTGTTCTGC
IL10RA 4-R	AGTCCATAAGGTGCTGCCAC
IL10RA 5-F	AAGTCTAAAACGGCTATTATCACTG
IL10RA 4-F	TCCGTGGACTAATTGTTCTGC
IL10RA 4-R	AGTCCATAAGGTGCTGCCAC
IL10RA5-F	AAGTCTAAAACGGCTATTATCACTG
IL10RA 5-R	AGCTGGAATTTGAGTTGGATG
IL10RA 6-F	GGCTCTGTTTCAGGGATTG
IL10RA 6-R	CATGTTGTCTGGAATTGGGC
IL10RA 7-6-R	TCCCACACATTATTACCTCC
IL10RB 1-F	AGGGTAAAGAAGACCTCAAA
IL10RB 1-R	CCTAGTTGCGTCTCAGCAG
IL10RB 2-F	GGAGAACCAAGTGCTGGATG
IL10RB 2-R	CAGACTCCCTTCTCTGTG
IL10RB 3-F	TTAACACAGTTTCCACTCCCG
IL10RB 3-R	AAGGCCATCCATTGTGG
IL10RB 4-F	TCCGTGGACTAATTGTTCTGC
IL10RB 4-R	AGTCCATAAGGTGCTGCCAC
IL10RB 5-F	AAGTCTAAAACGGCTATTATCACTG
IL10RB 5-R	AGCTGGAATTTGAGTTGGATG
IL10RB 6-F	GGCTCTGTTTCAGGGATTG
IL10RB 6-R	CATGTTGTCTGGAATTGGGC
IL10RB 7-1F	TCCAGCCAGGAGTTCTGTG
IL10RB 7-1R	GCTGAAAATTACACTCTCAGTGG
IL10RB 7-2F	CTCCAGACCCTGGACTTAG
IL10RB 7-2R	TCACTTTGTACCCAGGC
IL10RB 7-3F	GATGGCGCATGCCTATAATC
IL10RB 7-3R	TGGACATCAAGATGGCAAAC

characteristics were defined according to the Montreal classification [10] through which 16 IBD patients were diagnosed as having CD and 60 IBD patients as having UC. The study patients represented a subgroup of 12 patients with defined primary immunodeficiency and 26 patients with a history of autoimmunity who presented with onset of severe endoscopically and histopathologically confirmed colitis. In this study, we included patients with routine immunosuppressive therapy regimen (56 patients) and also patients resistant to immunosuppressive therapy (14 patients). Furthermore, we registered the consanguinity condition in 34 patients and 14 healthy donors.

Main clinical characteristics of the patients included in the study are presented in Table 2.

We identified 13 SNPs in all the IL10R exons in our IBD patient cohort and healthy controls.

Identified SNPs in *IL10RA* were rs2228055, rs752413943, rs2256111, rs3135932, rs145975996, rs755029073, rs2229113,

Table 2: Main clinical characteristics of IBD patients and HCs.

	CD	UC	HC
Number	16	60	50
Female, n (%)	10 (62.5)	26 (43.3)	26 (52.0)
Male, n (%)	6 (37.5)	34 (56.7)	24 (48.0)
Age, years, mean (SD)	22 (12.5)	21 (15.1)	19 (16.4)
Age at diagnosis, years, mean (SD)	16.6 (10.3)	13 (6.5)	–
Corticosteroid-dependent, n (%)	12 (75)	44 (73.3)	–
Corticosteroid-refractory, n (%)	4 (25)	17 (26.7)	–
Autoimmunity, n (%)	4 (25)	22 (36.6)	–
Immunodeficiency, n (%)	2 (12.5)	10 (16.6)	–
Consanguinity, n (%)	10 (62.5)	24 (40.0)	17 (34)

CD, Crohn's disease; UC, ulcerative colitis; HC, healthy control.

rs747711495, rs200254237, rs746081732, rs751283486 and rs768512760 and in *IL10RB*, rs2834167.

SNP functions were in following order: missense (I 224 Val), missense (Ala 153Val), synonymous (Ala153Ala), missense (Ser159Gly), missense (Arg263Gln), missense (Arg284Cys), missense (Arg351Gly), synonymous (Gln376Gln), missense (Thr416Ile), missense (Ala493Val), synonymous (Ala511Ala), synonymous (Ser563Ser) and missense (K47E).

The findings indicate that the frequencies of SNPs rs3135932 and rs2229113 in *IL10RA* are significantly different in IBD patients compared to healthy controls (p-value: 0.003 and 0.009, respectively).

A statistical analysis was conducted to compare patients from consanguine families with those from non-consanguine ones with regard to the two SNPs rs3135932 and rs2229113. The rationale behind the selection of the above SNPs lies in their statistically high correlation with IBD incidence in patients. Interestingly, a significantly higher rate of the incidence of SNPs was observed in patients from consanguine families (p-value=0.001).

In contrast, SNPs rs2228055, rs752413943, rs2256111, rs145975996, rs755029073, rs747711495, rs200254237, rs746081732, rs751283486 and rs768512760 of the *IL10RA1* and *IL10RB1* genes were not significantly different in patients compared to healthy controls; all those consisting of single base pair substitutions were identified and confirmed by direct sequencing (Table 3).

Discussion

In this case-control study, we determined the frequency of SNPs in the *IL10R* gene in IBD patients and healthy controls from Iran. The polymorphism rs3135932 in exon 4 and

Table 3: Frequency of SNPs in IBD patients.

SNP	Chr. position	Alleles	Ancestral allele	Prevalence in patients	Prevalence in healthy donors	p-Value (χ^2 test)	Previous IBD report
rs2834167	117994131	A/A	A	3	0	0.150	[11]
rs2228055	117994131	A	G	1	0	0.409	[12]
rs752413943	117993331	C/T	Not reported	2	33	0.009	Not reported
rs2256111	117993332	A/G	A	25	43	0.000	Not IBD reported
rs3135932	117993348	A/G	A	11	0	0.003	[13]
rs145975996	117995688	A/G	G	5	1	0.226	[12]
rs755029073	117998754	C/T	Not reported	1	0	0.409	Not IBD reported
rs2229113	117998955	A/G	G	23	8	0.009	[14]
rs747711495	117999032	A/G	Not reported	3	12	0.000	[14]
rs200254237	117999151	C/T	C	4	0	0.095	Not IBD reported
rs746081732	117999382	C/T	Not reported	1	12	0.000	Not reported
rs751283486	117999437	C/T	Not reported	1	31	0.000	Not reported
rs768512760	117999593	A/G	Not reported	1	20	0.000	Not reported

Chr., chromosome.

rs2229113 in exon 7 of the *IL10RA1* gene were significantly associated with IBD incidence in patients.

In contrast, polymorphisms in the *IL10R* gene (rs752413943, rs2256111, rs755029073, rs200254237, rs746081732, rs751283486, rs768512760) were not associated with any form of IBD.

The immune response has evolved to protect the host from a broad range of pathogenic microorganisms, control immune responses and prevent reactivity to self, all required to limit host damage in order to maintain hemostasis [15].

Intestinal homeostasis is a highly dynamic process requiring sensitivity to mount appropriate immune responses toward microbial or food antigens, yet necessitating the regulation of these responses in order to prevent chronic inflammation.

Cytokines have an important role in the pathogenesis of IBD; they control multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines in IBD could cause the resolution of inflammation and tissue destruction [16].

IL-10 as an anti-inflammatory cytokine plays a role in maintaining gut homeostasis [15] secreted by various cells, including monocytes, macrophages, T and B lymphocytes, dendritic cells, epithelial cells and mast cells. IL10 mediates its anti-inflammatory effects through IL-10R-dependent signals emanating from the cell surface.

The IL-10R is a hetero-tetramer that consists of two subunits of IL-10R α and two subunits of IL-10R β . The IL-10R α subunit is unique for IL-10 signaling, but the IL-10R β subunit is shared by IL-22, IL-26 and interferon

(IFN)- λ receptor [17]. After binding IL-10 to its receptor, it activates JAK1 and Tyk2, leading to the phosphorylation of STAT-3, the activation of downstream target genes and finally the expression of anti-inflammatory effectors [7].

In the study by Shim and Seo [11], the incidence of p.(T84I) was reported as a trigger to IBD pathogenesis. Moran et al. confirmed the association of p.(I224V) variant with IBD. The study by Glocker et al. indicated that IBD may be a monogenic disorder and homozygous mutations of p.(W159X) in IL-10RB were found in Kurdish siblings with consanguineous parents [18]. Interestingly, similar to Glocker et al.'s report, this variant was detected in nine patients of consanguineous origin in our IBD cohort. In our study, we could find p.(T84I), p.(I224V) and p.(W159X) variants in our patients. Genetic alteration which is caused by p.(R263Q) variant can affect IL10 receptor intracellular signaling and is related to IBD pathogenesis [14]. In five patients, P.(R263Q) variant was detected in our study. P.(R351Q) genetically predisposed IBD incidence [15], the very variant that we also determined in our cohort which was significantly associated with IBD initiation in our study. To the best of our knowledge, P.(A153V), P.(A153A), P.(R284C), P.(T416I), P.(A493V), P.(A511A) and P.(S563S) variants were not reported in IBD patients.

SNPs that affect IL-10 production have been associated with CD and/or UC. For the first time, our results confirmed that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding *IL10* receptors in Iranian IBD patients. These variants, alone, could only give rise to a sub-clinical manifestation of IBD.

Conclusions

In Iran, as a result of a high rate of consanguinity, the incidence of genetic disorders is relatively high; therefore, evaluation of *IL10R* mutation in IBD patients is necessary. This recommendation may help patients to have precise diagnosis and tailored therapy.

Author contributions: R.K.H., F.V. and S.N. designed and performed experiments and evaluated and interpreted data; M.N. carried out statistical analyses; M.E., H.S., H.T. and P.A. referred patients to our hospital; M.Y., N.N. and M.B., collected clinical data and samples, and screened cohorts. R.S.H., A.R., D.K. and C.K. gave conceptual advice. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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