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Prospective Study

Genetic polymorphisms predict response to anti-tumor necrosis factor treatment in Crohn's disease

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Author contributions: Netz U, Eichenberger MR and Galandiuk S designed the research; Netz U, Carter JV, Eichenberger MR, Dryden GW and Galandiuk S collected the data; Netz U, Carter JV and Eichenberger MR performed the genetic labwork; Netz U, Carter JV, Eichenberger MR, Pan J, Rai SN and Galandiuk S analyzed and interpreted the data; all authors drafted the manuscript and approved the final version.

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Abstract**AIM**

To investigate genetic factors that might help define which Crohn's disease (CD) patients are likely to benefit from anti-tumor necrosis factor (TNF) therapy.

METHODS

This was a prospective cohort study. Patients were

recruited from a university digestive disease practice database. We included CD patients who received anti-TNF therapy, had available medical records (with information on treatment duration and efficacy) and who consented to participation. Patients with allergic reactions were excluded. Patients were grouped as ever-responders or non-responders. Genomic DNA was extracted from peripheral blood, and 7 single nucleotide polymorphisms (SNPs) were assessed. The main outcome measure (following exposure to the drug) was response to therapy. The patient genotypes were assessed as the predictors of outcome. Possible confounders and effect modifiers included age, gender, race, and socioeconomic status disease, as well as disease characteristics (such as Montreal criteria).

RESULTS

121 patients were included. Twenty-one were non-responders, and 100 were ever-responders. Fas ligand SNP (rs763110) genotype frequencies, TNF gene -308 SNP (rs1800629) genotype frequencies, and their combination, were significantly different between groups on multivariable analysis controlling for Montreal disease behavior and perianal disease. The odds of a patient with a Fas ligand CC genotype being a non-responder were four-fold higher as compared to a TC or TT genotype ($P = 0.009$, OR = 4.30, 95%CI: 1.45-12.80). The presence of the A (minor) TNF gene -308 allele correlated with three-fold higher odds of being a non-responder ($P = 0.049$, OR = 2.88, 95%CI: 1.01-8.22). Patients with the combination of the Fas ligand CC genotype and the TNF -308 A allele had nearly five-fold higher odds of being a non-responder ($P = 0.015$, OR = 4.76, 95%CI: 1.35-16.77). No difference was seen for the remaining SNPs.

CONCLUSION

The Fas-ligand SNP and TNF gene -308 SNP are associated with anti-TNF treatment response in CD and may help select patients likely to benefit from therapy.

Key words: Anti-tumor necrosis factor; Fas ligand; Antibody; Response; Crohn's disease; Single nucleotide polymorphisms; Genotype; Tumor necrosis factor gene

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Core tip: Predicting the subset of patients who do not respond to anti-tumor necrosis factor (TNF) treatment is important clinically and economically. Patients with Crohn's disease who received anti-TNF therapy were grouped as ever-responders or non-responders. Genomic DNA was extracted from peripheral blood, and 7 single nucleotide polymorphisms (SNPs) were assessed. 121 patients were included. Twenty-one were non-responders, and 100 were ever-responders. Fas ligand SNP (rs763110) genotype frequencies, TNF gene -308 SNP (rs1800629) genotype frequencies, and their combination, were significantly different between groups on multivariable analysis and may help select

patients likely to benefit from anti-TNF therapy.

Netz U, Carter JV, Eichenberger MR, Dryden GW, Pan J, Rai SN, Galandiuk S. Genetic polymorphisms predict response to anti-tumor necrosis factor treatment in Crohn's disease. *World J Gastroenterol* 2017; 23(27): 4958-4967 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i27/4958.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i27.4958>

INTRODUCTION

Crohn's disease (CD) is a transmural chronic inflammatory disease that can affect any part of the alimentary tract, but which often involves the distal ileum.

Anti-tumor necrosis factor- α (anti-TNF- α) monoclonal antibodies are generally used for inducing and maintaining remission and can be used alone or in combination with other drugs^[1]. The most common drugs in this group, for CD, are infliximab (chimeric murine - human IgG1 monoclonal antibody targeting TNF- α), adalimumab (fully humanized IgG1 anti-TNF- α monoclonal antibody), and certolizumab pegol (a humanized monoclonal Fab' fragment with a high binding affinity for TNF- α)^[2,3].

Although the majority of patients benefit from anti-TNF treatment, approximately one-third of patients treated with an induction dose of anti-TNF do not improve clinically, termed primary non-response^[3]. An additional significant population who initially respond to treatment eventually lose responsiveness, termed a secondary non-response.

Identifying patients who will fail treatment with anti-TNF agents is of significant importance both from a clinical and economic perspective. Anti-TNF drugs have been associated with an increased risk of opportunistic infections, melanoma, and lymphoma^[4-6]. Anti-TNF treatment is also very expensive, with 2013 annual per patient costs for adalimumab and infliximab at approximately \$25000 and \$24000 respectively^[7,8].

Factors associated with the success of anti-TNF treatment include shorter disease duration, inflammatory (as opposed to fibrostenotic) disease phenotype, isolated colonic disease, young age, non-smoking status, as well as a serum high C-reactive protein that returns to normal after initiation of treatment^[9-11]. Non-response can be due to multiple factors such as an alternative non-TNF mediated pathway of inflammation, due to a differential role of TNF in certain stages of disease and/or due to the presence or development of anti-drug antibodies. Additionally, individual differences in drug bioavailability and pharmacokinetics can be factors associated with non-response^[9].

A possible conduit to predict response to anti-TNF therapy could be through genetic testing. Several genes have been implicated in the pathogenesis of

CD, including NOD2 and ATG16L1^[12,13]. There is, however, limited data on the ability to predict anti-TNF treatment response in CD based upon genetic data. Some genes have been investigated without success^[14]. Our aim was to investigate genetic factors that might help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment. We hypothesized that specific single nucleotide polymorphism (SNP) genotypes are associated with anti-TNF treatment response in patients with CD. We chose to examine a series of SNPs within genes that have been linked either with CD and/or with anti-TNF treatment response in order to determine whether these could aid in predicting response to anti-TNF treatment in CD patients.

MATERIALS AND METHODS

This study complies with the STROBE guidelines and the extension for genetic association studies^[15].

Patient recruitment and data collection

This is a prospective cohort study approved by the University of Louisville Institutional Review Board. All patients signed a written informed consent. Consecutive patients with a diagnosis of CD were identified from a large prospectively maintained genetic database, from a large University digestive disease practice, encompassing the period 1/1998 to 4/2016. Inclusion criteria were CD patients who had received anti-TNF therapy, and whose medical records were available, with information about receipt of anti-TNF therapy, its duration, efficacy, and cessation where applicable. Included patients received appropriate drug doses and had a follow-up of at least 12 mo following treatment initiation^[16,17]. Patients were excluded if anti-TNF treatment was stopped due to side-effects, local and/or systemic allergy, or if it was impossible to distinguish from the medical records whether the drug worked.

Additional data collected from the medical records included gender, race, socioeconomic status (patient's zip code of residence was used to obtain median household income based on United States census data from the American Community Survey 2014 - 5 year estimates)^[18], surgical history, and clinical state of the disease according to the Montreal classification for CD, including age at diagnosis, location, disease behavior, and the presence or absence of perianal disease^[19].

The main outcome measure (following exposure to the drug) was response to therapy. Participants were grouped as ever-responders if they had initial response to anti-TNF treatment (even if this was later lost due to antibody formation) or non-responders in accordance with the treating physician decision. The patient genotypes (see below) were assessed as the predictors of outcome. Possible confounders and effect modifiers included age, gender, race, and socioeconomic status disease, as well as disease characteristics (such as Montreal criteria).

DNA extraction

Peripheral blood was collected by venipuncture (after written informed consent) in EDTA-vacutainers (BD, Franklin Lakes, NJ, United States) and stored at 4 °C until further use.

Genomic DNA was extracted from blood samples using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Pittsburgh, PA, United States) using the manufacturer's protocol^[20]. Briefly, the blood was initially diluted with PBS buffer. Blood was then lysed: 1 µL of diluted blood was lysed with 1 µL of cell lysis solution (400 mmol/L KOH, 10 mmol/L EDTA, 100 mmol/L DTT), followed by the addition of 1 µL of neutralization buffer (400 mmol/L HCl, 600 mmol/L Tris-HCl, pH 7.5). Whole genome amplification was then performed: 17 µL of master mix [7 µL sample buffer, 9 µL reaction buffer, and 1 µL enzyme mix from the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Pittsburgh, PA, United States)] was added to each sample for a total reaction volume of 20 µL. Amplification was performed according to the following program: 30 °C for two hours, followed by 65 °C for 10 min, then cooled to 4 °C. Following whole genome amplification, DNA concentration was determined using NanoDrop® 2000 spectrophotometry. The samples were diluted and stored at -20 °C until analysis.

SNP genotyping

SNPs selection: A PubMed literature search was conducted using the keywords "tumor necrosis factor-alpha", "anti-TNF", "infliximab", "adalimumab", "polymorphism", "Crohn's disease", "response", "biomarker" using Boolean operators (AND), (OR), (NOT). Results were narrowed down to original studies investigating SNPs including frequency of alleles and genotypes for different groups. We included SNPs that had demonstrated association with CD or anti-TNF treatment, those that had biological relevance, and those that had an expected minor allele frequency \geq 5%. Both new genetic associations and previously described efforts were investigated. SNPs were excluded if they had been extensively investigated and if there was no prognostic value for the combination of CD and anti-TNF treatment response. As a result of this search, the following seven SNPs within 5 genes were selected for study and assessed in each patient's DNA sample: ATG16L1 (rs10210302, T300A rs2241880), Fas ligand (-843 rs763110), IBD5 (rs2522057), FCGR 3A (rs396991), and TNF (-308 rs1800629, -238 rs361525).

SNP assessment was performed using TaqMan® predesigned genotyping assays (Life Technologies®, Carlsbad CA)^[21]. The TaqMan® genotyping assays were diluted to a 20× working stock solution with 1× TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0, in DNase-free, sterile-filtered water) and stored at -20 °C, as recommended by the manufacturer.

MicroAmpR Fast Optical 96-well reaction plates (Applied Biosystems, Foster City, CA, United States)

Table 1 Hardy weinberg equilibrium n (%)

SNP	Genotype distribution		Hardy Weinberg equilibrium	
	Genotype	Frequency	Expected frequency (n)	P value ¹
ATG16L1 rs10210302	CC	25 (21.0)	24	NS
	TC	57 (47.9)	59	
	TT	37 (31.1)	36	
ATG16L1 rs2241880	AA	27 (22.5)	26	NS
	GA	57 (47.5)	60	
	GG	36 (30.0)	35	
Fas Ligand rs763110	CC	42 (36.5)	41	NS
	TC	54 (47.0)	55	
	TT	19 (16.5)	18	
IBD5 rs2522057	CC	35 (29.2)	29	0.045
	GC	49 (40.8)	60	
	GG	36 (30.0)	31	
FCGR 3A rs396991	AA	70 (58.3)	62	0.0005
	AC	33 (27.5)	48	
	CC	17 (14.2)	9	
TNF gene (-308) rs1800629	AA	4 (3.4)	3	NS
	GA	31 (26.1)	33	
	GG	84 (70.6)	83	
TNF gene (-238) rs361525	AA	2 (1.7)	1	NS
	GA	15 (12.5)	17	
	GG	103 (85.8)	102	

¹Calculated using χ^2 . TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

were used. Six microliters of master mix was used for each assay (5.5 μ L of TaqMan[®] Universal Master Mix II, no UNG [Applied Biosystems[™]] together with 0.5 μ L of 20 \times working assay [TaqMan[®] predesigned Genotyping Assays]). Five microliters of DNA (4.5 ng/ μ L) was added to the plate. PCR reactions were performed using a Step-One Plus[®] RT-PCR System (Life Technologies[®], Carlsbad, CA, United States) and the following program: 95 $^{\circ}$ C for 10 min, followed by 40 cycles of 95 $^{\circ}$ C for 15 s, and then 60 $^{\circ}$ C for 1 min. Analysis was performed using Step-One Plus[®] software v2.1 (applied Biosystems, Foster City, CA, United States). Each genotype was independently assigned by two investigators. In cases of disagreement, assignment was reached by consensus. All laboratory work and genotyping was done at the Price Institute of Surgical Research, Louisville Kentucky, United States.

Statistical analysis

Descriptive and analytical statistics were performed using SAS version 9.4 statistical software^[22]. Genotype frequencies, demographic, and disease characteristics were compared using a χ^2 test (or Fisher’s exact test for 2 \times 2 tables). Socioeconomic status was calculated according to the national percentile of the patient’s median household income divided into quartiles (0-25, 26-50, 51-75 and 76-100) and compared using a χ^2 test. Comparison of continuous variables was performed using a two-sample *t*-test or ANOVA. In order to explore for the presence of bias in the cohort, a group of contemporary subjects who did not receive anti-TNF treatment were compared with patients

included in this study. Following this, characteristics between ever-responders and non-responders in the study group were then compared^[23].

Hardy-Weinberg equilibrium was determined for each SNP (Table 1). Univariable logistic regression was modeled for the probability of anti-TNF treatment failure for each covariate. Multivariable logistic regression models were used for separate SNPs and covariates exhibiting a trend towards a significant difference ($P < 0.15$)^[24]. Final models included OR and 95%CI. A *P*-value of < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Pan J and Rai SN.

RESULTS

Patient demographics

Table 1 shows a flow diagram of patient selection; 121 patients were selected for study. Of these, 21 (17.4%) patients were primary non-responders to anti-TNF treatment and 100 (82.6%) patients were ever-responders to anti-TNF treatment. A quarter of these initial ever-responders (25/100) lost response at a later time and were termed secondary non-responders. The patient population was predominantly Caucasian (92.6%), with a higher proportion of women (58.7%) (Table 2). With regards to clinical parameters, 90/121 (74.4%) patients were diagnosed between the ages of 17 and 40 years of age (Montreal A2). Most CD patients, 74/121 (61.2%), had combined ileocolonic disease (Montreal L3), whereas 15/121 (12.4%) had isolated ileal disease (Montreal L1), and 32/121 (26.4%) had only colonic disease (Montreal L2). Only 3/121 (2.5%) patients had upper GI (Montreal L4) involvement, all of whom were responders. Montreal L4 disease was analyzed separately from L1-3, due to the fact that, according to the Montreal classification, it is not mutually exclusive and can be added to any of the other locations when concomitant upper GI disease is present^[19]. The population was fairly evenly distributed with respect to disease behavior with 36/121 (30%) patients having non-stricturing, non-penetrating disease (Montreal B1), 45/121 (37%) patients having stricturing disease (Montreal B2), and 40/121 (33%) patients having penetrating disease (Montreal B3). In addition, 37 of 121 (31%) patients had perianal disease (Montreal P designation). Table 2 shows the clinical and demographic data of the participants, as well as these data for the non-responder and ever-responder groups. None of the clinical or demographic characteristics were significantly different between these 2 groups.

Presence of bias

When comparing the characteristics of the patients who received anti-TNF treatment and included in the study ($n = 121$) with those who did not receive anti-TNF treatment ($n = 152$) in order to ascertain the

Table 2 Clinical and demographic patient characteristics *n* (%)

Variables	Total	Anti-TNF treatment		P value
		Non-responders	Ever responders	
Total	121 (100)	21 (17)	100 (83)	NA
Patient demographics				
Gender				
Female	71 (59)	15 (71)	56 (56)	NS
Male	50 (41)	6 (29)	44 (44)	
Race				
Caucasian	112 (93)	21 (100)	91 (91)	NS
African American	9 (7)	0 (0.0)	9 (9)	
Socioeconomic status ¹				
1 st Quartile	24 (20)	4 (19)	20 (20)	NS
2 nd Quartile	40 (33)	6 (29)	34 (34)	
3 rd Quartile	29 (24)	3 (14)	26 (26)	
4 th Quartile	28 (23)	8 (38)	20 (20)	
Montreal classification				
Age of onset (A)				
A1 - below 16 years old	14 (12)	1 (5)	13 (13)	NS
A2 - between 17 and 40 years old	90 (74)	17 (81)	73 (73)	
A3 - above 40 years old	17 (14)	3 (14)	14 (14)	
Location (L)				
L1 - ileal	15 (12)	1 (5)	14 (14)	NS
L2 - colonic	32 (26)	5 (24)	27 (27)	
L3 - ileocolonic	74 (61)	15 (71)	59 (59)	
Location (L4) upper				
No upper GI disease	118 (98)	21 (100)	97 (97)	NS
L4 - upper GI disease	3 (2)	0 (0)	3 (3)	
Behavior (B)				
B1 - non-stricturing, non-penetrating	36 (30)	4 (19)	32 (32)	0.1
B2 - stricturing	45 (37)	12 (57)	33 (33)	
B3 - penetrating	40 (33)	5 (24)	35 (35)	
Behavior (p) perianal disease				
No perianal disease	84 (69)	18 (86)	66 (66)	0.08
p - perianal disease present	37 (31)	3 (14)	34 (34)	
Anti-TNF treatment type				
Drugs received				
Infliximab	46 (38)	10 (48)	36 (36)	NS
Adalimumab	45 (38)	5 (24)	40 (40)	
Infliximab and Adalimumab ²	29 (24)	6 (29)	23 (23)	
Certolizumab pegol	5	0	5	

¹Calculated according to the national percentile of median household income; ²Received sequentially. TNF: Tumor necrosis factor.

presence of bias, no difference was found in 3 of the 4 variables examined: gender ($P = 0.27$), race ($P = 0.95$), or socioeconomic status ($P = 0.23$). The patients included in the study who received anti-TNF treatment were, however, younger (41.6 years old, 95%CI: 39.2-44.0) than those that did not receive anti-TNF treatment (49.4 years old, 95%CI: 47.1-51.7) ($P < 0.001$).

We assessed 7 different SNPs associated with 5 different genes and observed less than 5% technical failure rate in all assays. Table 3 shows the SNPs tested as well as their genotype and allele distribution. Comparison of genotypes between ever responders and non-responders (Table 4) identified a significant difference in the Fas ligand SNP rs763110 genotypes ($P = 0.042$). Patients with a CC genotype (as compared to those with a TC or TT genotype) were more likely to be non-responders to anti-TNF treatment, ($P = 0.016$; OR = 0.31, 95%CI: 0.11-0.83). Genotypes of another SNP, such as -308 (rs1800629), within the

TNF gene demonstrated a trend towards correlation with response to anti-TNF treatment ($P = 0.088$) when grouping genotypes AA and GA compared to genotype GG ($P = 0.093$, OR = 2.29, 95%CI: 0.85-6.17).

The vast majority of participants were Caucasian. Only 9 patients were African American, all of whom were ever-responders. Analyzing the Caucasians separately as a sensitivity analysis achieved similar results for the grouped Fas ligand SNP ($P = 0.029$) and for the grouped -308 TNF gene SNP ($P = 0.049$). No significant difference was observed for the remaining SNPs studied: ATG16L1 (rs10210302, T300A rs2241880), IBD5 (rs2522057), FCGR 3A (rs396991), and TNF (-238 rs361525).

Results of the univariable comparisons are shown in Table 5. In univariable analyses, the Fas ligand SNP (rs763110) demonstrated a difference between ever-responders and non-responders with borderline significance ($P = 0.058$) and significance when grouping TC and TT genotypes together ($P = 0.020$). The

Table 3 Single nucleotide polymorphisms tested with genotype and allele distribution for entire patients group *n* (%)

SNP	Location relative to gene (Chromosome number)	Nucleotide change	Assay successful	Genotype distribution		Allele distribution	
				Genotype	Frequency	Allele	Frequency
ATG16L1 rs10210302	2 kb upstream (2)	C/T	119 (98)	CC	25 (21.0)	Total	238
				TC	57 (47.9)	C	107 (45.0)
				TT	37 (31.1)	T	131 (55.0)
ATG16L1 rs2241880	Thr300Ala (2)	A/G	120 (99)	AA	27 (22.5)	Total	240
				GA	57 (47.5)	A	111 (46.3)
				GG	36 (30.0)	G	129 (53.7)
FAS ligand rs763110	-843 (1)	C/T	115 (95)	CC	42 (36.5)	Total	230
				TC	54 (47.0)	C	138 (60.0)
				TT	19 (16.5)	T	92 (40.0)
IBD5 rs2522057	Intergenic region (5)	C/G	120 (99)	CC	35 (29.2)	Total	240
				GC	49 (40.8)	C	119 (49.6)
				GG	36 (30.0)	G	121 (50.4)
FCGR 3A rs396991	Phe175Val (1)	C/G	120 (99)	AA	70 (58.3)	Total	240
				AC	33 (27.5)	A	173 (72.1)
				CC	17 (14.2)	C	67 (27.9)
TNF gene (-308) rs1800629	promotor region (6)	A/G	119 (98)	AA	4 (3.4)	Total	238
				GA	31 (26.1)	A	39 (19.6)
				GG	84 (70.6)	G	199 (80.4)
TNF gene (-238) rs361525	promotor region (6)	A/G	120 (99)	AA	2 (1.7)	Total	240
				GA	15 (12.5)	A	19 (7.9)
				GG	103 (85.8)	G	221 (92.1)

TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

Table 4 Single nucleotide polymorphism genotypes according to anti-tumor necrosis factor treatment response

SNP	Genotype	Anti-TNF treatment response		<i>P</i> value ¹
		Non-responders	Responders	
ATG16L1 rs10210302	CC	5 (25.0)	20 (20.2)	NS
	TC	9 (45.0)	48 (48.5)	
	TT	6 (30.0)	31 (31.3)	
ATG16L1 rs2241880	AA	5 (23.8)	22 (22.2)	NS
	GA	10 (47.6)	47 (47.5)	
	GG	6 (28.6)	30 (30.3)	
FAS Ligand rs763110	CC	12 (60.0)	30 (31.6)	0.042
	TC	5 (25.0)	49 (51.6)	
	TT	3 (15.0)	16 (16.8)	
FAS Ligand rs763110 (grouped)	CC	12 (60.0)	30 (31.6)	0.016 OR = 3.23, 95%CI: 1.20-8.78
	TC + TT	8 (40.0)	65 (68.4)	
IBD5 rs2522057	CC	6 (28.6)	29 (29.3)	NS
	GC	9 (42.9)	40 (40.4)	
	GG	6 (28.6)	30 (30.3)	
FCGR 3A rs396991	AA	11 (52.4)	59 (59.6)	NS
	AC	6 (28.6)	27 (27.3)	
	CC	4 (19.0)	13 (13.1)	
TNF gene (-308) rs1800629	AA	2 (10.0)	2 (2.0)	0.088
	GA	7 (35.0)	24 (24.2)	
	GG	11 (55.0)	73 (73.7)	
TNF gene (-308) rs1800629 (grouped)	AA + GA	9 (45.0)	26 (26.3)	0.093 OR = 2.29, 95%CI: 0.85-6.17
	GG	11 (55.0)	73 (73.7)	
TNF gene (-238) rs361525	AA	0 (0.0)	2 (2.0)	NS
	GA	2 (10.0)	13 (13.0)	
	GG	18 (90.0)	85 (85.0)	

¹Calculated using χ^2 . TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

comparison of -308 SNP (rs1800629) genotypes between ever-responders and non-responders ($P = 0.130$) became more different when grouping AA and GA genotypes together ($P = 0.099$). Univariate variables with $P < 0.15$ were included in the multiv-

ariable analysis for the comparison between anti-TNF treatment ever-responders and non-responders. Both Montreal disease behavior ($P = 0.125$) and perianal disease classification ($P = 0.086$) were included in the multivariable analysis.

Table 5 Univariable logistic regression data regarding factors associated with anti-tumor necrosis factor treatment failure

Covariate	P value
Demographic variables	
Gender	NS
Race	NS
Montreal Classification	
Age of onset (A) Montreal	NS
Location (L) Montreal	NS
Location (L4) upper GI Montreal	NS
Behavior (B) Montreal	0.13 ¹
Behavior (p) perianal Montreal	0.09 ¹
SNP variables	
ATG16L1 rs10210302	NS
ATG16L1 rs2241880	NS
FAS Ligand rs763110	0.057 ¹
FAS Ligand rs763110 (TC + TT grouped)	0.02 ¹
IBD5 rs2522057	NS
FCGR 3A rs396991	NS
TNF gene (-308) rs1800629	0.13 ¹
TNF gene (-308) rs1800629 (AA + GA grouped)	0.099 ¹
TNF gene(-238) rs361525	NS

¹Covariates included in the subsequent multivariable analysis. TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

Logistic multivariable regression models were developed for the Fas ligand (rs763110) SNP, the *TNF* gene -308A/G (rs1800629) SNP, and their combination. The multivariable logistic regression models included genotype data for each of these two SNPs (with genotypes grouped as described above), the Montreal disease behavior classification, and the Montreal perianal disease classification (Table 6). The Fas ligand SNP (rs763110) CC genotype was predictive of non-response, as compared to the TC and TT genotypes ($P = 0.009$, OR = 4.30, 95%CI: 1.45-12.80). In the -308 *TNF* gene (rs1800629) SNP multivariable model, the AA and GA genotypes were significantly predictive of non-response as compared to the GG genotype ($P = 0.049$, OR = 2.88, 95%CI: 1.01-8.22). Patients with the combination of the Fas ligand (rs763110) CC genotype and presence of the TNF -308 A allele (genotypes AA or GA as opposed to GG) had nearly five-fold higher odds of being non-responders ($P = 0.015$, OR = 4.76, 95%CI: 1.35-16.77). This occurred in 16 (13%) of our patients. Montreal disease behavior and the presence of perianal disease were not found to be predictive in any of the multivariable models.

DISCUSSION

We identified two SNPs, Fas Ligand SNP (rs763110) and the *TNF* gene -308 (rs1800629), as being associated with CD patient response to anti-TNF treatment.

The Fas ligand SNP (rs763110) genotype frequencies were significantly different between non-responders and ever-responders ($P = 0.042$). This association became more significant when grouping the TC and TT genotypes as compared to the CC genotype ($P = 0.016$). According to our multivariable

analysis, the odds of a patient with a Fas ligand CC genotype being a non-responder were four-fold higher as compared with a TC or TT genotype ($P = 0.009$, OR = 4.30, 95%CI: 1.45-12.80), when controlling for both Montreal disease behavior and perianal disease classification.

Abnormal regulation of apoptosis is one of the mechanisms of CD pathogenesis. Apoptosis (programmed cell death) can be induced through both extrinsic and intrinsic pathways^[25]. The extrinsic pathway is controlled through plasma membrane receptors belonging to the TNF receptor superfamily that include, among others, the Fas/Fas ligand which has been implicated in inflammatory bowel disease (IBD)^[26,27]. The SNP that we examined (rs763110) in the -843 position, which was located in a binding motif for the transcription factor CAAT/enhancer-binding protein β , has been implicated in carcinogenesis through the dysregulation of apoptosis^[28]. Higher basal expression of Fas ligand has been significantly associated with the C allele compared with the T allele of this SNP^[29]. The mechanism of action of anti-TNF drugs is complex; affecting many pathways, involving both soluble and membrane - bound TNF^[30]. Currently, anti-TNF treatments have not been linked directly with Fas ligand; however, an interaction is possible either directly with the Fas ligand or indirectly by affecting the cells upon which the Fas ligand acts. Hlavaty *et al.*^[31] examined response to infliximab and the Fas ligand (rs763110) SNP and found the TT genotype to be correlated with non-response.

The *TNF* gene SNP was also found to be associated with response to anti-TNF treatment. Two polymorphisms: -308 (rs1800629) and -238 (rs361525), both in the promotor region have previously had conflicting data reported with respect to response to anti-TNF treatment in rheumatoid arthritis and in IBD^[11,32-35]. The -308 (rs1800629) SNP has been shown previously to affect regulation of TNF α synthesis, with the minor allele (A) being a powerful transcriptional activator associated with increased TNF α production with the common allele (G)^[36,37]. Our study demonstrated a possible correlation with anti-TNF treatment response with the -308 (rs1800629) SNP. Separately, -308 (rs1800629) when examined by itself demonstrated only a trend towards significance, but when combined with disease behavior and perianal disease, a significant correlation was demonstrated ($P = 0.049$, OR = 2.88, 95%CI: 1.01-8.22). The presence of the AA and GA genotypes (A being the minor allele) was correlated with non-response, implying a 2.88 higher odds of being a non-responder to anti-TNF treatment if the patient has an A allele. The fact that the A allele is observed fairly infrequently (19.6% in our study, and 9%-16% in others^[38]) may explain the borderline statistical significance of this finding. Patients with the combination of the Fas ligand CC genotype and the TNF -308 A allele had nearly five-fold higher odds of being non-responders ($P = 0.015$, OR = 4.76, 95%CI: 1.35-16.77). This combination gives an

Table 6 Multivariable logistic regression models predicting anti-tumor necrosis factor treatment failure

Model	Variable	Class	Estimate	Global P value	OR (95%CI)
a	FAS ligand rs763110 (TC + TT grouped)	CC	1.460	0.009	4.30 (1.45-12.80)
		TC+TT	0		
	Montreal behavior (B)	B1	-0.083	NS	0.92 (0.21-3.96)
		B2	1.158		
		B3	0		
	Montreal behavior (p) perianal	No	0.964	NS	2.62 (0.68-10.09)
Yes		0			
b	TNF gene (-308) rs1800629 (AA+GA grouped)	AA+GA	1.056	0.049	2.88 (1.01-8.22)
		GG	0		
	Montreal Behavior (B)	B1	-0.153	NS	0.86 (0.20-3.59)
		B2	0.826		
		B3	0		
	Montreal Behavior (p) perianal	No	1.213	NS	3.36 (0.87-12.93)
Yes		0			
c	FAS ligand (CC genotype) and TNF gene -308 (AA or GA genotype) combined	CC and AA or GA	1.560	0.015	4.76 (1.35-16.77)
		Other	0		
	Montreal behavior (B)	B1	-0.022	NS	0.98 (0.23-4.22)
		B2	0.950		
		B3	0		
	Montreal behavior (p) perianal	No	0.970	NS	2.64 (0.69-10.10)
Yes		0			

additive effect compared to each SNP separately, most likely acting by different mechanisms. Considering that they are also found on different chromosomes (1 and 6), they are also in all probability inherited independently. The -238 TNF gene SNP (rs361525) did correlate with response to anti-TNF treatment. This combination was found in 16 patients in our study (13%).

The *ATG16L1* gene has been well described in CD and has an important role in autophagy^[39]. A SNP in this gene (rs2241880) has been linked to diminished autophagy, predisposing to CD^[40]. Our study did not show an association with anti-TNF treatment response for this SNP, nor for another SNP on this gene (rs10210302) that had shown promise as a predictor for response to adalimumab in Slovenian CD patients^[41].

Another region, the *5q31* gene cluster (IBD5), has been described to confer CD risk in some populations^[42-44]. A SNP in this cluster (rs2522057) may be associated with response to infliximab in CD^[45]. We were, however, unable to demonstrate such a correlation.

Antibody-dependent cell-mediated cytotoxicity is important in the mechanism of action of anti-TNF drugs. It requires leukocyte receptors for the Fc portion of IgG. A polymorphism (rs396991) in the gene encoding FCGR 3A expressed on macrophages and natural killer cells was associated with the response to rituximab in follicular non-Hodgkin's lymphomas^[46]. This polymorphism has also been associated with the response to infliximab in CD^[47], but this was not able to be confirmed in our study.

Our study population included patients from a large university digestive practice. The patients included in the study and receiving anti-TNF drugs were younger than the excluded non-anti-TNF treated patients but were comparable with respect to race, gender, and socioeconomic status. Their younger age can be

explained by the fact that younger patients tend to have a more severe disease^[1] and would probably require anti-TNF drugs more frequently. Since patients were comparable in other aspects, we believe there was no selection bias. All CD patients were followed by gastroenterologists and surgeons, and as such, had a higher incidence of complicated CD. This may be a source of potential bias. This may also explain why we did not observe a difference with respect to likelihood of anti-TNF treatment response and disease behavior which has been observed in prior studies^[9]. The clinical allocation into groups was performed by experienced clinicians (Galandiuk S, Dryden GW), who deal with this population on a daily basis. The assessment was made on the basis of the patient history, physical exam, patient follow-up, lab-work and endoscopy/pathology when clinically indicated. This study assessed non-response (no improvement whatsoever) vs ever-response. Since this was not a formal clinical trial and since only a quarter of patients had colonic disease, colonoscopy was not performed at defined intervals, but at the discretion of the treating physician. This could be a source of bias, but we believe that clinical assessments based on the above criteria are valid.

In summary, identification of patients whose anti-TNF treatment will fail is important, both from a clinical and from an economic perspective. We have identified two functional SNPs, Fas ligand (rs763110) and the *TNF* gene -308 (rs1800629), associated with non-response to anti-TNF treatment. Genotyping these SNPs from DNA obtained from peripheral blood may help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment by avoiding expensive therapy that is likely to fail and permitting selection of other treatments

more likely to succeed.

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COMMENTS

Background

Anti-tumor necrosis factor (TNF) agents will not be effective in a subset of patients with Crohn's disease (CD). Predicting the subset of patients who do not respond to anti-TNF treatment is important clinically and economically.

Research frontiers

A possible conduit to predict response to anti-TNF therapy could be through genetic testing. The authors chose to examine a series of single nucleotide polymorphisms (SNPs) within genes that have been linked either with CD and/or with anti-TNF treatment response in order to determine whether these could aid in predicting response to anti-TNF treatment in CD patients.

Innovations and breakthroughs

Two SNPs Fas ligand and TNF gene -308 were associated with response to anti-TNF treatment.

Applications

Genotyping these SNPs from DNA obtained from peripheral blood may help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment by avoiding expensive therapy that is likely to fail and permitting selection of other treatments more likely to succeed.

Terminology

Single nucleotide polymorphisms are a type of genetic variation in which a change is found in a single nucleotide at a specific position in the genome.

Peer-review

The authors investigate genetic factors that might help define which CD patients are likely to benefit from anti-TNF therapy. This is an interesting paper.

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