Egyptian Journal of Medical Human Genetics

RESEARCH

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Evaluation of adropin, fibroblast growth factor-1 (FGF-1), and Toll-like receptor-1 (TLR1) biomarkers in patients with inflammatory bowel disease: gene expression of TNF-α as a marker of disease severity

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Abstract

Background Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of unknown etiology and unpredictable course. The aim of the work was to assess the levels of adropin, fibroblast growth factor-1 (FGF-1), and Toll-like receptor-1 (TLR1) biomarkers in IBD patients compared to controls and evaluate the gene expression of TNF-α as a marker of disease severity.

Methods Adropin, fasting serum FGF-1 levels, TLR1, and TNF-α were measured in 60 IBD patients. They were also compared with 58 healthy controls matching age and gender. Moreover, the blood cells cDNA copy number of TNF-α were determined as a marker of severity.

Results Adropin and TLR1 levels were significantly lower in patients than controls. FGF-1 was reduced but not statistically significant. The expression of TNF-α gene in the IBD patients was significantly increased (42%) in comparison with control samples ($P < 0.001$).

Conclusions Adropin, IGF-I, and Toll-like receptor-1 biomarkers may have a role in the intricate pathophysiology of IBD and may possibly operate as predictors of disease activity. Thus, they may be therapeutic targets for IBD. Moreover, the expression of TNF-α gene can be used as a marker of severity.

Keywords Adropin, cDNA, FGF-1, Inflammatory bowel disease, TLR1

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Introduction

Inflammatory bowel diseases (IBD) are chronic intermittent inflammatory gastrointestinal disorders of unknown etiology but a clear genetic predisposition [1, 2]. Inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, are chronic and relapsing conditions characterized by massive damage of the epithelium and the underlying mesenchyme of the intestine that pose a growing burden on healthcare systems worldwide [3]. IBD is hypothesized to develop as a result of interplay between environmental, microbial, and immune-mediated factors [4]. It is related with significant morbidity in Western countries and is becoming more prevalent in the developing countries, causing an increasing strain on global healthcare systems [5]. The pathophysiology of inflammatory bowel disease (IBD) has been linked to the biomarkers as adropin, IGF-I, and TLR1. They play a role in the control of immunity, metabolism, and inflammation as well as the prediction of disease activity in IBD.

Adropin is a peptide hormone produced by fat cells, it controls a variety of processes, such as inflammation, insulin sensitivity, vascular protection, and energy metabolism. Adropin is produced in the liver and brain, heart, and gastrointestinal tract. It assists in the reduction of inflammation by acting on immune cells [6].

Impaired epithelium healing is a crucial aspect of inflammatory bowel disease (IBD). Fibroblast growth factor-1 (FBG-1) plays a significant part in the pathophysiology of the IBD disease. Their downstream consequences are linked to several cellular functions comprising epithelial healing in response to injury [7]. The growth factors are considered as possible tools for the controlling and repairing intestinal inflammation. They have a key role in cellular differentiation, angiogenesis, and proliferation [8].

TLR1 is a group of proteins that play a critical role in the pathogenesis of IBD and are involved in the innate immune response. When TLR1 binds to its ligand, it triggers a cascade of events that lead to the production of inflammatory cytokines and the activation of immune cells [9]. In patients with IBD, it is thought that TLR1 levels are lower due to dysregulation of TLR1 signaling pathways, increased production of anti-TLR1 antibodies, and reduced expression of TLR1 on immune cells. The lower levels of TLR1 in patients with IBD may contribute to the development and progression of the disease.

Tumor necrosis factor (TNF)-α is a multifunctional cytokine that plays a crucial role in the pathophysiology of inflammatory, autoimmune, and malignant diseases by promoting inflammatory responses[10, 11]. High serum TNF-α levels in IBD blood and tissue samples indicate the critical role of TNF-α in cell-mediated immunity. Several in polymorphisms exist TNF-α, most of which are found in the promoter region, and some of which affect the gene's expression level [12].

The present study aimed to estimate the levels of adropin, fibroblast growth factor-1 (FGF-1) and Toll-like receptor-1 (TLR1) biomarkers in IBD patients compared to controls and assess the gene expression of TNF-α.

Methods

The report included 60 patients with IBD. They were compared with 58 healthy controls matching age and gender. They were enrolled from the IBD Clinic, National Hepatology and Tropical Medicine (NHTMRI), Egypt. The NHTMRI Research Ethics Committees gave its approval to the study. An informed consent was demanded from the patients to contribute to the research. The informed consent was given by the parents or guardians of the younger patients. The current study conforms to the guidelines established by the NHTMRI Research and Ethics Committees.

A pedigree analysis was performed for all cases.

Diabetes, cardiovascular conditions, and corticosteroid therapy within three months of the start of the trial were the exclusion criteria. Additionally, each member of the control group underwent a thorough medical examination and any participants who displayed any indication of inflammation were disqualified.

Biochemical parameters

Assessment of adropin

Serum concentration of adropin was measured using enzyme-linked immune-sorbent assay (ELISA) according to SinogeneClon Biotech Co.

Assessment of fibroblast growth factor-1, Acidic (FGF-1)

Fibroblast growth factor-1 was determined using the enzyme-linked immunosorbent assay (ELISA) DLdevelop kits.

Estimation of the level of Toll-like receptor-1(TLRs)

Level of Toll-like receptor-1(TLRs) in the serum estimated according to R&D Systems, Minneapolis, MN, USA, using ELISA Kit, Catalogue Number:SL4063Hu.

Gene expression of TNF-α gene

Real-time quantitative PCR technique

Isolation of RNA and reverse transcription (RT) process

In accordance with the manufacturer's instructions, total RNA was isolated from the blood cells of control individuals and patients with inflammatory bowel disease (IBD) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) supplemented with DNaseI (Qiagen) digestion step. After digesting DNA residues with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany), isolated total RNA was resuspended in water treated with DEPC, and photospectrometry at 260 nm was used to measure the results. A total RNA sample's purity was determined by measuring its 260/280 nm ratio, which ranged from 1.8 to 2.1. Additionally, formaldehyde-containing agarose gel electrophoresis was used to ensure integrity through the analysis of the 28S and 18S bands using ethidium bromide stain [13]. Aliquots were kept at -80 °C unless they were used right away for reverse transcription (RT).

RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) was used to reverse transcribe complete Poly(A) + RNA isolated from blood cells into cDNA in a total volume of 20 µl. A master mix comprising 50 mM MgCl2, 10 × RT buffer, 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (a 50 kDa recombinant enzyme to inhibit RNase activity), and 50 IU MuLV reverse transcriptase was used in conjunction with 5 µg of total RNA. The RT reaction was conducted using the method of 10 min at 25 °C and 1 h at 42 °C. [14] and concluded with a 5-min denaturation step at 99 °C. The reaction tubes holding the RT preparations were then flash-cooled in an ice chamber prior to being utilized for quantitative real-time polymerase chain reaction (qRT-PCR) cDNA amplification.

Real-time PCR (qPCR)

Using the StepOneTM Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA), the cDNA copy number of blood cells was determined. 6.5 µL of distilled water, 5 µL of cDNA template, 12.5 µL of 1 × SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL of 0.2 µM sense primer, 0.5 µL of 0.2 µM antisense primer, and 0.5 µL of distilled water were used to set up the PCRs in 25 µL reaction mixtures [15]. There were three steps assigned to the response program. The first step was three minutes at 95.0 °C. The second step comprised 40 cycles, with each cycle being divided into three steps: (a) 15 s at 95.0 °C, (b) 30 s at 55.0 °C, and (c) 30 s at 72.0 °C. The third step consisted of 71 cycles which started at 60.0 °C and then increased about 0.5 °C every 10 s up to 95.0 °C. A control of distilled water was included in every experiment. The sequences of specific primer of the cytokine gene tumor necrosis factor alpha (TNF-α) was designed and is listed in Tables 2. The $^{2-\Delta\Delta CT}$ method was utilized to ascertain the target's relative quantification in relation to the reference [13].

The primer blast results are as follows:

```
Template masking not selected
No mispriming library specified
Using 1-based sequence positions
WARNING: Unrecognized base in input sequence

OLIGO            start  len      tm      gc%   any_th   3'_th hairpin seq
LEFT PRIMER       1753   20   59.00   50.00     0.00    0.00    0.00 atgtggcaagagatggggaa
RIGHT PRIMER      1911   20   59.02   55.00     0.00    0.00    0.00 ctcacaccccacatctgtct
SEQUENCE SIZE: 4011
INCLUDED REGION SIZE: 4011

PRODUCT SIZE: 159, PAIR ANY_TH COMPL: 13.74, PAIR 3'_TH COMPL: 5.73
```

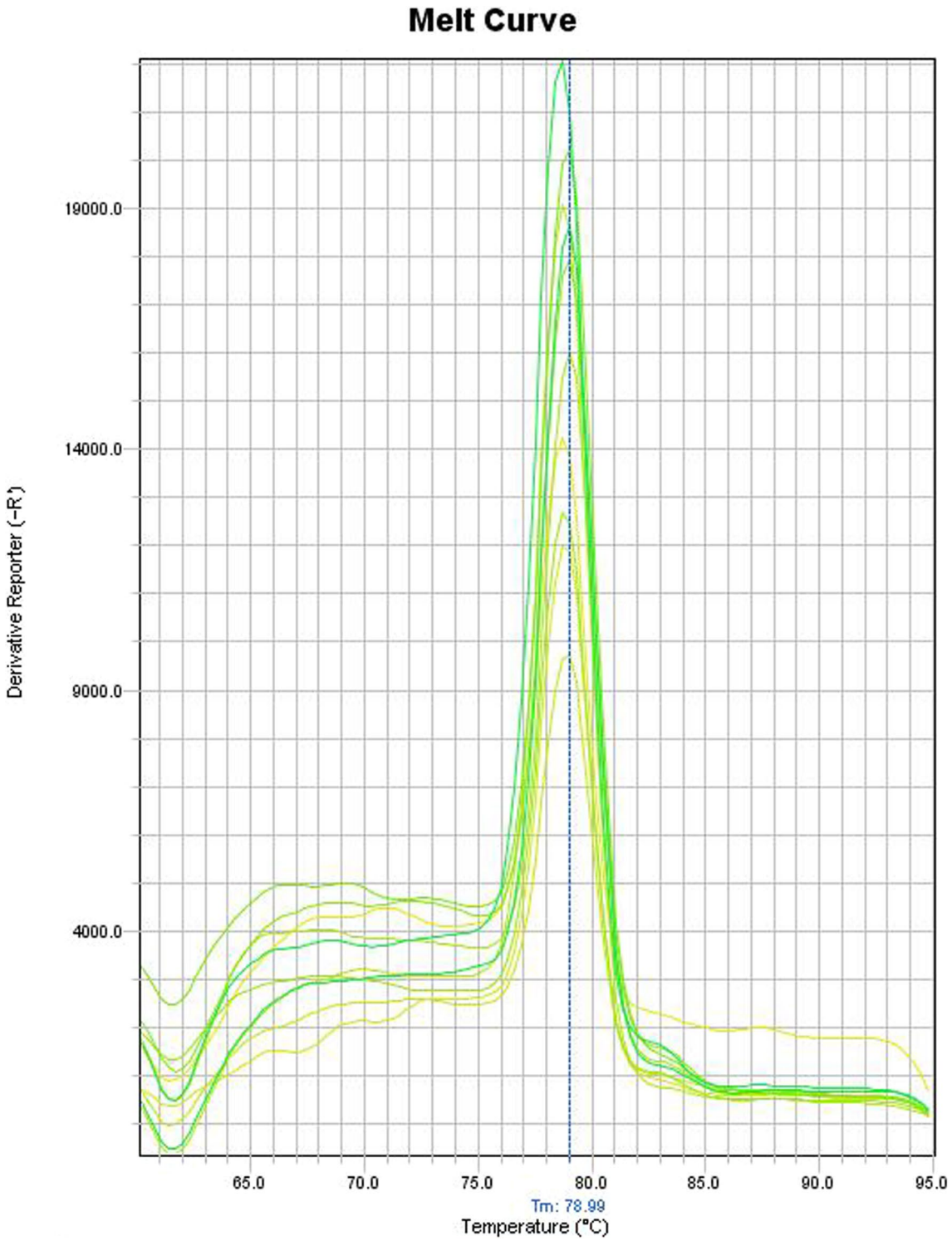

Fig. 1 Metling curve of TNF-α specific primers

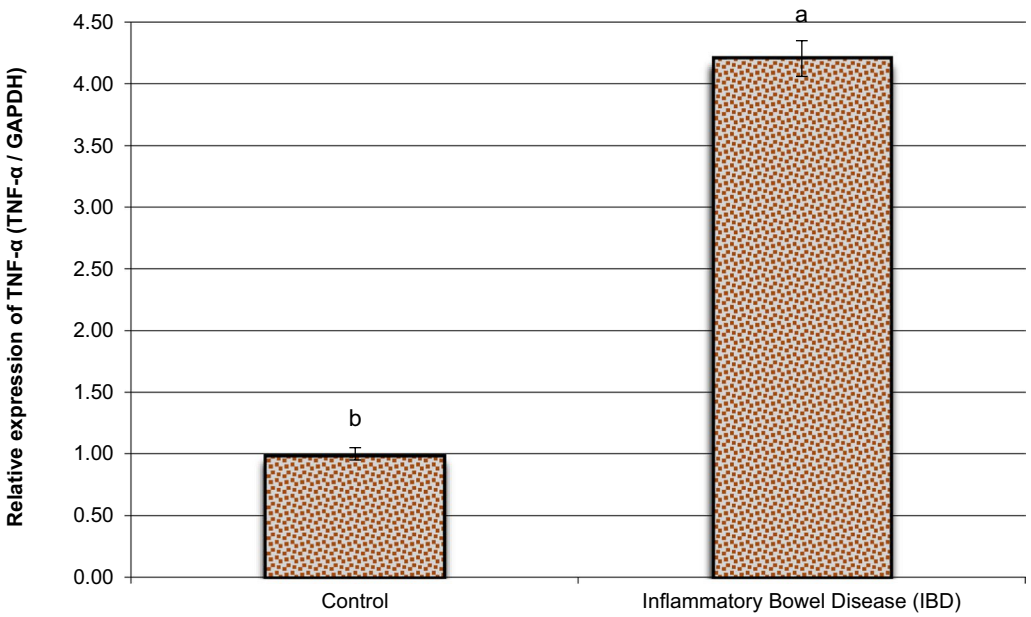

Fig. 2 The alterations of *TNF-α gene* in blood samples of inflammatory bowel disease (IBD) patients. Data are presented as mean ± SEM. [a,b]: Mean values with unlike superscript letters were significantly different

As the primer was designed, we got the annealing temperature which was used in the qRT-PCR program. Due to qRT-PCR accuracy and sensitivity versus conventional PCR, melt curve analysis provides the most confidence instead of gel electrophoretic in assessment of primer specificity. So, according to the following obtained melting curve from the current experiment indicating that the single peak observed for an amplicon from TNF-α specific primers (Fig. 1) is typically interpreted as representing a pure, single amplicon which is more accurate than the gel electrophoretic.

Statistical analysis

The statistical SPSS software for Windows, version 20.0 (SPSS, USA), was used to analyze the data. The means ± standard deviation (SD) is presented for the results. Data that were not paired were compared using the independent sample t test. P-values less than 0.05 were regarded as noteworthy.

Results

The age of patients ranged between 20 and 53 years (32.54 ± 10.02), whereas the age of controls was 34.00 ± 10.14 ($P = 0.67$). The ratio of female to male was 38:22. There was positive consanguinity in 18 patients (30%) and similarly affected family members in 19 (31.7%).

Compared to the control group, patients with IBD had significantly lower serum adropin levels (73.80 ± 5.99 vs 88.32 ± 6.48 µg/g, $P = 0.001$).

IBD patients' serum fibroblast growth factor-1 levels were lower than those of the control group (5.30 ± 5.44 vs 7.83 ± 7.66 µg/g, $P = 0.409$).

Compared to the control group, patients with IBD had significantly lower serum levels of Toll-like receptor-1 (328.77 ± 57.604 vs 587.24 ± 67.422 µg/g, $P = 0.002$) (Table 1). The expression levels of TNF-α gene were increased significantly ($P < 0.01$) in IBD blood sample compared with healthy control samples (Fig. 2). They were: about 80% of cases had abdominal pain, diarrhea, vomiting, weight loss and depression. Bleeding was found in 24% of cases, while arthritis was noted in 83% of cases. Elevated levels of TNF-α gene expression have been related to the occurrence of gastrointestinal dysfunction such as arthritis (70%), abdominal pain (80%), diarrhea (70%) vomiting(60%) as

Table 1 The biochemical parameters in IBD and control group

	IBD (n = 60)	Controls (n = 58)	*P*
Adropin (µg/g)	73.80 ± 5.99	88.32 ± 6.48	0.001
Fibroblast growth factor-1 (FBG-1) (pg/ml)	5.30 ± 5.44	7.83 ± 7.66	0.409
Toll-like receptor-1 (pg/ml)	328.77 ± 57.604	587.24 ± 67.422	0.002

Table 2 Primers sequence used for qRT-PCR of TNF-α gene

Gene	Primer sequence	GenBank (accession no)
TNF-α	F: ATG TGG CAA GAG ATG GGG AA R: CTC ACA CCC CAC ATC TGT CT	MH180383.1

Table 3 Correlation between *TNF*-α gene, adropin, FBG-1 and Toll- like receptor-1 in patients with IBD

Parameters	TNF-α gene	
	r	*p*
Adropin (μg/g)	−0.54	< 0.001
Fibroblast growth factor-1 (FBG-1) (pg/ml)	−023	0.45
Toll-like receptor-1 (pg/ml)	−0.57	< 0.001

r = Pearson correlation coefficient

well as with the reduced levels of adropin and Toll-like receptor-1 in IBD cases (Tables 2, 3).

Discussion

Immune-mediated diseases typically show a female preponderance. In our cohort, females predominated males (38:22). Studies from different countries vary in their findings regarding the association between gender and IBD [16, 17]. There are numerous reasons why these differences might exist. It is likely that different countries have variable environmental reasons of IBD. Another explanation is that certain groups have genetic variations that make specific individuals more prone to IBD than others. Understanding the causes of the disparities in results between studies of gender and IBD requires additional investigations. But it is undeniable that the gender plays a key role in the emergence of IBD.

Consanguinity is a risk factor for IBD [18]; in our research, 18 patients (30%) reported consanguineous marriage of their parents, and 19 patients (31.7%) had similarly affected family members.

Previous studies have shown that adropin levels are reduced in patients with inflammatory bowel disease [6, 19]. This suggests that adropin might be involved in the progression of IBD. Supporting the results of an earlier report, it was found that inflammatory bowel disease (IBD) patients had considerably lower serum adropin levels compared to the control group (73.80 ± 5.99 vs 88.32 ± 6.48 μg/g, $P = 0.001$). A precise explanation for how adropin level is decreased in IBD remains unknown. Though, inflammation is considered to be a potential factor that causes the damage of adropin-producing cells. More studies are needed to elucidate the role of adropin in the pathophysiology or prognosis of IBD.

IGF-I is a hormone that plays a role in growth, development, and metabolism [6].

In our study, serum fibroblast growth factor-1 levels were lower in IBD patients in comparison with the control group (5.30 ± 5.44 vs 7.83 ± 7.66 μg/g, $P = 0.409$). Similarly previous study [20] suggested that reduced serum levels of growth factor I (IGF-I) are common in patients with IBD, most likely due to gastrointestinal dysfunction, growth hormone resistance, and chronic inflammation.

Confirming the findings of previous studies, this report found that serum Toll-like receptor-1 levels were significantly lower in IBD patients in comparison with the control group (328.77 ± 57.604 vs 587.24 ± 67.422 μg/g, $P = 0.002$). TLR1 may be a contributing factor to the development and progression of IBD. Therefore, potential therapeutic targets for TLR1 in IBD could motivate the development of new therapies. TNF-α is the proinflammatory cytokine that has been studied the most and has been proven to play a role in IBD [21]. The etiology of IBD, a complicated multifactorial illness, is poorly understood. It has been demonstrated that allelic variations in cytokine genes affect gene expression, which in turn affects the severity and susceptibility to inflammatory disorders. The innate and adaptive immune systems are regulated by a vast family of cytokines. TNF-α causes proinflammatory consequences in chronic intestinal inflammation, angiogenesis, T cell and macrophage activation, and epithelial cell destruction.

Conclusion

Adropin, IGF-I, and Toll-like receptor-1 (TLR1) biomarkers may play a role in the complex pathophysiology of inflammatory bowel disease (IBD) and potentially serve as predictors of disease activity. As such, they may be therapeutic targets for IBD. Moreover, TNF-α gene expression plays a critical role in the pathogenesis of IBD and can be used as a marker of severity, which may be targeted by existing immunomodulatory therapies. Therefore, anti-TNF-α antibodies likely exert their therapeutic effects in inflamed intestinal mucosal tissues. However, larger-scale studies are needed to assess the significance of these findings. The limitations of the present study are small sample size and the cross-sectional design study which disables establishment of causal relationship.

Acknowledgements

Theodor Bilharz Research Institute, National Hepatology and Tropical Medicine Research Institute.

Author contributions

MZ contributed to writing manuscript and interpretation of data; HAO, ERY and MM contributed to biochemical investigation; HFB and WKBK

contributed to gene expression; WW, KAE-A, KH and HTE-B contributed to clinical investigation.

Funding
None.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
No competing of interest.

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Received: 12 December 2023 Accepted: 21 May 2024
Published online: 05 June 2024

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