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Association of the T allele of an intronic single nucleotide polymorphism in the colony stimulating factor I receptor with Crohn's disease: a case-control study

Adriana Zapata-Velandia^{1,4}, San-San Ng^{2,4}, Rebecca F Brennan¹, Neal R Simonsen⁵, Mariella Gastanaduy^{1,4}, Jovanny Zabaleta^{3,4}, Jennifer J Lentz², Randall D Craver³, Hernan Correa³, Alberto Delgado³, Angela L Pitts^{2,4}, Jane R Himel^{1,4}, John N Udall Jr¹, Eberhard Schmidt-Sommerfeld¹, Raynorda F Brown¹, Grace B Athas³, Bronya B Keats⁴ and Elizabeth E Mannick*^{1,4}

Address: ¹Louisiana State University Department of Pediatrics, New Orleans, LA 70112, USA, ²Louisiana State University Department of Genetics, New Orleans, LA 70112, USA, ³Louisiana State University Department of Pathology, New Orleans, LA 70112, USA, ⁴Stanley S. Scott Cancer Center, New Orleans, LA 70112, USA and ⁵Louisiana State University School of Public Health, New Orleans, LA, 70112 USA

Email: Adriana Zapata-Velandia - azapat@lsuhsc.edu; San-San Ng - sng@lsuhsc.edu; Rebecca F Brennan - rbrenn@lsuhsc.edu; Neal R Simonsen - nsimon@lsuhsc.edu; Mariella Gastanaduy - emanni@lsuhsc.edu; Jovanny Zabaleta - jzabal@lsuhsc.edu; Jennifer J Lentz - jlentz@lsuhsc.edu; Randall D Craver - rcrave@lsuhsc.edu; Hernan Correa - hcorre@lsuhsc.edu; Alberto Delgado - adelga@lsuhsc.edu; Angela L Pitts - apitts@lsuhsc.edu; Jane R Himel - mhimmel52@yahoo.com; John N Udall - judall@lsuhsc.edu; Eberhard Schmidt-Sommerfeld - eschmi@lsuhsc.edu; Raynorda F Brown - rbrown@lsuhsc.edu; Grace B Athas - gathas@lsuhsc.edu; Bronya B Keats - bkeats@lsuhsc.edu; Elizabeth E Mannick* - emanni@lsuhsc.edu

* Corresponding author

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Abstract

Background: Polymorphisms in several genes (NOD2, MDRI, SLC22A4) have been associated with susceptibility to Crohn's disease. Identification of the remaining Crohn's susceptibility genes is essential for the development of disease-specific targets for immunotherapy. Using gene expression analysis, we identified a differentially expressed gene on 5q33, the colony stimulating factor I receptor (CSFIR) gene, and hypothesized that it is a Crohn's susceptibility gene. The CSFIR gene is involved in monocyte to macrophage differentiation and in innate immunity.

Methods: Patients provided informed consent prior to entry into the study as approved by the Institutional Review Board at LSU Health Sciences Center. We performed forward and reverse sequencing of genomic DNA from 111 unrelated patients with Crohn's disease and 108 controls. We also stained paraffin-embedded, ileal and colonic tissue sections from patients with Crohn's disease and controls with a polyclonal antibody raised against the human CSFIR protein.

Results: A single nucleotide polymorphism (A2033T) near a RunxI binding site in the eleventh intron of the colony stimulating factor I receptor was identified. The T allele of this single nucleotide polymorphism occurred in 27% of patients with Crohn's disease but in only 13% of controls ($X^2 = 6.74$, $p < 0.01$, odds ratio (O.R.) = 2.49, $1.23 < \text{O.R.} < 5.01$). Using immunohistochemistry, positive staining with a polyclonal antibody to CSFIR was observed in the superficial epithelium of ileal and colonic tissue sections.

Conclusions: We conclude that the colony stimulating factor receptor I gene may be a susceptibility gene for Crohn's disease.

Introduction

Crohn's disease is a chronic intestinal disorder of unknown etiology characterized by weight loss, abdominal pain, diarrhea, arthritis and the development of fistulae and abscesses. It causes significant morbidity and affects approximately 1 in 1000 individuals in the developed world. Crohn's disease is believed to ensue from the action of an environmental trigger(s) including alteration in host intestinal flora on a genetically susceptible host mucosal immune system and intestinal epithelial barrier [1]. A variety of Crohn's disease susceptibility loci have been identified by genetic mapping studies. The first Crohn's disease susceptibility gene, NOD2, was identified definitively in 2002 by positional cloning and linkage disequilibrium mapping as well as candidate gene approaches [2,3]. NOD2 encodes an intracellular receptor for muramyl dipeptide, a component of the peptidoglycan moiety of bacterial cell walls, and triggers a cascade of signaling events resulting in the activation of NF-kappa B and the host innate immune system [4]. NOD2 is expressed in monocytes and in intestinal epithelial cells, including Paneth cells [5]. Crohn's disease-associated mutations in NOD2 result in defective NF kappa B activation, suggesting that Crohn's disease may represent, in part, a defect in innate immunity [4].

A second gene that has recently been linked to Crohn's disease and ulcerative colitis susceptibility is the multidrug resistance transporter 1 (MDR1). A single nucleotide polymorphism (SNP) in the coding region of the gene (Ala893Ser/Thr) has been found to occur more frequently in patients with Crohn's disease and a second SNP (C3435T) has been associated with ulcerative colitis susceptibility [6,7]. The MDR1 gene encodes an ATP-binding cassette (ABC) family member that pumps neutral and cationic hydrophobic molecules out of the cell and plays a role in resistance to chemotherapy. Intriguingly, MDR1-/- mice develop spontaneous colitis in the presence of normal intestinal bacteria [8]. The MDR1 protein may also play a role in host defense against intracellular bacteria by extruding them from the cell, explaining its protective role in intestinal inflammation, but this hypothesis remains to be proven [8].

Identification of additional Crohn's disease susceptibility genes is important to complete the puzzle of Crohn's disease pathogenesis and to develop specific, targeted immunotherapies. A region of broad susceptibility to inflammatory bowel disease has been identified on chromosome 5q31-5q33 and is known as IBD5 [9]. Within this region, a Crohn's disease susceptibility haplotype comprising a cytokine cluster on 5q31 has been identified [10]. Interestingly, a missense substitution in SLC22A4, a gene in this region that is a downstream target of the transcription factor, Runx1, is associated with susceptibility to

Crohn's disease [11]. Moreover, an intronic SNP in a Runx1 binding site of SLC22A4 has been found in rheumatoid arthritis, an autoimmune disease that sometime occurs in individuals and families affected by Crohn's disease [12]. Polymorphisms in the promoter of the CD14 gene, which plays a critical role in lipopolysaccharide signaling and is located downstream from the cytokine cluster, have been linked to Crohn's disease susceptibility in a case-control study [13].

To our knowledge, no Crohn's disease-related polymorphisms in genes located in the 5q32 or 5q33 region have been reported. Using microarray analysis to examine gene expression in endoscopic colonic biopsies from patients with newly diagnosed, untreated Crohn's disease, we identified an overexpressed gene on 5q33, CSF1R (unpublished data). We hypothesized that this gene was a candidate gene for Crohn's disease susceptibility. The CSF1R is a tyrosine kinase receptor proto-oncogene involved in monocyte to macrophage differentiation [14]. Although expression of CSF1R has been detected in epithelial cells of other organs, the expression of CSF1R in the intestine has not been well documented [15-18]. Here we report the results of a case-control study of Louisiana patients with Crohn's disease and ethnically similar controls showing increased prevalence of the T allele of a SNP (A2033T)* near an intronic Runx1 binding site in the CSF1R gene in patients with Crohn's disease. We also show, using immunohistochemistry, that the CSF1R protein is expressed in the superficial epithelium of the ileum and colon.

*This SNP occurs 2033 base pairs from the 3' end of the eleventh exon of the CSF1R gene.

Methods

Patients

Patients (n = 111) and controls (n = 108) were recruited in the study from Children's Hospital of New Orleans and private practices in Southeastern Louisiana and Western Mississippi after Louisiana Health Sciences Center Institutional Review Board (IRB) approval and informed consent and assent. Patient and control DNA were also obtained from archival colonic tissue blocks after IRB approval.

DNA extraction and purification

Genomic DNA was obtained from one of 3 sources for all subjects: peripheral blood buffy coat, buccal swab or paraffin-embedded archival tissue blocks. For blood, ten ml of whole blood was collected in purple top, EDTA tubes and buffy coats prepared using red blood cell lysis buffer (NH₄Cl, NH₄HCO₃, H₂O), pellet buffer (1 M Tris HCl pH 8.0, 0.5 M EDTA, NaCl, H₂O), 10% SDS and Proteinase K. Buffy coats were heated in a water bath overnight at 56°C

and stored at -20°C. DNA was extracted using phenol:chloroform:isoamyl alcohol, followed by chloroform, and precipitated in 100% ethanol. After air drying, the pellet was resuspended in TE buffer and its quantity and integrity were verified by 1% agarose gel and spectrophotometry (Beckman Coulter, DU640B).

DNA was extracted from buccal swabs (Epicentre Technologies, Madison, Wisconsin) following the manufacturer's instructions. Briefly, swabs were placed in DNA extraction solution, mixed for ten seconds and incubated at 60°C for 30 min, then a total of 16 min at 98°C. After centrifugation at 10,000 × g at 4°C, the supernatant was transferred to a clean tube and stored at -20°C.

For archival tissue blocks, 3 sections of 10 µm were cut and incubated twice with 1 ml of n-octane (Sigma, St. Louis, MO) at 56°C for 15 min. After centrifugation at 10,000 × g at room temperature (RT), the pellet was resuspended in 1 ml 100% EtOH and then in 1 ml 75% EtOH. After the last centrifugation, the pellet was resuspended in 85 µl of pellet buffer (10 mM Tris-HCL, pH 8.0, 10 mM EDTA, pH 8.0, 150 mM NaCl) followed by 5 µl of Proteinase K (20 mg/ml) (Invitrogen, Grand Island, NY) and 10 µl 10% SDS (Invitrogen). The samples were incubated overnight at 56°C. One hundred µl of phenol chloroform:iso-amylalcohol (50:1) (Sigma) was added and the sample was centrifuged at 10,000 × g for 5 min at RT. The aqueous phase was transferred to a clean tube and 100 µl of chloroform were added (Sigma). The sample was centrifuged at 10,000 × g for 5 min and the aqueous phase transferred to a clean tube and mixed with 200 µl 100% ethanol (Aldrich) and incubated at -70°C for at least 1 hr. The DNA was precipitated by centrifugation and resuspended in TE buffer. The DNA concentration was determined by UV spectrophotometry.

PCR

Forward and reverse primers to amplify DNA in the vicinity of the SNP of interest were designed using the Primer QuestSM (Integrated DNA Technologies (IDT), Coralville, IA) program and ordered from IDT. The primer sequences are: (F) 5'TTC TCT GAG CAG CTC CAA TG3' and (R) 3'CCA CAG ACA GGC CAC TTC TT5'.

Master Mix for PCR was prepared using Taq polymerase, dNTPs and other reagents from Invitrogen (Carlsbad,

CA). After optimization of conditions, PCR reactions were carried out in a Bio-Rad I-cycler. The PCR product was resolved on a 1% agarose gel and purified using Qiaquick DNA Purification Kit (Qiagen, Valencia, CA).

DNA Sequencing

Forward and reverse DNA sequencing to detect the A2033T SNP in intron 11 of the CSF1R gene was performed in the LSU Sequencing Core. Briefly, in a 0.2 mL PCR tube, DNA template, primer, BigDye Terminator Ready Reaction Mix (PE Applied Biosystems, Foster City, CA), 5X sequencing mix, and HPLC water were combined to 20 µl.

Tubes were placed in a thermal cycler (GeneAmp PCR 9700) set to the following program.

30 cycles 96°C – 10 seconds

58°C – 5 seconds

60°C – 4 minutes

Extension products were purified by adding 3 M NaOAc, pH 4.6 and 95% EtOH to reaction tubes for 20 m and spinning tubes upright at 3600 rpm for 30 m. Tubes were then inverted and spun at 700 rpm for 1 m. After washing the pellet in 70% EtOH, tubes were spun at 3600 for 10 m. The procedure beginning with inversion of tubes was repeated, tubes centrifuged at 700 rpm for 1 m and air dried. To analyze the sequencing reaction, formamide was added to each tube and denatured for 3 m at 95°C, followed by wet ice. The sequencing gel was prepared using urea, HPLC water, Long Ranger 50% (PE Applied Biosystems) and 10X TBE buffer, stirring for 1 h. 10% APS and TEMED were added to the filtered gel solutions and gel was loaded into a cassette with glass plates in an ABI 3100 automated sequencer equipped with ABI PRISM Data Collection Software. 1X TBE was used as running buffer for gel electrophoresis. Fluorescent dye labels were used to incorporate into DNA extension products. Four different dyes were used to identify the A, C, G, and T extension reactions using an argon laser.

Immunohistochemistry

Table 1: Crohn's Disease Status vs CSF1R A2033T SNP: All Patients

	CD-	CD+	Total
T Allele Absent	94 (87%)	81 (73%)	175
T Allele Present	14 (13%)	30 (27%)	44
Total	108	111	219

$\chi^2 = 6.74$ ($p < 0.01$) $O.R.: 2.49$ ($1.23 < O.R. < 5.01$)

Table 2: A2033T SNP Allele by Ethnicity

CROHN'S PATIENTS	T Allele Absent	T Allele Present	Total
Acadian	10 (53%)	9 (47%)	19
African-American	17 (85%)	3 (15%)	20
Caucasian	44 (75%)	15 (25%)	59
Hispanic	2 (100%)	0 (0%)	2
Jewish	4 (80%)	1 (20%)	5
Unknown	4 (67%)	2 (23%)	6
Total	81	30	111
CONTROL PATIENTS	T Allele Absent	T Allele Present	Total
Acadian	36 (84%)	7 (16%)	43
African-American	4 (57%)	3 (43%)	7
Caucasian	32 (91%)	3 (9%)	35
Hispanic	7 (87%)	1 (13%)	8
Jewish	3 (100%)	0 (0%)	3
Unknown	12 (100%)	0 (0%)	12
Total	94	14	108

Table 3: Ethnicity vs CSFIR A2003T SNP: Crohn's Patients

	Acadian	Non-Acadian	Total
T Allele Absent	10 (53%)	71 (77%)	81
T Allele Present	9 (47%)	21 (23%)	30
Total	19	92	111

$\chi^2 = 4.81$ ($p < 0.05$) O.R.:3.04 (1.09 < O.R. < 8.47)

Table 4: Crohn's Disease Status vs CSFIR A2033T SNP: Non-Acadian Patients

	CD-	CD+	Total
T Allele Absent	58 (89%)	71 (77%)	129
T Allele Present	7 (11%)	21 (23%)	28
Total	65	92	157

Fisher exact test ($p = 0.025$) O.R.:2.45 (0.97 < O.R. < 6.17)

Slides cut from paraffin-embedded tissue blocks were deparaffinized, hydrated, and blocked with 3% hydrogen peroxide at RT for 15 min. After rinsing in distilled water, they were placed in PBS for 2 min and then blocked with Biocare's Background Sniper (Biocare Medical, Walnut Creek, CA) for 10 min at RT. Slides were incubated with primary antibody (rabbit polyclonal antibody to human *c-fms*, Cymbus Biotechnology, Ltd., Chandlers Ford, Hants, UK) at a dilution of 1:100 for 60 min at RT and, after rinsing with PBS, incubated with secondary antibody (MACH 2 Rabbit-HRP Polymer, Biocare Medical) for 30

min at RT. After rinsing with PBS, slides were placed in diaminobenzamide for 7 min at RT, rinsed in 2 changes of distilled water, counterstained with hematoxylin, dehydrated and mounted with resinous medium.

Data analysis

Numbers of patients with the T allele of the A2033T SNP were compared to numbers of controls using a chi-square statistic. An odds ratio with 95% confidence interval was calculated using SAS software (SAS, Cary, NC).

Results

Association of the T allele of the A2033T SNP with Crohn's disease especially in patients of Acadian descent

A SNP (A2033T) was detected in the eleventh intron of the CSF1R gene that was located 77 base pairs downstream from a Runx1 binding site (TGTGGT). Forward and reverse sequencing of this SNP was performed in 111 patients with Crohn's disease and 108 controls (Table 1). Thirty patients with Crohn's disease (27%) but only fourteen controls (13%) had the T allele of the SNP ($\chi^2 = 6.74$, $p < 0.01$, O.R. = 2.49 with 95% confidence interval, $1.23 < \text{O.R.} < 5.01$). Data from the stratification of Crohn's patients and control patients by ethnicity (Table 2) suggest that the differential expression of the SNP was more pronounced in some ethnic groups (Acadian) than in others (African American). In the case of Crohn's patients of Acadian descent, the rate of the T allele (47% vs. 23%) was significantly higher than the rate of the T allele in all other ethnicities combined (Table 3; $\chi^2 = 4.01$, $p < 0.05$, O.R. = 3.04 with 95% confidence interval, $1.09 < \text{O.R.} < 8.47$). Since there were more controls of Acadian descent than patients of Acadian descent (Table 2), it is unlikely that the higher rate of the T allele of the A2033T SNP noted in Crohn's patients in general (Table 1) can be attributed to ethnicity. However, to exclude the possibility that Acadian ethnicity was a confounding variable, we compared rates of the T allele in non-Acadian patients with Crohn's disease to those of non-Acadian controls (Table 4). In this analysis, patients with Crohn's disease still had significantly higher rates of the T allele than controls.

The CSF1R protein is expressed in the superficial epithelium of the ileum and colon

Paraffin-embedded, formalin-fixed tissue sections from control patients were stained with a rabbit anti-human polyclonal antibody to CSF1R and read by a pathologist. Negative controls were stained with secondary antibody alone. Positive cytoplasmic staining was noted in the superficial epithelium of the ileum and colon with differentiated cells being sloughed off into intestinal lumen staining most vividly (Figure 1A,1B,1C). Examination of the intracellular staining pattern revealed a characteristic staining pattern with the terminal web and the lateral junctions of intestinal epithelial cells (Figure 1A).

Discussion

Based on gene expression data, chromosomal location and biological function, we have evidence that the colony stimulating factor 1 receptor gene may contribute to Crohn's disease susceptibility. Using a case-control study, we have shown that a SNP in an intron of this gene is associated with Crohn's disease. Whether this SNP is in close proximity to another disease-causing SNP in the same (or a neighboring) gene or is itself disruptive of gene func-

tioning in a way that increases susceptibility to Crohn's disease will be the focus of future studies.

The CSF1R gene is an intriguing candidate gene for Crohn's disease susceptibility for several reasons. First, it is involved in innate immunity and host defense against fungi and certain bacteria such as *Listeria* that have been postulated to play a role in Crohn's disease pathogenesis [19,20]. Second, CSF1R is involved in an intracellular signal transduction cascade linking the G alpha i2 receptor to the transcription factor Stat3. In NIH3T3 cells expressing a dominant negative G alpha i2, Stat3 phosphorylation by v-fms (oncogenic CSF1R) was inhibited [21]. This is significant because targeted disruption of either the G alpha i2 gene or the Stat3 transcription factor (in monocytes) results in inflammatory bowel disease in rodents [22,23]. These data suggest that a signaling pathway involving G alpha i2, Stat3 and CSF1R is critical for protection against intestinal inflammation. One possible mechanism is the regulation of IL-10, a cytokine known to be essential for normal intestinal homeostasis [24]. Third, acute myelogenous leukemia and myelodysplasia, two conditions that may occur with increased frequency in the context of Crohn's disease, are associated with polymorphisms or deletions in the CSF1R gene [25-27].

There is a paucity of literature regarding the expression of the CSF1R protein in the intestine despite documentation of its presence in the epithelium of a variety of other tissues including breast, ovary, endometrium, lung and prostate [15-18,28]. Therefore, we examined its expression by immunostaining and found it to be expressed in the cytoplasm of certain epithelial cells of the superficial epithelium and villous tips of the ileum and colon, including cells that were being sloughed into the lumen. Because of this superficial location of staining, it is tempting to hypothesize that the CSF1R protein plays a role in differentiation of intestinal epithelial cells as it does in macrophages. The most intense cytoplasmic staining occurred in the terminal web of the epithelial cell and in the lateral junctions of the cells. The localization of CSF1R in actin-rich areas of the cell is not surprising in view of data from in vitro studies demonstrating that the CSF1R protein mediates morphological changes in macrophages through the regulation of paxillin and focal adhesions [29]. What role the CSF1R protein might play in cytoskeletal regulation in either mononuclear cells or in intestinal epithelial cells the intestine as well as its expression pattern and pathogenic role in inflammatory bowel disease remain to be investigated further.

Studies of the prevalence of NOD2 polymorphisms in patients with Crohn's disease point to a subset of patients with ileal and fibrostenotic disease who are more likely to have the NOD2 genotype [30]. Moreover, specific NOD2

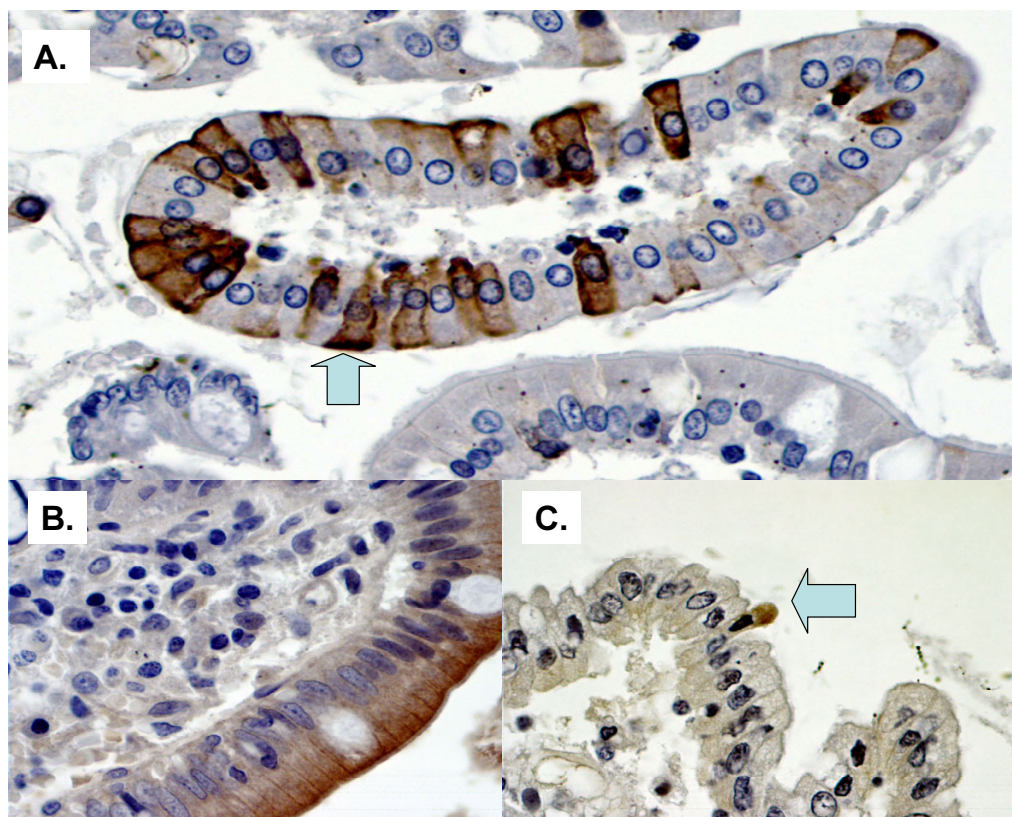


Figure 1

Sections from paraffin-embedded tissue blocks of normal human ileum and colon were stained with polyclonal rabbit anti-human antisera to CSF1R and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody with diaminobenzamide used as chromagen. **A.** Superficial epithelium of the terminal ileum with cytoplasmic staining of the terminal web and lateral junctions of the epithelial cells (green arrow). **B.** Staining of the superficial epithelium of the colon. **C.** Staining of the superficial epithelium of the ileum with an epithelial cell being sloughed off into the lumen (green arrow). 100 \times .

polymorphisms are more prevalent in some ethnic groups [31,32]. The numbers of patients enrolled in the current study do not permit conclusive analysis of disease subtype or ethnicity. However, we did find that patients of Acadian descent (descendants of émigrés from French Canada) have a higher prevalence of the disease-associated SNP in CSF1R. This is interesting because the population in which the IBD5 susceptibility locus was originally identified was, in part, French Canadian [9].

We do not know what the significance, if any, is of the location of the A2033T SNP near a binding site for the

transcription factor, RUNX1. It is intriguing to note, however, that SNPs in RUNX1 binding sites and in the RUNX1 gene itself have been associated with a variety of autoimmune conditions, including psoriasis, systemic lupus erythematosus, type I diabetes mellitus and rheumatoid arthritis [11,33-35]. Since the CSF1R gene is a target of RUNX1 and has multiple RUNX1 binding sites in several introns [35], complete sequencing of each of these sites will be performed to investigate the hypothesis that defective RUNX1 binding is related to Crohn's disease susceptibility [36].

Conclusions

In a case-control study of Louisiana patients with Crohn's disease, we have detected a SNP (A2033T) in the eleventh intron of the CSF1R gene that is significantly associated with the disease. We propose that the CSF1R gene is a candidate gene for Crohn's disease.

Competing interests

None declared.

Authors' contributions

AZV performed the molecular genetic studies and assisted in the recruitment of patients, the data analysis and the drafting of the paper. SSN performed the sequencing. RB recruited patients and ensured compliance with the IRB. NS performed the statistical analyses. MG assisted in data collection, entry and sequence alignment. JZ assisted with primer design and optimization. JL recruited patients and entered data. RC furnished archival tissue blocks. HC read and photographed stained slides. AD performed immunohistochemistry. AP performed sequencing. J.H. recruited patients. JU referred patients. ESS referred patients. RB referred patients. GA recruited patients and reviewed the manuscript. BK provided technical advice and assistance related to study design and analysis. EM conceived of the study, recruited patients, performed the data analysis and drafted the manuscript.

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