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Linkage of Bipolar Affective Disorder to Chromosome 18 Markers in a New Pedigree Series

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Summary

Several groups have reported evidence suggesting linkage of bipolar affective disorder (BPAD) to chromosome 18. We have reported data from 28 pedigrees that showed linkage to marker loci on 18p and to loci 40 cM distant on 18q. Most of the linkage evidence derived from families with affected phenotypes in only the paternal lineage and from marker alleles transmitted on the paternal chromosome. We now report results from a series of 30 new pedigrees (259 individuals) genotyped for 13 polymorphic markers spanning chromosome 18. Subjects were interviewed by a psychiatrist and were diagnosed by highly reliable methods. Genotypes were generated with automated technology and were scored blind to phenotype. Affected sib pairs showed excess allele sharing at the 18q markers D18S541 and D18S38. A parent-of-origin effect was observed, but it was not consistently paternal. No robust evidence of linkage was detected for markers elsewhere on chromosome 18. Multipoint nonparametric linkage analysis in the new sample combined with the original sample of families supports linkage on chromosome 18q, but the susceptibility gene is not well localized.

Introduction

Reports of linkage in psychiatric disorders have proliferated in recent years, with few replicated findings. This has promoted skepticism about all linkage findings in psychiatric disorders, despite the acknowledgment that the replication of true linkage findings for psychiatric disorders will be a difficult task (Risch and Botstein 1996). The range of phenotypes that share genetic risk factors, the number of genes involved, and the mode of inheritance are all unknown. Nevertheless, until susceptibility genes are identified, replication remains the most important indicator of valid linkage findings.

Linkage studies of bipolar affective disorder (BPAD) have now led to positive findings at nearby loci in two or more samples for chromosome 21 and chromosome 18. Detera-Wadleigh et al. (1996) published support for a prior linkage finding on chromosome 21q (Straub et al. 1994). For chromosome 18, Berrettini et al. (1994) reported significant evidence of linkage to markers in the pericentromeric region, with a peak identical by descent (IBD) score of .58 ($P = .0004$), although parametric LOD scores in the total sample were all negative. Our group (Stine et al. 1995) followed up these results in a set of 28 families selected for the presence of affected phenotypes in only one parental lineage (Simpson et al. 1992). Our findings showed a peak in the linkage statistic on 18p11 (IBD = .64, $P = .0003$), which overlapped with the previously reported findings, and another peak 40 cM distant on 18q21 (IBD = .57, $P = .02$) (Stine et al. 1995). These results appeared to strengthen those of Berrettini et al. (1994), but we could not draw firm conclusions as to either the number of loci involved or their precise location. Three other groups have since published supportive evidence for an 18q locus in BPAD (Coon et al. 1996; de Bruyn et al. 1996; Freimer et al. 1996). These studies all report LOD scores in the range of 1–2, although de Bruyn et al. found one 18q22 marker linked at the $P < .0007$ level in an affected-sib-pair analysis and Freimer et al. obtained a maximum LOD score of 4.06 for an 18q23 marker in a "joint linkage and association analysis."

The reports by Berrettini et al. (1994) and by Stine et al. (1995) both suggest loci that confer a relative risk <3 (Risch 1987). Linkage replication for loci conferring such a modest increased disease risk may be a considerable challenge. Indeed, several published reports have failed to detect linkage between BPAD and chromosome 18 markers (Kelsoe et al. 1995; Maier et al. 1995; Pauls et al. 1995; Smyth et al. 1995; Detera-Wadleigh et al.

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1997). A locus conferring a small relative risk may require larger samples than those used in these reports, to ensure adequate power for replication (Suarez 1994). Even when replication is achieved, the results may not correspond well to the site of the original findings, since the peak of the linkage statistic for complex phenotypes may not correspond to the site of the actual disease locus (Kruglyak and Lander 1995).

The linkage findings on chromosome 18 are further complicated by an apparent parent-of-origin effect. In our previous sample, most of the evidence for linkage derived from families with affected phenotypes in only the paternal lineage and from marker alleles transmitted on the paternal chromosome. Heterogeneity reflected in the sex of the apparently transmitting parent was suggested by our earlier clinical studies (McMahon et al. 1995), in which an excess of affected maternal relatives and a higher-than-expected proportion of families with no paternal transmission were observed. However the post hoc application of the parent-of-origin effect to the linkage analysis introduced additional df and underscored the need for replication. Other groups have since shown a paternal effect on the linkage evidence in the pericentromeric region of chromosome 18 (Gershon et al. 1996; Nöthen et al. 1996), but, to date, no confirmatory reports of a paternal effect on 18q have appeared.

In order to test the hypothesis that BPAD is linked to markers on chromosome 18, we studied 13 highly polymorphic markers spanning the chromosome in a new, 30-family set ascertained and analyzed in a manner similar to that used for the first sample of families. The results support our previously reported linkage of BPAD to chromosome 18q, but the parent-of-origin effect is less consistent than that in the original sample, and no robust evidence of linkage was detected for markers elsewhere on chromosome 18 in this relatively small set of families.

Subjects and Methods

Family Ascertainment and Evaluation

Clinics and inpatient units in Baltimore and Iowa City were screened for treated probands with bipolar I disorder (BPI) as described elsewhere (Simpson et al. 1992). Probands who, on the basis of family history, had either two or more two sibs with a major affective disorder or one sib and only one parent with a major affective disorder were enrolled for study. Informed consent was obtained from all participants.

Probands and their relatives were interviewed by a psychiatrist trained in the use of the Schedule for Affective Disorders and Schizophrenia—Lifetime Version, a semistructured psychiatric interview of established reliability and validity (Endicott and Spitzer 1978). Every effort was made to interview the parents, aunts, uncles, and grandparents, so that any affected phenotypes could be detected in both parental lineages. Such "bilineal" families were not evaluated further. Later, the interview data were reviewed, along with family-informant data and any available medical records, by two additional psychiatrists who assigned a best-estimate diagnosis based on research diagnostic criteria (Spitzer et al. 1975). When the best-estimate reviewers could not agree that a subject was affected with BPAD, or when a subject was assigned phenotypes that have been related to BPAD in family studies, that subject was classified as "phenotype uncertain" (for details, see Simpson et al. 1992). Our best-estimate diagnoses have very high reliability (see Stine et al. 1995).

The 23 completely evaluated families meeting ascertainment criteria as of August 1, 1996, were selected for genotyping. These families consisted of 9 "paternal" pedigrees (in which the proband's father or one of his sibs or parents is affected) and 14 "maternal" pedigrees (in which the proband's mother or one of her sibs or parents is affected), as defined elsewhere (see Stine et al. 1995). This set of families alone did not fulfill the threshold of 90% power to detect linkage, which we had set as a requirement for commencement of the confirmation study. Therefore we selected for genotyping seven additional families, before commencing genotyping. Since our previous linkage findings on 18q had suggested a paternal parent-of-origin effect, the additional families were selected for a paternal pattern of transmission. Although clinically similar to the other families, these families did not meet the original ascertainment criteria, for one or more of the following reasons: (1) they were considered "bilineal," since (in addition to the affected paternal relative) the proband's mother or a maternal aunt, uncle, or grandparent was found to have recurrent unipolar disorder (RUP) (two families); (2) the mother, although reportedly unaffected, could not be interviewed (one family); (3) the proband was not felt by the best-estimate diagnosticians to have typical BPI (three families); or (4) only an affected sib pair was available for study (one family).

In this total sample, of the 251 subjects for which a best-estimate diagnosis could be assigned, 56 (22.3%) were diagnosed with BPI, 38 (15.1%) with bipolar II disorder (BPII) plus recurrent major depression, 38 (15.1%) with RUP, and 4 (1.6%) with schizoaffective manic disorder (SAM). Of the remaining subjects, 51 (20.3%) were considered unaffected, and 56 (22.3%) were classified as phenotype uncertain. This is similar to the distribution of diagnoses in the original family sample (Stine et al. 1995). No best-estimate diagnosis could be assigned for eight subjects, because of inadequate phenotype data. These subjects and those assigned a best-estimate diagnosis of phenotype uncertain were

considered phenotype unknown for the purposes of linkage analyses.

Marker Selection

Two sets of markers were genotyped: the first set of 13 markers, spanning the chromosome, was selected in order to test confirmation of linkage on chromosome 18. The second set was selected with the goal of improving localization by saturation of the region surrounding the linkage finding on 18q. For the confirmation study, we chose to genotype five of the markers that had given the strongest linkage evidence in previous studies (markers D18S53, D18S37, D18S41, D18S64, and D18S38; Berrettini et al. 1994; Stine et al. 1995). In addition, eight markers covering other regions of the chromosome were selected from the Weber 6 set (Research Genetics), in order to take advantage of automated genotyping methods. For the subsequent 18q saturation study, 41 polymorphic markers between D18S41 and D18S70 were selected. Primer sequences were obtained from the Genome Database.

DNA Preparation and Genotyping

Genotyping was performed at laboratories at Johns Hopkins University and at Sequana Therapeutics. At Sequana, the methods employed have been detailed by Hall et al. (1996). In brief, lymphoblastoid-cell-line DNA was prepared in standard concentration, and samples were assembled into 96-well master plates. Each plate lot was tested for quality by a panel of 10 microsatellite markers.

PCR reactions were performed in an automated manner by a robotic workstation. Each well contained a 20-μl total-reaction volume. Touchdown PCR was performed in PTC-100 Peltier-effect thermal cyclers (MJ Research) by use of a standard protocol (Hall et al. 1996).

Multiplexed PCR products were electrophoresed on ABI Prism 377 sequencers. The gel-file output was checked for correct tracking, and automated size-standard-validation software checked the allele size determination. A control DNA sample was run on each gel, in order to correlate allele sizes among gels. Genotypes were read by GeneScan 2.1fc2 and Genotyper 1.1 programs. Once allele sizes for each microsatellite marker had been determined for all DNA samples in the study, arbitrary allele numbers were assigned, and the data were checked for correct Mendelian-inheritance patterns. At this stage, data from four markers in the saturation set were dropped, because of inconsistent binning.

Similar DNA-preparation and genotyping methods were employed at Johns Hopkins, except that DNA concentrations were determined spectrophotometrically. PCR reactions were performed manually and were optimized for each pair of primers. Initial allele scoring was done automatically and was checked manually. Genotypes were binned and tested for inheritance by use of GAS 2.0 (Genetic Analysis System, version 2.0; Young 1995). Alleles that fell outside the fixed bin sizes or that did not segregate were rechecked; the data from that portion of the pedigree were deleted if discrepancies could not be resolved.

Statistical Methods

Linkage was tested by a limited number of analytic methods, in order to avoid the problem of multiple tests. Nonparametric methods were chosen, since they are more powerful when genetic parameters are unknown.

Power analyses indicated that our sample had >90% power to detect linkage in affected sib pairs, at the $P \leqslant .01$ level, when subjects with BPI, BPII, RUP, or SAM were included in the analysis. The software package SLINK was used to generate simulated data on the basis of the pedigree structure, phenotype information, and genotype availability of the 30 pedigrees. A dominant model and a marker with six equally frequent alleles linked to the disease locus at a recombination fraction (θ) of .01 in all families was assumed. One thousand replicates of the simulated data were then analyzed by SIBPAL.

Since some cases of RUP ascertained through BPI probands may have distinct etiologies (Blacker and Tsuang 1993; McMahon et al. 1994), we also performed analyses excluding RUP subjects. On the basis of our simulations, the sample excluding RUP subjects had 89.5% power to detect linkage, at the $P \leqslant .01$ level.

The SIBPAL module of SAGE was used to test the null hypotheses of 50% allele sharing by affected sib pairs, in the total sample and in paternal pedigrees. The SIBDES module of GAS was used to test the null hypothesis of 50% allele sharing, by affected sib pairs, of 18q markers on paternally transmitted chromosomes. *P* values were adjusted for nonindependent sib pairs (Hodge 1984).

To improve our estimate of linkage localization, the data from 32 of the markers genotyped in the total sample of 58 families were analyzed by the all-relative-pair option in GENEHUNTER (Kruglyak and Lander 1995). For this analysis, we selected the most informative markers—that is, those with the fewest missing genotypes. GENEHUNTER haplotypes identified 11 single-marker apparent double recombinants in six families; genotypes from these markers were zeroed out in these families. In our sample, allele frequencies were determined by use of subjects without parents.

For the confirmation study, map order was determined with CRIMAP and agrees with the order on published maps. For the saturation study, the order of all such

Table 1

Results of Affected-Sib-Pair Analyses for Sibs Affected with BPI, BPII, SAM, or RUP, in 30 Families

Marker	Cumulative Sex Average (cM)	BPI/BPII/SAM			BPI/BPII/SAM/RUP		
		No. of Pairs	IBD[a]	P[b]	No. of Pairs	IBD[a]	P[b]
D18S63	0	69	.45	NS	150	.48	NS
D18S452	12.3	69	.51	NS	147	.53	NS
D18S53	31.3	72	.43	NS	154	.48	NS
D18S37	34.3	74	.50	NS	152	.52	NS
D18S847	45.3	69	.50	NS	149	.52	NS
D18S548	60.0	64	.50	NS	126	.56	.0013
D18S487	71.0	73	.52	NS	156	.51	NS
D18S41	73.9	62	.52	NS	130	.52	NS
D18S849	77.4	68	.51	NS	149	.51	NS
D18S64	81.3	74	.53	NS	157	.52	NS
D18S38	84.5	73	.55	.0584	154	.54	.0352
D18S541	99.1	72	.62	.0003	154	.54	.0670
D18S844	111.3	74	.53	NS	157	.53	NS

NOTE.—All P values <.1 are shown.

[a] Mean proportion of marker alleles shared IBD (by SIBPAL).

[b] NS = not significant.

closely spaced markers could not be determined unambiguously in this data set. However, the order of those markers which could be placed at 1,000:1 odds agrees with the standard chromosome 18 reference map (Collins et al. 1996).

Results

Two-Point Affected-Sib-Pair Analyses

Results are presented in table 1. Excess allele sharing by affected sib pairs was observed at the 18q markers D18S38 and D18S541. At D18S541, sib pairs affected with BPI, BPII, or SAM shared a mean 62% of alleles IBD (P = .0003). When RUP sibs were included, excess allele sharing was also seen at one 18p marker, D18S548 (IBD = .56, P = .0013), but with no evidence of linkage at the flanking markers D18S847 and D18S487, 15 cM and 11 cM distant, respectively.

As in the previous sample (Stine et al. 1995), the greatest excess allele sharing was seen for paternally transmitted chromosomes (table 2). At D18S541, 77.9% of paternal alleles (P = .015) and 59.5% of maternal alleles (P not significant) were shared IBD by sib pairs affected with BPI, BPII, or SAM. In contrast to the previous sample, evidence of linkage appeared greater in "maternal" pedigrees, although stratification by parent of origin caused the sample sizes to become very small (table 2). Similar results were seen when RUP sibs were included (data not shown).

Multipoint Affected-Relative-Pair Analysis

To improve localization of the linkage finding on 18q, genotype data from a total sample of 58 pedigrees, consisting of the original, 28-pedigree set described elsewhere (Stine et al. 1995) and the new, 30-pedigree set used for the replication study described above, were analyzed. The results for the narrower definition of the affected phenotype are reported, but they did not differ substantially when RUP sibs were included.

The multipoint results were consistent with the two-point findings (fig. 1). The peak nonparametric LOD (NPL) score was observed at D18S38 (2.84; $P < .0019$). Although strongly supportive of linkage, this result was not well localized. The NPL score is associated with $P < .01$ over an interval of ~14 cM.

Discussion

In order to test our previous linkage findings for BPAD on chromosome 18, we have studied 13 polymorphic markers spanning the chromosome in a new, 30-pedigree series ascertained and analyzed in a manner similar to that used with the first sample of families. The results support our previously reported linkage of BPAD to chromosome 18q, but the parent-of-origin effect is less consistent than that in the original sample, and no robust evidence of linkage was detected for markers elsewhere on chromosome 18 in this relatively small set of families.

Lander and Kruglyak (1995) have proposed criteria for "suggestive," "significant," and "confirmed" linkages in a genomewide scan. These statistical thresholds may be too conservative (Sawcer et al. 1997), and their application to complex phenotypes has been criticized since "they might not provide an accurate picture of the multifactorial situation" (Witte et al. 1996, p. 355). By the Lander and Kruglyak (1995) thresholds, our results

Table 2

Results of Affected-Sib-Pair Analyses for Sibs Affected with BPI, BPII, or SAM

	Total Sample						Families Stratified on the Basis of Parent of Origin					
	Paternal Chromosome			Maternal Chromosome			Paternal Families			Maternal Families		
Marker	No. of Pairs[a]	IBD[b]	P[c]	No. of Pairs[a]	IBD[b]	P[c]	No. of Pairs	IBD[d]	P[c]	No. of Pairs	IBD[d]	P[c]
D18S63	30.1	.45	NS	31.8	.42	NS	26	.39	NS	22	.49	NS
D18S452	33.1	.52	NS	31.3	.47	NS	27	.44	NS	21	.63	.022
D18S53	26.4	.45	NS	27.4	.42	NS	29	.41	NS	22	.40	NS
D18S37	12.8	.41	NS	14.4	.55	NS	30	.51	NS	23	.50	NS
D18S847	22.4	.51	NS	12.5	.53	NS	26	.47	NS	22	.50	NS
D18S548	15	.47	NS	15.5	.52	NS	28	.50	NS	20	.49	NS
D18S487	25.2	.53	NS	27.4	.47	NS	30	.47	NS	22	.50	NS
D18S41	18.8	.47	NS	16.2	.58	NS	23	.46	NS	23	.50	NS
D18S849	32.7	.46	NS	33.9	.54	NS	28	.51	NS	22	.56	NS
D18S64	28.6	.57	NS	26.2	.36	NS	30	.47	NS	23	.61	.028
D18S38	25.6	.68	.054	25.4	.53	NS	30	.51	NS	22	.61	.01
D18S541	18.9	.78	.015	25.8	.59	NS	29	.57	NS	22	.64	.024
D18S844	26.2	.56	NS	28.4	.43	NS	30	.56	NS	23	.50	NS

Note.—All P values <.1 are shown.

[a] Informative affected sib pairs, weighted by the method of Hodge (1984).

[b] Proportion of alleles shared IBD in informative matings (by GAS).

[c] NS = not significant.

[d] Mean proportion of marker alleles shared IBD (by SIBPAL).

would not qualify as a "confirmed" linkage, even though our affected-sib-pair results reach the confirmatory ($P \leqslant .01$) threshold of significance at D18S541. This is because the 18q linkage results in the first, 28-family sample did not reach the Lander and Kruglyak (1995) threshold for significant linkage ($P \leqslant 2.2 \times 10^{-5}$), except when paternally transmitted chromosomes in paternal families were analyzed, introducing additional df. Nevertheless, our results in this independent sample of 30 families again detect evidence of linkage to 18q and, in this sense, support our original finding.

Has the overall evidence of linkage on 18q been strengthened by the results in the new sample—and, if so, by how much? Significant evidence of linkage between BPAD and 18q markers is seen in both the original, 28-family sample and the new, 30-family sample reported herein, but the peak IBD score in the original sample (81% of paternal alleles shared; $P = .00002$) occurred at D18S41, whereas the peak IBD score for the new sample (78% of paternal alleles shared; $P = .015$) occurred at D18S541, ~25 cM distant from D18S41. This variation in the linkage peak is not resolved in the multipoint analysis, so it does not appear to result solely from variation in marker informativeness (fig. 1). Direct comparisons between individual markers in different samples may be problematic, since, in complex phenotypes, different sets of families contain different proportions of cases that are phenocopies or genetically heterogeneous with respect to a given susceptibility locus. Thus, peak allele sharing is expected to vary randomly around the locus, with some markers rising above—and other, nearby markers falling below—the criterion for significance (Kruglyak and Lander 1995). In complex phenotypes, true linkage peaks have been observed to shift over genetic distances as large as 20 cM (e.g., see Hall et al. 1990; Easton et al. 1993). Still, the question of whether the results in the new sample confirm linkage at the same location as was observed in the previous sample cannot be fully resolved by our data.

Our results are limited by the relatively small sample size. Small sample sizes could result in imprecise P values, especially in the SIBPAL analysis, which relies on asymptotic P values. This is particularly problematic in the parent-of-origin analyses, in which the range of effective sample sizes is 20–30 affected sib pairs. In order to increase sample size, we added seven families to the sample prior to genotyping. Although these families were recruited under less-stringent rules of ascertainment, they were clinically very similar to the other families, and excluding these families does not have a substantial impact on either the two-point or multipoint results.

The sample size may explain the failure to detect robust evidence of linkage to pericentromeric markers in this sample. Our simulations indicated that our sample of 30 pedigrees had good power to detect linkage, but the model under which our simulated data was generated may not fit the putative pericentromeric locus. Risch (1990) simulated the sample size needed to detect linkage to loci of varying relative risk, using a sample of affected sib pairs. On the basis of his results, our sample would have ~90% power to detect a locus conferring a relative risk $\geqslant 3$. The putative pericentromeric locus may confer a smaller relative risk in this particular sample and thus would not be detectable. It is also possible that our linkage finding at D18S548 (IBD = .56, $P = .0013$), ob-

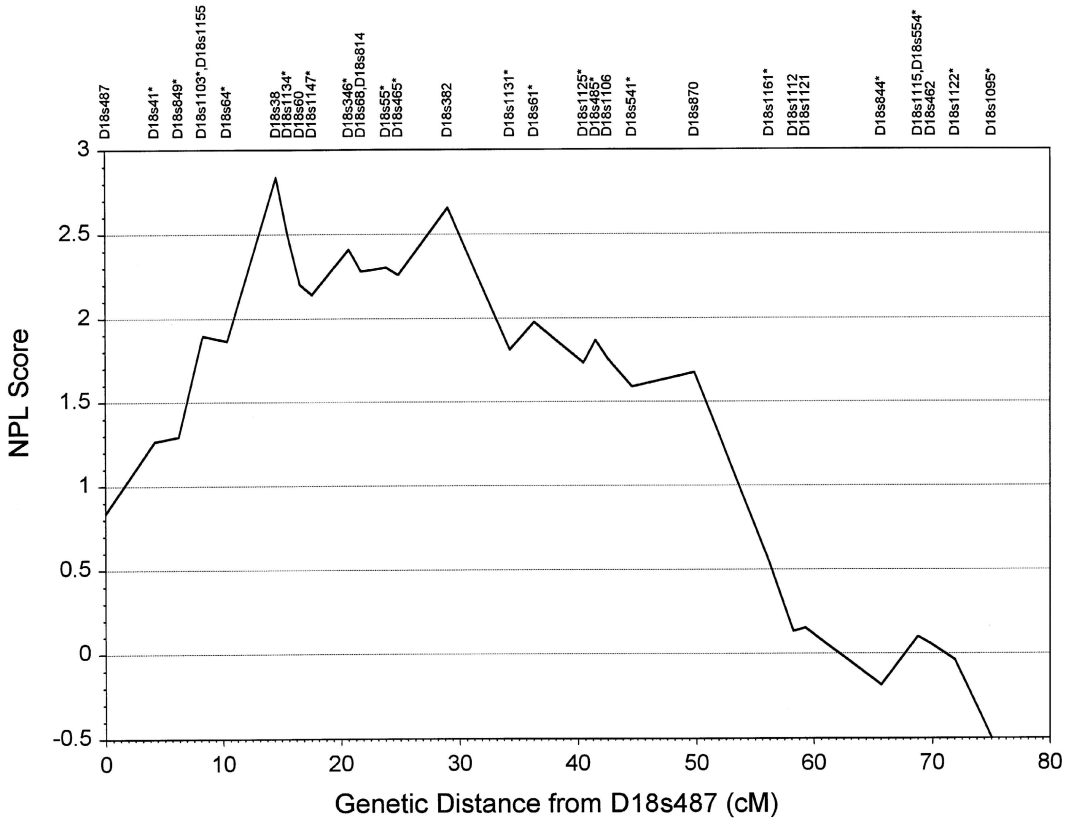

Figure 1 Results of multipoint nonparametric linkage analysis between BPAD and 31 markers on chromosome 18q in 58 families. Genetic distance (in cM) from the most centromeric marker, D18S487, is shown on the lower *x*-axis. Each marker used in the analysis is shown on the upper *x*-axis, according to map order and distance. Markers placed with ⩾1,000:1 odds are denoted by an asterisk (*); all other markers are placed in their most likely location. The nonparametric linkage statistic given by the all-pairs option in GENEHUNTER (i.e., the NPL score) is shown on the *y*-axis.

served only when RUP sibs were included in the sample, reflects the same pericentromeric locus reported by Berrettini et al. (1994) and Stine et al. (1995). According to the Location Database Map (Collins et al. 1996), D18S548 is located <2 male cM and ~12 female cM qter from D18S56, which is the closest significantly linked marker in the Berrettini et al. (1994) report, and <1 male cM and ~4 female cM qter from D18S34, which yielded $P < .1$ in the Stine et al. (1995) report. This issue might be clarified by genotyping more markers in this region.

Previously we reported a parent-of-origin effect in BPAD. This was manifested clinically both by an increased rate of major affective disorder among maternal relatives and by a higher than expected proportion of families with no paternal transmission, consistent with imprinting or mitochondrial inheritance (McMahon et al. 1995). A parent-of-origin effect was also evident in the linkage between BPAD and chromosome 18 markers, in which most of the linkage evidence derived from families with an affected or apparently transmitting father (Stine et al. 1995). On 18q a parent-of-origin effect was also seen in the form of higher IBD scores and stronger linkage evidence for marker alleles on paternally transmitted chromosomes (Stine et al. 1995). Similar parent-of-origin effects have since been observed by groups studying linkage to the pericentromeric region of chromosome 18 in other family samples (Gershon et al. 1996; Nöthen et al. 1996). Gershon et al. (1996) reanalyzed the 22 families studied in the original report of linkage between BPAD and chromosome 18 (Berrettini et al. 1994) and concluded that there was a higher than expected proportion of families with no paternal transmission—and that the linkage evidence on chromosome 18 derived mostly from those families which did show apparent paternal transmission; the linkage results for paternally transmitted chromosomes were not reported.

A parent-of-origin effect was again seen in the present sample, but the effect was not consistently paternal (table 2). This unexpected finding may be the spurious result of small sample sizes. This finding may also reflect the complex genetics of BPAD, which make it impossible, on purely clinical grounds, to identify a transmitting parent. Although we relaxed the criteria for unilin-

eality for 3 of the 16 paternal families in the new sample, eliminating these families does not significantly change the results. It is also possible that detection of linkage to paternal and not to maternal chromosomes is influenced by the decreased male versus female recombination rates on chromosome 18q (Collins et al. 1996).

The site of linkage on 18q, first reported by Stine et al. (1995) and supported by this study, may or may not overlap with the putative 18q23 locus reported by another group (Freimer et al. 1996). Many of the same markers showed evidence of linkage in both samples (McInnes et al. 1996), and we cannot rule out the possibility that the same disease locus is being detected in both family samples. The Freimer et al. (1996) analysis was based only on subjects with BPI; our analysis, like those of Berrettini et al. (1994) and Stine et al. (1995), included cases of BPII and RUP. Genetic heterogeneity may be contributing to the difference in linkage localization. The low relative risk apparently conferred by this locus suggests that fine mapping will be difficult with linkage methods alone, unless much larger sample sizes are used (Kruglyak and Lander 1995).

We have here reported new evidence that BPAD is linked to chromosome 18q. These results strengthen the earlier evidence that a susceptibility gene for BPAD resides on the long arm of chromosome 18. Further studies are needed to clarify the nature of the observed parent-of-origin effect and to identify the inferred gene through positional cloning methods.

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