Fine-scale mapping of a locus for severe bipolar mood disorder on chromosome 18p11.3 in the Costa Rican population

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We have searched for genes predisposing to bipolar disorder (BP) by studying individuals with the most extreme form of the affected phenotype, BP-I, ascertained from the genetically isolated population of the Central Valley of Costa Rica (CVCR). The results of a previous linkage analysis on two extended CVCR BP-I pedigrees, CR001 and CR004, and of linkage disequilibrium (LD) analyses of a CVCR population sample of BP-I patients implicated a candidate region on 18p11.3. We further investigated this region by creating a physical map and developing 4 new microsatellite and 26 single-nucleotide polymorphism markers for typing in the pedigree and population samples. We report the results of fine-scale association analyses in the population sample, as well as evaluation of haplotypes in pedigree CR001. Our results suggest a candidate region containing six genes but also highlight the complexities of LD mapping of common disorders.

It has been hypothesized that mapping genes for genetically complex disorders may be simplified by investigating isolated populations, in which a substantial proportion of affected individuals may share susceptibility alleles identical by descent from a common ancestor (3, 4). We have been conducting genetic mapping studies of bipolar disorder (BP)-I, a distinct and highly heritable complex phenotype (5), by using both pedigree and population approaches in the isolate of the Central Valley of Costa Rica (CVCR) (1, 2, 6). Work on Alzheimer's disease, another complex neuropsychiatric trait, has supported the strategy of using both pedigree and population samples (7), and this approach may be particularly fruitful in genetic isolates. In such populations, it may be possible to pinpoint the location of a disease susceptibility gene by observing conserved marker haplotypes among affected individuals in extended pedigrees and by identifying linkage disequilibrium (LD) among affected individuals sampled independently from the population.

In a genome-wide linkage study (1), we observed a logarithm of odds (lod) score of 1.43 in Costa Rican pedigree CR001 at marker D18S59, in 18p11.3. This lod score reflected sharing of a single allele by all nine of the BP-I patients in this family. In the other pedigree that we studied (CR004), there was no evidence of linkage or allele sharing among affecteds at this locus. In a subsequent study of LD on chromosome 18 in a population sample of 69 BP-I patients from the CVCR (2), the same D18S59 allele was associated with BP-I. An adjacent marker, D18S476 (less than 3 cM centromeric to D18S59), also showed a positive lod score in CR001 and gave evidence of association in the population sample. Further genotyping of the 69 affected individuals by using four publicly available microsatellite markers delineated a segment of maximal LD with BP-I, covering about 331 kb (ref. 8). Evaluation of a larger sample (227 patients and relatives and 26 independent control trios) by using these markers showed continuing evidence of LD and haplotype sharing in this sample for this region (8). We initiated fine mapping of a BP-I susceptibility locus in this 331-kb region by constructing a physical map, as described here. The current analysis is of the larger CVCR BP-I sample (227 affecteds), by using a much more detailed set of markers that we developed, and on the basis of the physical map of the region that we constructed.

Methods

Sample Collection. Details regarding the composition, ascertainment, and diagnostic procedures for the population sample analyzed in this paper can be found in refs. 2 and 9. Details regarding the recruitment and composition of the control sample

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Abbreviations: BP, bipolar disorder; LD, linkage disequilibrium; BLD, background LD; CVCR, Central Valley of Costa Rica; STS, sequence tagged site; BAC, bacterial artificial chromosome; SNP, single-nucleotide polymorphism.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. NM_014410, *Homo sapiens* clusterin-like 1 (retinal) (*CLUL1*) and mRNA gi 7657489 ref NM_014410.1 (7657489)].

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can be found in ref. 8. Briefly, the patient sample was composed of 227 CVCR BP-I individuals (including the set of 69 patients from ref. 8 that gave the original association evidence in 18p) and their available first-degree relatives (total, $n = 563$). All affected individuals had at least two psychiatric hospitalizations, with the first hospitalization by age 50. A second sample was comprised of these 563 individuals and a set of controls [52 unrelated parents of students recruited from the University of Costa Rica who were selected for CVCR ancestry (at least five of eight great-grandparents from the CVCR)].

Radiation Hybrid and Sequence Tagged Site (STS)-Content Mapping of Markers Within the Candidate Interval. Genetic and physical mapping information was initially obtained from Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research (http://www-genome.wi.mit.edu), Stanford Human Genome Center (http://www-shgc.stanford.edu), GÉNÉTHON Human Genome Research Center (http://www.genethon.fr/genethon_en.html), and the Cooperative Human Linkage Center (http://lpg.nci.nih.gov/CHLC). Radiation hybrid (RH) mapping (10) was used extensively in the early phase of this study to resolve discrepancies in marker order between maps. Specifically, the 83 Stanford G3 radiation hybrid panel was used to map all genetic and STS markers available from public database, as well as those developed specifically for the project. In addition to RH mapping, STS-content mapping using BAC (bacterial artificial chromosome) clones from the region of interest was also used routinely to determine the marker order and to complete the BAC contig.

BAC Library Screening, End Sequencing, and Contig Building. Microsatellite and STS markers obtained from public databases were used to screen the human BAC library from Research Genetics (Huntsville, AL) by PCR or to the BAC library from Genome Systems (St. Louis) screen by hybridization according to manufacturers' protocols. BAC DNA from positive clones was prepared using Qiagen tip 2500 columns following Qiagen Mega Prep protocol (Qiagen, Valencia, CA) with minor modifications. Sequences of the BAC ends were obtained by cycle sequencing the BAC DNA directly with vector primers T7 and SP6, respectively. Reactions were analyzed on an ABI 377 DNA sequencer (Perkin–Elmer Biosystems, Foster City, CA). PCR primers were designed from nonrepetitive end sequences and used as STS markers to improve the physical map and the BAC contig construction. The outlying markers from each side of the contigs were used to screen for overlapping BAC clones to extend the contigs.

Construction of Randomly Sheared Libraries from BACs. BAC DNA was sheared to small fragments of desired size range by using a nebulizer (CIS-US, Bedford, MA) in a buffer containing 50–100 μg DNA, 25% glycerol, 55 mM Tris, and 15 mM MgCl2. The mixture was added to the nebulizer, and gas pressure was determined by condition worked out on comparable salmon sperm DNA in a pilot experiment. After shearing, the libraries were constructed as previously described (11).

Microsatellite and Single-Nucleotide Polymorphism (SNP) Marker Development. Microsatellite markers were generated by hybridizing oligonucleotide probes for di-, tri-, and tetranucleotide repeats to randomly sheared sublibraries made from BAC clones by using Quicklite nonisotopic enzyme-induced chemiluminescent reagents from Lifecodes (Stamford, CT), following the manufacturer's instructions. Positive clones were sequenced to identify microsatellite sequences, and primers were then designed from flanking unique DNA sequence. Primers for amplifying STS markers were also designed using BAC end sequences and random sequences available within the candidate interval when extensive sequencing of the randomly sheared libraries were done. Primer sequences are given in Table 2, which is published as supporting information on the PNAS web site, www.pnas.org.

SNPs were identified using SSCP (single-strand conformational polymorphism) analysis of STS markers (all <300 bp in length). The PCR product (2.5 ml) was mixed with 4 ml of blue dye (95% formamide/20 mM EDTA/0.05% Bromophenol blue/0.05% Xylene cyanol FF), denatured at 100°C for 10 min, and immediately chilled on ice. The product (2.5 ml) was run on a 6% SSCP gel in 0.5 × TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) in the gel apparatus (Life Technologies, Rockville, MD) for about 16 h at 4°C. The gel was stained with SYBR green I nucleic acid and SYBR green II RNA gel stain (Molecular Probes) and visualized using the Fluorimager 575 (Amersham Pharmacia). When shifted bands were observed, the nucleotide basis for the polymorphism was determined by directly sequencing the PCR product. We used four unrelated individuals to screen for each SNP.

Sequencing of the Candidate Interval and Identification of the Candidate Genes. In the candidate interval of <3 cM suggested by the LD results from ref. 8, randomly sheared libraries prepared from BACs covering this region were sequenced at 10× coverage to discover all sequence information and identify all genes within the interval. More than 10,000 individual sequences from the region were compared by BLAST20 with sequences from publicly available databases and were analyzed by using GRAIL21 to identify potential coding sequences. In addition, sequences were assembled by using PHRAP22, 23, 24 in a single DNA strand of ≈331 kb. The whole sequence was again analyzed by using BLAST and GRAIL to aid in gene prediction. These data were displayed in ACEdb (data available from ncbi.nlm.nih.gov) to visualize predicted exons and their relationships to each other.

Genotyping of Microsatellites. We genotyped the four new microsatellites identified by us in sequencing the region. Primer sequences are available on request. Genotyping procedures for the microsatellites were performed as previously described in ref. 12. In brief, one of the two primers was labeled radioactively with a polynucleotide kinase, and PCR products were separated, by electrophoresis, onto polyacrylamide gels. Autoradiographs were scored independently by two raters without knowledge of affection status of the samples. Data for each marker were entered into the computer database twice, and the resultant files were compared for discrepancies and non-Mendelian errors.

SNP Genotyping. We genotyped the SNPs in patient and control samples by using the single-strand conformational polymorphism procedures described above.

Statistical Analyses. We applied a modified version of Terwilliger's (13) likelihood ratio test of LD to the four novel microsatellites and 26 SNPs that spanned our 331-kb candidate region. For each of these 30 markers, we applied this test twice, once in the sample of 227 patients and their available relatives, and also with the addition of the independent controls to the 227 patients and relatives. This likelihood ratio test estimates a single parameter, λ, which quantifies the overrepresentation of an associated marker allele on disease chromosomes versus control chromosomes. λ is related to the common epidemiological parameter of population attributable risk. If the frequency of an associated allele on disease and normal chromosomes is given by p_D and p_N, respectively, then λ is calculated by $(p_D - p_N)/(1 - p_N)$. Only positive associations with disease are permitted, and λ ranges from 0 (under the null of no association) to 1.0 (all disease chromosomes carry the associated allele). Others have shown that λ is the most closely related to the recombination fraction with disease and less influenced by marker allele frequencies

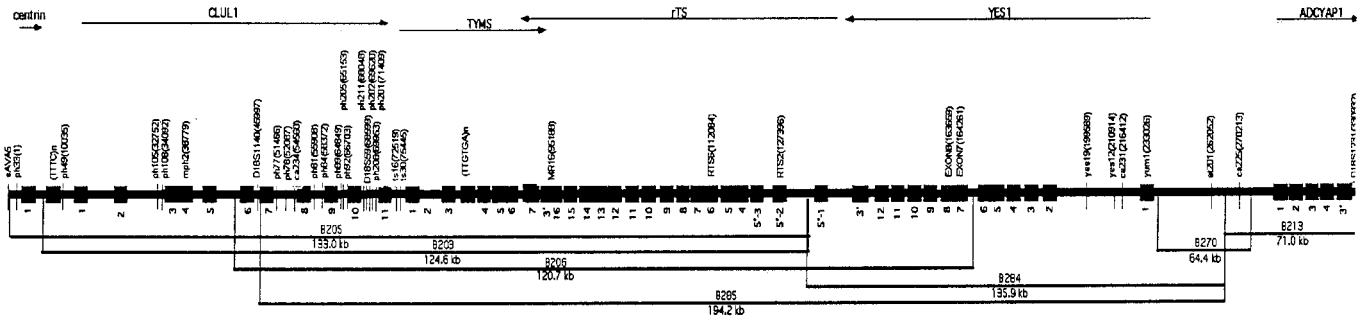

Fig. 1. Map of the genes contained in the 331-kb BP-I candidate interval on 18p11.3. The vertical lines indicate the location of the SNPs giving evidence for association to BP-I including (*Left* to *Right*, or telomere to centromere) PH33, PH84, PH205, PH202, PH208, TS16, and TS30.

than other measures of LD (14). Because we do not know which chromosome of an affected individual harbors the disease locus, we incorporated a genetic model of disease transmission in the procedure of Terwilliger. Using this model also enabled us to employ data from additional family members other than parents, if they were not available. The same genetic model (mostly dominant with reduced penetrance) was used as in our previous LD papers (2, 8) and in the genome screen of the Costa Rican pedigrees described in McInnes *et al.* (1). In this model, one chromosome of the affected individual is used as a control chromosome. The use of a model is likely to increase the power of the test and the precision of the estimates of λ when the inheritance pattern is approximately known (13). By using simulated data, Terwilliger shows that his test is conservative.

Results

Marker Development and Physical Map. On the basis of our previous results (1, 8), we focused marker development and physical mapping efforts (including direct sequencing) in the <3 cM region between sAVA5 and D18S1231. Within this region, we identified four new microsatellite markers and 26 SNPs to add to the four publicly available microsatellite markers already used (8) (Fig. 1). On the basis of the extent of haplotype sharing in pedigree CR001 and LD results from the previously used markers, we focused our detailed investigation on the region of about 331 kb between PH33 and D18S1231 (although in public databases, this segment is estimated as being 378 kb in length, contig NT_011005). By using several sequence analysis tools and database mining procedures (see *Methods*), we determined that this interval contained six known genes [*CENTRIN*, *CLUL1*, *TYMS*, *rTS*, *YES1*, and *ADCYAP1*, ordered from telomeric to centromeric, with *TYMS* and *rTS* overlapping each other (Fig. 1)]. This order differs in the public database (*CENTRIN*, *CLUL1*, *YES1*, *rTS*, *TYMS*, and *ADCYAP1*, with no overlap between *rTS* and *TYMS*). All of the genes except "clusterin-like 1 (retinal)" gene (*CLUL1*) have been well characterized previously (15–20). *CLUL1* was originally identified during a screen of a human retinal cDNA library for retina-specific genes (15). The function of this gene is not known; however, Northern blot analysis reveals that it is highly expressed in retina with much lower yet detectable expression in several other tissues including brain, kidney, and testes.

Genotyping Results. We genotyped these 30 markers in pedigree CR001 and in the CVCR patient and control samples. Fig. 2 shows the extent of haplotype sharing between the patients in CR001. The minimal region of 18.5 kb shared is contained between exons 7 and 11 of *CLUL1*. This shared 11-marker haplotype is also observed in some individuals not affected with BP-I who are shown in this pedigree, although most of these individuals cannot simply be considered "unaffected." This group includes individuals who transmit the haplotype to BP-I affected patients (3003, 3004, 4017, 4227, and 5021), as well as individuals affected with hypomania and/or major depressive disorder (4015, 4227, 4229, 5030, 5041).

Results of the LD analysis for these markers (and the four previously available markers reported in ref. 8) are displayed in Table 1. Of the 34 markers presented in Table 1, 16 showed association ($\lambda > 0$) with BP-I in at least one of the two samples (that with 227 patients/relatives and that with 227 patients/relatives and the addition of 52 controls). The *P* value associated with the estimate of λ was <0.01 for five of these 16 markers, and for four of the five markers, the magnitude of association was greater in the sample containing the population controls. All five of these markers (PH84, PH205, PH202, PH208, and TS30), had estimates of λ near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele. As reported and discussed in ref. 8, D18S59, the marker that showed linkage evidence in CR001 and association in the original CVCR population sample, shows only nonsignificant association evidence in the current samples. The markers showing LD are clustered in the 19-kb segment between exon 8 of *CLUL1* and exon 1 of *TYMS*. This segment also contains the minimal region of haplotype sharing within CR001, and for each marker in this segment, the associated alleles seen in the population samples are the same alleles in the shared haplotype in CR001 (last column in Table 1).

The observation of LD between BP-I and several clustered markers in the 331-kb segment that we evaluated could partially reflect LD between the markers themselves, i.e., background LD (BLD). To evaluate BLD in this region, we assessed LD between all adjacent markers in both the nontransmitted chromosomes of parents of affected individuals ($n = 226$ independent chromosomes) and in the control chromosomes ($n = 104$ independent chromosomes), by using a Monte Carlo approximation to Fisher's Exact Test (21, 22). Most marker pairs were in strong LD ($P < 0.0001$) in the nontransmitted and control chromosomes. Six pairs of markers were not in LD in either sample (sAVA5-PH33, PH49-PH105, PH105-PH108, PH108-MPH2, PH77-PH78, and PH84-PH89).

Discussion

The results of fine-scale genotyping of pedigree and population samples from the CVCR provide complementary evidence for a BP-I locus in a small segment within 18p11.3, on the basis of visual inspection of haplotypes and LD analyses. Four of the five markers with the strongest association evidence ($P < 0.01$ and $\lambda > 0.85$) in population samples were within *CLUL1*. Three of these markers, PH84, PH205, and PH202, were also in the region shared by all of the affected individuals in pedigree CR001. These mapping data suggest *CLUL1* as the most likely candidate

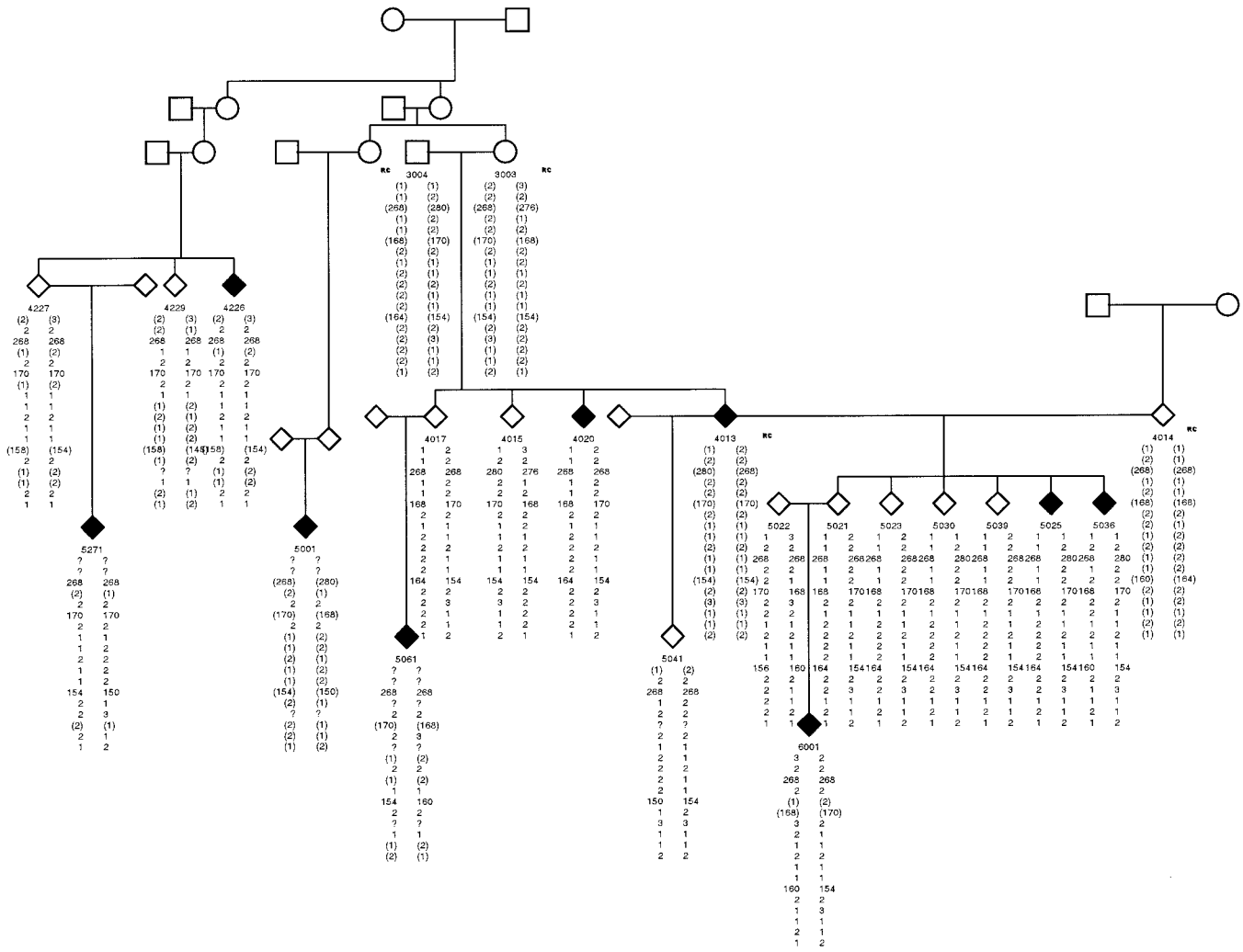

Fig. 2. Pedigree CR001. The identification numbers of individuals correspond to those in the legend of Table 1. The 18 markers shown in this figure are those shown in Table 1 with an X in the column headed CR001 Group B. All haplotypes were reconstructed by hand. Bracketed alleles indicate that assignment of phase cannot be certain. RC indicates that the haplotypes for these persons were reconstructed as no sample was available for genotyping. A ? indicates data missing. The gender of some individuals was made indeterminate to help protect the confidentiality of the family.

gene in this area and make it the focus of mutation detection efforts in BP-I samples.

Several factors complicate the interpretation of haplotype and LD data and investigation of candidate genes within the region we have targeted in 18p11.3. These factors include the accuracy of the physical map of the region, the extent of BLD within the region, the uncertainty regarding appropriate standards of significance for LD tests, and the possibility that patient and control samples derive from different subpopulations within the CVCR.

Despite extensive physical mapping and sequencing, the size of the 18p candidate region and the order of genes within it remain uncertain, given the discrepancies between our results and those contained in the public database. Our physical map is likely more accurate than the map in the database, as it is based on several-fold greater redundancy in sequencing. For interpreting fine-scale LD mapping results in identifying disease susceptibility genes, it is essential that the relative order of genes in the candidate region be unequivocal. For some such regions, as in 18p11.3, it may not be possible, for this purpose, to rely on the currently available information in the public databases.

The BLD analyses indicate that the LD results for the different markers cannot be considered independently from each other. There are no clear guidelines for interpreting the significance of the LD test (LRT) when the single marker results across the candidate region are correlated. Additionally, it is not clear how to consider the degree of evidence for BP-I localization provided by the observation that the alleles in the haplotype shared by the affected individuals in CR001 are the same as the alleles associated with BP-I in the population sample given the degree of BLD in the region. The initial finding that the D18S59 allele possessed by BP-I patients in the pedigree was over-represented in the population sample predicted it would be likely that the alleles at other markers would be the same in the two samples.

It is also not clear how to evaluate the degree of significance of the LD results. Genome-wide criteria for significance are not yet available for LD testing in founder populations and are also not appropriate when the search for a candidate gene is limited to a candidate chromosomal region.

Addition of the control chromosomes strengthened the association data for all markers except for PH84. The most straightforward explanation for this observation is that the inclusion of the extra control chromosomes provides more power to the LD analyses, compared with those analyses that use only the nontransmitted parental chromosomes as controls. It is possible that

Table 1. Fine mapping results from 18p

Intermarker physical distance	Marker	227 λ	χ^2	*P* value	227+ λ	χ^2	*P* value	CR001 Group A	CR001 Group B	CR001 Group C
Unknown	sAVA5*	0.00			0.00					
1058	PH33	0.00			0.66	2.81	0.047			
8977	Centrin gene									
7636	PH49	0.00			0.00					
9850	*CLUL1* Exon 1									
5231	*CLUL1* Exon 2									
1340	PH105	0.00			0.00					
3457	PH108	0.00			0.00			X	X	
1230	*CLUL1* Exon 3									
1224	MPH2 in *CLUL1* Exon 4	0.00			0.00			X	X	
5507	*CLUL1* Exon 5									
487	*CLUL1* Exon 6									
3723	D18S1140*	0.00			0.00			X	X	
1746	*CLUL1* Exon 7									
621	PH77	0.21	0.35	0.278	0.12	0.33	0.284	X	X	X
2473	PH78	0.43	1.04	0.154	0.00			X	X	X
963	CA234†	0.00			0.00			X	X	X
385	*CLUL1* Exon 8									
2464	PH81	0.00			0.00			X	X	X
5447	PH84	0.90	10.29	0.0007	0.78	4.40	0.018	X	X	X
1030	*CLUL1* Exon 9									
304	PH89	0.00			0.57	0.85	0.179	X	X	X
1550	PH205	1.00	3.98	0.023	1.00	7.14	0.004	X	X	X
494	PH92	0.00			0.59	0.46	0.249	X	X	X
851	*CLUL1* Exon 10									
551	PH211	0.00			0.10			X	X	X
1021	D18S59*	0.35	0.65	0.211	0.00			X	X	X
333	PH202	0.99	2.26	0.066	1.00	9.03	0.001	X	X	X
1456	PH208	0.96	2.20	0.069	1.00	5.96	0.007		X	
586	PH201	0.00			0.00				X	
522	*CLUL1* Exon 11									
2928	TS16	0.00			0.84	4.78	0.014		X	
3756	TS30	0.00			0.88	7.31	0.003		X	
15987	TYMS Exon 1									
16896	MR16	0.00			0.00					
15312	RTS6	0.00			0.00					
36263	RTS2	0.00			0.00					
602	EXON 8	0.46	0.59	0.220	0.00					
35308	EXON 7	0.00			0.00					
11345	YES19	0.28	1.37	0.121	0.27	1.89	0.085			
5498	YES12	0.00			0.00					
16614	CA231†	0.00			0.00					
29026	YUM1	0.28	0.28	0.297	0.00					
8161	AT201†	0.42	2.07	0.075	0.32	0.99	0.160			
60719	CA225†	0.00			0.00					
	D18S1231*	0.00			0.00					

227 λ indicates the estimate of λ for the 227 patients analyzed with relatives. The χ^2 and *P* value are associated with the estimates of λ. 227+ λ includes patients, their relatives, and controls. The three right-most columns of the table indicate the markers where alleles are shared identically by descent with BP-I patients from CR001. Group A indicates haplotypes shared by CR001 identification numbers 4020, 6001, and 5061. Group B includes CR001 identification numbers 4226 and 5271. Group C includes identification numbers 5025 and 5036. Of note, all eight of the predominantly phase known or reconstructed BP-I individuals from CR001 also shared haplotypes surrounding this region of at least 5 cM within their group. Intermarker distances are indicated in base pairs interpreted as follows: the end of marker PH33 is 1,058 bp from the start of Centrin, and so forth.

*Publicly available microsatellite markers [reported previously in Escamilla *et al*. (8)].

†Novel microsatellites.

these results reflect a mismatch of cases and controls; however, this is unlikely, as we previously performed an analysis of heterogeneity on the controls versus the nontransmitted parental chromosomes and determined that there were no significant differences in allele frequencies between these groups (data not shown).

Additional power in delineating a region of maximum LD in 18p11.3 could be provided by analysis of haplotype data from the population sample of BP-I patients; such an analysis could incorporate modeling of the correlation between adjacent markers. Currently available methods for such analyses (23–25), however, are contingent on determining genetic phase for affected individuals. For fine-mapping studies, the reliance on SNPs rather than more informative microsatellite markers introduces substantial uncertainty and potential bias to haplotype reconstruction.

Although the pattern of LD over several markers suggests *CLUL1* as the most likely candidate for a BP-I gene in the 18p11.3 region, this suggestion should be treated cautiously. In this region and in many other genome regions, LD is unevenly distributed (26), particularly over small physical distances (27), and the pattern of LD may indicate the vicinity of a disease gene without pinpointing its exact location. For example, in Werner's syndrome, maximal LD was detected with markers that were about 200 kb distant from the causative gene (28, 29). In a region as gene-dense as the 331-kb segment discussed here, the associations observed may reflect LD with a sequence variant affecting the function of one of the other genes in the region rather than *CLUL1*.

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