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Association of *NFKB1*, which encodes a subunit of the transcription factor NF-κB, with Alcohol Dependence

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ABSTRACT

A broad region on chromosome 4q has been linked to alcohol dependence (alcoholism). We hypothesized that such broad linkage regions represent the combined action of multiple genes. Seeking to identify genes within that region that are associated with alcoholism, we have tested the association of *NFKB1,* located at 4q24, with alcoholism. *NFKB1* encodes a 105 kDa transcription inhibitor that is cleaved to the 50 kDa DNA binding subunit of the ubiquitous transcription factor NF-κB. NF-κB regulates many genes relevant to brain function, and its actions can be potentiated by ethanol; thus *NFKB1* is an excellent candidate gene for alcoholism. Nineteen SNPs in and near *NFKB1* were analyzed in a sample of 219 multiplex alcoholic families of European American descent. Family based association analyses detected significant evidence of association with eight SNPs and marginal evidence for five more. The association was driven by the affected individuals with earlier onset of alcoholism (55% of the sample with onset ≤ years). Further analysis of the age of onset as a quantitative variable provided evidence for the association of 12 SNPs in this gene. Thus variations in *NFKB1* appear to affect the risk for alcoholism, particularly contributing to an earlier onset of the disease.

INTRODUCTION

Alcohol dependence (alcoholism) is a common, complex disease with both genetic and environmental contributions to its etiology. Identifying specific genes in which variations contribute to the risk for alcoholism is a difficult task, but there has been much progress in recent years (1). The Collaborative Study on the Genetics of Alcoholism (COGA) performed whole genome linkage analyses in a sample of families that include at least three alcoholic first degree relatives (2-6). A broad region on chromosome 4q was found linked to the phenotype of alcohol dependence in these families (3, 4, 7). The evidence for linkage to this chromosomal region came from several types of analyses. There was disproportionately low allele sharing among siblings discordant for alcohol dependence (3), and there was evidence of increased allele sharing among members of these densely-affected families who were not alcohol dependent (4). A variance component analysis of alcoholism provided strong evidence of linkage to this region (7), and a bivariate analysis that included both DSM-IV diagnosis of alcoholism and the amplitude of the P3 component of the event-related potential provided increased evidence of linkage (7). We have already identified *ADH4* as one gene in this chromosomal region that is associated with alcoholism (8); other studies have also implicated *ADH4* as a susceptibility gene for alcohol dependence (9-11). *SNCA*, encoding α-synuclein (another candidate gene in this region, based upon data from a rat model of alcoholism (12, 13)), was not associated with alcoholism but was associated with an endophenotype, craving for alcohol (14). We hypothesized that linkage peaks encompassing a large chromosomal region, such as the one on chromosome 4q, reflect the combined contribution of several genes. Therefore, we have continued to analyze additional candidate genes in this broad linkage region, to test whether additional genes in that region are associated with alcoholism susceptibility in the COGA families.

NFKB1, located within the linkage peak, encodes a 105 kDa Rel-family protein; in its full-length form it is an inhibitor of transcription, which is cleaved into the 50 kDa DNA-binding subunit of NF-κB, a ubiquitous transcriptional activator in mammalian cells (15-21). Although initially discovered in the immune system (22), NF-κB has since been found in virtually all mammalian cells, including in the brain, where new roles are being elucidated (15, 23). NF-κB is expressed in neurons, glia and Schwann cells of the central and peripheral nervous systems (15, 23-28). NF-κB is found in synapses, and moves to the nucleus by retrograde transport upon activation (24, 29-31). NF-κB is activated by a wide range of extracellular signals, including cytokines, T-cell and B-cell mitogens, viral infection, phorbol esters, bacterial lipopolysaccharide, oxidative stress, and excitatory neurotransmitters (17, 18, 32). Activation of NF-κB occurs by phosphorylation of IκB, which leads to the ubiquitination and degradation of the IκB, freeing NF-κB to move to the nucleus and bind to its target sequences (17-19). Active NF-κB is comprised of dimers of Rel-family proteins; the human Rel proteins includes NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), c-Rel, RelA (p65) and RelB (17-19). The different dimeric forms bind to a consensus sequence GGGRNNYYCC (22, 33, 34) with different affinities (17, 33-36). The most common heterodimer in neurons (24) was the first one to be identified, RelA:NFKB1 (p65:p50) (22). Disruption of *Nfkb1* in mice does not lead to developmental abnormalities, although it does lead to defects in immune responses (37); in contrast, disruption of the RelA gene is lethal during late embryonic development (38). The *NFKB1* gene is autoregulated by NF-κB (39).

In addition to cytokines, oxidative stress and the other general activators (17, 18, 32), NF-κB is activated by nerve growth factor, β-amyloid, μ-opioid receptor agonists, and excitatory

neurotransmission (16, 24-26, 28, 40-45). Basal levels of neurotransmission appear to account for the "constitutively active" NF-κB activity in neurons, which can be blocked by blocking NMDA receptors and L-type Ca2+ channels (15, 24, 30). Glutamate further activates NF-κB, probably through a Ca^{2+} signaling cascade involving CaMKII (15, 16, 41). Activation of dopamine D2 receptors may activate NF-κB through a MAPK pathway (15, 46). Both NF-κB and CaMKII are found in synapses, where CaMKII plays an important role in long-term adaptation (15). Brain genes regulated by NF-κB include μ-opioid receptors (47), brain derived neurotrophic factor (48), neural cell adhesion molecule (49, 50), and inducible nitric oxide synthase (51).

Activation of NF-κB is associated with synaptic plasticity (21). NF-κB is activated in response to signaling patterns that produce long term potentiation (25, 52). Mice in which the RelA gene is disrupted show a defect in spatial learning tasks related to hippocampal function (15). Mice in which NF-κB activity in the forebrain is inhibited by expression of a dominant negative IκBα variant show impaired spatial learning and retention, along with impaired long term potentiation (53).

Activation of NF-κB prevents neuronal apoptosis (21). *Nfkb1*$^{-/-}$ mice are more sensitive to neurodegeneration at lower doses of trimethyltin hydroxide; p50 was activated in the surviving cells and remained elevated (54). *Nfkb1*$^{-/-}$ mice have higher levels of oxidative stress after glutamate exposure, and are more sensitive to excitotoxic injury from kainate or glutamate (55). Mice in which NF-κB function is inhibited in the forebrain are more sensitive to excitotoxic cell death (56). Excitotoxic cell death in hippocampal-entorhinal cortex slices caused by glutamate (and blocked by NMDA receptor antagonists) is potentiated by TNFα; this potentiation appears

to act through NF-κB activation and can be blocked by an NF-κB inhibitor (PTD-p65) or butylated hydroxytoluene (BHT), an antioxidant (57). Further work in that system demonstrated that ethanol increased NF-κB binding to DNA and also potentiated the neurotoxicity due to the combination of glutamate and TNF (58). It has recently been found that in rats treated for 4 days with high levels of ethanol (to model binge drinking) NF-κB binding to DNA in rat brains increased and there was substantial brain damage; BHT blocked NF-κB binding, binge-ethanol induced brain damage, and inhibition of neurogenesis (59).

Because of its key role in the regulation of so many genes, and interaction with ethanol in the brain, we considered *NFKB1* to be a good candidate gene within the linkage peak on chromosome 4q. We genotyped 19 SNPs across *NFKB1* to analyze its association with alcoholism.

RESULTS

NFKB1 extends 116 kb along chromosome 4q24. SNPs were chosen to span *NFKB1* from 8.3 kb upstream of exon 1 to 11.5 kb downstream of exon 24 (Figure 1); the average minor allele frequency (MAF) of the 19 SNPs analyzed was 0.40 (Table 1). There was high LD (D'>0.92) across the entire gene (Figure 1). As an additional measure of SNP coverage, the genotyped SNPs were submitted to Tagger (60); the set of 16 SNPs that it could evaluate (because they were in the HapMap dataset) gave a mean r^2 of 0.73 with the 99 HapMap SNPs that had MAF >0.05 in the CEU (CEPH European) population and 0.87 with the 84 SNPs that had MAF •0.10 in the region we spanned. The 3 additional SNPs we genotyped would further increase the average r^2. Thus, the SNPs analyzed in this study provide very good coverage of common genetic variation in *NFKB1* and its immediate flanking region.

Eight of the 19 SNPs were significantly associated with alcoholism ($p<0.05$) in this group of European American families, and an additional 5 yielded suggestive evidence ($p•0.10$) (Table 1). The associated SNPs spanned the entire gene, from 3.5 kb upstream of exon 1 to intron 23, with a suggestive SNP 6.8 kb downstream from the 3' non-translated region (Figure 2). Two SNPs were still significant even after incorporating a conservative correction for multiple testing based upon the number of effectively independent SNPs (61), which yielded an experiment-wide significance threshold of 0.01.

In our previous work, the association of *GABRA2* and *CHRM2* with alcoholism was strongest in the most severely affected half of the sample (62, 63). Earlier age of onset is one such measure of severity. Therefore, to further explore the association finding, we separately analyzed the affected individuals who met DSM-IV criteria for alcohol dependence earlier or later, split based upon the median age of onset of dependence among those in this sample who met the lifetime diagnosis (21 years of age). Although the number of affected individuals was nearly halved (55% met diagnosis at • 21 years), the significance of the association was even greater among the earlier onset group than in the whole sample (Table 1): ten SNPs were significantly associated, with 4 others suggestive. In contrast, no SNPs were significantly associated with the individuals who met diagnostic criteria later. Given this finding, we also analyzed the age of onset of alcoholism as a quantitative variable; age of onset was significantly associated with 12 of the 19 SNPs (Table 1).

DISCUSSION

We previously reported a broad region of linkage for alcohol dependence on chromosome 4q (3, 4, 7); the evidence for linkage was strongest when alcoholism was defined by DSM-IV criteria. We followed up that finding with a comprehensive analysis of the association of SNPs

with 7 genes encoding alcohol dehydrogenases, located at 4q22, and reported that variations in *ADH4* were associated with alcohol dependence (8). We hypothesized that the strong and broad linkage peak we had detected in our genome screen (7) represented the combined contribution of more than one gene in the region. Therefore, we are analyzing additional candidate genes within the linkage peak. Here we report the analysis of 19 SNPs across *NFKB1*, the gene encoding the p50/p105 subunit of NF-κB.

Eight SNPs across *NFKB1* were significantly associated with alcohol dependence. The only coding SNP in the gene, rs4648072, had very low MAF (0.007) and was omitted from the analysis for lack of power. There was high LD across the gene, so the 19 SNPs we analyzed carry information about most of the common ungenotyped SNPs in the region. Several SNPs were still significant even after incorporating a conservative correction for multiple testing based upon the number of effectively independent SNPs (61), which yielded an experiment-wide significance threshold of 0.01. Haplotype analysis, performed using a sliding window of 3 adjacent SNPs, did not strengthen the evidence for association, nor narrow the region (data not shown). This could indicate that there are multiple causal variations embedded in different haplotypes or that the variants are old and relatively common.

HapMap data show that LD (as measured by r^2) declines sharply at both sides of *NFKB1*; the decline is particularly sharp just to the 5' (pter) side of *NFKB1*. Six of the 8 associated SNPs in the *NFKB1* region have r^2 less than 0.4 with the 3'-most SNP (rs7677509) we genotyped (data not shown). HapMap data on a European American population (CEU) show that LD extends only weakly to the neighboring gene in that direction: the 3' end of *MANBA* (mannosidase, beta A, lysosomal), which lies to the right of *NFKB1* in opposite orientation (Figure 3). Therefore, we think it unlikely that the association we detected with SNPs in *NFKB1* could be due to LD with variants in adjacent genes.

Further exploration of the association with *NFKB1* demonstrated that the evidence came primarily from those individuals who met criteria for alcoholism earlier, based on a median split of this sample; earlier onset is associated with more severe disease (62). The reduced number of affected individuals analyzed after these splits would be expected to reduce the evidence for association if the association was not correlated with the trait on which the sample was split. Therefore, the finding that the association was even stronger in the alcoholics with earlier onset suggests that the variations in *NFKB1* have a particularly strong impact on earlier onset alcoholism. This is reinforced by our analysis of the age of onset of alcohol dependence as a quantitative variable, in which 12 of the 19 SNPs in *NFKB1* were significantly associated. This finding is consistent with our analyses of other genes associated with alcoholism in this high-risk family sample. We found that for *GABRA2* and *CHRM2* the association was also driven primarily by the most severely affected among the alcoholics, as indicated by earlier onset of alcohol dependence (62), co-occurring dependence on illicit drugs (63), and other traits including higher number of DSM-IV symptoms endorsed (62).

NFKB1 encodes a subunit of a ubiquitous transcription factor that is involved in many cellular processes, including response to oxidative damage and other signaling, and in neuronal growth and pruning by apoptosis. NF-κB activity, due to heterodimers between p50 (a product of NFKB1) and p65, is widely distributed in rodent brain (23). NF-κB is important in NMDA-mediated neuroprotection, possibly by increasing expression of BDNF (64); NF-κB expression may in turn be activated by BDNF in an autocrine loop (64, 65). Ethanol attenuates the NMDA-mediated increase in BDNF (66). The effects of ethanol and NF-κB on neurodegeneration are complex and not well understood. Binge exposure to ethanol that causes neurotoxicity actually

increased NF-κB activity (58); both effects were prevented by butylated hydroxytoluene, an antioxidant, but not by other antioxidants (59).

Another intriguing biological link of NF-κB is with the κ-opioid system. A prodynorphin-derived peptide, Big dynorphin, produces anxiolytic behavior, enhanced locomotion and noiceceptive behavior in mice, mediated by NMDA receptors (67). Homodimers of p50 (encoded by *NFKB1*) bind to a site within the coding sequence of prodynorphin and may inhibit gene expression (68).We have recently found that variations in *PDYN* (encoding prodynorphin) and *OPRK1* (encoding the κ-opioid receptor, which binds dynorphin) affect the risk for alcoholism (69). The risk for alcoholism is likely influenced by interactions among many genes; these appear to include *NFKB1*, *PDYN* and *OPRK1*.

Here we have identified an additional gene within this linkage peak that is associated with alcohol dependence: *NFKB1*. These findings lend support to the idea that the broad linkage peak on chromosome 4q is indeed a result of contributions of multiple genes.

MATERIALS AND METHODS

Subjects

Subjects were collected at six centers in the United States: Indiana University, State University of New York Health Science Center, University of Connecticut, University of Iowa, University of California/San Diego, and Washington University, St. Louis (2, 4, 5). Probands were identified through alcohol treatment programs. After providing informed consent, probands and their relatives were administered a validated poly-diagnostic instrument, the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) interview (70, 71). Details of the

ascertainment and assessment have previously been published (2, 4, 5) and are available in detail at zork.wustl.edu/niaaa/coga_instruments/resources.html. The institutional review boards of all participating institutions approved the study.

Families in which the proband and at least two first-degree relatives met both DSM-IIIR criteria for alcohol dependence (72) and Feighner criteria for definite alcoholism (73) were studied in more detail, and a genetically informative subset were selected for genotyping, as described in more detail by Foroud et al. (5). Because the linkage results in these families (7) were most significant using the DSM-IV criteria (74) for alcohol dependence (which could be derived from the SSAGA data), we have employed these criteria as our primary phenotype. The subjects in this study were from 219 European-American families, of which 1923 individuals were genotyped. Among these genotyped individuals, 753 were alcohol dependent, 1047 unaffected, and 123 unknown (subjects without a completed SSAGA diagnostic interview).

SNP genotyping

SNPs across *NFKB1* were selected from public databases (dbSNP, HapMap), with a preference for those having high minor allele frequencies (MAF > 0.3). SNP positions are from the NCBI reference human genome, build 36.1. Assays were designed for the Sequenom MassArray system (Sequenom, San Diego, CA) using MassArray Assay Design Software. Most were done using the homogeneous Mass Extension reaction, and the remainder using iPLEX assays (Sequenom, San Diego, CA); in both cases, alleles are discriminated by mass spectrometry. Assays were tested on two groups of 40 unrelated individuals from the Coriell European-American and African-American samples. SNPs that were not in Hardy-Weinberg equilibrium in both populations were not genotyped in the sample. All SNPs were tested for Mendelian inheritance using the program PEDCHECK (75). Marker allele frequencies and

heterozygosities were computed using the program USERM13 (76). The only coding SNP, rs4648072, had a MAF of only 0.007, and was omitted from the analyses.

Statistical analyses

Linkage disequilibrium among genotyped SNPs was evaluated using HAPLOVIEW (77). Coverage of the gene was additionally examined using Tagger (60) to determine the correlation between genotyped SNPs and all known SNPs in the HapMap I dataset.

Family based association analyses were performed using the Pedigree Disequilibrium test (PDT) (78) as implemented in the program UNPHASED (version 2.404) (79). The PDT utilizes data from all available trios in a family, as well as discordant sibships; this was most appropriate, because our earliest analyses (3) showed significantly reduced marker allele sharing among siblings discordant for the alcohol dependence phenotype. We report results using the PDTaverage option, which weighs each family equally in computing the overall test statistic. The effective number of independent tests and thus the experiment-wide significance threshold was computed using the method of Li and Ji (61) which is based on the spectral decomposition of the matrix of pairwise LD between SNPs (80).

We have previously found that the association of *GABRA2* and *CHRM2* with alcoholism was driven primarily by the most severely affected half of the dataset (62, 63). Therefore, we divided the affected individuals into two groups, earlier onset and later onset, based upon the median age at which DSM-IV criteria were met in this sample, 21 years. For the analysis of earlier onset alcoholism, the individuals who met criteria before age 22 were considered affected (n = 426), and those who met criteria later were coded as unknown. To analyze late onset alcoholism, those who met criteria after age 22 were considered affected (n = 337), and those who met criteria earlier were coded as unknown. Age of onset of alcoholism was also analyzed as a quantitative

variable using the Quantitative Pedigree Disequilibrium Test (QPDT) (81) as implemented in UNPHASED (79).

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CONFLICTS OF INTEREST STATEMENT

No authors have reported a conflict of interest.

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LEGENDS TO FIGURES

Figure 1: *NFKB1*: gene structure and linkage disequilibrium (D') among SNPs. Top: Gene structure, showing location of exons (vertical bars on line) and SNPs (bars below line); the SNPs associated with alcohol dependence (DMS-IV) are underlined; transcription is from left to right. Bottom: LD (D') among SNPs genotyped in and flanking *NFKB1*, based on Haploview (Barrett et al., 2005) analysis.

Figure 2: Association of SNPs in *NFKB1* with alcohol dependence. Data are shown as $-\log_{10}(p)$ *vs*. distance along the chromosome. Diamonds: DSM-IV; circles, DSM-IV with earlier onset (median split) ; dashed line represents p=0.05.

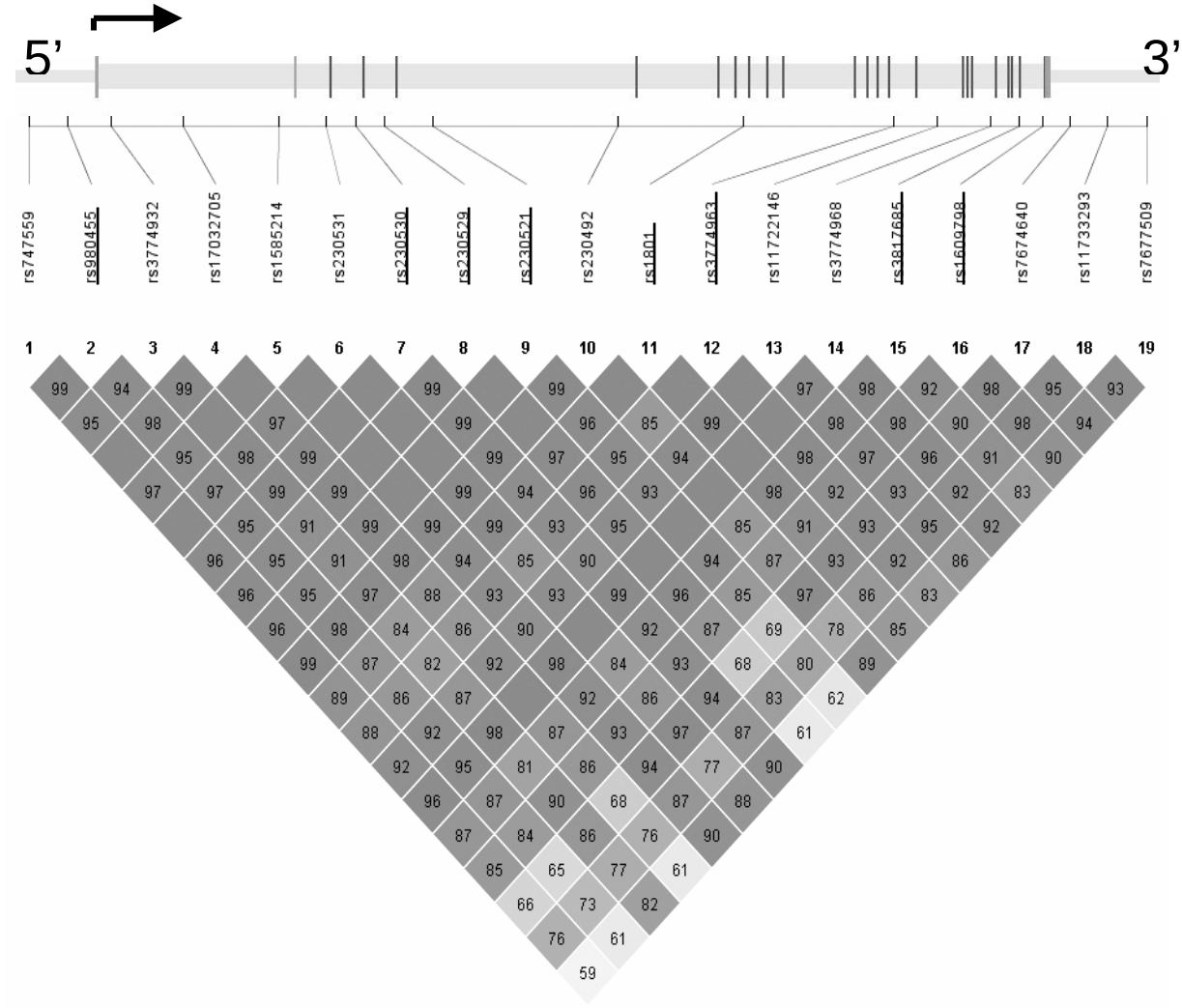
Figure 3: Linkage disequilibrium between *NFKB1* and *MANBA*. HapMap data (Release 21a/phaseII) for the region from bp 103,780,000 to 104,020,000 showing linkage disequilibrium (r^2) for CEU (CEPH European, Utah) in the region of *NFKB1* and *MANBA*. LD is reasonably high within each gene but there is a distinct break between the two genes.

TABLE 1

SNP	Position[a]	Function	MAF[b]	DSM-IV (p value)	Earlier onset[c] (p value)	Later onset[c] (p value)	Age of onset[d] (p value)
rs747559	103,633,207	upstream	0.42	0.10	0.07	0.22	0.22
rs980455	103,637,989	upstream	0.42	***0.03**	***0.02**	0.26	***0.02**
rs3774932	103,643,223	IVS1	0.44	0.14	***0.03**	0.13	***0.01**
rs17032705	103,652,004	IVS1	0.41	0.18	0.11	0.18	0.11
rs1585214	103,663,563	IVS1	0.43	0.06	***0.02**	0.20	***0.02**
rs230531	103,669,407	IVS2	0.36	0.12	0.08	0.19	0.06
rs230530	103,673,010	IVS3	0.43	***0.05**	***0.01**	0.22	***0.02**
rs230529	103,676,448	IVS4	0.43	***0.03**	***0.01**	0.56	***0.03**
rs230521	103,682,357	IVS5	0.43	***0.02**	***0.02**	0.32	***0.04**
rs230492	103,704,817	IVS5	0.35	0.13	0.13	0.14	0.10
rs1801	103,720,092	IVS8	0.39	***0.03**	***0.04**	0.22	***0.04**
rs3774963	103,738,403	IVS15	0.35	***0.01**	***0.02**	0.41	***0.02**
rs11722146	103,743,667	IVS16	0.33	0.08	0.12	0.42	***0.05**
rs3774968	103,750,150	IVS19	0.42	0.07	***0.03**	0.21	***0.02**
rs3817685	103,753,606	IVS22	0.35	***0.01**	***0.04**	0.80	***0.04**
rs1609798	103,756,488	IVS23	0.34	***0.05**	0.08	0.59	***0.05**
rs7674640	103,759,828	downstream	0.48	0.21	0.14	0.15	0.17
rs11733293	103,764,301	downstream	0.36	0.06	0.06	0.62	0.22
rs7677509	103,769,054	downstream	0.48	0.32	0.33	0.15	0.30

[a]Position based on dbSNP126, Genome build 36.1
[b]Minor allele frequency
[c]DSM-IV with earlier or later onset, defined based on a median split.
[d]Analyzed as a quantitative variable.
*Significant ($p<0.05$)

Figure 1


5'
3'
rs747559
rs980455
rs3774932
rs17032705
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rs230530
rs230529
rs230521
rs230492
rs1801
rs3774963
rs11722146
rs3774968
rs3817685
rs1600798
rs7674640
rs11733293
rs7677509
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
99 94 99 99 99 99 97 98 92 98 95 93
95 98 97 99 96 85 99 98 98 90 98 94
95 98 99 99 97 95 94 98 97 96 91 90
97 97 99 99 99 94 96 93 98 92 93 92 83
95 91 99 99 99 93 95 85 91 93 95 92
96 95 91 98 94 85 90 94 87 93 92 86
96 95 97 88 93 93 99 96 85 97 86 83
96 98 84 86 90 92 87 69 78 85
99 87 82 92 98 84 93 68 80 89
89 86 87 92 86 94 83 62
88 92 98 87 93 97 87 61
92 95 81 86 94 77 90
96 87 90 68 87 88
87 84 86 76 90
85 65 77 61
66 73 82
76 61
59

Figure 2

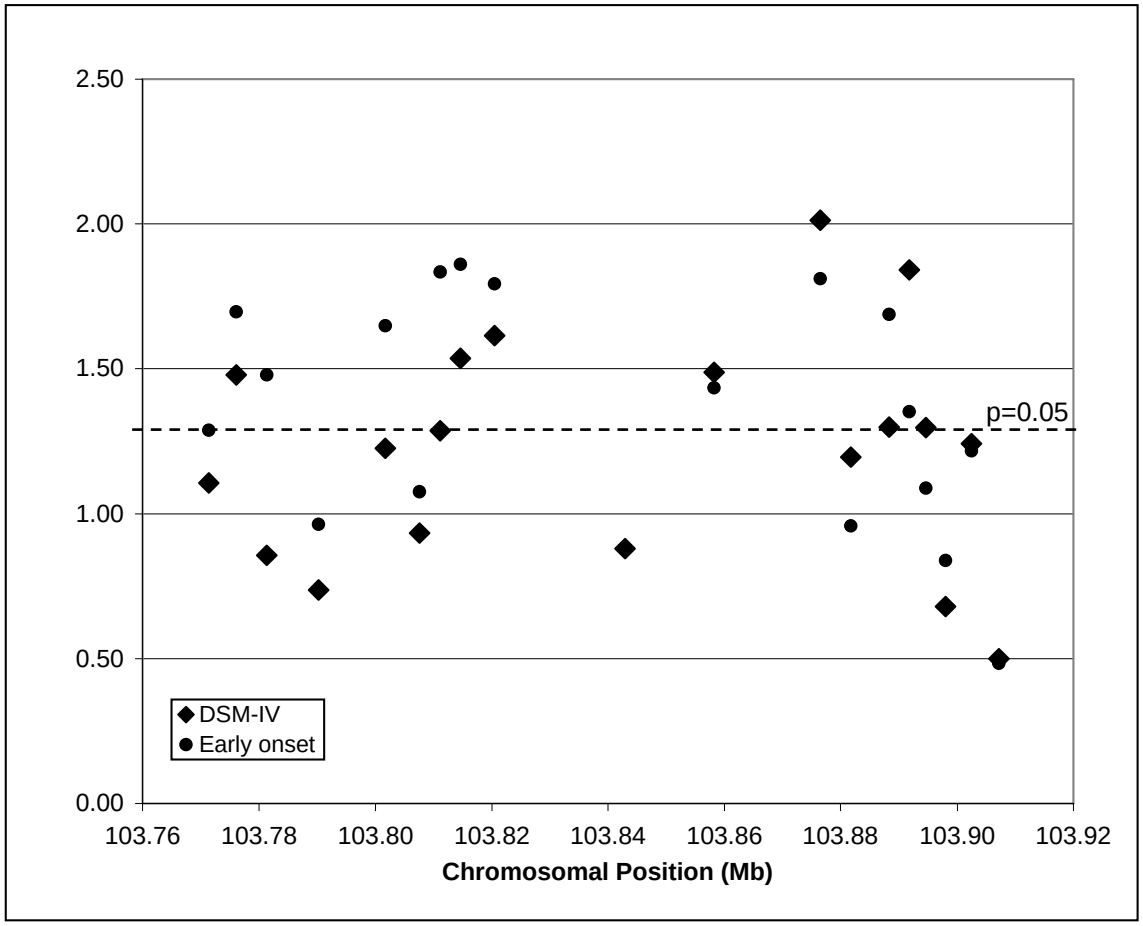
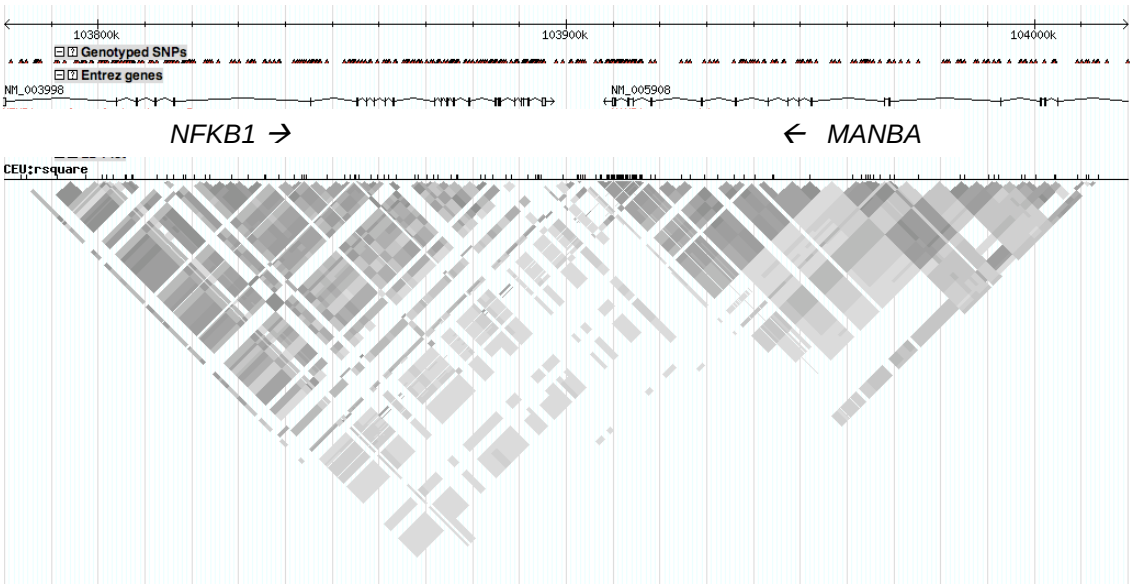

2.50
2.00
1.50
1.00
0.50
0.00
p=0.05
DSM-IV
Early onset
103.76
103.78
103.80
103.82
103.84
103.86
103.88
103.90
103.92
Chromosomal Position (Mb)

Figure 3


103800k
103900k
104000k
Genotyped SNPs
Entrez genes
NM_003998
NM_005908
NFKB1 →
← MANBA
CEU:rsquare