

Common NOD2 mutations are absent in patients with Crohn's disease in India

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Background: Crohn's disease is being increasingly diagnosed in the Indian subcontinent. Three apparently common mutations in the NOD2 gene are found in up to 30% of sporadic patients with Crohn's disease in western countries. We examined whether such mutations are also found in Indian patients with Crohn's disease.

Methods: Venous blood was collected from 82 patients (age range: 7–65 years, 53 men) with Crohn's disease and 149 control subjects; DNA was extracted and subjected to polymerase chain reaction using specific primers. The amplified fragments of size 185, 163 and 151 bp for R702W, G908R and 1007fs, respectively, were digested with MspI, HhaI and ApaI, and the restriction pattern noted after electrophoresis.

Results: Twenty-eight patients had ileocolonic disease, 26 ileal disease, 20 colonic disease and 8 had disease limited to proximal small bowel or stomach. None of the 82 patients showed any of the three NOD2 mutations. The control subjects (93 men) had a variety of chronic gastrointestinal disorders (ulcerative colitis 52, irritable bowel syndrome 30, intestinal tuberculosis 20, colon cancer 7, miscellaneous 37). None of the control subjects showed a mutation in any of the three NOD2 mutation analyses.

Conclusion: The three NOD2 gene mutations described above are uncommon in Indian patients with Crohn's disease. This study complements information provided by recent studies on NOD2 mutations in Indians.

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In 2001, mutations in the NOD2 gene were identified as a possible cause of Crohn's disease.^{1,2} This gene is present in the IBD1 region of chromosome 16q12 and its product belongs to the NOD1/APAF1 protein family, defects in which lead to a variety of auto-inflammatory diseases. The NOD2 gene product is an intracellular protein that is a pattern recognition receptor for muramyl dipeptide (MDP), the minimal motif in all peptidoglycans and serves as a general sensor of bacteria. It is expressed in the cytoplasm of myelomonocytic and intestinal epithelial cells, enhances host defense by inducing production of antimicrobial peptides such as human beta-defensin 2, and influences the function of effector or regulatory T cells.^{3,4} Specific mutations in the NOD2 gene lead to defective innate immune recognition of luminal microbes and result in intestinal inflammation.⁵

Three common mutations of this gene, all localized in the leucine-rich repeat region, are detected in patients with sporadic Crohn's disease in the West. These are the R702W, G908R and 1007fs mutations. They are present in approximately 20% of western patients with Crohn's

disease in whom these mutations are specifically associated with ileal and ileocolonic disease, early onset disease, and fibrostenotic disease.^{6,7} Crohn's disease, hitherto rare in the Indian subcontinent, has been diagnosed in increasing numbers in India in the past decade.⁸ The present study was undertaken to evaluate the frequency of these three mutations (R702W, G908R and 1007fs) of the NOD2 gene in patients with Crohn's disease.

Methods

Eighty-two consecutive patients with Crohn's disease and 149 patients (controls) with a variety of other gastrointestinal diseases attending the outpatient clinics of the Department of Gastrointestinal Sciences, Christian Medical College, Vellore between July 2003 and September 2005 were studied. The patients (53 men) with Crohn's disease ranged in age from 7 to 65 years (mean [SD] age 34.8 [12] years). The diagnosis of Crohn's disease was based on typical clinical findings supported by ileocolonoscopy and mucosal biopsy showing evidence of granulomatous bowel disease consistent with CD, or a surgical resection

specimen showing transmural inflammation and/or non-caseating granulomas in the bowel wall and/or mesenteric lymph nodes. All patients had a negative tuberculin skin test, and did not have pulmonary or lymph nodal tuberculosis. The diagnosis was confirmed by follow up for at least one year showing response to therapy with mesalazine and/or corticosteroid and azathioprine or 6-mercaptopurine. Disease extent was assessed by upper and lower gastrointestinal endoscopy with segmental biopsy, and imaging of the small intestine consisting of barium meal follow through study, computed tomography scan or both, and in some instances by operative findings and surgical pathology of the resected specimen. Nine milliliters venous blood was collected in 9 mL EDTA-coated Vacutainer tubes from all subjects after obtaining written informed consent. The protocol was approved by the Research Committee of our institution.

DNA was extracted from whole blood by digestion with proteinase K followed by phenol/chloroform extraction. PCR was carried out using specific primers shown in the Table. Touchdown PCR was done in a PTC-150 MiniCycler (MJ Research, USA). The PCR protocol for R702W comprised initial denaturation for 30 sec at 95°C, 20 cycles of touchdown PCR comprising denaturation (30 sec, 94°C), annealing (30 sec, 70°C to 66°C in 0.2°C decrement every cycle), and extension (30 sec, 72°C), followed by 20 cycles comprising denaturation (20 sec, 94°C), annealing (20 sec, 65°C) and extension (20 sec, 72°C), followed by final extension at 72°C for 10 min. The PCR protocols for G908R and 1007fs were similar except that the annealing temperatures varied from 65°C to 61°C during the initial 20 touchdown cycles and 60°C in the final 20 cycles. PCR products were resolved using 2% agarose gel electrophoresis to confirm specific amplification.

Restriction fragment length polymorphism (RFLP) analysis was carried out as described by Helio *et al.*⁹ Briefly, fragments corresponding to R702W, G908R and 1007fs were digested with 2U of MspI, HhaI and ApaI (MBI Fermentas, USA), respectively, at 37°C for 16

hours. The digested products were resolved on 3% agarose gels and the bands noted using a Gel Doc 2000 gel documentation system.

Sample size was calculated using the western data on NOD2 mutation frequencies. A sample size of 64 cases was calculated assuming that at least one of the three mutations would be found in 4% of control subjects and in 20% of patients with Crohn's disease (cases), with an alpha error of 0.05 and a power of 90% based on 2:1 control: case allocation. The sample size was exceeded to allow for dropouts resulting from change in diagnosis at follow up or loss to follow up.

Results

The mean (SD) duration of illness was 35 (26) months. The disease location was ileocolonic (28), ileal (26), colonic (20) and proximal small bowel or stomach (8). Sixteen patients gave a history of smoking; the rest denied tobacco consumption in any form. The presentation symptoms were diarrhea, hypoproteinemia and anemia (n=35), severe intermittent abdominal pain suggestive of partial small bowel obstruction (35), iron deficiency anemia (6), fever of unknown origin (2), and frank bleeding per rectum (3). Seven patients had fistulizing disease (3 perianal, 4 abdominal fistulae). Forty patients had received anti-tuberculous treatment prior to diagnosis of Crohn's disease. All the patients received mesalazine; in addition, 28 received steroids, and 10 received azathioprine. The mean (SD) follow up duration was 38 (28) months. In 24 patients, the diagnosis was confirmed by surgical pathology following resection of bowel – small bowel (12), right hemicolectomy (10), and colonic resection (2). Extraintestinal manifestations included sacroiliitis (4), enteropathic arthropathy (6), erythema nodosum (2), spondyloarthropathy involving the lumbosacral spine (1) and episcleritis (1).

The control subjects (94 men, 56 women; mean age 41.3 [SD 12.8] years) included patients with ulcerative

Table: Primers, PCR product sizes, restriction enzymes and restriction digest product sizes for the three NOD2 mutations

Mutation	Forward and reverse primer sequences	PCR product size	Restriction enzyme	Digestion product size
R702W	5'-AGATCACAGCAGCCTTCCTG-3' 5'- CACGCTCTTGGCCTCACC-3'	185 bp	MspI	Wild type: 20, 35, 54 and 76 bp Heterozygous: 20, 35, 54, 76 and 130 bp Mutant: 20, 35 and 130 bp
G908R	5'-CTCTTTTGGCCTTTTCAGATTCTG-3' 5'-CAGCTCCTCCCTCTTCACCT-3'	163 bp	HhaI	Wild type: 163 bp Heterozygous: 27, 136 and 163 bp Mutant: 27 and 136 bp
1007fs	5'-GGCAGAAGCCCTCCTGCAGGGCC-3' 5'- CCTCAAATTCTGCCATTCC-3'	151 bp	ApaI	Wild type: 151 bp Heterozygous: 20, 131 and 151 bp Mutant: 20 and 131 bp

colitis (52), irritable bowel syndrome (30), intestinal tuberculosis (24), colon cancer (7), anemia (6), solitary rectal ulcer (4), infective colitis, diabetic diarrhea, polyps, and habitual constipation (3 each), cirrhosis and hemorrhoidal bleed (2 each), hepatitis A, alcoholic liver disease, carcinoid tumor, rectal prolapse, short gut syndrome, vasculitis, gallstones, leiomyoma colon, non-ulcer dyspepsia, tuberculous ascites (1 each).

The PCR-RFLP analyses for G908R, R702W and 1007fs mutations did not show any abnormalities in any of the patients or controls.

Discussion

The genesis of Crohn's disease in south Asians remains unknown. Studies of Indian and Bangladeshi immigrants to the UK show that the genetic background for development of Crohn's disease exists in this population.^{10,11} Three common mutations in the NOD2 gene, found in about 20% of western patients with Crohn's disease, are particularly associated with ileal disease, penetrating disease and a need for surgery.^{6,7,9} The present study suggests that these particular NOD2 mutations are not important in the genesis of Crohn's disease in Indians.

A recent study from northern India examined 298 patients with ulcerative colitis, 25 patients with Crohn's disease and 262 controls, and found that the above three mutations were extremely infrequent.¹² Only the G908R mutation was found in 2 patients with ulcerative colitis. Reports from other Asian countries confirm that these three common NOD2 gene mutations are rare or absent in patients with Crohn's disease.^{13,14,15} The last mentioned study compared 65 patients with Crohn's disease to 63 patients with ulcerative colitis and 70 patients with dyspepsia. The present study used hospital patients with a diagnosis other than Crohn's disease as the control subjects, and found that none of the cases or the controls had any of the three NOD2 mutations that account for NOD2-related Crohn's disease in the West.

Phenotypically, Crohn's disease in our patients was similar to that described in Western patients. Importantly, the study group included 28 patients with ileocolonic disease and 26 patients with ileal disease, the patterns associated with the NOD2 mutations in Western populations. The absence of the common NOD2 mutations in any of these patients indicates a need to search further for the etiology of Crohn's disease in Indians.

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