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**Henning Frenzel · Jochen Hampe · Klaus Huse
Silvia M. Mascheretti · Peter J.P. Croucher
Nicholas J. Lynch · Reiner Siebert · Matthias Platzer
Stefan Schreiber**

Mutation detection and physical mapping of the *CD11* gene cluster in association with inflammatory bowel disease

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Abstract A genetic component in the etiology of inflammatory bowel disease (IBD) has clearly been demonstrated by epidemiological and genetic linkage studies. Linkage to IBD on proximal Chromosome (Chr) 16p is well established and replicated. A stratification experiment showed that the recent identification of a disease gene on the q arm does not interfere with the approach on the p arm, and the linkage peak is still significant. Here we present a candidate gene study of the alpha integrins (*CD11A-D*) on Chr 16. The alpha integrins play a key role in inflammatory processes, including leukocyte adhesion and migration. Their genes are located on the p arm of Chr 16, and therefore represent excellent positional and functional candidates. Since the assignment of the *CD11* genes in the genome was not clear, we performed physical, radiation hybrid, and fluorescent in situ hybridization mapping of the gene family. All *CD11* genes map on Chr 16p11–12. *CD11B–D* are arranged in a gene cluster within 300 kb and *CD11A* is located about 2.5 Mb telomeric. Thirteen new single nucleotide polymorphisms (SNPs) and eight SNPs from databases were identified through full-length sequencing. Case-control statistics demonstrated an association lead in the *CD11* gene cluster, which was not confirmed in further family based association/linkage analyses using single markers and haplotypes. It is unlikely that the *CD11* genes play an important role in the pathogenesis of IBD. The marginally significant results could indicate a disease gene in the vicinity of the gene cluster.

Keywords Integrin · CD11 · Inflammatory bowel disease · Polymorphism · Linkage disequilibrium mapping

H. Frenzel · J. Hampe · S.M. Mascheretti · P.J.P. Croucher
S. Schreiber (✉)
Department of Internal Medicine I, University Hospital,
Christian-Albrechts-University, Schittenhelmstrasse 12,
24105 Kiel, Germany
e-mail: s.schreiber@mucosa.de
Tel.: +49-431-597-1279, Fax: +49-431-597-1842

K. Huse · M. Platzer
Institute for Molecular Biotechnology, Beutenbergstrasse 11,
07745 Jena, Germany

N.J. Lynch
Department of Microbiology and Immunology,
University of Leicester, University Road, Leicester LE1 9HN,
UK

R. Siebert
Institute of Human Genetics, University Hospital,
Schwanenweg 24, 24105 Kiel, Germany

Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing disease of the gastrointestinal tract with two major forms: Crohn's disease (CD) and ulcerative colitis (UC) (Hamilton 1987; Podolsky 1991). A genetic factor in the etiology of IBD has clearly been shown by epidemiological and genetic linkage studies. There is consistent evidence for a clustering within families (Orholm et al. 1991) and an increased concordance of the IBD phenotype in monozygotic twins (Thompson et al. 1996; Tysk et al. 1988).

Genome-wide linkage analyses have identified several susceptibility regions on different chromosomes. The linkage region on Chromosome (Chr) 16 was detected through a genome-wide linkage analysis in 1996 and designated *IBD1* (from inflammatory bowel disease 1) (Hugot et al. 1996). This region has subsequently been replicated in several independent populations (Annese et al. 1999; Brant et al. 1998; Cavanaugh 2001; Curran et al. 1998). The region extends over both sides of the centromere and has multiple peaks of linkage on both the p and the q arm. The first disease gene predisposing to CD has been identified on Chr 16q12 near the microsatellite *D16S3136* (Hampe et al. 2001a; Hugot et al. 2001; Ogura et al. 2001a). A series of mutations in the *NOD2* gene are highly associated with CD but not with UC. A frame shift mutation through a C-insertion in exon 13

conveys the strongest risk of developing CD. It leads to the truncation of the NOD2 protein, which appears to be associated with an altered activation of NFkB by NOD2 in response to lipopolysaccharide (Ogura et al. 2001a, b). While mutations in the *NOD2* gene can explain the genetic risk of developing CD in a subset of patients, other genetic variations will be responsible for the remaining population of patients with IBD.

Several studies (Annese et al. 1999; Brant et al. 1998; Cho et al. 1998; Curran et al. 1998) have demonstrated linkage of IBD to the proximal p arm of the chromosome, whereas *NOD2* is located on the q arm. A formal exclusion analysis demonstrated that the linkage peak observed in this region is independent of a contribution by the *NOD2*-positive patients (Hampe et al. 2001b). Additional candidate gene studies in this area to detect additional disease-associated genetic variations on 16p are therefore warranted.

Integrins are heterodimeric integral membrane glycoproteins composed of one alpha and one beta chain in non-covalent binding. The integrin alpha L (CD11A), integrin alpha M (CD11B), integrin alpha X (CD11C), and integrin alpha D (CD11D) chains share a uniform integrin beta chain (CD18). CD11/CD18 is expressed on blood leukocytes and is known for the key role in leukocyte adhesion to blood vessels and migration into inflamed tissue lesions (Kishimoto et al. 1989). There are 16 alpha and eight beta chains located on different chromosomes. The *CD11* genes are located on the proximal p arm of Chr 16p11.2 with neighboring positions of *CD11C* and *CD11D* (Callen et al. 1998; Corbi et al. 1988; Noti et al. 2000; Shelley et al. 1998). Contrary to these publications, the annotation information provided by the Human Genome Project Working Draft, National Center for Biotechnology Information (NCBI), Ensemble, and Celera databases have profound differences in their physical maps, even in the chromosomal localization. Therefore the construction of a physical map was needed prior to a genetic investigation.

While *CD11* genes are excellent positional candidates, there is also functional evidence to suggest their involvement in the pathophysiology of intestinal inflammation. Increased expression of CD11 has been shown in colonic and ileal tissue of patients with CD (Bernstein et al. 1998; Malizia et al. 1991). Differential regulation is seen between patients with CD and UC (Vainer et al. 2000). Signaling through CD11/CD18 influences cellular inflammation pathways involving MAPK and IL1-ß (Rezzonico et al. 2000; Schmidt et al. 2001), JNK activation by TNF-alpha (Avdi et al. 2001) or NF-kappaB, MIP-1alpha and MIP-1beta (Rezzonico et al. 2001). Most of these inflammation pathways and molecules have been suggested to be important in the pathophysiology of IBD. In addition, compounds inhibiting members of the integrin-ICAM family appear to be promising therapeutic candidates for CD (Gordon et al. 2001; Schreiber et al. 2001; Yacyshyn et al. 1998). Genetic variations in the integrin genes can have a large impact on the function of the protein. Functional studies showed that single amino acid changes can cause a restored inside-out function of CD11A (Bleijs et al. 2001) and the deletion of the functional I domain can change the receptor function (Leitinger and Hogg 2000). Therefore the full-length screening of these genes for sequence variations and investigation for disease association is important.

In this study physical, radiation hybrid (RH), and fluorescent in situ hybridization (FISH) mapping data confirm the arrangement of *CD11B, C, D* in a gene cluster on Chr 16p. By complete screening of the *CD11A-D* genes, 13 new sequence variations and eight single nucleotide polymorphisms (SNPs) suggested by database search were identified and evaluated as predisposing factors for IBD.

Materials and methods

Family ascertainment and phenotypes

The cohort was recruited in Germany at the Charité University Hospital (Berlin, Germany) and the Department of Internal Medicine I, University Hospital, Kiel, Germany. All study participants gave informed, written consent. The recruitment protocols were approved prior to study initiation by the institutional review boards. The diagnosis of IBD and the classification into CD and UC were determined by standard diagnostic criteria (Lennard-Jones 1989; Truelove and Pena 1976). Ascertainment criteria were determined prior to the initiation of patient collection. Clinical, radiological, and endoscopic (type of lesions, distribution) examinations were performed to unequivocally confirm the diagnosis of either UC or CD. Histological findings also had been confirmatory or compatible with this diagnosis. In case of uncertainty, the diagnosis of indeterminate colitis was assigned and the patient excluded from the study. If possible, the patients were directly examined by one or more of the principal investigators. Alternatively, two written records containing a detailed disease history and results of all diagnostic procedures were obtained for each patient and reviewed by the principal investigators. A venous blood sample was obtained from the affected siblings and their parents, if possible. Normal controls were recruited through the blood donor system at the University Hospital in Kiel. An overview of the family cohort is given in Table 1.

Mapping

RH mapping was performed using the Stanford G3 and TNG panels. Sequence tagged site-polymerase chain reactions (STS-PCRs) to all integrins were performed in the panels. RH vectors were analyzed using the Stanford RH mapping server (at http://shgc.stanford.edu/) and by using the RADMAP extension of the MULTI-

Table 1 Cohorts investigated: affected sibling pairs are ordered according to sibship size and disease type [mixed refers to sibships with both ulcerative colitis (*UC*) and Crohn's disease (*CD*)] within the family. The number of sibships and affected sib pairs (in parentheses) is given in each category

Sibship size	CD	UC	Mixed	Total
1 (trios)	304	65	–	369
2	94 (94)	44 (44)	27 (27)	165 (165)
3	7 (21)	–	5 (15)	12 (36)
Total	419	109	42	570
Controls	–	–		380

Table 2 Single nucleotide polymorphism (*SNP*) markers identified in this study. Positions are given in the corresponding intron (*i*) or exon (*e*). Allele frequencies are based on healthy controls. The novel SNPs have been submitted to dbSNP

SNP	Position	Nucleotide/ amino acid	Allele frequency	SNP-ID	Genotyped?
CD11A-i08	+132	C/T	T=45.1%	New, ss4318992	Yes
CD11A-i11–1	+9	T/G	G=0.5%	New, ss4318993	No
CD11A-i11–2	+54	G/A	A=42.4%	New, ss4318994	Yes
CD11A-i12	+137	C/T	T=8.3%	New, ss4318995	Yes
CD11A-i18	+927	G/A	A=29.1%	New, ss4318996	Yes
CD11A-e21–1	+6	G/C, Arg/Thr	C=27.8%	New, ss4318997	Yes
CD11A-e21–2	+61	C/T, synonymous	T=10.7%	New, ss4318998	Yes
CD11A-i21	+95	C/A	A=2.5%	New, ss4318999	No
CD11A-i22	+360	G/A	A=22.1%	New, ss4319000	Yes
CD11B-e03	+96	G/A, Arg/His	A=11.9%	Confirmed, rs1143679	Yes
CD11B-e10	+20	C/A, Ser/Arg	A=0.3%	New, ss4319001	No
CD11B-e12	+109	T/C, Met/Thr	C=16.3%	Confirmed, rs1143680	Yes
CD11B-e16	+106	C/T, synonymous	T=2.5%	Confirmed, rs1143681	No
CD11B-e20–1	+108	C/A, synonymous	A=2.5%	New, ss4319002	No
CD11B-e20–2	+136	G/A, synonymous	A=33.4%	Confirmed, rs1143682	Yes
CD11B-e21	+68	C/T, Ala/Val	T=17.5%	Confirmed, rs1143683	Yes
CD11B-e29	+87	C/T, synonymous	T=2.5%	Confirmed, rs1143677	No
CD11B-e30	+49	C/T, Pro/Ser	T=17.3%	Confirmed, rs1143678	Yes
CD11C-e09	+44	G/A, Ala/Thr	A=20.3%	New, ss4319003	Yes
CD11C-e15	+50	C/G, Pro/Arg	G=31.6%	New, ss4319004	Yes
CD11C-I31	+75	A/G	G=17.6%	Confirmed, rs2929	Yes

MAP program. Marker order and the distance separating each marker were defined using information derived from our pedigrees using the automated mapping program MULTIMAP v. 2.0. (Matise et al. 1994). The maps were confirmed by comparison with published genetic maps (CHLC and GENETHON at http://www.ncbi.nlm.nih.gov).

The physical map was built by PCR-based screening of CIT-B (California Institute of Technology Human BAC library B: CIT978SK) clone library. We used gene-specific primers based on Genebank sequences (NM_000632, NM_000887, U37028). The contig was verified by end sequence-specific PCR of identified clones (AF449707/-12). All sequences were masked using REPATMASKER (http://repeatmasker.genome.washington.edu). Additionally, BLAST searches against publicly available databases (http://www.ncbi.nlm.nih.gov/BLAST/) was performed to identify previously sequenced clones and genes. The orientation of the genes and the fine mapping of markers were based on sequence data.

FISH mapping of clones CIT-B 178P10 and CIT-A 635H12 was performed with SpectrumOrange (SO, Vysis, Downers Grove, USA) or SpectrumGreen (SG, Vysis) labeled BAC-DNA using standard protocols (Schlegelberger et al. 1999).

Mutation detection

The *CD11A* gene was screened by genomic re-sequencing in 24 unrelated individuals with IBD, including all exons with their flanking intron structure, based on a GenBank sequence (AC002310). The *CD11B-D* genes were screened by cDNA sequencing (oligo-dT primed, reversed transcribed mRNA from Epstein-Barr virus transformed B-lymphocyte cultures) in the same 24 unrelated individuals, based on GenBank sequences (NM_000632, NM_000887, U37028). There were insufficient genomic sequences available for genomic sequencing. Fluorescent sequencing of templates was carried out using Big Dye Cycle Sequencing (PE Biosystems, Foster City, Calif., USA) and products were analyzed on an ABI PRISM 3700 automated DNA sequencer (PE Biosystems).

Genotyping

All SNPs were genotyped using the Taqman (PE Biosystems) system. The primers we used are shown in Table 2. Previously published SNPs (http://www.ncbi.nlm.nih.gov/SNP) were included in our study. Genomic DNA was prepared from whole blood using the Puregene system (Gentra Systems, Minneapolis, Minn., USA). DNA samples were arrayed in 96- and 384-well microtiter plates and subjected to Taqman-PCR. The products were directly read in an ABI 7700 and ABI7900 (Applied Biosystems). The data were managed and checked for mendelian inheritance errors through an integrated database system. (Hampe et al. 2001c).

Statistical analysis

Genetic analyses were conducted using the two standard diagnostic categories CD and UC, as described. The IBD category represents all affected individuals as a single phenotype for analysis. Case-control analyses were performed using chi-squared and Fisher's exact tests, with one affected individual picked at random from multiplex families.

Family-based linkage and association analyses were calculated using the TRANSMIT program (Clayton 1999; Clayton and Jones 1999) with the robust variance estimator option. Significance was verified using 10,000 bootstrap samples.

Haplotype frequencies for pairs of SNPs were estimated using EH (Terwilliger and Ott 1994). Linkage disequilibrium (LD) coefficients $D'=D/D_{max}$ between pairs of SNPs were obtained according to Devlin and Risch (1995).

Results

Map

The RH mapping experiments suggested a distance of 108 cR (G3 panel) between *CD11A* and *CD11B-D*, corresponding to 2.5 Mb. *CD11B-D* were located within 130 cR (TNG panel), corresponding to 516 kb. *CD11A* was telomeric to *CD11B-D*.

The genomic structure was known for the *CD11A* gene, which was located on a sequenced bacterial artificial chromosome (BAC) (CIT-A 635H12/AC002310). The relative positions of *CD11B-D* were previously not known. As the RH mapping suggested a cluster of the *CD11B-D* genes within 500 kb, we built a physical map

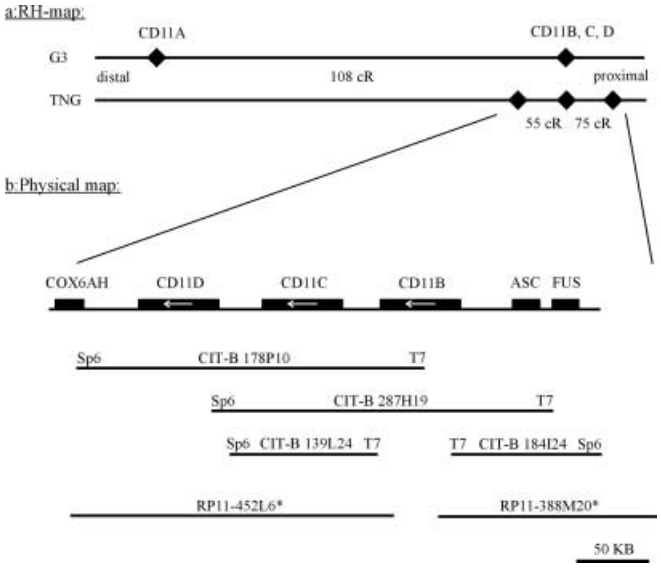

Fig. 1 **a** Radiation hybrid map. G3 and TNG map of *CD11* genes, distances given in centirays. **b** Physical map. Genomic structure of the *CD11* gene cluster; *arrows* indicate the orientation of genes. BAC clones are indicated by *horizontal lines*. CIT clones were identified by PCR. RP11 clones were identified by BLAST; * working draft sequence. *178P10-sp6* aligns with *Cox6ah* gene, 287H19-sp6 aligns with the putative intron 5 of *CD11D*. Orientation of *ASC* and *FUS* is unknown

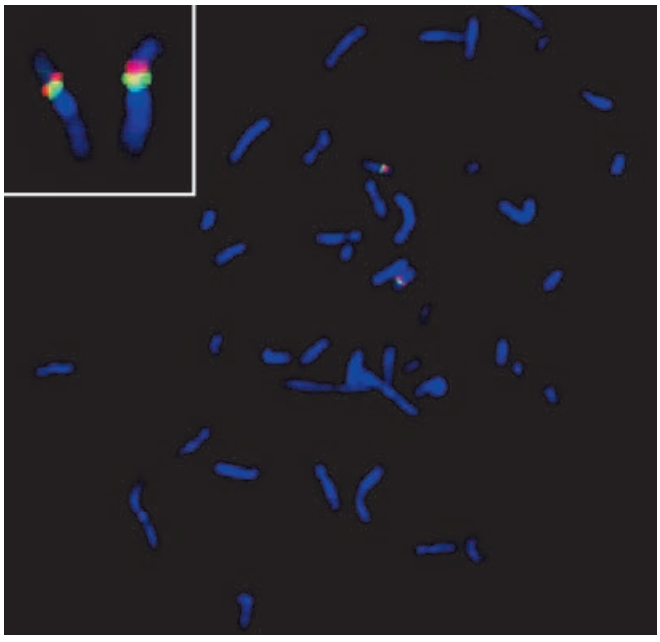

Fig. 2 Chromosomal assignment of the *CD11A* gene and the *CD11B-D* gene cluster by fluorescent in situ hybridization. Both clones *635H12* (*red fluorescence*) and *178P10* (green fluorescence) containing the *CD11A* gene and the *CD11B-D* gene cluster, respectively, show hybridization signals only in the short arms of both Chromosomes 16

Table 3 The primers and probes of the TAQMAN assays genotyped in this study. Some assays are designed on the reverse strand

SNP	Primers	Probes
CD11A-i08	F: TCTCCTTGGTCTGAAAATGCTTT R: TGCTGCTTCCTGCATCATATCT	G: AGGGAGTGGAGAGTGAGGAGCTGAAATCC A: AGGGAGTGGAGAGTGAAGAGCTGAAATCC
CD11A-i11	F: AGAGCAGGCTATTTGGGTGAGT R: GAGCCACCAGGACCAGCTAC	A: TCAGATGGTCACTCAGCCTGGCTTCC G: TCAGATGGTCGCTCAGCCTGGCTT
CD11A-i12	F: GTGGGCTCTTTGAGGTTCAGTT R: GCAGCCCCTGTGCTAAACAT	G: AGGGCTTACCTCTGTGTGTTATGAGGGATAAATGT A: AGGGCTTACCTCTGTGTATTATGAGGGATAAATGTGA
CD11A-i18	F: GTATGTCCTTGCCCTGCTGAG R: GCACAAATGGCAGTAGGCTGA	C: AAGGAGAGCCGGAGGGGCAGG T: TGAAGGAGAGCTGGAGGGGCAGG
CD11A-e21–1	F: TTACTCAGGCTCAGCTCCACAGA R: CCATCTTTCCCTGATCATCCC	C: TTAGACGCAGGGCTCTGGATCTGTGG G: TTAGACGCAGGGCTGTGGATCTGTGG
CD11A-e21–2	F: CTTGCGGAAGGAGAGTCCC R: TCTAACTGCTTTTGCCAGCCTC	G: AGTAAGCATCTTCTTCCAAGTTACTCAGGCTCAGC A: AGTAAGCATCTTCTTCCAAATTACTCAGGCTCAGC
CD11A-i22	F: GAGCTGTTTACCAGTGTATTAAACATCA R: TTGGTTGCCTCATCTTTGCA	C: TGAGGAGCAGCCTCATGGCCC T: CATGAGGAGCAGTCTCATGGCCCC
CD11B-e03	F: TGCCTGGAAAGTCCCTATGTG R: CAGGAGATAGTGGCTGCCAAC	C: CCCTGCAGGCGGATGGGCT T: CACCCTGCAGGTGGATGGGCTC
CD11B-e12	F: GGTGCCCTTGACATTAGCGT R: ATCGGCCTGGTAGCGATGT	C: AGGCAGAACACTGGCACGTGGGAGT T: AGGCAGAACACTGGCATGTGGGAGTC
CD11B-e20–2	F: CTGTGAGAAATGATGGTGAGGACT R: GTGAGCGCTGGTTCTGGAAG	G: CGGAGGGTGTCCACGCTCCAGGTA A: CGGAAGGTGTCCACACTCCAGGTAGC
CD11B-e21	F: CCTGGCCTGTGAGTCTGCCT R: GGGGTGGTTTATGCTGCAGC	C: CGAAGTGTCTGGGGCCTTGAAGAGC T: CCGAAGTGTCTGGGGTCTTGAAGAGCA
CD11B-e30	F: CAAGCGGCAATACAAGGACAT R: CCGTCACCTACCCACACGC	T: ATGAGTGAAGGGGGTTCCCCGG C: ATGAGTGAAGGGGGTCCCCCG
CD11C-e09	F: TAGGCACCGATTGTTCCATG R: TGTCGCCTTCTTTCTTCCCA	A: CCGTAGGGATGCCACCAAAATTCTCT G: CCGTAGGGATGCCGCCAAAATTC
CD11C-e15	F: TTCTGCAGTGGAGAAGGTGGT R: AAACAGGTAGACAGCACCCCG	C: AGGGCCACCCCTGGGGTCG G: AGGGCCACCGCTGGGGTCG
CD11C-i31	F: TCCCCACTTACTTACCCTCACC R: AAGCAGGCCCATCCCAG	G: CTGACGGGGAGGAACCACTGGAC A: CTGACGGGGAAGAACCACTGGACC

Table 4 Statistical analyses. In the case-control statistic only significant values are given; for all other markers there was no significant difference between cases and controls. For marker frequencies see Table 2. Single marker and haplotype TRANSMIT analyses were not significant after bootstrap simulation; * indicates marginally significant haplotypes (four-marker window) before bootstrap in CD patients

Marker	Case-control		Single	Haplotype
CD11A-i08	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-i11	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-i12	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-i18	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-e21–1	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-e21–2	*P*>0.05		*P*>0.05	*P*>0.05
CD11A-i22	*P*>0.05		*P*>0.05	*P*>0.05
CD11B-e03	*P*>0.05		*P*>0.05	*P*>0.05*
CD11B-e12	Allele frequency (C): 16.3% (NC) 13.9% (CD), *P*>0.05 13.9% (UC), *P* >0.05 15.1% (IBD), *P* >0.05	Genotype frequency (TT/TC/CC): 71.3%/24.8%/3.9% (NC) 72.6%/28.9%/0.5% (CD), *P*=0.002 73.9%/24.2%/1.8% (UC), *P* >0.05 71.1%/27.6%/1.3% (IBD), *P*=0.03	*P*>0.05	*P*>0.05*
CD11B-e20–2	*P*>0.05		*P*>0.05	*P*>0.05
CD11B-e21	*P*>0.05		*P*>0.05	*P*>0.05
CD11B-e30	*P*>0.05		*P*>0.05	*P*>0.05*
CD11C-e09	p >0.05		*P*>0.05	*P*>0.05
CD11C-e15	Allele frequency (G): 31.6% (NC) 37.8% (CD), *P*=0.01 34.2% (UC), *P*>0.05 36.8% (IBD), *P*=0.02	Genotype frequency (CC/CG/GG): 45.1%/46.6%/8.3% (NC) 38.7%/47.0%/13.3% (CD), *P*=0.02 43.5%/44.0%/12.5% (UC), *P* >0.05 39.6%/47.1%/13.3% (IBD), *P*=0.04	*P*>0.05	*P*>0.05
CD11C-i31	*P*>0.05		*P*>0.05	*P*>0.05

using BAC clones. In order to establish a genomic contig carrying the *CD11B-D* genes, we screened a BAC library with gene-specific PCR primers. We identified four clones carrying the entire *CD11* gene cluster, spanning a region of approximately 300 kb. The contig was verified with end-sequence and multiple gene-specific PCRs. BLAST search in the NCBI database identified two more clones and three other genes in that region. They were confirmed by specific PCR.

The order of genes is *CD11B*, *CD11C*, and *CD11D*, all in the same orientation. The *COX6AH* gene (U66875) is downstream of *CD11D* in the same orientation. The genes *ASC* (NM_013258) and *FUS* (NM_004960) are located on the clone RP11–388M20. along with the first six exons of *CD11B*. An overview of the mapping results is given in Fig. 1.

We confirmed the neighboring position of *CD11C* and *CD11D* as described before (Noti et al. 2000; Shelley et al. 1998) and also showed that the *CD11B* gene is adjacent to this cluster. FISH mapping of BAC clone CIT-B 178P10 and CIT-A 635H12 confirmed their assignment to chromosome region 16p11–12 (Fig. 2). Double-color FISH showed CIT-B 178P10 to be located centromerically of CIT-A 635H12 (data not shown).

Novel mutations in the *CD11* genes

We screened a total of 14 kb of genomic sequence and identified nine novel SNPs in the *CD11A* gene. We screened a total 12 kb of cDNA and identified two novel SNPs in the *CD11B* gene and confirmed the published SNPs (rs1143677–1143683). Two novel SNPs in the *CD11C* gene were identified and the known SNP (rs2929) was confirmed. The published SNPs (rs1574566–1574568) of the *CD11C* gene were not observed in our cohort or controls. According to our data, these SNPs do not exist in the German population or the published mRNA (NM_000887) is incorrect (ss4319005). The correct sequence at position 684/685 is GC instead of CG, and the correct amino acid at position 209 is Ser instead of Thr. We did not identify any new SNPs in the *CD11D* gene by cDNA sequencing. The SNPs identified in this study are listed in Table 2. SNPs inducing amino acid changes or having a frequency >10% were chosen for genotyping and analyses in our patient cohort.

Genetic analysis

A total of 177 multiplex families of German descent, 369 German trios (sporadic cases and their families), and 380 German controls were analyzed. Primers and probes used for genotyping by TaqMan are detailed in Table 3.

Between the genotyped markers in the *CD11* genes the following LD values were observed. The exonic markers of *CD11B-D* are in strong pairwise LD, $|D'|>0.7$. The intronic marker is in weak LD with the exonic markers, $|D'|<0.3$. In the *CD11A* gene there are two LD groups with $|D'|>0.8$ with a break between the markers CD11A-i18 and CD11A-e21–1.

An exploratory case-control analysis with all genotyped markers identified a series of potential association leads (Table 4). The SNP in exon 15 of *CD11C* demonstrated a significantly increased allele frequency of the mutated allele and of the homozygote genotype in CD patients, with 59 observed over 47 expected patients (*P* value=0.02). The SNP in exon 20–2 of *CD11B* is in strong LD, $|D'|=0.93$ and shows a non-significant trend

in the same direction (*P* value=0.08). The SNP in exon 12 of *CD11B* shows a significantly decreased frequency of the rare homozygote genotype in CD patients, with two observed over eight expected patients (*P* value= 0.002). No effect was observed in allele frequency. No significance was seen in the other markers.

In order to verify the observed significances in the case-control statistics a single marker TDT was run. We used TDT as implemented in the TRANSMIT program. The family based linkage and association was negative for all single markers.

In haplotype analyses we observed marginally significant results in the *CD11B-D* genes. These haplotypes have a population frequency of 0.5–2% and show an increased transmission in CD patients. Under strong simulation conditions using the bootstrap option the significance dropped. There was no consistency between the case-control statistic and the association/linkage analysis; the significant markers CD11C-e15 and CD11B-e12 show no effect in transmission.

Discussion

Genetic linkage to Chr 16 is well established and results in multiple linkage peaks. Recent studies using positional cloning as well as candidate gene approaches in different populations led to the identification of the first IBD disease gene on the q arm of Chr 16. Through formal exclusion of the contribution of *NOD2* variant patients to the linkage findings, an independent linkage peak on Chr 16p can be demonstrated (Hampe et al. 2001b). The identification of *NOD2* in the French population (Hugot et al. 2001) cannot explain the entire linkage findings on Chr 16 with mutations on the q arm. Therefore the alpha integrins as excellent positional and functional candidates had to be evaluated.

In this study a physical map of all alpha integrins on Chr 16p11.2 is shown. The genes *CD11B-D* are adjacent in a cluster of approximately 300 kb. We confirm the neighboring position *CD11C* and *CD11D* and found evidence that *CD11B* is part of the cluster, whereas *CD11A* is located at a distance of 2.5 Mb towards the telomere. The map shown in Fig. 1 describes the exact position and genomic arrangement of these four genes, which is correcting the human genome project working draft of the international public consortium and the Celera assemblies. BLAST search identified two recently submitted working draft BAC clones (RP11–388M20, RP11–452L6), which support the mapping results. Our laboratory based results correct the automated annotation of an *ITGAD-like gene* to the clone RP11–452L6. We conclude that the *ITGAD-like gene* is identical to *ITGAD* (*CD11D*) itself. The arrangement of *CD11B-D* in a gene cluster and the positioning of *CD11A* away from that cluster fit with a duplication model for integrin evolution. The clustered genes share a significantly higher homology with each other than with CD11a, 63% versus 35%. Therefore the hypothesis is that a primordial gene for the leukocyte alpha integrin subunit duplicated and gave rise to *CD11A* and a primordial cluster gene. Further duplication of the cluster gene gave rise to the *CD11B-D* genes (Kishimoto et al. 1989).

In order to thoroughly investigate the role of genetic variations for IBD all four genes were re-sequenced. Full-length mutation detection on genomic DNA for *CD11A* and on cDNA for *CD11B-D* was performed (1) to give us a dense map of genetic variations for linkage analyses covering the region of interest and (2) to detect hypothetical disease-causing or population-specific mutations. We confirmed eight known and identified 13 new SNPs (Table 2). There was a difference in the number of exonic SNPs in the genes, with *CD11B* showing a much higher number of SNPs than the other genes. No SNPs are listed in the database for *CD11D* and we also did not detect any sequence variations. The SNPs were chosen for genotyping on the basis of information content (allele frequency >10%) and functional relevance (causing amino acid changes). Because the two IBD subphenotypes (CD and UC) may be associated with different genetic determinants, separate and combined statistical analyses were carried out to determine whether different frequency of genotypes and excess transmission of alleles or haplotypes of these markers could be detected.

Exploratory analyses with case-control statistics yielded two leads. One was in the genotype and allele frequency of the SNP in exon 15 of *CD11C* and one was in the genotype frequency of the SNP in exon 12 of *CD11B*. We felt that the polymorphism CD11C-e15 was most meaningful, as a linked additional SNP (CD11B-e20–2) showed a similar although not significant trend. Confirmatory association/linkage analyses of these and other single markers were negative. Sliding window haplotype analyses of all markers were calculated with TRANSMIT. The initial analysis showed higher transmissions than expected for a few haplotypes with a low population frequency. Under simulation conditions using the bootstrap option of TRANSMIT the significance did not hold. Therefore, these distortions of transmissions have to be regarded as a statistical artifact or alternatively can be seen as week signals of a nearby relevant genetic variation.

Given these results it is unlikely that the alpha integrin sequence variations on 16p themselves have a major influence on the development of IBD, although we cannot completely exclude false-negative results. It is possible that marginally significant results in case-control and haplotype analyses could point to a disease-relevant variation in the vicinity of the *CD11* gene cluster. Although not relevant in the etiology of IBD, the *CD11* genes appear to be important elements of disease pathophysiology and may be important therapeutic targets. The mapping results of this study and the SNPs described here may provide an excellent basis for specific investigations of other inflammatory diseases.

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References

Annese V, Latiano A, Bovio P, Forabosco P, Piepoli A, Lombardi G, Andreoli A, Astegiano M, Gionchetti P, Riegler G, Sturniolo GC, Clementi M, Rappaport E, Fortina P, Devoto M, Gasparini P, Andriulli A (1999) Genetic analysis in Italian families with inflammatory bowel disease supports linkage to the *IBD1* locus – a GISC study. Eur J Hum Genet 7:567–573

Avdi NJ, Nick JA, Whitlock BB, Billstrom MA, Henson PM, Johnson GL, Worthen GS (2001) Tumor necrosis factor-alpha activation of the c-Jun N-terminal kinase pathway in human neutrophils. Integrin involvement in a pathway leading from cytoplasmic tyrosine kinases apoptosis. J Biol Chem 276:2189–2199

Bernstein CN, Sargent M, Gallatin WM (1998) Beta2 integrin/ICAM expression in Crohn's disease. Clin Immunol Immunopathol 86:147–160

Bleijs DA, Duijnhoven GC van, Vliet SJ van, Thijssen JP, Figdor CG, Kooyk Y van (2001) A single amino acid in the cytoplasmic domain of the beta 2 integrin lymphocyte function-associated antigen-1 regulates avidity-dependent inside-out signaling. J Biol Chem 276:10338–10346

Brant SR, Fu Y, Fields CT, Baltazar R, Ravenhill G, Pickles MR, Rohal PM, Mann J, Kirschner BS, Jabs EW, Bayless TM, Hanauer SB, Cho JH (1998) American families with Crohn's disease have strong evidence for linkage to chromosome 16 but not chromosome 12. Gastroenterology 115:1056–1061

Callen DF, Chen LZ, Nancarrow J, Whitmore SA, Apostolou S, Thompson AD, Lane SA, Stallings RL, Hildebrand CE, Harris PG, Sutherland GR (1998) Current state of the physical map of human chromosome 16. Cytogenet Cell Genet

Cavanaugh J (2001) International collaboration provides convincing linkage replication in complex disease through analysis of a large pooled data set: Crohn disease and chromosome 16. Am J Hum Genet 68:1165–1171

Cho JH, Nicolae DL, Gold LH, Fields CT, LaBuda MC, Rohal PM, Pickles MR, Qin L, Fu Y, Mann JS, Kirschner BS, Jabs EW, Weber J, Hanauer SB, Bayless TM, Brant SR (1998) Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. Proc Natl Acad Sci USA 95:7502–7507

Clayton D (1999) A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. Am J Hum Genet 65:1170–1177

Clayton D, Jones H (1999) Transmission/disequilibrium tests for extended marker haplotypes. Am J Hum Genet 65:1161–1169

Corbi AL, Larson RS, Kishimoto TK, Springer TA, Morton CC (1988) Chromosomal location of the genes encoding the leukocyte adhesion receptors LFA-1, Mac-1 and p150,95. Identification of a gene cluster involved in cell adhesion. J Exp Med 167:1597–1607

Curran ME, Lau KF, Hampe J, Schreiber S, Bridger S, Macpherson AJ, Cardon LR, Sakul H, Harris TJ, Stokkers P, Van Deventer SJ, Mirza M, Raedler A, Kruis W, Meckler U, Theuer D, Herrmann T, Gionchetti P, Lee J, Mathew C, Lennard-Jones J (1998) Genetic analysis of inflammatory bowel disease in a large European cohort supports linkage to chromosomes 12 and 16. Gastroenterology 115:1066–1071

Devlin B, Risch N (1995) A comparison of linkage disequilibrium measures for fine-scale mapping. Genomics 29:311–322

Gordon FH, Lai CW, Hamilton MI, Allison MC, Srivastava ED, Fouweather MG, Donoghue S, Greenlees C, Subhani J, Amlot PL, Pounder RE (2001) A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active Crohn's disease. Gastroenterology 121:268–274

Hamilton SR (1987) The differential diagnosis of idiopathic inflammatory disease by colorectal biopsy. Int J Colorectal Dis 2:113–117

Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, Deventer S van, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG (2001a) Association between insertion mutation in *NOD2* gene and Crohn's disease in German and British populations. Lancet 357:1925–1928

Hampe J, Frenzel H, Mirza M, Croucher P, Cuthbert A, Mascheretti S, Huse K, Platzer M, Bridger S, Meyer B, Nürnberg P, Stokkers P, Krawczak M, Mathew CG, Curran M, Schreiber S (2001b) Evidence for an independent susceptibility locus for inflammatory bowel disease on chromosome 16p. Proc Natl Acad Sci USA (in press)

Hampe J, Wollstein A, Lu T, Frevel H, Will M, Schreiber S (2001c) An integrated database for Taqman based high throughput SNP genotyping. Bioinformatics (in press)

Hugot JP, Laurentpuig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, Naom I, Dupas JL, Vangossum A, Orholm M, Bonaitipellie C, Weissenbach J, Mathew CG, Lennardjones JE, Cortot A, Colombel JF, Thomas G (1996) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 379:821–823

Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411:599–603

Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA (1989) The leukocyte integrins. Adv Immunol 46:149–182

Leitinger B, Hogg N (2000) Effects of I domain deletion on the function of the beta2 integrin lymphocyte function-associated antigen-1. Mol Biol Cell 11:677–690

Lennard-Jones JE (1989) Classification of inflammatory bowel disease. Scand J Gastroenterol [Suppl] 170:2–6; discussion 16–19

Malizia G, Calabrese A, Cottone M, Raimondo M, Trejdosiewicz LK, Smart CJ, Oliva L, Pagliaro L (1991) Expression of leukocyte adhesion molecules by mucosal mononuclear phagocytes in inflammatory bowel disease. Gastroenterology 100: 150–159

Matise TC, Perlin M, Chakravarti A (1994) Automated construction of genetic linkage maps using an expert system (MultiMap): a human genome linkage map. Nat Genet 6:384–390

Noti JD, Johnson AK, Dillon JD (2000) Structural and functional characterization of the leukocyte integrin gene CD11d. Essential role of Sp1 and Sp3. J Biol Chem 275:8959–8969

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH (2001a) A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. Nature 411:603–606

Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G (2001b) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 276: 4812–4818

Orholm M, Munkholm P, Langholz E, Nielsen OH, Sorensen IA, Binder V (1991) Familial occurrence of inflammatory bowel disease. N Engl J Med 324:84–88

Podolsky DK (1991) Inflammatory bowel disease. II. N Engl J Med 325:1008–1016

Rezzonico R, Chicheportiche R, Imbert V, Dayer JM (2000) Engagement of CD11b and CD11c beta2 integrin by antibodies or soluble CD23 induces IL-1beta production on primary human monocytes through mitogen-activated protein kinase-dependent pathways. Blood 95:3868–3877

Rezzonico R, Imbert V, Chicheportiche R, Dayer JM (2001) Ligation of CD11b and CD11c beta(2) integrins by antibodies or soluble CD23 induces macrophage inflammatory protein 1alpha (MIP-1alpha) and MIP-1beta production in primary human monocytes through a pathway dependent on nuclear factor-kappaB. Blood 97:2932–2940

Schlegelberger B, Metzke S, Harder S, Zuehlke-Jenisch R, Zhang Y, Siebert R (1999) Classical and molecular cytogenetics of tumor cells. In: Wegner R (ed) Diagnostic cytogenetics. Springer, New York Berlin Heidelberg, pp 151–185

Schmidt A, Caron E, Hall A (2001) Lipopolysaccharide-induced activation of beta2-integrin function in macrophages requires Irak kinase activity, p38 mitogen- activated protein kinase, and the Rap1 GTPase. Mol Cell Biol 21:438–448

Schreiber S, Nikolaus S, Malchow H, Kruis W, Lochs H, Raedler A, Hahn EG, Krummenerl T, Steinmann G (2001) Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. Gastroenterology 120:1339–1346

Shelley CS, Da Silva N, Georgakis A, Chomienne C, Arnaout MA (1998) Mapping of the human CD11c (*ITGAX*) and CD11d (*ITGAD*) genes demonstrates that they are arranged in tandem separated by no more than 11.5 kb. Genomics 49:334–336

Terwilliger JD, Ott J (1994) Handbook of human genetic linkage. The Johns Hopkins University Press, Baltimore, Md. pp 245–250

Thompson NP, Driscoll R, Pounder RE, Wakefield AJ (1996) Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ 312:95–96

Truelove SC, Pena AS (1976) Course and prognosis of Crohn's disease. Gut 17:192–201

Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B (1988) Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut 29:990–996

Vainer B, Nielsen OH, Horn T (2000) Comparative studies of the colonic in situ expression of intercellular adhesion molecules (ICAM-1, -2, and -3), beta2 integrins (LFA-1, Mac-1, and p150,95), and PECAM-1 in ulcerative colitis and Crohn's disease. Am J Surg Pathol 24:1115–1124

Yacyshyn BR, Bowen-Yacyshyn MB, Jewell L, Tami JA, Bennett CF, Kisner DL, Shanahan WR Jr (1998) A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. Gastroenterology 114:1133–1142