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Case Control Study

Association of miR-146 rs2910164, miR-196a rs11614913, miR-221 rs113054794 and miR-224 rs188519172 polymorphisms with anti-TNF treatment response in a Greek population with Crohn's disease

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Abstract**AIM**

To investigate the correlation between rs2910164, rs11614913, rs113054794, and rs188519172 polymorphisms and response to anti-TNF treatment in patients with Crohn's disease (CD).

METHODS

One hundred seven patients with CD based on standard

clinical, endoscopic, radiological, and pathological criteria were included in the study. They all received infliximab or adalimumab intravenously or subcutaneously at standard induction doses as per international guidelines. Clinical and biochemical response was assessed using the Harvey-Bradshaw index and CRP levels respectively. Endoscopic response was evaluated by ileocolonoscopy at week 12-20 of therapy. The changes in endoscopic appearance compared to baseline were classified into four categories, and patients were classified as responders and non-responders. Whole peripheral blood was extracted and genotyping was performed by PCR.

RESULTS

One hundred and seven patients were included in the study. Seventy two (67.3%) patients were classified as complete responders, 22 (20.5%) as partial while 13 (12.1%) were primary non-responders. No correlation was detected between response to anti-TNF agents and patients' characteristics such as gender, age and disease duration while clinical and biochemical indexes used were associated with endoscopic response. Concerning prevalence of rs2910164, rs11614913, and rs188519172 polymorphisms of miR-146, miR-196a and miR-224 respectively no statistically important difference was found between complete, partial, and non-responders to anti-TNF treatment. Actually CC genotype of rs2910164 was not detected in any patient. Regarding rs113054794 of miR-221, normal CC genotype was the only one detected in all studied patients, suggesting this polymorphism is highly rare in the studied population.

CONCLUSION

No correlation is detected between studied polymorphisms and patients' response to anti-TNF treatment. Polymorphism rs113054794 is not detected in our population.

Key words: MicroRNA; Crohn's disease; Polymorphisms; Anti-TNF; Biomarkers

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Core tip: Anti-TNF agents are the cornerstone of inflammatory bowel disease (IBD) treatment strategy so far though not effective in one third of patients in the first months of administration. Biomarkers for prediction of each patient's treatment response are vigorously sought in the era of personalized medicine. MicroRNAs have been studied as possible predictors of response to therapy in cancer and autoimmune diseases including IBD. MiRNA polymorphisms though have never been studied in IBD as markers of anti-TNF response. Our results suggest that for rs2910164, rs11614913, rs113054794, and rs188519172 no association to anti-TNF agents' response in patients with Crohn's disease can be established.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing disease of unknown etiology. It is hypothesized that it arises from a combination of genetic susceptibility and environmental factors that trigger an inappropriate mucosal inflammatory response^[1]. Anti-TNF agents have revolutionized IBD therapy since their induction in the market almost 20 years ago and are nowadays considered the cornerstone of IBD treatment strategy^[2]. Although their undisputable effectiveness, almost one third of patients will never respond in the first 3-6 mo of therapy (primary non response)^[3]. Taking into consideration their known side-effects and the cost-effectiveness of such expensive medications it becomes clear that research and identification of novel reliable biomarkers of response is of paramount importance. Moreover, with several new therapeutic drugs lying ahead of us [anti-interleukin (IL)-12/23 monoclonal antibodies, janus kinase inhibitors, agents targeting leukocyte trafficking] options will expand rendering prediction of response to a specific drug crucial for the patient. Until now, existing clinical or serologic markers have failed to accurately predict a patient's response to anti-TNF treatment^[4]. Genetic or epigenetic markers are under vigorous study in an attempt to improve our understanding of the disease and enhance the prospect of personalized medicine according to each patient's likelihood to respond to different drug classes, especially anti-TNF agents^[5,6].

MicroRNAs (miRNAs) are small, single stranded, non-coding RNA molecules comprising of 19-25 nucleotides exerting post-transcriptional gene expression regulation in response to cellular or environmental changes^[7]. MiRNAs have begun to attract scientists' attention as biomarkers of prognosis or response to treatment in various diseases in part due to some unique advantages they possess: They are practically noninvasive, stable in serum, and can be promptly and repeatedly detected from archived sera^[8].

A series of studies have focused on miRNA expression and its impact on response to anti-TNF agents in autoimmune diseases - sharing many commons with IBD - such as rheumatoid arthritis^[9,10] or psoriasis^[11,12]. MiRNA expression has been investigated in differentiation of CD from UC^[13,14] or in distinction of CD phenotypes^[15,16] while only one study in Asian population has been done so far searching an association between miRNA expression and response to infliximab in patients with CD^[17]. As far as miRNA single nucleotide polymorphisms (SNPs) in mature

or pre-miRNA are concerned no assiduous research has been done trying to unravel a possible association between autoimmune diseases and anti-TNF response. That is in contrast with cancer, where a wealth of studies exists upon miRNA variants and response to treatment^[18-22], with promising results that haven't though found their role yet in clinical every day practice. Regarding autoimmune diseases and SNPs predicting treatment efficacy, only one recently published study seeks to correlate miR-146a expression and rs2910164 polymorphism to rheumatoid arthritis development and clinical outcome after anti-TNF therapy^[23].

It is well known that miR-146a is implicated in regulation of immune responses through NF- κ B pathway and has been extensively studied in autoimmune diseases pathogenesis^[23-27] including IBD^[28,29]. Concerning miR-196, apart from having been extensively studied in cancer^[18-20], it has also been reported to negatively regulate IGRM, a gene associated with autophagy, thus facilitating epithelial inflammation in CD^[30] while its variant rs11614913 has been recently related as possibly contributing to IBD-related colorectal cancer development^[31]. Last, miR-221 and miR-224 have been detected to be up-regulated after anti-TNF treatment in patients with CD in Fujioka *et al.*^[17] work while both have been shown to interfere in IBD related pathways; miR-221 as a down regulator of *ICAM1* gene the protein of which has been widely studied in IBD pathogenesis^[32-34] and miR-224 inducing cell proliferation in ovarian murine cells through SMAD/TGF- β pathway^[35]. Assuming that SNPs in the aforementioned miRNAs would exert an alteration in their functional capacity, we chose to examine whether rs2910164 of miR-146a, rs11614913 of miR-196a, rs113054794 of miR-221, and rs188519172 of miR-224 can predict response to anti-TNF treatment in a cohort of Greek patients with CD.

MATERIALS AND METHODS

Patients

One hundred and seven patients diagnosed with CD attending the IBD Clinic at Aretaieio Hospital, Athens, Greece were enrolled in the study. The diagnosis of CD was based on standard clinical, endoscopic, radiological, and pathological criteria^[36]. Patients, who were due to receive anti-TNF therapy-infliximab (IFX) or adalimumab (ADA) - and were naïve to these or any other anti-TNF agent, were eligible for the study. Patients could receive in parallel other disease related drugs as long as there was no dose change 8 wk before enrollment. Patients with the following characteristics were excluded from the study: < 18 or > 80 years old, IBD-unclassified, and malignancy.

IFX was administered intravenously at a dose of 5 mg/kg at weeks 0, 2, 6 and every 8 wk thereafter. ADA was administered subcutaneously at a dose of 160 mg at week 0, 80 mg at week 2 and 40 mg every 2 wk thereafter. Clinical and serological response was assessed with Harvey-Bradshaw Index (HBI) and CRP, respectively at various time points: At baseline

(before 1st infusion or injection), the day before each subsequent drug administration and at week 12 of treatment. Ileocolonoscopy was performed at baseline and after 12-20 wk of therapy to assess mucosal healing. Changes of endoscopic image compared to baseline were classified in four categories and patients were classified as responders or not to anti-TNF therapy as previously described^[37].

Genotyping

Genomic DNA from whole peripheral blood containing EDTA was extracted using validated techniques (NucleoSpin Blood kit; Macherey-Nagel, Germany). PCR-RFLP was used to determine the rs2910164 and rs11614913 was performed using T-ARMS-PCR assay as described previously genotypes as previously described^[38,39]. Regarding the rs113054794 we used PCR-RFLP method. Forward primer: 5'CAGAAACATTATAGGGGTAGCA3' and reverse: 5'GGTAGTAGGTAAGTCCCAGCA3'. Annealing was done at 62 °C. PCR products were digested with MvaI. For rs188519172 polymorphism we used allele-specific PCR. Two different PCR reactions are performed with one or the other allele specific primer. The primers used were a common forward 5'CCTCAAGAATCCTCCTCACT3', and a reverse for the G-allele: 5'GTGGTTCGTTTAGTAGATGAC3' and for the A-allele: 5'GTGGTTCGTTTAGTAGATGAT 3'.

Statistical analysis

Genotype frequencies were compared with the χ^2 test with Yate's correction using S-Plus (v.6.2 Insightful, Seattle, WA, United States). Odds ratios (OR) and 95%CI were obtained with GraphPad (v.300, GraphPad Software, San Diego, CA, United States). The *P* values are all two-sided. *P* values of < 0.05 were considered to be significant.

RESULTS

Patients' demographic and clinical characteristics are summarized in Table 1. From the 107 patients included in the study, 104 (97.19%) received infliximab while the rest received adalimumab. Seventy two (67.29%) were classified as complete responders while 22 (21.57%) as partial responders to anti-TNF induction treatment. Thirteen patients (14.74%) did not respond and were considered non-responders. No correlation was detected between complete or partial responders and non-responders to anti-TNF therapy as far as patients' characteristics, like age, gender or behavior, are concerned. Clinical and serological indexes used - HBI and CRP-were consistent with endoscopically assessed response.

The prevalence of rs2910164, rs11614913, and rs188519172 in patients with CD who responded fully, partially and those who didn't respond to anti-TNF treatment are depicted in Table 2.

Regarding the first polymorphism studied, rs2910164 C allele was not found to be significantly different between complete, partial, and non- responders (*P* =

Table 1 Patient demographic and clinical characteristic according to response to anti-TNF treatment

Characteristics	Responders	Partial responders	Primarily non-responders
n (%)	72 (67.29)	22 (21.57)	13 (14.74)
Age (yr, mean \pm SD)	34.10 \pm 11.63	32.23 \pm 13.31	39.09 \pm 15.60
Sex (%)			
Male	30 (41.67)	14 (63.64)	10 (76.92)
Female	42 (62.69)	8 (36.36)	3 (23.08)
C-reactive protein (mg/dL, mean \pm SD)			
Baseline	3.10 \pm 2.03	4.13 \pm 2.31	6.86 \pm 2.89
After treatment	0.88 \pm 1.84	2.21 \pm 2.69	3.96 \pm 2.81
δ C-reactive protein (%)	80.44 \pm 22.42	71.94 \pm 45.74	58.04 \pm 22.63
Duration of disease (yr)	6.38 \pm 5.91	5.71 \pm 3.77	4.00 \pm 3.13
Infliximab dose (mg/kg)	5	5	5
Location			
L2	24 (33.34)	3 (13.64)	2 (15.38)
L3	45 (62.50)	19 (86.36)	11 (84.62)
L4	3 (4.48)	0	0
Behavior			
B1	31 (43.06)	7 (31.82)	5 (38.46)
B2	13 (18.06)	6 (27.27)	2 (15.38)
B3	28 (38.89)	9 (40.91)	6 (46.15)

0.55, OR = 1.67; 95%CI: 0.14-19.32 and $P = 0.39$, OR = 2.92; 95%CI: 0.25-34.76 respectively) while CC genotype was not found in any of the patients.

Concerning rs11614913, again neither T allele nor TT genotype was found to be statistically associated with response to anti-TNF. Specifically, T allele was not found to be different between complete, partial, and non- responders ($P = 0.11$, OR = 2.7; 95%CI: 0.86-8.39 and $P = 1$, OR = 1.03; 95%CI: 0.27-3.91 respectively); similarly for TT genotype ($P = 0.18$, OR = 3.78; 95%CI: 0.8-17.73 and $P = 0.34$, OR = 2.83; 95%CI: 0.54-4.69 respectively).

No significant difference was found for rs188519172 as well, between complete, partial, and non- responders. G allele was not statistically different between these groups ($P = 0.44$, OR = 1.56, 95%CI: 0.56-4.36; and $P = 0.75$, OR = 0.73, 95%CI: 0.19-2.78 respectively) with GG genotype not being statistically different either ($P = 0.61$, OR = 1.78, 95%CI: 0.28-11.33; and $P = 0.58$, OR = 2.86, 95%CI: 0.35-15.05). The rs113054794 SNP of miR-221 was not detected at all in our population.

DISCUSSION

Recent studies highlight the emerging role of circulating microRNAs as potential biomarkers in the pathogenesis or response to treatment of cancer and autoimmune diseases^[9-22]. In the era when personalized medicine becomes the ultimate goal, vigorous research is carried out towards identification of biomarkers able to predict the exact outcome a therapy may have to a specific patient, according to his unique genetic fingerprints. In IBD, until today, no marker has achieved to fully foresee how patients will respond to anti-TNF treatment, the most popular therapy, which though will be ineffective in one out of three patients during the first months of drug administration^[3].

Recently, a study from Japan investigated serum

miRNA expression in CD patients receiving induction therapy with infliximab. They concluded that, among others, miR-221 and miR-224 increased during induction therapy with infliximab in patients considered as responders^[17]. Castro-Villegas *et al.*^[9] studied serum miRNA levels as possible biomarkers of response to 6-month anti-TNF α therapy in patients with rheumatoid arthritis and concluded that, among others, miR-146a increased after anti-TNF therapy in patients who responded. In addition, Bogunia-Kubik *et al.*^[23] have also recently assessed miR-146 expression along with its rs2910164 polymorphism and their possible connection to rheumatoid arthritis pathogenesis and therapeutic outcome after 3 mo of anti-TNF administration. Their results showed initially reduced miR-146 levels in patients compared to controls and restoration of these levels in patients receiving a 3 mo course of anti-TNF. Moreover they concluded that although rs2910164 variant could be associated with miR-146 levels after treatment, overall this genetic variant didn't influence neither predisposition to the disease nor efficacy of anti-TNF therapy, in accordance to our results.

This is the first to our knowledge study to examine the association of polymorphisms in either pre- or mature miRNAs with response to induction therapy with anti-TNF agents in patients with CD.

Our results showed that of the SNPs genotyped, rs2910164 of miR-146, rs11614913 of miR-196a, and rs188519172 of miR-224 had no statistically significant association to anti-TNF treatment response in Greek patients with CD. Moreover, rs113054794 SNP of miR-221 was not detected in our population, indicating that this polymorphism is probably highly rare in Caucasian populations. In accordance to our results, Nguyen-Dien *et al.*^[40] have also demonstrated this SNP's absence in a Caucasian population studied for correlation of miRNA variants and risk of breast cancer.

Response to anti-TNF therapy was assessed by

Table 2 Genotype and allele frequencies of rs2910164, rs11614913, and rs188519172 polymorphisms in Crohn's disease patients according to response to anti-TNF treatment

Genotype	Complete responders (n = 72)	Partial responders (n = 22)	P value; OR (95%CI)	Non-responders (n = 13)	P value; OR (95%CI)
miR-146a, rs2910164					
GG	70 (97.22)	21 (95.45)	1.0 (reference)	12 (92.31)	1.0 (reference)
GC	2 (2.78)	1 (4.54)	0.55; 1.67 (0.14-19.32)	1 (7.69)	0.39; 2.92 (0.25-34.76)
CC	0	0	-	0	-
miR-196a, rs11614913					
CC	33 (45.83)	5 (22.73)	1.0 (reference)	5 (38.46)	1.0 (reference)
CT	32 (44.45)	13 (59.09)	0.11; 2.7 (0.86-8.39)	5 (38.46)	1.0; 1.03 (0.27-3.91)
TT	7 (9.73)	4 (18.18)	0.18; 3.78 (0.8-17.73)	3 (23.08)	0.34; 2.83 (0.54-14.69)
miR-224, rs188519172					
AA	38 (52.78)	9 (40.9)	1.0 (reference)	7 (53.84)	1.0 (reference)
AG	29 (40.28)	11 (50)	0.44; 1.60 (0.59-4.37)	4 (30.76)	0.75; 0.75 (0.20-2.80)
GG	5 (6.94)	2 (9.09)	0.62; 1.69 (0.28-10.16)	2 (15.38)	0.59; 2.17 (0.35-13.51)

clinical, serological and endoscopic markers. Endoscopy at the end of the study was performed to measure primary response to therapy with the most objective marker of treatment efficacy, which is mucosal healing^[3].

MiR-146a has been demonstrated to be an integral part of the immunological responses observed in many autoimmune diseases through NF- κ B pathway. Specifically, it participates in a negative feedback system induced by microbial constituents like LPS or other pro-inflammatory elements resulting in inhibition of protein production by specific genes. These genes were shown to be interleukin-1 receptor-associated kinase (IRAK) 1 and tumor necrosis factors receptor associated factor (TRAF) 6^[24]. Moreover, miR-146a was shown to be overexpressed - upon nitric oxide(NO) trigger - in the nucleotide-binding oligomerization domain (NOD2) signaling pathway thus facilitating further activation of various inflammatory genes like IL-12, TNF- α , IL-6^[28]. Both abovementioned mechanisms have been implicated in IBD pathogenesis^[41,42] with NF- κ B pathway actually being one of the targets of anti-inflammatory effects exerted by steroids and anti-TNF agents^[41,43].

MiR-196a has been demonstrated to be related to IBD pathogenesis^[29] and IBD phenotype^[44] with the target molecular pattern through which it exerts its effect probably being related to autophagy. Specifically it has been reported to negatively regulate Immunity Related GTPase M (IGRM)^[30], a gene that has been associated to IBD susceptibility^[45]. In addition, its rs11614913 SNP has only recently been implicated in IBD related colorectal cancer progression^[31] while its association to other forms of cancer, mainly colorectal, has already been established^[46].

MiR-221 has been shown to mediate down regulation of ICAM-1 translation in human cholangiocytes with ICAM-1 playing a major role in regulation of a balanced inflammatory response in biliary cells. MiR-221 related ICAM-1 expression has also been implicated in T cells adhesion during local inflammation^[33]. Furthermore, ICAM expression in human umbilical vein endothelial cells was demonstrated to be regulated by miR-221

in response to HIV again influencing monocytes adherence^[47]. Zhao *et al.*^[48] have connected miR-221 to TLR4 mediated production of pro-inflammatory cytokines in lung cells with simultaneous increased TNF α and IL-6 expression through NF- κ B signaling, a key pathway in IBD pathogenesis.

Apart from the aforementioned cell lines, miR-221 has also been studied in colonic epithelial cells with similar results. Fang *et al.*^[49] have shown that down regulation of miR-221 leads to amplification of experimental colitis and increase of TNF α in histological specimens. All the above highlight a plausible role of miR-221 in inflammatory response either through ICAM regulation, a molecule suggested to interfere with IBD inflammation facilitation^[34,50,51], either through still unraveled mechanisms.

Last, miR-224 expression has been shown to be up-regulated in hepatocellular cancer patients with its possible target being apoptosis inhibitor-5 (API-5), thus mediating its role by inducing apoptosis^[52]. Over expression of miR-224 has also been displayed in T cells of systematic lupus erythematosus patients again through suppression of API-5 leading to T cell apoptosis^[53]. Interestingly, one report has presumed API5 involvement in IBD inflammation and progression to neoplasia, as quick epithelial cell turnover, cell proliferation and finally apoptosis are present in both these situations^[54]. Moreover, Olaru *et al.*^[55] have proved involvement of miR-224 in down-regulation of p21, a tumor suppressor gene through which miR-224 coordinates neoplasia initiation and progression through dysplasia to IBD related colorectal cancer. MiR-224 is implicated in inflammatory pathways, also linked to IBD pathogenesis. Scisciani *et al.*^[56] reported p65/NF- κ B to be a target pathway of miR-224 in liver cells while TNF α inflammatory pathway is activated with up-regulation of miR-224. Two other reports have demonstrated that SMAD4 is the target of miR-224 with SMAD4 being a pivotal component of TGF- β pathway leading to cell proliferation^[35,57]. TGF- β pathway dysregulation has long been shown to be a contributor to IBD pathogenesis^[58].

Hypothesizing that miRNAs regulating IBD susceptibility genes would be a logical initial thought, we chose to study SNPs of such miRNAs, such as miR-196a, and their association to anti-TNF response in patients with CD. Among many, we presumed that SNPs of miR-146a, miR-221, and miR-224 would additionally be ideal based on previous work showing alteration of their expression after anti-TNF treatment in patients with CD and rheumatoid arthritis^[9,17,23]. However, even though anti-TNF treatment interferes in pathophysiologic pathways regulated by those miRNAs, no correlation was found. This is in agreement to what Lee *et al.*^[59] have concluded in a very recent genome-wide association study in CD. Their data support the idea of different genetic loci contributing in susceptibility compared to prognosis - and thus potential therapeutic interventions - in adult patients with CD. Interestingly, this had been already shown in pediatric patients with CD being treated with anti-TNF agents^[60]. Furthermore, absence of studied SNPs' association to treatment response may denote that other SNPs or other transcriptional (ex, methylation of gene promoters) and post-transcriptional mechanisms (concerning miRNA stability or processing) interfere with alterations in miRNA expression. In addition, in the only study conducted in IBD patients assessing miRNA expression alterations and anti-TNF response, the population studied was of a different ethnic group - Japanese-compared with ours. Ethnic and geographic differences representing distinct genetic and environmental background may influence frequency or even variety of polymorphisms detected.

We chose to include in our study patients receiving both infliximab or adalimumab. It has been established that their clinical and endoscopic efficacy is similar in Crohn's disease^[61,62]. Nevertheless their different structure could have interfered with our results. Notwithstanding, with only 3 patients receiving adalimumab, we believe that our findings have only minimally been influenced.

Lastly, another factor contributing in our inability to show a positive association between studied SNPs and anti-TNF response may be that due to the small effect these variants may pose, we would require a larger sample to identify a statistically significant result. IBD genetic background has not been fully elucidated but we know that a variety of risk factors contribute with a small or modest effect and not one highly penetrant, suggesting a difficulty of uncovering this effect.

Nevertheless, we believe that miRNAs constitute a small but rather attractive pawn in our effort to delineate epigenetic regulation of gene expression and its contribution to IBD susceptibility, prognosis and therapeutic possibilities. Genetic markers may need to be used as biomarkers of therapy response in combination to other clinical or serological ones to attain the maximum benefit and accurately distinguish the ideal patient for each therapeutic treatment.

In conclusion, our results demonstrate for the first time that mir-146 rs2910164, miR-196a rs11614913, miR 221 rs113054794 and miR-224 rs188519172 are not

correlated with anti-TNF treatment response in Caucasian patients with CD. Hence, they cannot be used as biomarkers to predict anti-TNF drug response in candidate patients with CD. Further independent studies are required to validate our findings in a larger scale or possibly to a different ethnic population.

COMMENTS

Background

Crohn's disease (CD) is a chronic debilitating disease related to poor quality of life, increased risk of surgery and prolonged hospital admissions. Anti-TNF drugs have revolutionized therapy by reducing but not eliminating complications. Biomarkers of anti-TNF therapy response constitute essential tools for physicians considering that one third of patients will not benefit by induction therapy as well as drugs' cost and side effects.

Research frontiers

Epigenetic alterations such as those exerted by microRNAs are now studied as contributors to pathogenesis and prognosis of many diseases including inflammatory bowel disease (IBD). MiRNA polymorphism associated response to therapy has been extensively studied in cancer with promising results though our knowledge about a possible association to anti-TNF treatment response in IBD is still limited.

Innovations and breakthroughs

This is the first study trying to unravel a correlation between microRNA polymorphisms and response to anti-TNF medication in Greek patients with CD. Expression of those miRNAs has been shown to correlate to anti-TNF response in rheumatoid arthritis and in IBD in Chinese population.

Applications

Studied polymorphisms of relevant miRNAs can be used as predictive markers for anti-TNF therapy response in patients with CD thus contributing to identification of ideal drug candidates for a costly treatment with potentially serious side effects.

Terminology

MicroRNAs are small non-coding RNA sequences of a few nucleotides exerting epigenetic regulation in gene expression.

Peer-review

The result of this study demonstrates for the first time that mir-146 rs2910164, miR-196a rs11614913, miR 221 rs113054794 and miR-224 rs188519172 are not correlated with anti-TNF treatment response in Caucasian patients with CD. Hence, these markers can be used as biomarkers to predict anti-TNF drug response in candidate patients with CD.

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