

INFLAMMATORY BOWEL DISEASE

DLG5 variants do not influence susceptibility to inflammatory bowel disease in the Scottish population

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Introduction: Recent data have suggested that specific haplotypic variants of the DLG5 gene on chromosome 10q23 may be associated with susceptibility to inflammatory bowel disease (IBD) in Germany. Haplotype D, notably characterised by the presence of a G→A substitution at nucleotide 113, was associated with susceptibility to Crohn's disease (CD) whereas an extended haplotype A conferred protection.

Aims: Association of DLG5 haplotypic variants with disease susceptibility, genotype-phenotype relationships, and epistasis with CARD15 was investigated in the Scottish population.

Patients and methods: A total of 374 CD, 305 ulcerative colitis (UC), and 294 healthy controls (HC) were studied. Genotyping for the variants rs1248696 (113A, representing haplotype D) and the single nucleotide polymorphism tag rs2289311 (representing haplotype A) were typed using the Taqman system.

Results: On analysis of the DLG5 variant 113A, there were no associations with IBD when allelic frequency (11.4% IBD v 13.2% HC; $p=0.30$) and carrier frequency (19.2% IBD v 24.6% HC; $p=0.069$) were analysed. No associations were observed between 113A variant allelic frequency ($p=0.37$), carrier frequency ($p=0.057$), and CD. In fact, 113A heterozygosity rates were lower in CD (16%) and IBD (16.9%) than in HC (23%) ($p=0.029$ and $p=0.033$, respectively). No associations between DLG5 and UC were observed. Haplotype A was not protective and there was no evidence of epistasis between DLG5 and CARD15.

Conclusions: The present data contrast strongly with previous data from Germany. DLG5 113A is not associated with disease susceptibility and haplotype A does not confer resistance. Further work is required to evaluate the significance of DLG5 in other populations from geographically diverse regions.

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Crohn's disease (CD) and ulcerative colitis (UC) are common causes of gastrointestinal morbidity in the developed world, and the incidence of early onset CD has continued to rise in Northern Europe.^{1–3} Increasing insight has been gained into the critical role of the dysregulated immune response to bacterial flora and it is clear that genetic susceptibility to environmental agents are central to the pathogenesis of chronic intestinal inflammation.^{4,5}

Genome wide scanning has identified six confirmed loci that confer susceptibility to CD and the first discovered and most consistently replicated critical mutations were found in the CARD15 (NOD2) gene on chromosome 16 (IBD1).^{6,7} The physiological role of the CARD15/NOD2 protein remains under detailed examination. In vitro data suggest that CARD15 functions as an intracellular sensor of muramyl dipeptide, a highly conserved peptidoglycan motif common to many intraluminal bacteria.^{8,9} These observations have now been complemented by studies of genetically engineered mouse models. Watanabe *et al* have suggested that CARD15^{-/-} mice lose negative control of Toll-like receptor 2 (TLR2) mediated activation of nuclear factor κ B, potentially offering an explanation for the Th1 phenotype characteristic of CD.¹⁰ However, recently compiled studies do not provide support for NOD2/CARD15 interaction with the TLR2 pathway and emphasise the complexity of NOD2/CARD15 activation.^{11,12}

Carriage rates of the three common NOD2/CARD15 mutations single nucleotide polymorphisms (SNPs) Gly908Arg, Arg702Trp, and the frame shift mutation

Leu1007fsinsC vary between 0% and 50% in different CD cohorts, with high rates reported in Central European populations⁶ and low rates in Northern Europe (Finland and Scotland).^{13,14} Genetic heterogeneity between the populations of North and Central Europe is an obvious reason for the difference in CARD15 carriage rates and as more susceptibility genes are being discovered this explanation has become increasingly pertinent.¹³

A locus on chromosome 10 was first implicated in genome wide scanning of a European cohort (UK, Germany, and the Netherlands) in 1999 as conferring susceptibility to inflammatory bowel disease (IBD) (LOD score 2.30).¹⁵ Recent data now suggest that the gene DLG5 (Drosophila discs large homologue 5) located on chromosome 10q23 may be responsible for the observed linkage and contains critical mutations that confer susceptibility to IBD.¹⁶

DLG5 is a member of the MAGUK (membrane associated guanylate kinase) gene family which encode cell scaffolding proteins and are also involved in intracellular signal transduction.¹⁷ MAGUKs interact with other proteins to create an assembly of large multiprotein complexes that bind transmembrane proteins at the cytoplasmic side to other signal transduction proteins, thus creating a platform for specific signal interactions.^{18,19} The DLG5 gene spans 79 kb,

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; HC, healthy controls; SNP, single nucleotide polymorphism; TLR, Toll-like receptor; DLG5, Drosophila discs large homologue 5; MAGUK, membrane associated guanylate kinase; PSC, primary sclerosing cholangitis

contains 32 exons, and is expressed most strongly in placental tissue and less so in heart, skeletal muscle, liver, small bowel, and colon.²⁰

Stoll and colleagues¹⁶ identified two extended DLG5 haplotypes that influenced disease susceptibility in the German population. The first haplotype (named haplotype D¹⁶) was especially notable for the presence of a G→A substitution at nucleotide 113 that resulted in an amino change at position 30 from arginine to glutamine (R30Q). On analysis of carrier frequency, Stoll *et al* found the 113A variant to be associated with CD (25% CD v 17% healthy controls; $p=0.001$) in a case control study and trends between 113A transmission and IBD ($p=0.09$) and CD ($p=0.065$) were observed on transmission disequilibrium testing.¹⁶ In silico analysis suggests that the 113A (R30Q) variant may impair DLG5 scaffolding function, but as yet no expression or functional studies in IBDs have been conducted. Evidence of epistasis between the 113A variant of DLG5 and CARD15 variants was also observed in the CD cohort.¹⁶

The second haplotype (haplotype A¹⁶) was tagged by eight marker SNPs and was observed to be significantly under transmitted in the IBD group ($p=0.006$), suggesting the haplotype may be protective.¹⁶ No phenotypic associations were investigated and as yet no replication data have been published.

In the present study, we have assessed the contribution of the DLG5 polymorphisms rs1248696 (113A) and rs2289311 (one of the marker SNPs for the protective haplotype A¹⁶) in determining genetic susceptibility to CD and UC in the Scottish population which has a high incidence of IBD. We have also investigated genotype-phenotype associations in our rigorously defined IBD population and assessed epistasis with established CARD15 mutations.

PATIENTS AND METHODS

A total of 679 patients with well characterised IBD (IBD) and 294 controls were recruited. All IBD patients attended the clinic at the Western General Hospital, Edinburgh, a tertiary referral centre for IBD in the South East of Scotland. The group comprised of 374 patients with CD and 305 with UC. The diagnosis of IBD adhered to the criteria of Lennard-Jones.²¹ CD patients were classified according to the Vienna

classification which involves age at diagnosis (A1, <40 years; A2, >40 years), location (L1, terminal ileum; L2 colon; L3, ileocolon; L4, upper gastrointestinal), and behaviour (B1, non-stricturing, non-penetrating; B2, stricturing; B3, penetrating).²² UC disease severity was judged by the criteria proposed by Truelove and Witts.²³ Phenotypic data were collected by patient questionnaire, interview, and case note review, and comprised of demographics, date of onset of symptoms and diagnosis, disease location, disease behaviour, progression, extraintestinal manifestations, surgical operations, smoking history, joint symptoms, family history, and ethnicity. The study protocol was approved by Lothian Research Ethics Committee (LREC 2000/4/192).

Demographics: CD and UC

The demographics of the CD and UC patients are shown in table 1. Duration of follow up was defined as the time from diagnosis to the time of the most recent clinic review (median duration 11.8 years in the CD group and 7.5 years in the UC group). The CD group consisted of 181 males and 193 females and the UC group 171 males and 134 females, with a median age at diagnosis of 34 years.

Vienna disease classification was available for 347 (93%) CD patients at diagnosis and 374 (100%) CD patients at follow up. Full phenotypic data were available for the UC cohort.

Control subjects

A total of 294 controls (163 blood donors from the south east of Scotland and 131 healthy controls subjects) were enrolled. Allelic frequencies of DLG5 variant SNPs 113A, rs2289311, OCTN1 variant rs1050152, OCTN2 variant rs26313667, and IBD5 marker SNP IGR2198 are shown in table 2.

Genotyping

Genomic DNA was extracted from peripheral venous blood by a modified salting out technique and resuspended in 1×TE (10 mM Tris (pH 8.0), 1 M EDTA (pH 8.0)) at a final concentration of 100 ng/μl. SNPs rs1248696 (113G→A representing haplotype D) and rs2289311 (chosen because of its reliability in genotyping to represent the protective haplotype A; M Stoll, personal communication) were typed using the Taqman system. IBD patients and controls were

Table 1 Demographics and clinical features of the Crohn's disease (CD), ulcerative colitis (UC), and control groups

	Crohn's disease (n = 374)	Ulcerative colitis (n = 305)	Controls (n = 294)
Sex (M/F)	181/193	171/134	143/151
Age at diagnosis (y) (median (IQR))	27.8 (20.9–40.4)	34 (25–50)	39 (27–52)
Duration of follow up (y) (median (IQR))	11.8 (6.5–20.2)	7.5 (3.35–13.38)	
Caucasian (%)	98.7%	98.3%	
CD location at diagnosis		UC disease extent	
Ileal disease	125 (36%)	Proctitis 105 (34.5%)	
Colonic disease	137 (39%)	Left sided colitis 116 (37%)	
Ileal and colonic	58 (14%)	Extensive colitis 84 (27.5%)	
Upper GI disease	30 (8.5%)		
Perianal disease	75 (21.4%)		
CD location at follow up			
Ileal disease	92 (25%)		
Colonic disease	130 (35%)		
Ileal and colonic	103 (28%)		
Upper GI disease	49 (13%)		
CD behaviour at diagnosis			
Inflammatory (Vienna B1)	258 (74.8%)		
Stricturing (Vienna B2)	30 (8.4%)		
Penetrating (Vienna B3)	59 (16.8%)		
CD behaviour at follow up			
Inflammatory (Vienna B1)	142 (38%)		
Stricturing (Vienna B2)	68 (18%)		
Penetrating (Vienna B3)	164 (44%)		

IQR, interquartile range.

Table 2 Demographics and allelic frequencies in the blood transfusion samples and healthy volunteer control samples

	Blood transfusion samples	Healthy control samples
Age (y) (median (IQR))	35 (26–47)	36 (29–51)
Sex (M/F)	83/79	52/55
Allelic frequency		
113A	14.4%	12%
rs2289311	30.4%	33%
rs1050152	43.5%	42.4%
rs26313667	49%	46.1%
IGR2198	42%	40.4%

IQR, interquartile range.

Allelic frequencies of DLG5 variant single nucleotide polymorphisms (SNPs) 113A, rs2289311, OCTN1 variant rs1050152, OCTN2 variant rs26313667, and IBD5 marker SNP IGR2198 are shown to illustrate the consistency between the blood transfusion controls and the healthy volunteer controls.

typed for polymorphisms of the CARD15 gene (R702W, G908R, and 1007fsinsC) using previously described methods.¹³ All genotyping except R702W was carried out using the Taqman system. R702W genotyping was performed by restriction fragment length polymorphism polymerase chain reaction. Restriction digestion was performed using 1 u MspI at 37°C overnight and polymerase chain reaction fragments run on 4% NewSieve 3:1 agarose gels. These were stained with ethidium bromide and viewed under ultraviolet light. An image was recorded digitally.

Data analysis

The two SNPs rs1248696 and rs2289311 were analysed for association with IBD overall, CD, UC, and disease phenotype. Allelic frequency, carrier frequency, heterozygosity, and homozygosity rates were studied. Each allele was shown to be in Hardy-Weinberg equilibrium. Genotype-phenotype associations were analysed by χ^2 analysis using the Minitab statistical software package version 13/02 (Minitab Ltd, Coventry, UK). To identify significant independent variables associated with genotype, univariate and multivariate analysis was carried out. Evidence for DLG5 epistasis with CARD15 was investigated by stratifying DLG5 variants by carriage of one or more of the three common CARD15 variants—R702W, G908R, and 1007fsinsC. Allelic frequencies of the DLG5 variants were compared between the subgroups of patients with and without CARD15 variants by χ^2 analysis. The null hypothesis was that the frequency of DLG5 variants did not differ between these subgroups. Phenotypic associations of DLG5 variants were also stratified for the presence and absence of CARD15 variants.

RESULTS

Disease susceptibility: haplotype D (113A)

On analysis of the DLG5 variant 113A there were no associations with IBD when allelic frequency (11.4% IBD v 13.2% healthy controls (HC); $p=0.30$), carrier frequency (19.2% IBD v 24.6% HC; $p=0.069$), and homozygosity rates (2.3% IBD v 1.5% HC; $p=0.48$) were analysed (table 3). A negative association was observed between heterozygous rates of 113A and IBD (16.9% IBD v 23% HC; $p=0.033$). Furthermore, a negative correlation was observed between heterozygous 113A variants and CD (16% CD v 23% HC; $p=0.029$). No associations were observed between 113A variant allelic frequency (11.4% CD v 13.2% HC; $p=0.37$), carrier frequency (18.3% CD v 24.6% HC; $p=0.057$), homozygosity rates ($p=0.36$), and CD. No associations between 113A and UC were observed—allelic frequency (12.8% UC v 13.2% HC; $p=0.34$), carrier frequency (20.3% UC v 24.6% HC; $p=0.23$), heterozygous rates (18% UC v 23% HC; $p=0.45$), and homozygous rates (2.3% UC v 1.5% CD; $p=0.5$).

DLG5 haplotype A

Haplotype A allelic frequencies, represented by rs2289311 variants, did not differ between HC (31.5%) and IBD (35%; $p=0.17$), CD (36.9%; $p=0.078$), or UC (33.4%; $p=0.51$) patients. No significant differences were observed between carriage rates of rs2289311 variants—HC (52%), IBD (57.2%; $p=0.18$), CD (60.9%; $p=0.052$), and UC (54%; $p=0.65$). The frequency of patients who were heterozygotes or homozygotes for rs2289311 polymorphisms did not differ between IBD and HC groups (heterozygote HC 41.1% v IBD 44.8% ($p=0.43$), CD 48% ($p=0.13$), and UC 42.1% ($p=0.83$); homozygote HC 10.9% v IBD 12.6% ($p=0.49$), CD 12.6% ($p=0.61$), and UC 12.9% ($p=0.50$)).

Phenotypic analysis

On univariate analysis of CD patients, no association was observed between DLG5 113A variants and the Vienna classification for age of diagnosis, location of disease, or disease behaviour. Location of disease and disease behaviour in CD patients was analysed at the time of diagnosis and at the most recent follow up and there was no association between DLG5 113A variants and disease progression. No association was observed between DLG5 113A variants and age at diagnosis in the IBD and UC groups and there was no association between DLG5 113A variants, disease extent, and severity in UC patients.

DLG5 113A variants displayed a trend towards being less common in IBD patients with joint problems (large joint arthralgias related to disease activity, small joint arthralgias unrelated to disease activity, ankylosing spondylitis, and sacroiliitis) ($n=127$) compared with those who had no joint problems when allelic frequency was analysed (7.5% v 11.8%;

Table 3 DLG5 113A variant allele frequency, carrier frequency, heterozygote frequency, and homozygote frequency in the inflammatory bowel disease, Crohn's disease, ulcerative colitis, and control populations

	Controls	IBD (p value)	Crohn's disease (p value)	Ulcerative colitis (p value)
Allelic frequency	13.2%	11.4%	11.4%	11.4%
Carrier frequency	63/256 (24.6%)	125/652 (19.2%) $p=0.30$	65/356 (18.3%) $p=0.37$	60/296 (20.3%) $p=0.34$
Heterozygosity rates	59/256 (23%)	110/650 (16.7%) $p=0.069$	57/356 (16%) $p=0.057$	53/294 (18%) $p=0.228$
Homozygosity rates	4/256 (1.5%)	15/650 (2.3%) $p=0.033$	8/356 (2.2%) $p=0.029$	7/294 (2.4%) $p=0.45$
		$p=0.48$	$p=0.36$	$p=0.50$

The p values shown are calculated between the control group and each respective disease group.

$p = 0.053$). When allelic frequency of 113A variants was analysed in UC patients with primary sclerosing cholangitis, a trend towards these patients having fewer 113A variants was observed (0% ($n = 7$) *v* 11.4%; $p = 0.17$). On analysis of the haplotype A, no genotype-phenotype associations were observed in the CD and UC patient groups. Multiple logistic regression analysis did not identify any variables that were independently associated with haplotype D (113A) or haplotype A.

There was no evidence of epistasis between DLG5 113A variants and carriage of the three common CARD15 variants Gly908Arg, Arg702Trp, and Leu1007fsinsC—CARD15 carriage positive DLG5 113A allelic frequency 9.7% ($n = 108$) versus CARD15 carriage negative DLG5 113A allelic frequency 11.6% ($n = 584$) ($p = 0.43$). When CD patients were stratified for CARD15 variant carriage, no significant genotype-phenotype relationships were found with DLG5 113A.

DISCUSSION

The present study has demonstrated that in the Scottish population, traditionally characterised by low rates of admixture, the DLG5 variant 113A, representing haplotype D, is not a critical determinant of susceptibility in either CD or UC. In fact, heterozygous rates of 113A were significantly higher in the healthy control population when compared with the IBD cohort and the CD cohort. Haplotype A represented by the SNP rs2289311 was not protective in our CD or UC population.

These data differ markedly from those of Stoll *et al* who showed that in a German population, DLG5 113A variants were overtransmitted to individuals with IBD, and in a case control study there were significantly higher rates of 113A carriage in the IBD group compared with the control group (25% *v* 17%; $p = 0.001$).¹⁶ Considerations, including sample size and phenotypic differences, between the present study and that of Stoll *et al*, may be responsible for the observed discrepancy in results but a more plausible explanation would be genetic heterogeneity between the populations of Germany and Scotland. This has been clearly illustrated by data now available with respect to the three common polymorphisms of the NOD2/CARD15 gene (G908R, R702W, and 100fsinsC) which are significantly more common in the Central European CD population^{6,24} than in the Northern European CD population.^{13,14,25} Furthermore, CARD15 polymorphisms are absent in Japanese and Chinese CD populations.^{26–28} The different incidences of the R702W polymorphism has also been shown in healthy volunteers in Europe, Africa, and Asia.²⁹ In the eight independent groups worldwide who have performed genome wide scans in IBD patients, chromosome 10 has not met the stringent criteria for significant linkage.^{30,31} This would suggest that if indeed DLG5 plays a role in the pathogenesis of IBD, its contribution may be limited to specific populations.

Further data illustrating genetic heterogeneity in European IBD patients have been observed on analysis of the Asp299Gly mutation of the TLR4 gene.³² TLR4 is a member of the Toll-like receptor family which are involved in recognition of pathogen associated molecular patterns by the immune system and TLR4 functions as an extracellular pattern recognition receptor for lipopolysaccharide which is common to many intraluminal bacteria.³³ The Asp299Gly variant has been shown to confer susceptibility to CD and UC in the Belgian population³⁴ but no association was observed between Asp229Gly variants and IBD in German and Scottish cohorts.^{13,35}

Univariate and multivariate phenotypic analysis showed no associations with DLG5 113A variants (haplotype D) and the Vienna classification of CD or with age of onset of disease or disease severity in UC. Trends were observed towards a

lower DLG5 113A variant frequency in CD patients with arthropathy and UC patients with primary sclerosing cholangitis (PSC) on univariate analysis. Both of these extraintestinal complications have been shown to have a molecular genetic basis. Susceptibility to axial arthropathy has been strongly associated with HLA-B*27 in patients with and without IBD,³⁶ and peripheral arthropathies in patients with IBD have been associated with HLA-DRB1*0103, HLA-B*35, HLA-B*27, and HLA-B*44.³⁷ In PSC, strong disease associations with extended HLA haplotypes have been observed,³⁸ and a functional variant of stromolysin (matrix metalloproteinase 3) has also been associated with susceptibility to PSC and with disease progression.³⁹ In the current investigation, the relatively small numbers of IBD patients studied with these specific extraintestinal manifestations mean that these results regarding any DLG5 effect in these subgroups should be regarded as exploratory observations. Replication studies in other cohorts may help shed some light on this question.

No evidence of epistasis between DLG5 113A variants and carriage of the three common CARD15 variants Gly908Arg, Arg702Trp, and Leu1007fsinsC was observed in patients with CD. Again, these data contrast with Stoll *et al* who found significantly greater transmission of DLG5 113A in patients with CD carrying one of the risk associated alleles of CARD15.¹⁶ CARD15 variants have consistently been associated with a younger age of onset of disease, ileal disease, and stricturing disease.⁴⁰ A possible explanation for the absence of epistasis between DLG5 and CARD15 could be the low incidence of CARD15 variants in the Scottish CD population (1007fsinsC = 4.7%, G908R = 1.8%, and R702W = 7.1%) and the combined population attributable risk these variants confer (11%).¹³ Furthermore, Moore has illustrated the problems in using statistical epistasis to interpret genetic and biological phenomena.⁴¹

Although the present data suggest these DLG5 variants are not important determinants in the Scottish IBD population, our own recent studies suggest that NOD2/CARD15, MDR1, and IBD5 variants are involved in disease susceptibility and behaviour.^{13,42} The identity of other genetic determinants in the Northern European IBD population remains under detailed investigation.

In conclusion, in our North European study population, we were unable to replicate Stoll's data that the DLG5 variant 113A confers susceptibility to IBD. Haplotype A represented by the SNP rs2289311 did not confer protection in our population. Further genetic studies of DLG5 polymorphisms in IBD populations are required to elucidate whether these variants play a role in the pathogenesis of IBD. These studies must be complemented by data regarding the expression and function of DLG5 in the gastrointestinal tract.

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Hopkins Endoscopy Prize 2006

Applications are invited by the Endoscopy Committee of the British Society of Gastroenterology who will recommend to Council the recipient of the 2006 Award. Applications (10 copies) should include:

- A manuscript (two A4 pages **only**) describing work conducted
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- An outline of the proposed content of the lecture, including title
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