ELECTRONIC LETTER

Angiotensinogen and transforming growth factor β 1: novel genes in the pathogenesis of Crohn's disease

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Background: Angiotensin peptides may act locally as cytokines in several organ systems with elevated mucosal levels present in Crohn's disease. A variant in the angiotensinogen gene promoter results in increased peptide production, while transforming growth factor β 1 (TGF β 1) codon 25 variants demonstrate variable peptide production, predisposing to fibrosis in several organs.

Aims: Conduct an Australian-based analysis of the angiotensinogen-6 variant in two independent inflammatory bowel disease (IBD) cohorts, and examine the role of angiotensinogen-6 and TGF β 1 codon 25 variants in shaping Crohn's disease phenotype.

Methods: IBD Patients (Crohn's disease = 347, ulcerative colitis = 147) and CD families (n = 148) from two cohorts, together with 185 healthy controls were genotyped for angiotensinogen-6. Genotype-phenotype analyses were performed for both angiotensinogen-6 and TGF β 1 codon 25.

Results: Angiotensinogen-6 AA genotype was significantly associated with Crohn's disease ($p = 0.007$, OR = 2.38, CI = 1.32–4.32) in cohort 1, but not in the smaller cohort 2 ($p = 0.19$). The association remained significant when the two cohorts were combined ($p = 0.008$), and in a TDT family analysis ($p = 0.03$). TGF 1 codon 25 was associated with stricturing Crohn's disease ($p = 0.01$, OR = 2.63, CI = 1.16–5.88) and a shorter time to intestinal resection ($p = 0.06$).

Conclusions: The association of the angiotensinogen-6 variant with Crohn's disease supports a potential role for angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists in disease treatment.

Key points

- Angiotensin and TGF β 1 may have a role in Crohn's disease.
- An angiotensinogen gene promoter variant was associated with Crohn's disease.
- A TGF β 1 variant was associated with stricturing Crohn's disease and a shorter time to intestinal resection.
- Supports a potential role for ACE inhibitors and angiotensin II receptor antagonists in disease treatment.

constrain bacterial survival.⁶ Interindividual variation in cytokine production is thought to represent another potential contributing factor to the exaggerated or dysregulated mucosal inflammatory response in IBD. Variability in the 5q31 cytokine gene cluster and the -857C promoter polymorphism in the tumour necrosis factor α (TNF α) gene have been independently associated with Crohn's disease and IBD, respectively.^{7–10} More recently, variations in the *OCTN1* and *OCTN2* cation transporter genes at the 5q31 locus have been linked to Crohn's disease. It is hypothesised that altered transport function may cause a defect in pathogen killing or fatty acid β -oxidation in epithelial cells.¹¹ Most recently, variation in the *DLG5* gene at 10q23, a member of the guanylate kinase gene family, has been associated with IBD. The gene encodes a scaffolding protein associated with in the maintenance of epithelial integrity.¹²

In addition to their role in the regulation of systemic blood pressure and blood volume, evidence suggests that the angiotensin peptides are produced and function locally as cytokines in several organ systems participating in the regulation of inflammation and fibrosis.¹³ Colonic mucosal levels of angiotensin I and angiotensin II (AII) in endoscopic biopsy specimens are markedly raised in Crohn's colitis compared with their levels in ulcerative colitis, other forms of colitis or controls.¹⁴ Molecular variation in the *angiotensinogen* gene has been associated with both essential hypertension and the level of hepatic fibrosis in hepatitis C, and the gene has been mapped to 1q42–q43 on the opposite arm to the IBD7 susceptibility locus at 1p36.^{3 15 16} The functional polymorphism is thought to be a nucleotide substitution (guanine→adenine) 6 bp from the transcription start site in

The genetic model proposed for the inflammatory bowel diseases (IBDs), Crohn's disease and ulcerative colitis describes related complex genetic disorders sharing some but not all susceptibility loci.¹ Both are characterised by chronic, relapsing and remitting intestinal inflammation. However, in most cases Crohn's disease can be distinguished from ulcerative colitis. Crohn's disease can affect the gastrointestinal tract anywhere from the mouth to the anus, with the inflammation transmural and often granulomatous in nature. By contrast, ulcerative colitis affects only the colon, with inflammation being confined to the superficial mucosal layers.

Although genomewide scanning has identified numerous susceptibility loci for IBD,^{2 3} few candidate genes have been clearly elucidated at the molecular level. Mutations in the *CARD15/NOD2* gene on chromosome 16 confer susceptibility to Crohn's disease but not to ulcerative colitis.^{4 5} *CARD15* is thought to be a key component of the innate mucosal immune response, with the variant protein less able to

Abbreviations: ACE, angiotensin-converting enzyme; All, angiotensin II; IBD, inflammatory bowel disease; PCR, polymerase chain reaction; RBWH, Royal Brisbane and Women's Hospital; TNBS, trinitrobenzene sulphonic acid; TDT, transmission disequilibrium test; TGF β 1, transforming growth factor β 1; TNF α , tumour necrosis factor α

the gene promoter, which results in increased production of the peptide.¹⁷

Angiotensinogen is cleaved by renin into angiotensin I, which is rapidly converted to AII by the angiotensin-converting enzyme (ACE). The angiotensin peptides play an important but imprecisely defined role in granulomatous inflammation.¹⁸ This might suggest that they will be more associated with intestinal inflammation in Crohn's disease than with ulcerative colitis. In addition, AII may increase the production of transforming growth factor β 1 (TGF β 1), an important regulatory cytokine in the intestinal mucosa. TGF β 1 is important for epithelial wound healing and is a member of several families of growth factors that have been implicated in the susceptibility of IBD mucosa to tissue injury and repair.¹⁹ A polymorphism in the signal sequence at codon 25 of the TGF β 1 gene results in variation in peptide production and has been associated with susceptibility to fibrosis in many organs, including the liver and lungs.^{16–20} Its role in shaping disease phenotype in Crohn's disease, in particular susceptibility to fibrotic complications, has not been clearly defined. In two small human cohorts with Crohn's disease, neither the TGF β 1 codon 25 nor the C509T promoter polymorphism has been shown to influence the behaviour of Crohn's disease. The gene has been mapped to 19q13.1–q13.3 on the arm opposite to the IBD6 susceptibility locus at 19p13.^{3, 19, 21}

In this study, we aim to investigate the frequency of the angiotensinogen-6 G→A promoter polymorphism in patients with well-characterised IBD, including patients with Crohn's disease and those with ulcerative colitis, compared with matched controls. We hypothesise that the polymorphism may contribute to an exaggerated immune and inflammatory intestinal mucosal response and hence confer a degree of susceptibility to IBD. In addition, we suspect it will have a more important role in Crohn's disease than in ulcerative colitis in view of the granulomatous nature of its inflammation. We also aim to investigate the role of the TGF β 1 codon 25 polymorphism in shaping the phenotype of Crohn's disease. We hypothesise that it may increase the risk of fibrotic complications, such as intestinal strictures.

PATIENTS AND METHODS

Subjects

This candidate gene association study was a case-control design using two separate Australian cohorts with IBD. Cohort 1 comprised patients recruited from the IBD Clinical and Research Programme at the Royal Brisbane & Women's Hospital (RBWH), Brisbane, Queensland, Australia (Crohn's disease, $n = 235$; ulcerative colitis, $n = 147$). Informed consent was obtained from all patients and the RBWH Human Research Ethics Committee approved the study protocol. Patients with Crohn's disease are a consecutive series of patients prospectively recruited into a longitudinal IBD research protocol from 1994. The RBWH is the only public referral centre for north Brisbane, which encompasses a population of approximately 500 000. All cases are recorded on an IBD database, together with relevant phenotypic information including disease distribution, behaviour and duration, surgery, smoking and histological data such as granulomas. Phenotypic characteristics were carefully determined by two investigators using clinical, endoscopic, radiological, histological and surgical data available in the patient's clinical records. The investigators were blinded to the patient's genotype during this process. Behaviour of Crohn's disease was based on the Vienna Classification, which describes predominantly stricturing, penetrating or inflammatory disease (non-stricturing, non-penetrating).²² Patients were also subdivided into those who had the

ever-stricturing always (or never-stricturing) phenotype independent of penetrating disease.

Controls were randomly selected from a community-based twin database that contains 3808 twin pairs from around Australia enrolled with the Australian twin registry. Between 1980 and 1982, these twin pairs participated in a health survey by mailed questionnaire. One twin from each pair was selected randomly as a control and matched by sex and 5-year age intervals to each patient with IBD.

Cohort 2 comprised 112 patients with Crohn's disease from the Medical Genetics Research Unit at the Canberra Hospital, Canberra, Australian Capital Territory, Australia. By contrast, these patients (unrelated probands only) were almost exclusively from multiplex families. These cases were collected from all over Australia with the assistance of the Australian Crohns and Colitis Association and collaborating clinicians who provided diagnostic confirmation of the disease using the Vienna Classification. Informed written consent was obtained from all participants and appropriate institutional ethical approval was obtained before data collection. Only data on age of diagnosis were available for analysis for this cohort. All cases were genotyped for the angiotensinogen-6 G→A promoter polymorphism.

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells using the salting-out technique.²³ Both polymorphisms were detected using a polymerase chain reaction (PCR)-restriction fragment polymorphism analysis. For the angiotensinogen-6 G→A polymorphism, the PCR was carried out using Platinum PCR Supermix (Invitrogen Australia, Mount Waverley, Victoria, Australia) and the primers 5' CTC AGT TAC ATC CTG AGA GAG ACA AGA CC 3' and 5' GTG TCG CTT CTG GCA TCT GTC CTT CTG G 3'.¹⁶ After amplification, the product was digested using *Bsi*E1 (New England Biolabs), Genesearch, Arundel, Queensland, Australia). The enzyme cuts the product when the G allele is present, allowing the two alleles to be identified on a 2.5% agarose gel. The TGF β 1 codon 25 polymorphism codes for arginine (A) or proline (P). Genotyping included PCR with the primers 5' CAC CAC ACC AGC CCT GTT C 3' and 5' CCG CAG CTT GGA CAG GAT 3', followed by digestion with *Bgl*II (New England Biolabs), which cuts at the site of the polymorphism and in another distinct location so that the two alleles can be easily distinguished on a 2.5% agarose gel, with ethidium bromide for visualisation.

Statistical analysis

Only one patient from the affected families was included in the case-control analysis. The data were statistically interrogated using a variety of statistical methods. The associations between genotype and categorical phenotypic factors (table 1) were tested using Pearson's χ^2 test or Fisher's exact test as appropriate. The associations between genotype and continuous factors were tested with the two-group *t* test or Wilcoxon's rank sum test as appropriate. Multiple regression models were used to test for significant associations after adjusting for other potentially relevant biological variables (ie, age at diagnosis, disease duration, family type, disease site, disease behaviour and presence of granulomas), which may influence disease expression and had a $p \leq 0.2$ at the univariate level. Binary or ordinal logistic regression models were used as required for categorical data, and analysis of variance regression models were used for continuous data. To further test for association, the transmission disequilibrium test (TDT) was carried out using the SIB-PAIR program V.0.99.9 to analyse data from all available families (<http://www.qimr.davidD/index.html>).²⁴

Table 1 Clinical characteristics (phenotype) of the Brisbane cohort with Crohn's disease (n = 235, unless indicated otherwise)

Clinical characteristic	Mean	SD	Median
Age at diagnosis (years)	28.0	12.5	24.8
Disease duration (years)	12.5	7.1	11.0
Smoking (pack-years)	18.9	18.3	13.0
	%	n	
Sex	Female	62.1	146
Multiplex	Yes	26.4	62
Bowel resection	Yes	69.4	163
Immunosuppression	Yes	61.7	145
Infliximab	Yes	13.6	32
Any CD surgery	Yes	78.7	185
Disease site at diagnosis (n = 234)	Ileal	42.7	100
	Ileocolonic	40.2	40
	Colonic	17.0	94
Perianal fistulae	Yes	6.0	14
Phenotype (n = 231)	Stricturing	30.3	70
	Penetrating	38.1	88
	Inflammatory	31.6	73
	Ever stricturing	48.9	113
	Never stricturing	51.1	118
Smoking at diagnosis (n = 221)	Current	52.9	117
	Ex	40.3	89
	Never	6.8	15

CD, Crohn's disease.

All clinical data were obtained from the Royal Brisbane and Women's Hospital database of patients with inflammatory bowel disease, comprising patients who were recruited from the Brisbane North region (cohort 1).

RESULTS

Clinical characteristics of patient cohorts

Cohort 1

The population with Crohn's disease from cohort 1 had a mean age of 28 years at diagnosis and a mean disease duration of 12.5 years (table 1). Only 27.7% of patients were from multiply-affected families, with the remainder being sporadic. More than half (51.4%) of the patients were smokers at diagnosis; 65.0% of them had required bowel resection at some point in their history.

Cohort 2

The phenotypic characteristics of this cohort were similar to those of cohort 1: 64.5% of patients were women and the mean (standard deviation) age at diagnosis was slightly younger at 25.5 (8.9) years. With respect to disease location, 39.2% of patients had ileal disease, 31.1% had ileocolonic and 25.7% had colonic disease. A total of 69.0% of these patients had undergone surgery compared with 75% from cohort 1, and almost all had required bowel resection. All patients in both cohorts were of Caucasian ancestry.

Angiotensinogen-6 G→A promoter polymorphism

In cohort 1, 235 patients with Crohn's disease, 147 with ulcerative colitis and 185 controls were genotyped for the angiotensinogen-6 polymorphism. The genotype data were in Hardy-Weinberg equilibrium. The variant A allele was present more frequently in patients with Crohn's disease from cohort 1 than in controls, with both the genotype distribution data ($p = 0.007$; AA v GG, odds ratio (OR) 2.38, confidence interval (CI) 1.32 to 4.32; table 2) and allele frequencies differing significantly ($p = 0.003$) between patients with Crohn's disease and controls. By contrast, we found no significant differences when comparing the population with ulcerative colitis and controls ($p = 0.72$; table 2).

When patients with Crohn's disease in cohort 1 were stratified for the presence of any of the three major *NOD2*/

Table 2 Angiotensinogen-6 genotype frequencies for expand cohorts 1 and 2, and controls

	AA	GA	GG
CD cohort 1	0.213 (50)	0.557 (131)	0.230 (54)
CD cohort 2	0.200 (22)	0.527 (59)	0.273 (31)
UC cohort 1	0.170 (25)	0.497 (73)	0.333 (49)
Controls	0.141 (26)	0.497 (92)	0.362 (67)
Combined CD cohorts	0.209 (72)	0.548 (190)	0.243 (85)

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

Genotype counts are in parentheses. CD cohort 1 versus controls, $p = 0.007$; CD cohort 2 versus controls, $p = 0.19$; UC cohort 1 versus controls, $p = 0.72$; combined CD cohorts 1 and 2 versus controls, $p = 0.008$.

CARD15 mutations associated with susceptibility to Crohn's disease, we found no significant difference in genotype distribution ($p = 0.8$; data not shown).

The second cohort of patients with Crohn's disease showed a similar genotype distribution similar to that of cohort 1 ($p = 0.7$; table 2), but when compared with the controls, this did not meet significance ($p = 0.19$). The combined data from both cohorts, however, confirmed the original positive association with Crohn's disease ($p = 0.008$).

Phenotype-genotype analysis

We found no significant relationships between the angiotensinogen-6 polymorphism and discrete clinical variables at the univariate or multivariate levels. There was a trend towards a lower mean age at diagnosis in patients carrying the variant A allele (AA 26.4 years, GA 28.1 years, GG 30.5 years; $p = 0.11$). Patients with Crohn's disease homozygous for the wild-type G allele were less likely to be from a multiply-affected family ($p = 0.11$).

Transmission disequilibrium test

To further investigate the observed association of the angiotensinogen-6 promoter polymorphism with Crohn's disease, a TDT was carried out using 148 families with Crohn's disease (ie, patients with Crohn's disease and two unaffected parents) from the two IBD centres. There were 104 transmissions of the A allele compared with 78 transmissions of the G allele. This showed a moderately significant overtransmission of the variant A allele from heterozygous parents to offspring with Crohn's disease ($p = 0.03$).

TGFβ1 A→P codon 25 polymorphism

A total of 235 patients with Crohn's disease from cohort 1 and 144 controls were genotyped for this polymorphism. We found no significant difference ($p = 0.74$) in the genotype frequencies between the groups.

Phenotype-genotype analysis

Analysis of phenotypic variables was carried out in relation to the TGFβ1 codon 25 genotype in patients with Crohn's disease from cohort 1. Significantly more patients who had a

Table 3 TGFβ1 codon 25 genotype frequencies for CD cohort 1 with Crohn's disease and controls ($p = 0.74$)

	AA	AP	PP
CD Cohort 1	0.868 (204)	0.119 (28)	0.013 (3)
Controls	0.854 (123)	0.139 (20)	0.007 (1)

AA, Arg/Arg; AP, Arg/Pro; PP, Pro/Pro; CD, Crohn's disease. Genotype counts are in parentheses.

stricturing phenotype were of the profibrotic AA genotype ($p = 0.01$; AA v AP/PP, OR = 2.63, CI 1.16 to 5.88); however, this was only approaching significance after multivariate analysis ($p = 0.06$). We found no other significant associations with disease phenotype at the univariate or multivariate levels. Patients with Crohn's disease homozygous for the profibrotic A allele of TGF β 1 codon 25 also tended to have a shorter median and mean (SD) time to intestinal resection (3, 5.32 (0.41) years) compared with those carrying the less common P variant (6 years, 7.35 years (1.13)), which was approaching significance ($p = 0.06$).

DISCUSSION

This study indicates that inherited variation in the angiotensinogen gene may contribute to susceptibility to Crohn's disease, but not to ulcerative colitis. The significant association observed in cohort 1 case-control analysis between Crohn's disease and the angiotensinogen-6 variant A allele was not replicated in the second smaller cohort 2. However, the genotype distribution data were statistically similar between the groups, and the combined data clearly showed significance ($p = 0.008$). These results were strengthened by the TDT analysis, with significant overtransmission of the angiotensinogen-6 variant A allele to offspring with Crohn's disease in both cohorts.

We also showed a novel association between the TGF β 1 codon 25 (Arg/Arg) genotype and surrogate markers of intestinal fibrosis, specifically stricturing behaviour and a more rapid progression to intestinal resection. We have not been able to show a statistically significant relationship between the angiotensinogen-6 variant A allele and a particular Crohn's disease phenotype, although a trend towards lower mean age at diagnosis was observed.

Biological evidence supports a potential role for the angiotensinogen gene in Crohn's disease. AII has been well documented to regulate the synthesis of proinflammatory cytokines and chemokines in other organs such as the kidney where it has been shown to increase the production of pro-inflammatory mediators such as TNF α in vivo.²⁵ AII has been shown to induce nuclear factor κ B activation through AII receptors in a variety of cell lines, and it has been suggested that nuclear factor κ B is the main mediator of the AII-induced increase in adhesion molecules and cytokine expression in models of vascular injury.^{26, 27} In addition, numerous studies have shown the therapeutic effects of AII blockade via both inhibition of ACE and direct AII receptor blockade. For example, the ACE inhibitor lisinopril alleviates chronic pancreatic inflammation and fibrosis in male WBN/Kob rats.²⁸

Even more recently, the administration of the ACE inhibitor captopril to rats with trinitrobenzene sulphonic acid (TNBS) colitis was shown to considerably reduce colonic levels of angiotensin II. It markedly ameliorated macroscopic and microscopic inflammation of the colon.²⁹ Rectal administration of TNBS induces chronic colitis, closely resembling the clinical, histopathological and immune characteristics of human Crohn's disease.³⁰ Therefore, in light of the documented overexpression of AII in human Crohn's colitis,¹³ it is conceivable that inherited variation of AII production in the setting of other genetic and environmental factors may contribute to susceptibility to Crohn's disease in a fashion similar to inherited polymorphisms in the promoter of the TNF α gene. Further support for this hypothesis has recently been shown by the amelioration of TNBS colitis in angiotensinogen knockout mice.³¹

Several in vitro and in vivo studies have shown a strong biological link between AII and TGF β .³² AII induces time and dose-dependent increases in both TGF β mRNA and TGF β activation in rat mesangial cells.³³ TGF β has a central role in

fibrotic diseases, and both animal and human studies support the role of AII in renal and cardiac fibrosis through the therapeutic effects of AII blockade, presumably via a reduction in TGF β . More recently, similar results have been shown in animal models of various gastrointestinal disorders, including pancreatic and hepatic fibrosis,^{28, 34} as well as TNBS colitis.²⁹ In this model, the administration of an ACE inhibitor markedly impairs expression of TGF β 1 mRNA and the collagen α 1 gene, as well as tissue hydroxyproline, a marker of collagen deposition.²⁹ These results support the hypothesis that intestinal fibrosis associated with TGF β 1 activation may be partly mediated by AII.

Although our study suggests that an inherited increase in TGF β production can predispose to a stricturing phenotype in Crohn's disease, a similar relationship could not be shown with the angiotensinogen-6 G $\rightarrow$ A promoter polymorphism. Several factors may have contributed to this. Firstly, there is no widely accepted or applied histological grading system of intestinal fibrosis. Clinical markers such as evidence of strictures and intestinal resection are required. These markers are inherently less sensitive than, for example, the histological grading systems for hepatic fibrosis.³⁵ Therefore, although it is still possible to show a relationship between behaviour of Crohn's disease and an inherited predisposition to increased TGF β production, a central fibrogenic cytokine, it may not be possible for those cytokines further upstream such as AII. Alternatively, the role of AII in intestinal fibrosis in Crohn's disease may be minimal. However, there is mounting evidence of its widespread influence in multiple organ systems, including the gastrointestinal tract. In our cohorts, a greater proportion of patients requiring immunosuppression were homozygous for the angiotensinogen-6 A allele. This treatment may also have partially masked the effect of this allele by therapeutically impairing the progression from intestinal inflammation to clinically relevant fibrotic disease.

These results may also be clinically relevant, as ACE inhibitors and AII receptor antagonists are widely available for oral administration with a well-established safety profile in human clinical practice. It remains to be shown whether they would reduce clinically relevant intestinal inflammation, strictures or delay intestinal resection in patients with Crohn's disease. In addition, a patient's genotype may help identify people more likely to respond to this form of treatment.

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