

Evolution of Crohn's disease-associated *Nod2* mutations

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Abstract Several lines of evidence have confirmed the importance of *Nod2* mutations for disease susceptibility in Crohn's disease. For tracing *Nod2* evolution, exons 4a, 4e, 8, and 12 mutations were screened in a collection of 1,064 DNA samples from 52 worldwide populations. The overall allele frequency was 7.5% for single nucleotide polymorphism (SNP)5, 0.2% for SNP8, 0.3% for SNP12, and 0.4% for SNP13. *Nod2* mutations are mainly Caucasian alleles with strong distribution dissimilarity between single populations and major geographical regions. This regional diversity of *Nod2* mutations within Europe points to the regional existence of selection pressure (possibly through dairy-associated bacterial infections within Neolithic cattle farming populations). The SNP5 gradient between Africa and the Middle East and its absence in Asian and Native American populations indicate that the evolution of this variant occurred in the Middle East. As mutations in exons

4e, 8, and 12 were only found in association with SNP5, this variant may have allowed selection pressure to arise.

Keywords *Nod2* · CARD15 · Crohn's disease · SNP

Abbreviations

IBD	inflammatory bowel disease
CD	Crohn's disease
SNP	single nucleotide polymorphism
CARD	caspase-activation and recruitment domain
NBD	nucleotide binding domain
LRR	leucine-rich repeat
NF- κ B	nuclear factor kappa B
Dhplc	denaturing high-performance liquid chromatography

Crohn's disease (CD) is a chronic transmural inflammatory disease of the gastrointestinal tract which mainly affects the small bowel and the colon. The disease process is characterized by T-cell-mediated tissue destruction and aberrant immune response to antigens derived from the intestinal microbial milieu (Lodes et al. 2004). The higher frequency of CD in certain ethnic groups and populations (particularly in Caucasians and Ashkenazi Jews), familial aggregation, and a greater disease concordance in monozygotic twins are strong indicators for genetic susceptibility in disease pathogenesis. The genetic trait of CD, however, is complex with genetic heterogeneity, several susceptibility loci, and incomplete phenotype penetrance.

The *caspase-activation and recruitment domain gene 15* (*CARD15*, also named *Nod2*) has been identified as the candidate gene on the IBD1 susceptibility locus (on chromosome 16q12) and confers risk for development of CD (Ogura et al. 2001). Its 1,040 amino acids are structured into four distinct domains: two N-terminal CARDS, a

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central nucleotide-binding and oligomerization domain (NBD or so-called NACHT domain), and a C-terminal leucine-rich repeat (LRR) domain (Meylan et al. 2006). Three variants within the LRR were initially described to be associated with CD (Hugot et al. 2001). Screening of the entire coding region of *Nod2* identified another 30 non-conservative missense mutations (Lesage et al. 2002). The three main variants are highly disease specific (Hugot et al. 2001; Schurmann et al. 2003; Steer et al. 2003; van der Paardt et al. 2003). They are present in up to 50% of Crohn's and 20% of healthy individuals (Lesage et al. 2002) and are not in linkage disequilibrium. However, they share a common ancestral allele, the so-called H5 haplotype that is characterized by the presence of an upstream coding variant (P268S or single nucleotide polymorphism (SNP)5) (Croucher et al. 2003; Hugot et al. 2001).

One thousand sixty-four genomic DNA samples from 52 different world populations that had been previously characterized by typing of 377 autosomal microsatellites (Rosenberg et al. 2002) were kindly provided by the Foundation Jean Dausset (Centre d'Etude du Polymorphisme Humain, CEPH) (Cann et al. 2002) and screened for the presence of mutations in exons 4a, 4e, 8, and 12 by denaturing high-performance liquid chromatography (dHPLC; WAVE®, Transgenomic, Inc.) (Lesage et al. 2002). These genomic regions had been selected on the basis of known CD-associated mutations within these exons (i.e., SNP5 (C802T, P268S), SNP8 (C2104T, R702W), SNP12 (G2722C, G908R), and SNP13 (3020insC, 1007fs)) (nomenclature according to Lesage et al. (2002)). Polymerase chain reaction (PCR) was performed with addition of equal amounts of wildtype DNA and one unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) as previously described (Lakatos et al. 2005; Lesage et al. 2002). Prior to dHPLC, heteroduplex formation was induced by denaturation of PCR products for 5 min at 94°C followed by slow cooling to room temperature. PCR products of 5 µl were automatically loaded on a DNASep Cartridge (Transgenomic Limited, UK). Run and gradient conditions were unchanged from our previous protocol (Lakatos et al. 2005). For each run, a heterozygous sample was used as internal control. Inconclusive samples were tested at least twice. The allele frequency of the wild-type (a) and mutant ($b=1-a$) allele was estimated by the percentage of wild-type individuals (a^2) assuming Hardy–Weinberg equilibrium in disease-free populations. “Africa” in this article refers to sub-Saharan Africa and “Middle East” includes the Mozabite population of Algeria. “Han” includes individuals ($n=10$) sampled from northern China by the Chinese Human Genome Diversity Project and individuals ($n=35$) born in China and sampled in the United States (San Francisco Bay area) by the laboratory of L. L. Cavalli-Sforza.

The amplification of 1,063/1,064 samples was informative, one sample (#1315 from Dai) was not. One hundred fifty-three samples showed heteroduplex formation at exon 4a (SNP5), 910 were wild type (estimated allele frequency, 0.075). Five of the 153 SNP5-positive samples were also positive for SNP8 (estimated allele frequency, 0.002), six for SNP12 (estimated allele frequency, 0.003), and nine for SNP13 (estimated allele frequency, 0.004). No heteroduplex formation was found in exons 4e, 8, or 12 in the 910 samples that were negative for SNP5.

In this collection, the distribution of *Nod2* variants showed a strong dissimilarity between single populations and major geographical regions (Table 1). European populations displayed the highest SNP5 frequency followed by Oceania, Middle East, and Central/South Asia (Fig. 1). SNP5 was almost absent in populations from East Asia and America. The three CD-associated variants were almost exclusively found in Europe (Table 1). Single positive cases were identified in Mozabites from Algeria, Pathan from Pakistan, Uyгур from China, and Maya from Mexico. A high frequency of SNP5 but no CD-associated variant was detected in Oceania (Fig. 1).

The gradient of SNP5 frequencies between Africa and the Middle East and the absence in East Asian and American populations indicate that the evolution of this SNP occurred somewhere in this region in a period after hunter gatherers had settled to Asia (i.e., “out of Africa 2”) (Cavalli-Sforza and Feldman 2003). The high frequency of SNP5 carriers in Oceania may reflect a founding effect. Within Africa the highest SNP5 frequency was found in Mbuti Pygmies from the Republic of Congo (14%) and none were found in Mandenka or Yoruba from West Africa (despite a rather large sample size). Significant local variation was also found in Middle East and Central/South Asia where the SNP5 allele frequency varies between 2% and 16%. In contrast, SNP8, 12, and 13 are almost exclusively Caucasian alleles that are absent in native populations from Africa, East Asia, Oceania, or America. These observations may be caused either by random genetic drift and/or natural selection within a certain geographical region. However, it seems unlikely that the three (independent) mutations occurred only on a single ancestral allele and only within a single geographic region. It rather points to the presence of some sort of prehistoric selection pressure within Europe and to the functional importance of SNP5, which has not been observed in previous studies testing its ability to activate nuclear factor kappa B (NF-κB) (Bonen et al. 2003; Chamailard et al. 2003). One study, however, showed a small increase in NF-κB activity after muramyl dipeptide stimulation at a certain concentration (Inohara et al. 2003). In addition, NF-κB is only one of several *Nod2* targets (Abbott et al. 2004; Barnich et al.

Table 1 Allele frequencies of *Nod2* variants in 52 worldwide populations

Region	Population	Geographic origin	Coordinates	Sample size	SNP5	SNP8	SNP12	SNP13	
Africa	Bantu South	South Africa	19–29S, 18–30E	8	–	–	–	–	
	San	Namibia	21S, 20E	7	0.07	–	–	–	
	Mandenka	Senegal	12N, 12W	24	–	–	–	–	
	Yoruba	Nigeria	6–10N, 2–8E	25	–	–	–	–	
	Biaka Pygmies	Central African Republic	4N, 17E	36	0.03	–	–	–	
	Mbuti Pygmies	Republic of Congo	1N, 29E	15	0.14	–	–	–	
	Bantu North-East	Kenya	3S, 37E	12	–	–	–	–	
North Africa	Mozabite	Algeria (Mzab)	32N, 3E	30	0.16	0.03	–	–	
Middle East	Bedouin	Israel (Negev)	31N, 35E	49	0.05	–	–	–	
	Druze	Israel (Carmel)	32N, 35E	48	0.04	–	–	–	
	Palestinian	Israel (Central)	32N, 35E	51	0.14	–	–	–	
Europe	Adygei	Russia Caucasus	44N, 39E	17	0.23	–	–	0.03	
	Russian	Russia	61N, 39–41E	25	0.23	–	0.02	0.06	
	Sardinian	Italy	40N, 9E	28	0.24	0.04	–	–	
	Tuscan	Italy	43N, 11E	8	0.39	–	0.06	0.06	
	North Italian	Italy (Bergamo)	46N, 10E	14	0.24	–	–	–	
	French Basque	France	43N, 0	24	0.26	–	0.02	–	
	French	France	46N, 2E	29	0.23	0.02	–	0.05	
Central/South Asia	Orcadian	Orkney Islands	59N, 3W	16	0.21	–	0.03	–	
	Balochi	Pakistan	30–31N, 66–67E	25	0.04	–	–	–	
	Brahui	Pakistan	30–31N, 66–67E	25	0.08	–	–	–	
	Makrani	Pakistan	26N, 62–66E	25	0.02	–	–	–	
	Sindhi	Pakistan	24–27N, 68–70E	25	0.15	–	–	–	
	Pathan	Pakistan	32–35N, 69–72E	25	0.08	–	0.02	–	
	Burusho	Pakistan	36–37N, 73–75E	25	0.08	–	–	–	
	Hazara	Pakistan	33–34N, 70E	25	0.08	–	–	–	
East Asia	Kalash	Pakistan	35–37N, 71–72E	25	0.02	–	–	–	
	Uygur	China	44N, 81E	10	0.11	–	0.05	–	
	Xibo	China	43–44N, 81–82E	9	–	–	–	–	
	Dai	China	21N, 100E	9	–	–	–	–	
	Lahu	China	22N, 100E	10	–	–	–	–	
	Naxi	China	26N, 100E	10	–	–	–	–	
	Tu	China	36N, 101E	10	0.05	–	–	–	
	Yizu	China	28N, 103E	10	–	–	–	–	
	Han	China	26–39N, 108–120E	45	–	–	–	–	
	Miaozi	China	28N, 109E	10	–	–	–	–	
	Tujia	China	29N, 109E	10	–	–	–	–	
	Mongola	China	48–49N, 118–120E	10	–	–	–	–	
	She	China	27N, 119E	10	–	–	–	–	
	Oroqen	China	48–53N, 122–131E	10	–	–	–	–	
	Daur	China	48–49N, 124E	10	–	–	–	–	
	Hezhen	China	47–48N, 132–135E	10	–	–	–	–	
	Japanese	Japan	38N, 138E	31	–	–	–	–	
	North-East Asia	Yakut	Siberia	62–64N, 129–130E	25	0.02	–	–	–
	South-East Asia	Cambodian	Cambodia	12N, 105E	11	0.05	–	–	–
	Oceania	Papuan	New Guinea	4S, 143E	17	0.16	–	–	–
Melanesian		Bougainville	6S, 155E	22	0.20	–	–	–	
America	Pima	Mexico	29N, 108W	25	–	–	–	–	
	Maya	Mexico	19N, 91W	25	0.02	–	–	0.02	
	Colombian	Colombia	3N, 68W	13	–	–	–	–	
	Karitiana	Brazil	10S, 63W	24	–	–	–	–	
	Surui	Brazil	11S, 62W	21	–	–	–	–	

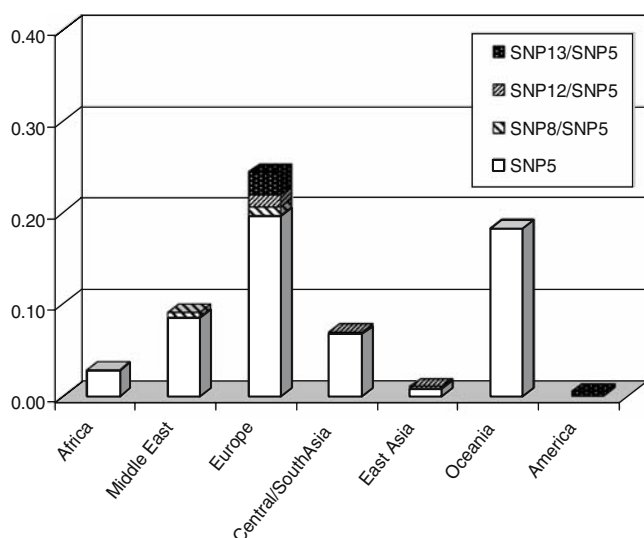


Fig. 1 Allele frequencies of the three CD-associated *Nod2* variants (SNP8, SNP12, and SNP13) and their common ancestral allele (SNP5) in different geographic regions. The genotype data from Table 1 were summarized for different geographic regions

2005) and might be a suboptimal read out to challenge the functional role of SNP5.

Nod2 is a member of the emerging family of Nod-like receptors (NLRs) that include *Nod1–5*, *NACHT*, *LRR*, and *PYD* containing protein (*NALP1–14*), *Ice* protease-activating factor (*IPAF*), class II transactivator (*CIITA*), and neuronal apoptosis inhibitory protein (*NAIP*) (Meylan et al. 2006). NLRs contain three distinct domains: an N-terminal effector domain (*CARD*, *Pyrin* domain (*PYD*)), a nucleotide-binding oligomerization domain (*NBD* or *NACHT*), and a C-terminal receptor domain consisting of a variable number of leucine-rich repeats. *Nod1* and *Nod2* were identified through their sequence homology to *Apaf-1* (Inohara et al. 1999), the master switch in the intrinsic apoptosis pathway, which activates caspase-9, a step that requires deoxyadenosine triphosphate (dATP)/(ATP) and cytochrome *c* (Li et al. 1997). Similar proapoptotic activity has been postulated for *Nod2* (Beutler 2001; Inohara and Nunez 2001). In the light of our findings, experimental data on the ability of *Nod2* variant to differentially activate apoptosis are needed. A recent study has found differential regulation of apoptosis-related genes in dendritic cells from CD patients carrying a variant *Nod2* genotype (Zelinkova et al. 2008).

The allelic frequency change in populations is owing to two factors: natural selection, which is the result of population variation among individual genotypes in their probability to survive and/or reproduce, and random genetic drift, which is due to a finite number of individuals participating in the formation of the next generation (Cavalli-Sforza and Feldman 2003). Both natural selection and genetic drift can ultimately lead to the elimination or the fixation of a particular allele. Migration is another impor-

tant factor in human evolution that can profoundly affect genomic variation in populations. If a migrating group is initially small but subsequently expands, by chance alone the frequency of alleles among founders will differ from the original population and affect the frequency of the new population. In this respect, group migration creates chances for genetic drift and divergence (Cavalli-Sforza 1973). Most of our current knowledge on environment-driven human evolution is derived from studies on genetic variants that are thought to provide reduced risk from malaria infection. These genes (such as α - and β -globin, Duffy factor, and glucose-6-phosphate dehydrogenase) are typically expressed in red blood cells and cause reduced red cell life span thereby reducing proliferation of *Plasmodium*. Mutations occurred in areas where *Plasmodium* was prevalent. Studies have shown that high diversity in human genes can evolve rapidly due to selection (Tishkoff et al. 2001). In the Middle East, plant and animal domestication started about 10,000 years ago and led to an increase in reproduction efficiency (more children could be fed) and population density (settlements were built). This may have provided a good basis for accelerating the speed of acquiring genetic mutations. However, the increase in population density through establishment of human settlements is the basis for epidemic disease. It is reasonable to hypothesize that epidemic or endemic disease was a major cause of positive selection for SNP5 and its associated C-terminal mutations (i.e., SNP8, SNP12, and SNP13) within Europe. Multiple founding effects within the Caucasian population and/or a type I error (as the number of individuals in each geographical area is limited) are alternative explanations.

The fact that *Nod2* is an intracellular muramyl dipeptide recognition protein that senses the presence of bacterial cell wall products makes such a hypothesis even more plausible. Similar to sickle cell anemia, heterozygote advantage may explain how *Nod2* mutations have evolved through increased resistance to bacterial infection. Since *Nod2* is highly expressed in specialized intestinal epithelial cells (such as Paneth cells), such bacterial infections may have been primarily intestinal (Ogura et al. 2003). Milk from domestic cows has been a valuable food source for over 8,000 years, especially in lactose-tolerant human societies that exploit dairy breeds (Holden and Mace 1997). The historic importance to feed from dairy products in Northern Europe is reflected by the geographic coincidence of Neolithic cattle farming sites, cattle milk protein gene diversity, and human lactase tolerance (Beja-Pereira et al. 2003). It is tempting to speculate that *Nod2* variants confer protection from dairy-associated bacterial infections such as *Listeria*, *Brucella*, or *Mycobacteria*. Resistance to such infections would carry a significant selective advantage in times before milk pasteurization was introduced, specifically in newborns and children. Young weanling *Nod2*

knockout mice were indeed more resistant to systemic challenge with bacterial endotoxin (Pauleau and Murray 2003) and the loss of *Nod2* was not associated with any disease phenotype (Kobayashi et al. 2005; Pauleau and Murray 2003). On the other hand, mutant *Nod2* mice exhibited elevated NF- κ B activation and more interleukin-1 β secretion (Maeda et al. 2005), which may allow enhanced immune activation after bacterial contact, shorter bacterial persistence, and reduced risk of bacterial invasion. In this *Nod2*-mutant mouse model, however, any complementary role of the human SNP5 is difficult to test. CD-associated *Nod2* mutations in humans have recently been associated with graft-versus-host disease and mortality after allogeneic stem cell transplantation (Holler et al. 2004), a process that seems to be related to defective defense against nonpathogenic intestinal bacteria because gastrointestinal decontamination protects from this outcome (Holler et al. 2006). Similarly, a higher mortality was also observed in patients with sepsis leading to the notion that the CD-associated *Nod2* mutations favor bacterial translocation from the gut (Brenmoehl et al. 2007), a finding that has its match in *Nod2* knockout mice (Kobayashi et al. 2005). In any case, the proposed protective effect of *Nod2* mutations would have been most relevant in young children who were exposed to a large number of bacterial pathogens in the absence of hygienic conditions.

In summary, our data indicate that the main CD-associated *Nod2* mutations have evolved on the SNP5 allele within a certain geographical region (i.e., Europe). The diversity of *Nod2* mutations points to the presence of a historic selection pressure in this geographical region and to a yet unrecognized functional role of SNP5.

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