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Pharmacogenetic Effect of Thiopurine Methyl Transferase (TPMT) Gene Expression and Serum TNF on the Imuran Response in Ulcerative Colitis (UC) Iraqi Patients

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Abstract

Background: Ulcerative colitis (UC), a chronic inflammatory bowel disease (IBD), exerts its impact on both rectal and colonic mucosa, with a growing incidence. This study aims to explore the pharmacogenetic influence of thiopurine methyl transferase (TPMT) gene expression and serum tumor necrosis factor (TNF) levels on the response to Imuran in Iraqi patients with UC.

Methods: Seventy individuals with chronic UC and 30 healthy controls were enrolled in this investigation. RNA extraction using the triazole method and enzyme-linked immunosorbent assay (ELISA) for TNF measurement were employed. Patients, aged 15-50 years, underwent Imuran treatment.

Results: Diverse responses to Imuran were observed among patients, with TPMT gene expression levels below 1 in 35 patients leading to side effects, while the remaining 35 patients exhibited positive responses with TPMT gene expression exceeding 1. Patients with varying degrees of severe, moderate, and mild UC associated with TNF showed a significant correlation with Imuran non-response.

Conclusions: A distinct correlation was identified between TPMT gene expression and Imuran therapy outcomes in UC patients. Further investigation is warranted to elucidate the underlying mechanism, positioning the TPMT gene as a potential therapeutic target for mitigating the impact of UC.

Keywords: Gene expression, Imuran response, Thiopurine Methyl Transferase, Tumor Necrosis Factor, Ulcerative Colitis.

Introduction

Ulcerative colitis (UC), a chronic inflammatory bowel disease (IBD), manifests as inflammation primarily in the colonic region of the gastrointestinal tract, often involving the rectum and potentially extending proximally through parts or the entirety of the colon. The complicated nature of UC necessitates a multidisciplinary approach for specialized patient care. Existing medical guidelines address UC symptoms in adults, while European Crohn's and Colitis Organisation (ECCO) recommendations are instrumental for the diagnosis and monitoring of UC (1). UC's clinical presentation varies based on the affected gastrointestinal tract region, showcasing a dynamic interplay between remission and flare-up phases, thereby contributing to the complexity of the disease (2). Risk factors, encompassing smoking, alcohol consumption, stress, spicy food, and genetic predispositions, further contribute to the intricate nature of UC (3). This article offers a comprehensive review of UC diagnosis and treatment from a primary care perspective (4).

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine, normally released due to inflammasome activation and strongly

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participating in the induction of inflammation in the intestine (5). Therefore, there is a high demand to block the TNF signal by anti-TNF monoclonal antibodies (mAbs) to provide effective therapy to severe steroid-refractory or dependent IBD patients (6). Additionally, TNF-α is a pro-inflammatory mediator that contributes to the pathogenesis of ulcerative colitis, known to be genetically associated (7). Modern therapeutic agents for UC have improved the targeting of pro-inflammatory molecules, effectively restricting the inflammatory cascade. 'Biological therapy' refers to selective therapeutic actions targeting specific inflammation mediators (8), unlike conventional drugs that tend only to affect the entire immune system.

Thiopurine methyl transferase (TPMT) gene has a potential role in encoding the enzyme that metabolizes thiopurine drugs. This is achieved considering that S-adenosyl-L-methionine is the S-methyl donor and S-adenosyl-L-homocysteine as a byproduct. It is worth mentioning that thiopurine drugs (e.g., 6-mercaptopurine) may be applied as chemotherapeutic agents. The TPMT gene is characterized as being 34 kb long and containing 10 exons, may be responsible for encoding the enzyme responsible for metabolizing thiopurine drugs, such as 6-mercaptopurine, which can be used as chemotherapeutic agents. Its autosomal co-dominant genetic polymorphism indicates its potential role in drug metabolism (9), which can lead to deficiency or lack of TPMT activity in either heterozygous or homozygous individuals (10). The TPMT activity phenotype in a person's red blood cells is inherited in the autosomal co-dominant mode, where 89% of which have boosted enzyme activity, as well as 11% and less than 1% having an intermediate and a total deficit of activities, respectively. Both the genetic origin of the TPMT activity polymorphism and elucidation of its molecular mechanisms have been confirmed by the analysis of the TPMT gene sequence (11). Thiopurine drugs undergo a complex metabolism process, resulting in the formation of 6-thioguanine nucleotides (6TGN) and azathioprine (AZA) and 6-mercaptopurine (6-MP). These drugs are activated intracellularly through three enzymes, with AZA partially converted into 6-MP in the liver (12). Thiopurines prevent adenine and guanine production, treat malignant tumors, rheumatic diseases, dermatological disorders, post-transplant organ rejection, and inflammatory gastrointestinal complaints. AZA dosage increases gradually (13). AZA is a pro-drug that is converted to 6-MP in vivo (14).

The aim of this work was to study the pharmacogenetic effect of TPMT gene expression and serum TNF on the Imuran response in UC Iraqi patients.

Materials and Methods

Subjects and blood sample collection

This study was conducted from December 2022 to April 2023, involving 70 patients with chronic ulcerative colitis, comprising both males and females aged 15-50 years. The study was carried out at Al-Yarmouk Teaching Hospital, Al-Imamin Al-Kadhimin Medical City, and the Digestive System Hospital in the Medical City. Peripheral blood samples of five cc were collected from each patient, with 600 microliters placed in Eppendorf tubes containing Triazole for ribonucleic acid (RNA) extraction. The remaining blood was transferred to a gel tube for the measurement of TNF. All cases underwent molecular study, and the blood samples were preserved with TRIzol.

In addition, 30 healthy volunteers aged 20-60 years were included in the study to determine TNF levels in normal human serum, plasma, culture media, or any other biological fluid. The amount of TNF in the serum of both healthy volunteers and patients with chronic ulcerative colitis was assessed using an enzyme-linked immunoassay (ELISA) device from SunLong Biotech Co., LTD.

Gene expression study on ulcerative colitis patients

Following blood collection, genomic RNA was extracted from fresh human blood using

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the Trizol method system, employing a kit from Bioneer Corporation (Korea Bio Park BLDG), in accordance with the manufacturer's instructions. The concentration of RNA and its purity were evaluated spectrophotometrically by measuring absorbance at 260 (A260) and 280 (A280) using a Nano spectrophotometer.

For reverse transcription (RT), a total of 2 g of RNA was utilized, and messenger ribonucleic acid (mRNA) sequences for the TPMT gene were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. The primer sequences for TPMT detection were as follows: forward primer 5′AGCCTGAAAATGTAATGGATGAAT′3 and reverse primer 3′TCACATCATAATCTCCTCTCCAAA′ 5, as shown in Table 1.

Real-time polymerase chain reaction (PCR), or QRT-PCR, amplification conditions consisted of 50 cycles at a temperature of 95 °C for 15s, 58 °C for 20s, and 72 °C for 1 min during the extension stage. The melt curves were obtained by increasing the temperature to 95 °C for 10 sec, then decreasing it to 25 °C for 30 sec. The expression level was calculated using the ΔΔCt method, with amplification conditions detailed in Table 2.

Table 1. The sequence Primers and molecular weight in QPCR additional expression profiling and validation TPMT gene and Housekeeping.

Primer name		Sequence (5' to 3')	Length (bp)	Annealing Temp.(ºC)	Product (bp)
TPMT	Forward	AGCCTGAAAATGTAATGGATGAAT	24	57	172
	Reverse	TCACATCATAATCTCCTCTCCAAA	24	57	172
RPL27	Forward	ATCGCCAAGAGATCAAAGATAA	22	57	123
	Reverse	TCTGAAGACATCCTTATTGACG	22	57	123

Table 2. The amplification reaction of QRT-PCR.

Steps	°C	Duration (min:Sec)	Cycle
RT Enzyme Activation	37	15:00	1
Initial Denaturation	95	05:00	50
Denaturation	95	00:20	
Annealing	58	00:20	
Extension	72	00:20	1

Data analysis and statistics

The SPSS software was utilized to conduct the statistical analysis. The data from this investigation were organized into distributions and statistical descriptions (mean, SE), as well as computerized data files and frequency distributions. The least significant difference (LSD) and analysis of variance (ANOVA) tests were applied with a probability threshold of <0.01 (p).

The approach (ΔΔCt relative Ct) was employed to quantify the level of gene expression. By measuring the ΔCt of the value of RPL32 for each sample from the target, the Ct values were calculated. The fold inductions were then determined using the formula 2^(ΔΔCt), where the threshold was ΔCt as a cycle, and ΔCt was calculated as Ct (for housekeeping gene) - Ct (for the target gene). ΔΔCt was defined as ΔCt (for treated) and − ΔCt (for control).

Software was used to evaluate the data to determine the relative gene expression in each sample. The ratios of derived expression regarding non-irradiated and irradiated

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samples were compared statistically using a single-sample t-test.

TNF was evaluated using the ELISA device from SunLong Biotech Co., LTD, specifically designed to measure TNF levels in the serum of chronic UC patients and healthy participants. Human TNF ELISA kits were employed to assay TNF levels in human serum.

Results

From the peripheral blood of all UC patients and control groups, total RNA was extracted. Through measuring their absorbance at 260 nm (A260) and 280 nm (A280) using a Nano spectrophotometer, the RNA concentration and purity were evaluated spectrophotometrically. ΔCt technique is used to measure the relative quantitative levels of TPMT gene expression in peripheral lymphocytes for UC patients. This gene showed higher expression differences between TPMT gene folding in UC patients and control groups. The TPMT gene was analyzed by RT-PCR. The current study includes 70 patients with chronic ulcerative colitis and 30 healthy individuals without chronic ulcerative colitis, the ages of the patients ranged from 15-50 years. 70 patients were subjected to Imuran treatment, 35 patients having gene expression of TPMT higher than 1 were noticed and had Imuran response while 35 patient having gene expression of TPMT of less than 1 were spotted and did no Imuran response (non-response) and the gene expression is low, and the results are shown in Table 3.

Table 3. Distribution of sample study according to therapy responding.

Treatment	Number (%)	Chi square (x^2)	P-value
Responding	35(50%)	0.0	1.0*
Non-responding	35(50%)		

*Non-significant.

Table 4. Relationship of TPMT gene expression between Imuran response therapies in patients.

Genotype TPMT	Ct (TPMT)	Ct (housekeeping)	ΔCT	ΔΔCt	Folding	T-test	P-value
Response	22.32±0.38	20.93±0.32	1.38±0.31	-2.97±0.31	8.71± 1.12	7.429	0.0001*
Non-response	25.67±0.33	19.19±0.30	6.48±0.27	2.12±0.27	0.35 ± 0.04		

*P≤0.01 highly significant.

The results of distribution of samples study according to the genetic factor group for patient detected that there is non-significant difference between patients (P=0.81).

The results of distribution of samples study according to the level of TNF in serum of study cases (patient and control) detected that there is a significant difference between patients and control groups (P*≤0.01) highly significant). The results of distribution of samples study according to the level of TNF in serum for patient group detected that there is a significant difference between patients for response and non-response (P*-value=0.0001) highly significant.

The results of the distribution of samples study according to the age group for patient and control detected that there is non-significant difference between patients and control (p-value=NS) as shown in Table 5.

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Table 5. Distribution of samples study according to the age group for patient and control.

Gender	Age (Year), Mean ± SE		T-test	P-value
	Patients	*Control*		
Male	32.66±1.56	36.29±3.65	-0.912	0.37
Female	33.03±2.0	38.38±2.18	-1.156	0.12
T-test	-0.146	-0.491		
P-value	0.88	0.62		
Mean	32.82±1.23	37.20±2.25	-1.82	0.07

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The results of the distribution of samples study according to the gender group for patient and control detected that there is non- significant difference between patients and control (P-value = 0.92 male and 0.91 female) as shown in Table 6.

Table 6. Distribution of samples study according to the gender group for patient and control.

Gender	Patients (Total=61) No (%)	Control (Total=58) No (%)	Chi square (x^2)	P-value
Male	39 (55.7%)	17 (56.6%)	0.009	0.92
Female	31 (44.3%)	13 (43.4%)	0.011	0.91
Chi square (x^2)	0.91	0.53		
P-value	0.34	0.46		

Discussion

70 patients were subjected to Imuran treatment, 35 patients having gene expression of TPMT higher than 1 were noticed and had Imuran response while 35 patient having gene expression of TPMT of less than 1 were spotted and did no Imuran response (non-response) and the gene expression is low. UC is also influenced by genetic factors. Constant antigen stimulation by the commensal enteric bacteria, fungi, and viruses that cause chronic inflammation in hosts with genetic disorders, deficiencies in the mucosal barrier' function, and immunoregulatory problems is the etiology of IBD (15). The immune responses lead to the increase and persistence of the intestinal inflammation. Over 240 genetic variations linked to IBD have been found in different studies. Those genetic variations contribute to defective bacterial handling, autophagy, and innate and adaptive immunity (16). The relationship of TPMT gene expression between Imuran response therapies in patients are high significant P*value =0.0001. The combination of oral azathioprine and intravenous infliximab has been significantly more effective when compared to infliximab or azathioprine alone in corticosteroid-free remission at week 16, demonstrating the importance of biological treatment for patients (17). At week 16, the

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combination was as efficacious to infliximab and superior to azathioprine in promoting mucosal healing (18). The results agreed with Mallick and Malik who find that the discrepancy in patient responses to AZA may be due to genetic variations in TPMT and Enzyme Nudix Hydrolase 15 (NUDT15) (19). TPMT activity is negatively correlated with the clinical response to AZA therapy (20). Increased hepatotoxicity and low response rates are brought on by high TPMT activity, which is also associated with lower 6-TGN and higher 6-MMP levels. In patients with high TPMT activity, the AZA dose could be rapidly increased to therapeutic medication levels with careful monitoring for developing hepatotoxicity. To prevent myelosuppression, the low TPMT activity requires a lower starting dose and a more progressive dose increase (21). About 10% of population has a genetic mutation in the TPMT gene, which causes decreased TPMT enzyme activity and higher 6-TGN levels, raising the risk of possibly fatal myelosuppression following AZA therapy (22).

Total RNA for gene expression of TPMT was accomplished with a purity range of 1.75 to 1.95. The first stage in RT-qPCR was the synthesis of complementary deoxyribonucleic acid cDNA, which was followed by the amplification of the target genes (23). Analysis of the double Ct was utilized for calculating the expression of the TPMT gene, with HK gene serving as the reference gene. The amplification was recorded as having a Ct value.

The results of distribution of samples study according to the genetic factor group for patient detected that there is non-significant difference between patients (P=0.81) These results are consistent with those obtained by Bramantya et al., who demonstrated the characteristics of ulcerative colitis of being a part of inflammatory bowel disease and a chronic illness characterized by diffuse inflammation in the colonic mucosa (15). Additionally, genetic factors have a role to cause ulcerative colitis. Continuous antigen stimulation by commensal enteric bacteria, fungi and viruses initiates pathogenesis of inflammatory bowel disease in turns causes chronic inflammation in host organs with a genetic disorder and defects in mucosal barrier function (15, 24).

Additionally, these results agree with those noticed by Sarlos et al. who found that the genetic factor participates up to 30% in the disease (25). In fact, is not yet well-understood the pathogenesis of inflammatory bowel disease (IBD). However, different reasons including increased and permanent intestinal inflammation, gut micro-biota, and environmental factors, are proposed to lead to IBD. Several studies have been conducted to recognize more than 240 genetic variants associated to IBD. The latter are indicated in innate and adaptive immunity, autophagy, defective bacterial handing (16).

The results of distribution of samples study according to the level of TNF in serum of study cases (patient and control) detected that there is a significant difference between patients and control groups (P value≤0.01) highly significant) These results are in accordance with Carlton et al. who discovered that the purine analog 6-TGN suppresses DNA replication by incorporating itself into nucleic acids. Eventual inhabitance of T-lymphocyte proliferation by 6-TGN is seen, resulting in immunosuppression. Additionally, AZA suppresses several genes related to intestinal inflammation and leukocyte trafficking to the gut. These include tumor necrosis factor (TNF)-related apoptosis-inducing ligand, TNF receptor superfamily member 7, and alpha-4-integrin, inactivated T-lymphocytes, or by T-cell apoptosis induction by preventing CD28-dependent Rac1 protein stimulation (26). TNF-α has a significant function in IBD pathogenesis considering that IL-1β, IL-6, and IL-33 expression can all be increased by TNF-α.

The results of distribution of samples study according to the level of TNF in serum for patient group detected that there is a significant difference between patients for response and non-response (P^*-value=0.0001) highly significant These results are in agreement with Lee et al. who found that the clinical severity

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of UC and CD were correlated with levels of TNF-α in the serum of IBD patients. TNF-α is produced by immune and non-immune cells in the gut of IBD patients and considered as the main pathogenic factor. This result is also in agreement with (27). These results agree with Sarlos et al. who realized that TNF is a pro-inflammatory cytokine, and responsible to cause epithelial barrier disruption in colonic epithelial cells. It is initiated at the IBD3 locus, and it is found at high levels in serum, stools, and inflamed bowel mucosa of patients with IBD (25).

Furthermore, increased TNF-α levels have been previously studied in patients with UC. TNF-α represents an important factor in the pathophysiology of UC, and hence efficient agent that targets TNF-α in UC. Recent studies have confirmed that biologic anti-TNF-α therapy is effective in UC. Soluble TNF-α receptors or biologic agents that prevent TNF-α production may also show therapeutic potential (7).

An association between TNF and IBD was first proven by publications demonstrated that patients with IBD have increased levels of TNF in serum, stool, or mucosal biopsy specimens (28). However, the use of TNF as a marker of IBD has been demanded upon establishing that TNF can be increased during infectious colitis. Also, TNF level may possibly be remained constant in patients with IBD. TNF content may be decreased in case of administration of certain medication such as cyclosporine A (29). Therefore, TNF-α represents main mediator in the inflammatory response (30). It is mostly secreted by monocytes, macrophages, and natural killer cells (31-33). However, the high levels of TNF-α tissue in both the mucosa and lamina propria of IBD patients renders odd pro-inflammatory response, which is normally related to the dysregulation of both mucosal immune cells and tissue damage (33, 34).

The interest in medical therapy in UC is widely growing and applying novel biological drugs has considerably caused major changes in the conventional principles of management. Infliximab is the earliest biological agent that has been applied as rescue therapy following failure of steroids, azthropurin in UC (35, 36).

The results of the distribution of samples study according to the age group for patient and control detected that there is non-significant difference between patients and control (p-value=NS). These results agree with Duricova et al. who find that the Age at onset in UC disease has been reported in two population-based studies from France and Hungary. Early onset disease is associated with more frequent disease extension, medical management differences, and a higher risk of cancer and mortality. This review aims to describe the differences in epidemiology, clinical characteristics, and natural history of pediatrics and elderly-onset inflammatory bowel disease (37).

The results of distribution of samples study according to the gender group for patient and control detected that there is non-significant difference between patients and control (P-value = 0.92 male & 0.91 female) The result agreed with Thomas Greuter et al. who find that the Gender differences in UC have been reported for epidemiology, disease presentation, course and complications, therapies, adherence, psychosocial functioning, and psychiatric co-disorders. Gastroenterologists should be aware of gender-specific issues to improve disease management and foster individualized treatment approaches (38). It was discovered that the percentage of males was higher than females, and this corresponds to Rustgi et al. (39). This result may be due to the limitations of small sample size, an optimum sample size needs to be employed to identify statistically significant differences if they exist and obtain scientifically valid results.

Thiopurines represent immunomodulatory agents that are extensively utilized in the therapeutic armamentarium and may also be utilized for treating IBD. We have discussed the pharmacology, mechanism of action, optimization techniques, effectiveness, toxicity, and cancer risk of thiopurines in this work. Family history has a very strong impact in causing chronic ulcerative colitis, as the

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percentage of patients carrying the genetic factor was greater. TNF was elevated in non-response therapy of patients with chronic ulcerative colitis than normal. TNF showed the severity of ulcerative colitis. The gene expression of the TPMT gene showed an overexpression in patient's response to Imuran therapy, it is higher than its expression in patient's nonresponse to Imuran treatment, detected that there is high significant (P*value ≤0.01 highly significant). In conclusion, all the above results have revealed a clear correlation between TPMT gene expression and therapy in the patients with UC. There is a role for the gene that needs further investigation to determine the mechanism by which it works, and it can be used as a therapeutic target to reduce the risk of UC affects.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.
Participants provided their informed consent to participate in this study, their consent was obtained to publish any data included in this study.

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