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• REVIEW •

Progress in searching for susceptibility gene for inflammatory bowel disease by positional cloning

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Abstract

Inflammatory bowel disease (IBD) includes two clinical subtypes: Crohn disease (CD) and ulcerative colitis (UC). The general prevalence is about 1.0-2.0 % in Western countries. It is predominantly regarded as a multifactorial disorder involving environmental factors and polygenic defects. The view was confirmed by a lot of evidences from clinical attributions and animal models, especially from epidemiological investigations. So the etiological study of IBD has been focused on searching for susceptibility genes by positional cloning, which consists of two steps: linkage analysis and association analysis. Linkage analysis has been an important method of searching for susceptibility genes to polygenic diseases as well as single-gene disorders. IBD, as a polygenic disease, has been widely investigated by linkage analysis for susceptibility gene since 1996. The paper reviewed 38 articles, which covered almost all original researches in relation to IBD and linkage analysis. So far, several loci, such as 16q, 12q, 6p and 3p, have been identified by the studies. The most striking is 16q12 (IBD1), which linked only with CD not UC in the majority of studies. Association analysis, as one essential step for positional cloning, is usually carried out by genotyping candidate genes selected by means of linkage analysis or other methods, for figuring out the frequencies of alleles and comparing the frequencies between IBD group and healthy control group to identify the specific allele. It has been established that IBD is implicated in immune disorder. So the studies were centered on the genes of NOD2/CARD15, HLA-II, cytokine, cytokine receptor and adhesion molecule. This paper reviewed 14 original articles on association between NOD2 and IBD that have been published since 2001. All results, with the exception of one report from a Japanese group, provide evidences that the three kinds of variants of NOD2 are susceptibility factors for IBD. This article also comprehensively analyzed 18 original researches of HLA gene polymorphism in IBD. We found extensive discrepancy among the conclusions and a novel hypothesis was put forward to explain the discordance. Most studies published recently on association between IBD and cytokine gene polymorphism were reviewed.

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INTRODUCTION

Inflammatory bowel disease (IBD) is composed of two clinical subtypes: Crohn disease (CD) and ulcerative colitis (UC). Its general prevalence is 0.1-0.2 % in Western countries[1]. There has been no epidemiological investigation of large scale for the prevalence or incidence of IBD in China so far, despite the facts that UC is common in China and CD has been more frequently diagnosed by clinical physicians in recent years[2]. The pathogenesis of IBD has not been clearly identified. Today, the most generally accepted pathogenesis of IBD is that IBD is resulted from abnormal immune response to enteric bacteria in individuals with susceptibility due to genetically polygenic defects. Therefore, investigators have searched human genome for the loci of susceptibility genes by linkage analysis and have achieved great success. On the other hand, association analysis, as one of the essential steps for positional cloning, was carried out by many investigators to identify the specific allele. It has established that IBD is implicated in immune disorder. Biochemical substances involved in immunoregulation are very rich and the corresponding genes are widely distributed in genome. Genome-wide linkage analysis has suggested multiple candidate regions in several chromosomes for IBD, therefore, considerable numbers of candidate genes should be selected for association analysis. In recent years, these studies were centered on the genes of NOD2/CARD15, HLA-II, cytokine, cytokine receptor and adhesion molecule. These studies were summarized in this review.

IBD IS A MULTIFACTORIAL DISEASE

There have been a number of hypotheses about the pathogenesis of IBD, but neither environmental factors, such as habit of diet and behavior, infection of microorganisms and contact of chemical or physical pathogenic agents, nor single-gene genetic disorder alone can fully explain its complex phenotypes. Thereby, it is thought to be a multifactorial disease. The view was supported by a larger amount of evidences from clinical attributions and animal models, especially epidemiologic investigations and linkage analyses.

Persuasive evidences of genetic contribution to IBD[3-12]

A. The first-degree relatives of affected individuals show about 20-50-fold increased risk of developing the disease compared with the general population for CD, and 10-20-fold increased risks for UC. Moreover, the affected siblings frequently present at similar ages and concordance rates reach up to 80 % for disease site, behavior and presence of extraintestinal manifestation. B. Twin studies have shown that the concordance rate of CD is about 20-44 % for monozygotic twins, and 3.8-6.5 % for dizygotic twins; the concordance rate of UC is about 6-16 % and 3 % respectively[6,7]. C. There are significant ethnic differences in disease frequency. For instance, the prevalence in Ashkenazi Jews is much higher than that in other races, even though they share similar living environment in the same community[8,9]. D. All genome-scanning linkage analyses detected some linkage loci, certain of which were subsequently confirmed by replication studies only involving certain

chromosomes; NOD2 was consistently identified as the susceptibility gene for CD in recent years. E. Simulation studies on animal models have showed that transgenic mice or gene-knockout mice are subject to colitis similar to human IBD, and that spontaneous colitis or hapten-induced colitis manifests fairly different in different strains of mice[10-12].

Evidence of environmental contribution

A. The concordance rate of IBD for monozygotic twins is much less than 100 %. The identical genotype with different phenotypes means that environmental factors take part in the pathogenesis of the disease[6,7]. B. Intestinal bacteria are suggested as the main environmental contributions demonstrated by many evidences: antibiotic therapy can usually induce temporary remission for most IBD cases[13], diversion of faeces stream can make distal improvement in patients with CD[14], some studies suggested that certain strains of intestinal bacteria were associated with IBD[15,16], colonization with normal enteric bacterial flora was required for the occurrence of disease in animals with CD irrespective of the underlying defect[10-12]. C. smoking is likely to be associated with the progress of IBD[17,18]. D. Migrant epidemiological studies demonstrated that population of identical ethnic background, when lived in different communities, showed discordant incidence[8,9,19,20].

IBD is not a disorder of simple mendelian inheritance[3,21-25]

Genetic disease of classic Mendelian model, which consists of Mendelian dominant and recessive genetic disorders, is a phenotype of single-gene defect and called single-gene disorder. IBD has previously been interpreted as genetic disease of Mendelian recessive model. But segregation analyses offered counter-evidence that IBD followed the principle of Mendelian inheritance. Parents of most IBD probands were healthy, frequency of siblings or children of the patients was much less than 50 %, the decline in frequency of affected second-degree relatives compared with first-degree relatives was greater than that predicted by autosomal dominant inheritance, in which the frequency was expected to decrease by 1/2 with each step. Incidence of IBD in children of affected spouses was sharply less than 100 % and a similar proportion of affected siblings and children of affected probands was inconsistent with autosomal recessive inheritance. Linkage analysis has detected several linkage loci that are distributed on a number of chromosomes.

LINKAGE ANALYSIS

It is very difficult to find the biochemical substances, which express qualitative difference between patients and healthy population by means of classical functional cloning. So linkage analysis, as the first step of positional cloning, may serve as a unique and practicable substitution for the time being. Figuring out genetic distance between marked loci and susceptibility gene by means of pedigree investigation and genotyping, then defining the approximate position of susceptibility gene in genomic map are the essential courses of linkage analysis. The dramatic progress of human genome project, which has located nearly 10 000 marker loci in genomic map, has greatly boosted positional cloning for complex genetic diseases. Epidemiological studies have identified striking genetic contributions to the etiology of IBD, but so far, studies with traditional biochemical methods have not yet identified the products with quantitative defects. Many investigators have turned to linkage analysis and have achieved great success. The important data from 38 original researches, which covered almost all articles in relation to IBD and linkage analysis that have been published since 1996, are listed in Table 1[26-63], and some aspects were reviewed as follows.

The common course of linkage analysis for IBD is: collecting families with affected sibling pairs (ASP) or affected relative pairs (ARP) $\geqslant$2 by strict ascertainment, genotyping of genome-wide or certain chromosomes according to microsatellite polymorphisms, figuring out multi-point maximal non-parametric LOD score (MLOD) and two-point LOD score by means of statistical software, inferring genetic distances of susceptibility genes to marker loci and locations in physical genome map, offering candidate genes for association analysis. The majority of investigations found certain suggestive linkage loci with various LOD score, but when defined according to different LOD thresholds, the locations or number of linkage loci were variable. In view of the traits of statistical software and quantity of subjects in most studies, we only displayed the results with MLOD $\geqslant$2.0 or 3.0, represented by ± and +. The chromosomes, on which the linkage loci strongly supported (MLOD$\geqslant$3.0) by at least one of 8 linkage analyses of genome-wide scanning located, include chromosomes 1, 3, 5, 6, 7, 12, 14, 16, 18 and 19, as well as chromosomes 4, 10, 17 and x with suggestive evidence (2.0$\leqslant$MLOD<3.0). Although there was striking discrepancy among the genome-wide scans in respect of linkage loci, almost all studies detected more than 3 linkage loci. This shows that the pathogenesis of IBD is involved in multiple genes and manifests obvious genetic heterogeneity. Several loci were supported by relative more studies, such as 16q, 12q, 6p, and 3p. Because Hugot *et al*[26] and Satsangi *et al*[27] detected strong linkage evidences for chromosomes 16, 12, 6, 3 and 7 in 1996, subsequent studies mainly focused on these chromosomes. It can be seen from Table 1 that more evidences were offered for these loci, with the exception of 16q, simply because these loci were investigated by more studies. Some loci supported by certain genome-wide scans, such as 14q, 5q, 19p, likely to harbor susceptibility genes, were less investigated.

Stratification studies demonstrated significant variances as to the degree and loci of linkage between families with severe IBD and those with only slight IBD, male patients and female patients, Jewish people and non-Jewish people, as well as between UC and CD. Some investigators examined families with CD patients only; others examined families with UC patients only, but most studies detected both families and those with mixed patients and compared the differences of linkage loci between the two groups. As shown in Table 1, there were some differences between UC and CD. The most striking is 16q12 (IBD1), which linked only with CD not UC in the majority of studies. This shows that CD and UC have some common susceptibility genes, as well as certain individual susceptibility genes. Three studies[32,47,48] found that certain loci linked only with the families with early onset of CD. All subjects examined by the studies listed in Table 1 included Caucasian or Jewish patients from Europe, Australia and northern America, but no Mongolian patients. Three studies[28,29,42] demonstrated significant differences between Jewish patients and non-Jewish patients. In respect of nationality of patients, it seems there are no remarkable differences among American, English, German, Australian, Canadian, Italian, Dutch and Belgian. But Paavola *et al*[40] examined chromosomes 1, 3, 7, 12, 14 and 16 in Finnish patients and did not find linkage loci. Fisher *et al*[33] found that some loci on chromosomes 6p, 1, 14 and 18 linked only with IBD of male sufferer. These results confirm the extensive genetic heterogeneity of IBD.

Linkage analysis is intended as an essential tactic to offer candidate genes for association analysis. We should focus our attention on the linkage loci containing some candidate genes, products of which have been suggested as pathogenic factors by other methods, as well as confirmed by subsequent replication studies. The loci meeting these conditions were briefly reviewed here.

Table 1 Data of linkage analysis

Ref	Author	Year	Subject	Scope	Linkage loci for IBD	Linkage loci for CD	Linkage loci for UC
R26	Hugot JP	1996	Caucasian CD	Autosome		16q(IBD1)+	
R27	Satsangi J	1996	Northern european IBD	Autosome	7+, 12+, 3±	7±, 12±, 3±	7+
R28	Cho JH	1998	American IBD[a]	Genome	(3q+,1p±)(non-Jewish), (3q±,4q±)[e]	16±	––
R29	Ma Y	1999	American CD[a]	Genome		14q±, 17q±[e], 5q±[e]	
R30	Hampe J	1999	European IBD[b]	Genome	1±, 6±, X±	10±, 12±, 16±	4±, X±
R31	Duerr RH	2000	American CD[a]	Genome		14q+	
R32	Rioux JD	2000	Canadian IBD	Genome	19p+, 5q+, 3p±, 6p±	5q+[f], 19p+	19p±
R33	Fisher SA	2002	European IBD	Genome	(6p+,1+,14+,18+)(male)	6p+(male)	6p+(male)
R34	Brant SR	1998	American CD[a]	3,7,12,16		16q(IBD1)±	
R35	Rioux JD	1998	Toronto IBD	3,7,12,16	– –	––	––
R36	Curran ME	1998	European IBD[b]	12,16	– –	12q±	––
R37	Annese V	1999	Italian IBD	3,6,7,12,16	16q±	16q±	16q±
R38	Vermeire S	2000	Belgian CD	3,7,12,16		––	
R39	Dechairo B	2001	European IBD	3,6,7,	6p+	––	––
R40	Paavola P	2001	Finnish IBD	1,3,7,12,14,16	– –	––	––
R41	Gavanaugh J	2001	IBD[c]	12,16	16q+	16q+	––
R42	Ohmen JD	1996	American IBD[a]	16	– –	16q±[e]	––
R43	Parkes M	1996	English IBD	16	– –	16q±	––
R44	CavanaughJA	1998	Australian CD	16		16q+(IBD1)	
R45	Mirza MM	1998	Northern european UC	16			16q±(IBD1)
R46	Porabosco P	2000	Italian IBD	16	16q+	16q+	16q+
R47	Brant SR	2000	American CD	16		16q+[f], 16q±	
R48	Akollar PN	2001	Jewish CD	16		16q+[f]	
R49	Van Heel DA	2002	European CD	16		––	
R50	Zouali H	2001	European CD	16		16q+	
R51	Hampe J	2002	European IBD	16	16p±	16q+, 16p±	––
R52	Satsangi J	1996	European IBD	6(MHC-II)	– –	––	6p(MHC-II)+
R53	Silverber MS	1999	Canadian CD	6		6p(MHC-II)±	
R54	Hampe J	1999	Northern european IBD[b]	6	6p+	6p+	6p+
R55	Yang H	1999	American CD	6		6p(MHC)+	
R56	Duerr RH	1998	Northern american IBD	12	12q±	––	––
R57	Yang H	1999	American IBD	12	– –	12q±	––
R58	Lesage S	2000	Northern european CD[d]	12		––	
R59	Parkes M	2000	American IBD	12	12q+	––	12q+
R60	Hampe J	2001	Northern european IBD[b]	3	– –	––	––
R61	Duerr RH	2002	American IBD	3	3p+	––	––
R62	Rioux JD	2001	American CD	5q		5q+	
R63	Vermeire S	2001	Belgian CD	X		Xq±	

Note: CD, CD-only family; UC, UC-only family; IBD=UC+CD+mixed family; +, convincing linkage (LOD≥3.0); ±, suggestive linkage (3.0>LOD≥2.0); —, no suggestive linkage (LOD<2.0); [a], including Jewish; [b], family from English, German and Dutch; [c], family from Northern American, European and Australian; [d], family from French and Belgian; [e], linkage only for Jewish; [f], linkage only for IBD with early onset.

Chromosome 16 As shown in Table 1, 14 out of 25 related studies found linkage loci for CD with MLOD more than 2.0 on the chromosome, additionally, some loci with suggestive score (MLOD between 1.0 and 2.0) were detected. Only 3 studies found linkage loci with UC, furthermore, 2 of them also detected linkage with CD, and the other one merely examined UC families. It can be inferred from these studies that chromosome 16 contains susceptibility gene for CD rather than UC. Chromosome 16 is comparatively short, with 98 Mb of physical length, 130.8 cm of genetic distance, and has been spaced by about 200 microsatellite markers[64]. The linkage loci suggested by the studies in Table 1 were distributed in most part of chromosome 16 (for instance, D16S409-419[26], D16S748-764[28], D16S411[42,43], D16S3136[50]), but only pericentromeric region on 16q was the most consistent linkage region. The important candidate genes in the region are NOD2, CD11integrins, CD19, Sialophorin, IL-4 receptor gene etc. NOD2 gene has been established as susceptibility gene to CD. It remains unanswered if there are other susceptibility genes in the chromosome. Hampe *et al*[51] examined additional regions with high-density experiment using 39 microsatellite markers and found three-peak logarithm of odds (LOD) scores of 2.7, 3.2, and 3.1 on proximal 16p, proximal 16q, and central 16q, respectively. Taking account of the differences of suggestive markers, it is probable that there are other susceptibility genes for CD in the chromosome.

Chromosome 12 Six out of 19 studies found suggestive linkage loci in the chromosome, 4 of them for CD, and one for UC. Though the studies with suggestive MLOD are rare, several studies found linkage loci with slightly suggestive significance (MLOD between 1.0 and 2.0) with IBD, especially with UC. This may result from the fact that the sample sizes in most studies were not large enough; furthermore, they were predominantly consisted of CD families. Parkes *et al*[59] examined 581 affected relative pairs, of which 252 were from CD-only families, 138 from UC-only families, and 191 from mixed families (the sample size was much larger than that in most of other studies, especially for UC), and found that MLOD at certain marker on chromosome 12 was 5.26 for all IBD, 3.91 for UC and 1.66 for CD. In summary, it is probable that

Table 2 Data of association analysis for NOD2

Ref year author	Subject	Main conclusion
R65, 2001	Europe CD, UC	A. Find P241S, R432R, R675W, G881R, IVS8-133delAinSCT, 980fs etc.31 variants
Hugot, JP		B. R675W, G881R, 980fs with CD, not with UC
		C. CD-GRR 3.0 at SHEM, 38.0 at HOM, 44.0 at CHEM
R66, 2001	American CD	A. 3020insC with CD
Ogura Y		B. CD-GRR 1.5 at SHEM, 17.6 at HOM
R67, 2001	German, English CD	A. 3020insC with CD, not with UC
Hampe J	UC	B. CD-GRR 2.6 at SHEM, 42.1 at HOM
R68, 2002	Europe CD, UC	A. Find 67 sequence variants, 9 of which gene frequency >5 %
Lesage S		B. R702W, G908R, 3020insC with CD, not with UC
		C. Support gene-dosage effect
R69, 2002	Europe CD, UC	A. R702W, G908R, 3020insC with CD, especially ileum CD, not with UC
Cuthbert AP		B. P628S linkage disequilibrium with the other three mutations
		C. CD-GRR 3.0 at SHEM, >22.0 at HOM or at CHEM
		D. Mutation frequency: familial CD > sporadic CD
R70, 2002	Dutch CD	A. 3020insC with CD, G2722C not with CD
Murillo L		B. No association with clinical phenotype
R71, 2002	German, Norwegian	A. R675W, G881R, 980fs with CD
Hampe J	CD	B. Especially with ileum CD
R72, 2002	Canadian CD	A. R702W, G908R, 1007fs with CD, especially ileum CD
Vermeire S		B. No difference between familial CD and sporadic CD
R73, 2002	German UC, CD	A. 3020insC with CD, not with UC
Radlmayr M		B. Association with fistula, fibrostenosis, ileocecum resection
R74, 2002	Japanese CD, UC	A. R675W, G881R, 3020insC not with CD or UC
Inoue N		
R75, 2002	Europe CD	A. R675W, G881R, 3020insC with CD
Vermeire S		B. Not with effect of Infliximb
R76, 2002	American CD	A. R702W, G908R, 1007fs with CD
Abreu MT		B. With fibrostenosis
R77, 2002	English CD	A. R702W, G908R, 1007fs with CD, especially ileum CD
Ahmad T		B. 3020insC with early onset of CD
		C. CD-GRR 2.4 at SHEM, 9.8 at HOM, 29.3 at CHEM
R49, 2002	Europe CD	A. R702W, 1007fs with CD, linkage disequilibrium with P628S
Van heel DA		B. Support gene-dosage effect

Note: CD=Crohn disease; UC=ulcerative colitis; CD-GRR=CD-genotype relative risk; SHEM=simple heterogeneous mutation; HOM= Homozygous mutation; CHEM=complex heterogeneous mutation; *P* value is uniformly set at $\leqslant$0.05; Nomenclatures were not uniform among the studies, R675W=R702W, G881R=G908R, 1007fs=980fs=3020insC.

chromosome 12 contains susceptibility genes both for CD and UC, but with only weak contributions. The most consistent microsatellite markers lay in 12q13, which is also called IBD2. The main candidate genes in the region are IFN-γ, natural resistance associated macrophage protein (MRAMP2), vitamin D receptor genes etc.

Chromosome 6 Eight out of 14 studies found linkage loci in the chromosome. There was no remarkable difference between CD and UC in terms of the number of suggestive studies or LOD score in most studies. This shows that common susceptibility genes for both CD and UC are probably located in chromosome 6. Linkage markers of most studies were distributed in 6p13, which was called IBD3 in some studies. The important candidate susceptibility genes in the region include HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, MIC-A, MIC-B, HLA-DR, HLA-DP, HLA-DQ, HLA-DM, LMP-2, LMP-7, transporterin antigen processing (TAP)-1, TAP-2, TNF-α, TNF-β, (LT-α), heat shock proteins (HSP), complement C4, C2 genes etc.

Chromosome 3 Four out of 16 related studies found linkage loci with LOD score more than 2.0. It should be noticed that the LOD scores in 4 studies for all IBD were always higher than that for CD or UC alone and no LOD score for CD or UC alone reached 2.0 in all related studies. It may be partly due to the fact that the sample sizes were much smaller after stratifications and could not reach the threshold of statistical significance. It is very likely that there are some common susceptibility genes for CD and UC, but they probably confer slight genetic contribution to IBD, since some studies found linkage loci in the chromosome only with weakly suggestive LOD score[30,39,60]. The considerable linkage region was proximal 3p. The principal candidate genes in the region include CCR2, CCR5, IL-4RA, IL-5RA, lactotransferrin, IFN-α A2, cathelicidin antimicrobial peptide genes etc.

Chromosome 5q It was confirmed by only 3 studies involved in the chromosome. The linkage loci were located in 5q32-35, which happened to be in the region of cytokine-rich cluster and was called IBD5 in some studies. The main candidate genes are IL-3, IL-4, IL-5, IL-13, CSF-2 genes etc. The cytokines have been accepted as playing important roles in initiating IBD. So further investigations are needed.

14q11(IBD4) and 19p13 They were suggested as linkage loci in some studies. 14q11 contains the immunoloregulation members TCR-α/δ gene and 19p13 contains ICAM1, C3, TBXA2 and LTB4H genes.

ASSOCIATION ANALYSIS

Association analysis, as an essential step for positional cloning, was usually carried out by genotyping candidate genes selected by means of linkage analysis or other methods, for figuring out the frequencies of alleles and comparing the frequencies

Table 3 Data of association analysis for HLA

Ref	Year	Author	Subject	Region	Association with CD	Association with UC
R83	1995	Duerr RH	American UC	HLA-DR2		DRB1*1601-
R84	1995	Nakajima A	Japanese CD	DR, DQ, DP	(DRB1*0405,0410,DQA1*03,DQB1 *0401,0402)+, (DQA1*0102,DRB1* 1501,1302,DQB1*0602) -	
R85	1996	Reishagen M	German CD	DRB1, DQA1, DQB1	DRB1*07+, DRB1*03-	
R86	1996	Hesresbach D	French CD	HLA-I, HLA-II, TAP	DRB1*1302+, DRB1*04+[c]	
R52	1996	Satsangi J	Europe IBD	DRB, DQB		DRB1*103+, DRB1*12+
R87	1996	Danze PM	French CD	DRB1, DQB1	(DQB1*0501, DRB1*07, 01)+ (DQB1*0602 / 0603, DRB1*03)-	
R88	1996	Heresbach D	French UC	HLA-II, TAP		DRB1*03-
R89	1997	Bouma G	Dutch IBD	HLA-DR		DRB1*15+, DRB1*13-
R90	1998	Fernandez AM	Spanish UC	DRB1		DRB1*1501+, DRB1*
R91	1998	Cariappa A	American CD	DRB, DQB, DPB	Haplotype DRB3*0301 / DRB1*1302+	1502(severe)+
R92	1999	Bouma G	Dutch UC	DR, TNF-α, LT-α		DRB1*0103+, DRB1*15(female)+
R93	1999	Yoshitake S	Japanese IBD	DQ, DR, DP	DQB1*0402+, DRB1*1502-	(DRB1*1502,DRB5*0102, DQA1*0103,DQB1*06011, DPA1*0201,DPB1*0901)+, (DRB4*0101,DQA1*0302) -
R94	1999	Stokkers PC	Meta-analysis	DR, DQ, TNF-α	(DR7,DRB3*0301,DQ4)+, (DR2,DR3)-	(DR2, DR9, DRB1*0103)+, DR4 -
R95	1999	Hirv K	Estonians UC	DR, DQ, TNF-α		DRB1*1501+
R96	2001	Seki SS	Japanese UC	HLA-I, HLA-II		(MICA-TM-STR6, B52, DR2)+
R97	2002	Lantermann A	IBD[a]	DPA1	DPA1*02021(German)+	
R98	2002	Orchard TR	Europe IBD	HLA-B, DR, TNF-α	Uveitis with (B*27,B*58,DRB1*0103)	
R77	2002	Ahmad T	CD[b]	HLA-I, HLA-II TNF-α, LT-α, HSP70	(DRB1*0701,CW*0802)+, DRB1*0103(fistula)+, DRB1*1501-	

Note: UC= Ulcerative colitis; CD= Crohn disease; IBD= UC+CD; [a], German, South Africa and South Korea IBD; [b], white people CD; [c], DRB1*04 is only associated with CD with no effect to cortexin; *P* value is uniformly set at $\leqslant$0.05; +, Positive association; -, Negative association.

between IBD group and healthy control group to identify the specific allele. It has established that IBD is implicated in immune disorders. So studies have been centered on genes of NOD2/CARD15, HLA-II, cytokine, cytokine receptor and adhesion molecule.

NOD2/CARD15 mutations

Identification of NOD2 as susceptibility gene for IBD is supported by linkage analysis, association analysis and immunological function analysis. In this review, crucial information from 14 original studies on the relationship between IBD and NOD2 mutations are listed in Table 2[49, 65-77] and were comprehensively analyzed as follows. NOD1 is an intracellular protein composed of a N-terminal caspase recruitment domain (CARD), a centrally located nucleotide binding domain (NBD), and a leucine rich repeat (LRR) domain at its C-terminus which could activate nuclear factor κB (NFκB) and also promote apoptosis[78]. NOD2 was identified by searching the public database for genes encoding similar proteins to NOD1. The gene happens to be located on chromosome 16q12, a domain called IBD1 supported by most linkage analysis. NOD2 has one more CARD at its N-terminal than NOD1. It is expressed primarily in monocytes and following stimulation by bacterial lipopolysaccharide (LPS), which occurs at LRR domain, activates NF-κB.

So far, approximately one hundred sequence variants have been detected in NOD2 gene, most of which were rare mutations, and located in LRR domain. Lesage *et al* discovered 67 sequence variants in the gene, 9 out of them with gene frequencies more than 5 %. This demonstrates that NOD2 gene has high polymorphism. Of these alleles, P268S, R702W, G908R and 1007fs are consistently confirmed to be the genetic susceptibility factors to IBD. Gene frequency of P268S in general population was about 20-28 %, a little higher than other three variants. While it was remarkably higher in CD patients, transmission disequilibrium test (TDT) suggested that this was very likely to be the result of transmission disequilibrium with other three variants, but not the independent susceptibility factor to CD. R702W, G908R and 1007fs are widely considered as independent susceptibility factors. 1007fs allele frequency was different among all reports: about 1.6-3.3 % in control population and 6.6-23.7 % in CD patients. All the studies, with the exception of a report from Japanese group, strongly support the association of 1007fs with CD. Allele frequency of R702W was slightly higher than that of 1007fs, and was significantly higher in CD patients than in control population. Allele frequency of G908R was about 1.5 % and has been suggested as an independent susceptibility factor to CD, but the statistical values in some reports did not reach significant level. This may be due to its lower gene frequency. The CD-genotype relative risk of the three mutations was about 3.0 for simple heterozygote mutation, and more than 20.0 for homozygote mutation or compound heterozygote mutation. This supports the fact that mutations in NOD2 gene show a gene-dosage effect and somewhat recessive trait. Five studies, in which subjects included UC patients, did not find any persuasive evidences that the three mutations were associated with UC. It' s worthy to point out that association of the three mutations with CD was not confirmed in study by the Japanese group. The discordance is usually explained by the view that CD is a genetic disease with high locus heterogeneity or allele heterogeneity among patients from different races or different geographic territories. With respect to the association of three mutations with clinical phenotypes, most reports suggested 3 mutations were only associated with ileum CD and certain reports found they were associated with fibrostenosis, fistula or effect of medicine.

How can the mutations of NOD2 initiate CD[65,66,79-82]? Some

studies have provided evidences that NOD2 is an intracellular receptor for bacterial pathogenic agents, and expresses only in monocytes. LRR of NOD2, after being activated by lipopolysaccharide (LPS), can trigger NF-κB signal pathway, which promotes the expression of certain proinflammatory cytokines. LRR can also promote apoptosis. So it is the most likely mechanism that the mutations in NOD2 either raise sensitivity of monocyte to bacterial pathogenic agents, with the result of overexpression of certain proinflammatory cytokines, or cause deficiency of apoptosis, leading to monocyte accumulation in intestinal mucosa and chronicity of the course. There are some questions about the relation between mutations in LRR and activity of NF-κB. Though high activity of NF-κB is always found in monocytes in lamina propria of intestinal mucosa from CD patients, it descends in cells with frame-shift mutation in LRR *in vitro* when stimulated by LPS. Functional study by Hugot *et al* demonstrated that expression of a NOD2 mutant form lacking the entire LRR region results in enhanced NF-κB activity, whereas the frame-shift mutation causing a truncated protein missing the final 33 amino results in low NF-κB activity. The potential explanation may be that the truncated protein leads to elevated NF-κB when stimulated by an untested bacterial LPS and that the frame-shift mutation may have a differential effect on caspase 9 induced apoptosis. To understand the mechanism how mutations in NOD2 confer susceptibility to CD, more functional analyses should be ingeniously performed. Identification of NOD2 was a great achievement in the history of exploring genetic susceptible factor for IBD. Detecting of the mutations may well have some clinical benefits for the prediction of onset risk, classification of disease, individualization of therapy and future gene therapy, but it should not be used as a tool for diagnosis since there is about 6-9 % overall allele frequency of the three single nucleotide polymorphisms (SNPs) in general population.

HLA gene polymorphisms

HLA gene is composed of three regions: HLA-I, HLA-II and HLA-III. HLA-I mainly includes HLA-A, HLA-B and HLA-C, and HLA-II mainly contains HLA-DR, HLA-DQ, HLA-DP. The primary immunity relative genes in HLA-III region are TNF-α, TNF-β, (LT-α), complement 2 (C2), complement 4(C4). Genes in the regions are highly polymorphic. HLA-II is expressed primarily in macrophages, dendritic cells and thymic epithelial cells, playing important parts in presenting exogenous antigen. Immunologic studies have shown strong evidences that IBD is closely associated with disturbance of Th lymphocyte subclass, and that the major environmental factor inducing immune disorder is intestinal bacterial flora. T lymphocytes accept enteric antigens presented by microphages, so it is naturally viewed that sequence variants of HLA-II are likely to cause disorder of antigen presenting and result in imbalance of Th lymphocyte subclass. HLA genes located in IBD3 (6p13) were identified by linkage analysis, it is sensible to select HLA gene as candidate gene for association analysis. This review collected 18 studies published since 1995 and listed the main results in Table 3[52,77,87-98]. Most studies published before 1995 usually typed HLA by examining serum HLA antigen through immunologic assay. The studies shown in Table 3 tried to identify HLA alleles by using reliable and precise molecular biological techniques, such as specific sequence primer polymerase chain reaction (PCR-SSP), specific sequence oligonucleotide probe assay (PCR-SSO) and gene sequencing.

It could be summarized from Table 3 that polymorphisms positively or negatively associated with UC or CD are mainly located in DRB1 or DQB1, which are the key regions to determine the polymorphism of peptide-binding cleft. The sequence variants in the regions could change the affinity for distinct antigen peptides. According to serum typing, results from more than two studies suggest that UC is positively associated with DR2, DR9 and negatively associated with DR4, and that CD is positively associated with DR7, DQ4, negatively with DR2, DR3. Further genotypings by molecular biological technique found that only DRB1*0103, DRB1*1501 and DRB1*1502 were associated with UC in more than two studies. Other variants in the regions have not been confirmed to be positively or negatively associated with UC or CD. Many investigators studied the association between polymorphism and clinical phenotype (p-ANCA, sex, extent, age of onset, site of disease, effect of certain medicine, complication, certain extraintestinal symptoms), but their conclusions were not consistent. The heterogeneity was obvious between UC and CD in respect of association with polymorphisms of HLA-II genes. The alleles associated with UC or CD were not associated with the others. On the contrary, DR2, positively associated with UC in some reports, was negatively associated with CD in other reports.

Stokkers *et al*[94] carried out a meta-analysis involving 29 pieces of related reports published from 1980 to 1999 (there were some overlaps between those and reports listed in Table 2). Taking a wider view of all these studies, we discovered a remarkable characteristic: the vast majority of studies discovered certain positively or negatively associated alleles, but all the suggested alleles showed significant discrepancy (there were always both evidences and counterevidences of the association as to any of the alleles among the studies). The contradiction cannot be explained by race heterogeneity because there are discrepancies both in white people and yellow people. It cannot be convinced that sequence variants in HLA-II gene are not associated with IBD and positive results are due to confusions either from linkage disequilibrium or from coincidences resulted from high polymorphisms. In view of the functional property of HLA-II and the widespread contradictions in association analysis, we consider that polymorphism in the regions, to some extent, plays a role in initiating IBD, but the involved alleles differ between different communities (in view of geographic situation, climatic condition and dietary culture) and even between individuals in the same community. The constituent characteristics (sort, ratio, total amount and time order of all antigens) of antigen compound (all kinds of antigens) derived from intestine in one community are distinct from that in an other community. The antigen presenting cells (APC) containing certain HLA-II allele, only when disposing and presenting the antigen compound with matching constituent characteristic, can cause pathologic response. There are different predominant antigen compound in different communities, so the predominantly matching alleles implicated in IBD might be different among communities, and therefore no particular or unchanged antigen and HLA-II allele can take part in pathologic lesions in all IBD. Nevertheless, some of them may play a more dominant role in one community than in another community.

Cytokine, cytokine receptor and adhesion molecule polymorphisms

IL-1β, excreted mainly by microphages, up-regulates the expression of HLA-II, adhesion molecule and IFN-γ in an autocrine manner. It can also, through paracrine, promote activity of Th lymphocyte and play an important part in triggering immune response. IBD is regarded as the result of imbalance of Th lymphocyte subclass, thereby, IL-1β, IL-1β receptor (IL-1R), IL-1 receptor antagonist (IL-1RA), balance of IL-1β/IL-1RA may be associated with IBD. Up to date, many investigators have tried to explore association between IBD and gene polymorphism, but studies in recent years did not

find any positive results[99-101]. Nemetz *et al* found that IL-1β-511*2 allele was associated with the overexpression of IL-1β and descent of bone mineral density[102], and that IL-1β(+3953,-511) allele is associated with pathological course and patient' s condition[103]. Mwantembe *et al*[104] examined the polymorphism of IL-1β, IL-1R and IL-1RA genes by *TaqI, Pst I* and VNTR and discovered IL-1R (*TaqI-*) allele frequency was significantly higher in white patients than that in white healthy controls and Negro patients, whereas IL-1R (*Pst I-)* allele frequency was higher in Negro patients than those in white patients. IL-1β and IL-1RA genes were not located in the region which was strongly supported by linkage analysis.

TNF-α is suggested as a pathogenic factor for IBD because its concentration is usually increased in intestinal mucosa of IBD patients, and therapy of anti-TNF-α antibody Infliximb has shown satisfactory effects on refractory IBD. The gene is located in IBD3 (6p13) which is supported by linkage analysis. Most studies in recent years discovered certain alleles were associated with IBD. Van Heel *et al*[105] found (-857C) TNF-α allele was associated with IBD. Sashio *et al*[106] found TNF-α (-308G/A, -238 G/A) allele was associated with UC. Mitchell *et al*[107] found that TNF-α (-308G/A) was associated with sclerosis cholangitis. Koss *et al*[108] found that different haplotypes of TNF-α gene were associated with the expression of TNF-α. Louis *et al*[109] discovered (-308) TNF-α was associated with certain clinical phenotypes of CD. Negoro *et al*[110] discovered (-1031C, -803A, -857T) TNF-α was associated with CD. These results show that sequence variants of TNF-α gene, especially (-308G/A) may take part in the pathogenesis of IBD by enhancing the expression of TNF-α and promoting activity and proliferation of Th lymphocytes.

IL-4, expressed mainly in Th2 lymphocytes, plays an important role in regulating the balance of Th lymphocyte subclasses and induces differentiation and proliferation of B lymphocytes or microphages, thereby it is regarded as a principal factor in initiating UC. IL-4 and IL-4 receptor (IL-4R) gene are separately located in 5q31-33 (IBD5) and 16q12 (IBD1) which are supported by linkage analyses. Klein *et al* [111] and Aithal *et al*[112] found that certain alleles of the genes were associated with CD. Peng *et al* examined IL-4 polymorphisms in Chinese people and found IL-4-RP2 allele frequency was obviously higher in UC patients than in healthy control, whereas RP1 allele frequency was higher in healthy control than in UC patients[99].

IL-10, mainly excreted by Th3 or Th2 lymphocytes, can suppress the expression of IL-12 and TNF-α in natural killer cells or microphages, restrain activity or proliferation of Th1 lymphocytes. The functional deficiency of IL-10 may be an important maintenance factor for chronicity of IBD. The fact that IL-10 gene knockout mice are subject to colitis similar to human IBD is a persuasive evidence. But studies in recent years did not discover any allele was associated with IBD (allele IL-10 (-627, -1117, -1082, -592, -819) etc.)[101,107,113,114]. Koss *et al*[108] found IL-10 (-1082G/A) allele was associated with the down-regulation of IL-10 expression.

ICAM-1 plays an important role in regulating the homing of lymphocytes. Overexpression of ICAM-1 and significant lymphocyte infiltration have been found in intestinal mucosa of IBD patients. ICAM-1 gene is located in 19p13, which is supported by some linkage analysis. Yang *et al*[115,116] found that R241 allele was associated only with ANCA-positive UC. Contrarily, Braun *et al*[117] reported that ICAM-1 R241 allele and R/G241 heterogeneous mutation were much more frequent in UC patients than in healthy control irrespective of ANCA-positive or ANCA-negative.

Other investigators examined E-selection, L-selection, CCR2, CCR5, IL-6, NRAMP1 and IFN-r genes and found they had no association with IBD[95,118-121].

PROBLEMS AND PERSPECTIVES

Taking a wide view of these studies, we could find extensive discrepancies, which are usually interpreted by the view that IBD is a genetic disease with widespread heterogeneity[122]. It means that the complicated clinical phenotypes of IBD are determined by interaction of multiple genes with environmental factors. Single gene contributes little to IBD, and only polygenic defects with corresponding conformation underlie the complicated phenotypes of IBD. One phenotype may be determined by more than one conformation models of polygenic defects. Nevertheless, we should take into account of other aspects to resolve the discordant results in linkage analyses. A. Entrance criteria and clinical classifications of subjects must be controled more strictly and uniformly, since a minor mistake may influence the outcomes[123,124]. B. The microsatellite markers used by different investigators were not uniform, some investigators selected high-density markers, and others used somehow lower density markers. This could result in discrepancy conclusion. C. The sizes of sample were different among the studies, ranging from about 100 patients to more than 600 patients. Stratification studies performed using small sample sizes may cause false-negative error. Cavanaugh *et al*[41] carried out an international multicenter study, which involved 613 families from 12 study centers. By pooling of data sets, which were acquired from 12 independent centers using the same statistical method, despite the lack of convincing evidence for linkage based on data from individual center, they discovered unequivocal linkage for IBD on chromosome 16 (MLOD 5.79). D. The principle of linkage analysis is based on the view that crossing over in meiosis I is random and physical distance on chromosomes is necessarily in accord with genetic distance. The findings that significant association was found, but no linkage was suggested for the same subject group and the same loci demonstrated somehow theoretical disability of linkage analysis[49,125,126]. E. Other molecular biological mechanisms such as epigenetics may also play a role in initiating IBD[127]. In addition, if IBD is thought as a genetic disorder like familial adenomatous polyposis (certain mutations were inherited from parents, then somatic mutations were accumulated in certain cells such as macrophages or epithelial cells in intestinal mucosa or lymphoid tissue), all phenomena observed so far would not produce counterevidence. Today, linkage analysis has shed lights on genetic diseases such as diabetes mellitus, hypertension, asthma, Alzheimer' s disease and arteriosclerosis, as well as single-gene disorders. As for IBD, we have identified several linkage loci, which harbor a number of important candidate genes pending further confirmation by association analysis and functional analysis.

At present, about 30 candidate genes have been investigated by means of association analysis, and the majority of them either have no association with IBD or have not been confirmed by replication studies. With respect to HLA gene, though the majority of investigations discovered certain positively or negatively associated alleles, all the suggested alleles showed significant discrepancy. The three mutations in NOD2 gene have been consistently confirmed to be the independent susceptibility factors for CD in all-14 original studies except for one from a Japanese group, but how the variants can cause CD remains to be answered. Taking a wide view of all reports, which reached statistic significance, we discovered that frequencies of any alleles ever suggested by association analysis did not manifest a great absolute difference between patients and healthy controls. For instance, only about 20 % CD patients carried at least one of the three alleles of NOD2, whereas about 4-7 % healthy population also carried one of them. This shows that no allele ever studied demonstrates high specifity and sensitivity of the association with IBD and other

alleles need to be explored. It should be noted that the results of most studies, irrespective of positive or negative, did not absolutely ascertain the involvement of genes as susceptibility genes to IBD, since these could be confounded by a number of factors such as type I error or type II error caused by linkage disequilibrium or coincidence. In addition, there are many sequence variants in most genes, some of them are rare mutations and can only be properly analyzed in study of very large samples. Most investigators only detected variants in certain regions of the genes, instead of sequencing of whole gene of these alleles, therefore these results cannot represent the whole genes. The associations of some alleles with clinical phenotype of IBD have been detected by stratification study in many studies, but studies on the associations with the expression of cytokines involving the regulation of Th lymphocytes, were far less and more studies need to be carried out. The biochemical substances, which were ever suggested to be pathogenic factors for IBD, are of great variety, so it' s important to select proper candidate genes for association analysis. Since 1996, nearly 40 linkage analyses have identified several linkage loci in different chromosomes, such as IBD1, IBD2, IBD3, IBD4, and IBD5. Therefore, the genes located in such loci, and their products widely established as pathogenic factors for IBD, should be preferentially selected as candidate genes. Association analysis is an important method for unraveling the pathogenesis of IBD at gene level and will contribute tremendously to the understanding of IBD in the near future.

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