Am. J. Hum. Genet. 61:873–881, 1997

Genetic Heterogeneity in Familial Acute Myelogenous Leukemia: Evidence for a Second Locus at Chromosome 16q21-23.2

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Summary

The identification of genes responsible for the rare cases of familial leukemia may afford insight into the mechanism underlying the more common sporadic occurrences. Here we test a single family with 11 relevant meioses transmitting autosomal dominant acute myelogenous leukemia (AML) and myelodysplasia for linkage to three potential candidate loci. In a different family with inherited AML, linkage to chromosome 21q22.1-22.2 was recently reported; we exclude linkage to 21q22.1-22.2, demonstrating that familial AML is a heterogeneous disease. After reviewing familial leukemia and observing anticipation in the form of a declining age of onset with each generation, we had proposed 9p21-22 and 16q22 as additional candidate loci. Whereas linkage to 9p21-22 can be excluded, the finding of a maximum two-point LOD score of 2.82 with the microsatellite marker D16S522 at a recombination fraction $\theta = 0$ provides evidence supporting linkage to 16q22. Haplotype analysis reveals a 23.5-cM (17.9-Mb) commonly inherited region among all affected family members extending from D16S451 to D16S289. In order to extract maximum linkage information with missing individuals, incomplete informativeness with individual markers in this interval, and possible deviance from strict autosomal dominant inheritance, we performed nonparametric linkage analysis (NPL) and found a maximum NPL statistic corresponding to a *P*-value of .00098, close to the maximum conditional probability of linkage expected for a pedigree with this structure. Mutational analysis in this region specifically excludes expansion of the AT-rich minisatellite repeat *FRA16B* fragile site and the CAG trinucleotide repeat in the E2F-4 transcription factor. The "repeat expansion detection" method, capable of detecting dynamic mutation associated with anticipation, more generally excludes large CAG repeat expansion as a cause of leukemia in this family.

Received May 6, 1997; accepted for publication July 23, 1997.

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0002-9297/97/6104-0015$02.00

Introduction

A number of genes have been found to be mutated in leukemia, largely through the cloning of somatic translocation breakpoints and the screening of known tumor suppressor genes, oncogenes, and other genes with cellular regulatory function (Sachs 1996). However, the pathogenic mutations that initiate the multistep evolution of leukemia have been more difficult to elucidate (Fialkow et al. 1991; Busque and Gilliland 1993). Rare autosomal dominant cancer families provide an opportunity to study genes involved in the earliest phases of malignancy (Knudson 1993).

Familial leukemia is exceptionally rare; we are aware of only three extant pedigrees that have been adequately sampled (Ho et al. 1996; Horwitz et al. 1996*b;* Olopade et al. 1996). The difficulty of ascertaining large leukemia families with multiple available affected members limits the power of a genomewide survey for linkage and makes evaluation of candidate loci a more feasible approach. In previous reviews of the clinical features of familial leukemia (Horwitz et al. 1996*a;* Horwitz 1997), we observed anticipation, in the form of a declining age at onset with each passing generation, and proposed potential candidate regions: In a family inheriting autosomal dominant myelodysplasia and acute myelogenous leukemia (AML) of multiple subtypes, which serves as the subject of the current study, we found that two affected individuals shared a constitutional cytogenetic euchromatic banding variation at chromosome 9p21-22 (Horwitz et al. 1996*b*), suggesting potential linkage to this region. Evidence supporting a chromosome 21 locus includes the finding of linkage for a platelet disorder and propensity to develop AML to 21q22.1-22.2 in one family (Ho et al. 1996), an increased incidence of leukemia in Down syndrome (Epstein 1995), and frequent somatic translocation in leukemia involving the *CBFA* gene on 21q22.3. We proposed 16q22 as a potential familial leukemia candidate region (Horwitz et al. 1996*a;* Horwitz 1997), in part because of a report of a father and daughter coinheriting leukemia and a fragile site in this region (Ferro et al. 1994) and because somatic inversion of the *CBFB* gene in the AML M4 subtype involves this band (Speck and Stacy 1995).

The family that we have described (Horwitz et al. 1996*b*) may be particularly valuable in the study of leukemia genesis because affected family members have developed myelodysplasia or AML of differing subtypes, indicating that the responsible gene operates at a primitive and signal step in myeloid differentiation. In the current article, we evaluate linkage of disease in this family to these proposed candidate loci and report provisional evidence for assignment of the disease to chromosome 16q21-23.2. In addition, we conduct preliminary mutational analysis of repetitive sequence elements in this region, because familial leukemia displays anticipation (Horwitz et al. 1996*a*). The only known mechanism of anticipation results from the instability of repeat sequences (La Spada et al. 1994; Sutherland and Richards 1995), although we have also proposed a model of anticipation in familial cancer based on polygenic inheritance of multiple de novo mutations secondary to a loss of a major gene regulating DNA replication fidelity (Horwitz 1997).

Subjects and Methods

Clinical Description and DNA Sources

Relevant individuals in the pedigree are shown in figure 1. Diagnostic criteria were previously established, and a full clinical description has been reported (Horwitz et al. 1996*b*). All affected individuals had frank hematopoietic maturation defects documented by bone marrow examination interpreted by at least one hematopathologist unaware of the family history. The proband is individual IV-1, who had M7 megakaryoblastic AML. Individuals II-3 and II-1 had M2 AML. Patients III-3 and III-4 had myelodysplastic syndrome terminating in AML. Because of limited clinical information, it was not possible to determine leukemia subtype on patient II-3. Individual IV-3 had myelodysplasia with monosomy of chromosome 7 in bone marrow cells. Patients II-4 and IV-2 had bone marrow abnormalities consistent with incipient myelodysplasia; in addition, a rare opportunistic mycobacterial infection contributed to the death of patient II-4.

DNA was obtained from various preparations as follows: buffy coat from peripheral blood from individuals IV-1 and IV-2; Epstein-Barr virus–transformed polyclonal lymphoblastoid cell lines derived from peripheral blood from individuals II-1, II-5, II-6, III-1, III-5, III-6, and IV-3; paraffin blocks of autopsy-derived liver on patient II-3, biopsied lymph nodes on patient II-4, bone marrow biopsy on patient III-2, skeletal muscle on patient III-3; and air-dried microscope slides of bone marrow aspirate on patient III-4. Archival DNA was extracted by the QIAGEN QIAamp tissue kit and was used according to the manufacturer's instructions. Archival material was not used for linkage analysis on chromosomes 9p21-22 and 21q22.1-22.2. No apparently unaffected, at-risk individuals, including obligate heterozygotes II-1, II-5, and III-5, consented to clinical and bone marrow examination; therefore, the sample available for linkage testing, for which clinical phenotype was available, consisted solely of affected individuals.

Linkage Analysis

Since all individuals at risk for leukemia included in the linkage analysis were either affected or obligate heterozygotes, and the overall incidence of AML (Ho et al. 1996) is low (.0001), it was possible to use an autosomal dominant model of inheritance that is essentially independent of penetrance. This avoids the consideration of anticipation and age-dependent penetrance. The data were initially examined under this single conservative model with gene frequency set to .0001, fixed prior to obtaining genotypes.

Genotype information was generated with PCR amplification of microsatellite repeat polymorphisms. PCR was employed with end-labeled primers or in the presence of [α^{32}P]dCTP, using protocols published in the Genome Database (Fasman et al. 1996), and the products were analyzed by 6% denaturing PAGE and autoradiography.

Two-point LOD scores were calculated using the MLINK subroutine of the LINKAGE software package, version 5.1 (Lathrop et al. 1984), and maximum likelihood estimate of θ was performed with the ILINK subroutine. Nonparametric linkage analysis was performed with GENEHUNTER software, version 1.1 (Kruglyak et al. 1996). Allele frequencies for markers were taken from the Genome Database, except for the chromosome 21 marker UT7582 and D16S398, for which information is not published. Allele frequencies for UT7582 were determined by genotyping 32 chromosomes from unrelated individuals in CEPH families (M. F. Leppert, unpublished data). Allele frequencies for D16S398 were derived using family-based allele frequency estimation (Boehnke 1991); LOD scores were determined using ILINK to estimate iteratively allele frequencies jointly with maximum likelihood estimation of θ (Terwilliger and Ott 1994, pp. 185–87), starting with the allele frequencies observed in the three genotyped founders, II-6, III-6, and IV-3. The LOD scores for the other chromosome 16 markers were separately evaluated using this method, to assess the influence of allele frequencies in this family in which genotypes of several founders are absent.

Mutational Analysis

PCR quantification of the AT-rich *FRA16B* minisatellite repeat (Yu et al. 1997) was performed in 16.5 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 0.65 mM EDTA, 0.17 mg/ml BSA, 10% (v/v) dimethyl sulfoxide (DMSO), 1.42 μM β-mercaptoethanol, 1.5 mM each of the four dNTPs, 5 μCi [α^{32}P]dATP, 50 ng

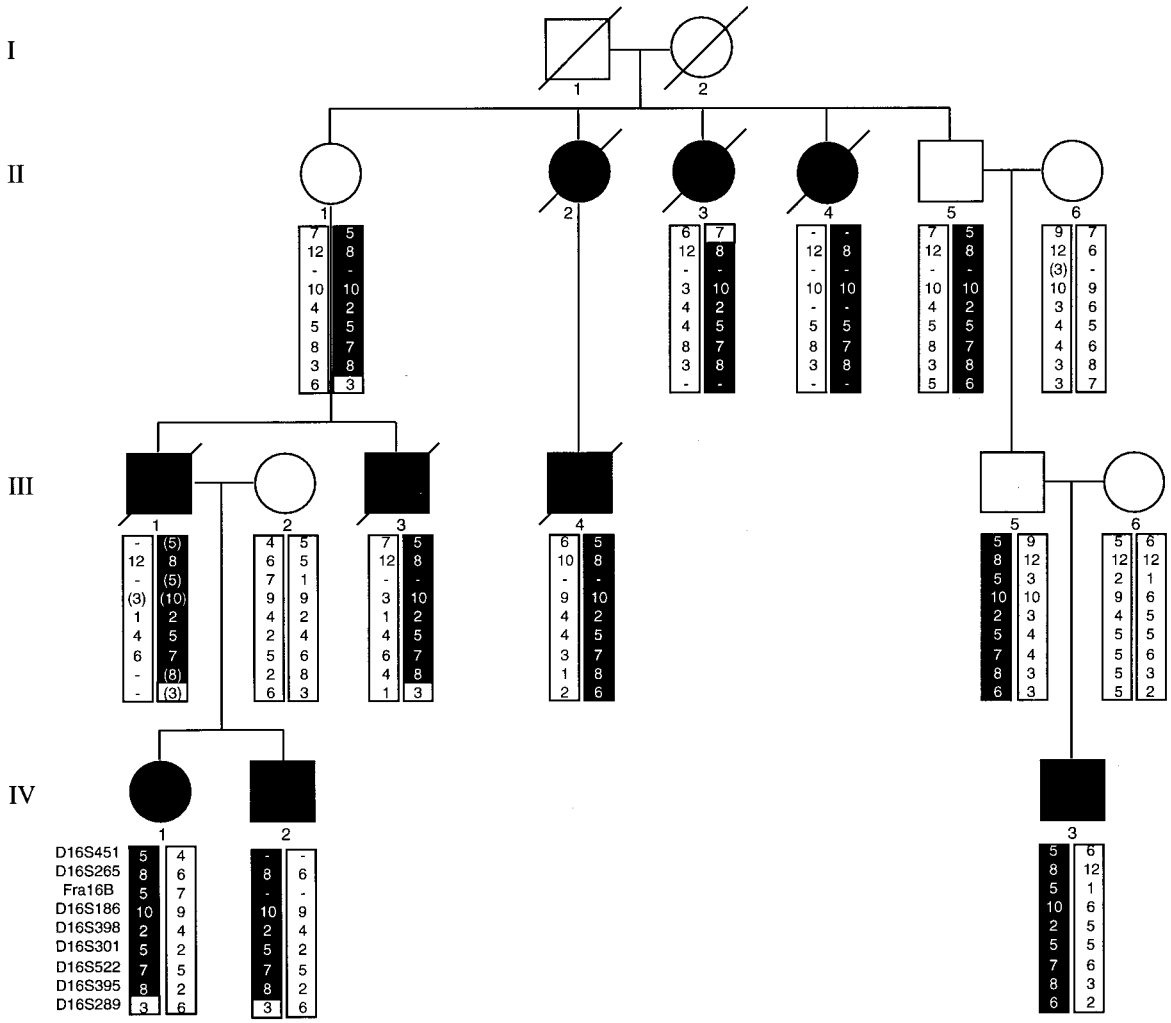

Figure 1 Pedigree and haplotype analysis for chromosome 16q21-23.2. Solid symbols denote affected individuals. The common haplotype is denoted in black. Dashes (-) indicate not tested or a failure to detect a PCR amplification product. Inferred genotypes are given in parentheses. (Inferred genotypes were not used in the linkage analysis.)

of DNA sample, 37.5 ng of each primer (5′-TGTAAAACGACGGCCCAGTGTACTATACTATACTATATTATACAG-3′ and 5′-CAGGAAACAGCTATGACCGTATTATATATTATCTAATTATATCTTATATATTGAATATTAC-3′), and 2.5 U *Taq* polymerase for 10 cycles of 88°C at 30 s, 45°C at 30 s, and 58°C at 3 min and 20 cycles of 88°C at 30 s, 50°C at 30 s, and 58°C at 3 min. Products were analyzed by denaturing PAGE and autoradiography.

PCR amplification of the E2F-4 CAG repeat was performed as described by Ginsberg et al. (1994). PCR amplification of the AF9 CAG repeat was performed as described by Walker et al. (1994).

Repeat expansion detection (RED) analysis was performed as described by Bleyl et al. (1995) with the following modifications: the $(CTG)_{15}$ oligonucleotide was purified by 19% nondenaturing PAGE after [γ^{32}P]ATP phosphorylation; 396 cycles of 94°C for 10 s and 80°C for 30 s were performed after an initial denaturation step of 94°C for 5 min; and products were analyzed by 7% denaturing PAGE with autoradiography.

Results

Exclusion of Linkage to 9p21-22 and 21q22.1-22.2

In the initial clinical evaluation of this family (Horwitz et al. 1996*b*), we observed a constitutional euchromatic banding variation of chromosome 9p21-22 in patient IV-3 and in a grandson of patient II-3 (implying its presence in patient II-3, who died before the era of routine cytogenetic testing in leukemia). One candidate gene in this region is *AF9*, which is the frequent site of reciprocal translocation with the *MLL* gene on chromosome 11 in recurrent t(9;11)(p22;q23) associated with AML (Nakamura et al. 1993). Furthermore, AF9 has a polymorphic polyserine-encoding CAG repeat (Walker et al. 1994) that is of interest in view of the observation of anticipation in familial leukemia (Horwitz et al. 1996*a*).

Linkage analysis with the markers D9S126 on 9p21 and IFNA on 9p22 (table 1) effectively excludes linkage to this region. In addition, karyotypes of individuals III-2 and IV-1 failed to reveal the cytogenetic variation, and PCR amplification of the CAG repeat in AF9 demonstrated that all individuals in this family were homozygous for the most common allele of 42 repeats (not shown).

In a large family inheriting platelet granule defects and a predisposition to AML, linkage to a 15.2-cM interval on chromosome 21q22.1-22.2 has been determined (Ho et al. 1996). That family differs from the subject family here by the presence in the prior family of platelet granule and aggregation defects, constitutive thrombocytopenia, an evident risk for solid tumors, and an apparently lower penetrance of AML that may occur after solid tumor treatment with chemotherapy (Dowton et al. 1985). However, this locus is an obvious candidate for other leukemia families, especially in light of the fact that it is the site for recurrent t(8;21)(q22;q22) of the *CBFA* gene in many cases of sporadic AML and the association of Down syndrome with leukemia (Epstein 1988). Two-point linkage analysis of six markers spanning an ~12.4-cM interval excludes linkage of disease in our family to 21q22.1-22.2 (table 1).

Evidence Supporting Linkage to 16q21-23.2

It has been proposed that inherited fragile sites might be involved in familial cancers (LeBeau and Rowley 1984; Richards and Yu 1996). The observation of a fragile site on 16q22 in one small leukemia family (Ferro et al. 1994) suggested that this region may contain a locus for familial leukemia (Horwitz et al. 1996*a;* Horwitz 1997). Therefore, we evaluated the distamycin-sensitive *FRA16B* site resulting from amplification of an AT-rich minisatellite sequence at the 16q21-22.1 boundary (Yu et al. 1997). Pathological expansion of the minisatellite to lengths productive of a fragile site was not detected in individuals in two branches of the family (fig. 2). However, the finding that allele 5 is coinherited by two affected second cousins (IV-1 and IV-3) prompted further investigation of linkage to this region.

Results of linkage analysis to multiple flanking markers in 16q21-23.2 are shown in table 2. A maximum two-point LOD score of 2.82 occurs with the marker D16S522 at $\theta = 0$. All affected individuals share a haplotype without recombination extending 23.5 cM (or 17.9 Mb, as determined from the chromosome 16 physical map [Doggett et al. 1995]) from D16S451 to D16S289 (fig. 1).

Linkage analysis in this family is limited by missing individuals and is therefore a function of the estimated population allele frequencies. To gauge the effect of this potential bias, we also calculated LOD scores with a family-based approach to estimate allele frequency (Boehnke 1991). Since the allele segregating with leukemia for several of the markers is not observed in the unrelated founders, we conclude the reported LOD scores to be conservative. For example, when this approach is used for D16S522 or D16S265, Z_{max} inflates to ~3.00.

Linkage analysis in this family is further limited by a lack of informativeness for all markers with each meiosis and the potential for model misspecification due to possible complex inheritance (Horwitz 1997). In order to extract maximum linkage information under these restrictions, we evaluated the genotype data utilizing nonparametric linkage analysis (NPL; Kruglyak et al. 1996) as shown in figure 3. The maximum NPL statistic in this interval of 10.13 corresponds to an exact *P* value of .00098 that the observed marker segregation would occur under the null hypothesis of no linkage.

Preliminary Mutational Analysis

A possible explanation for the observation of anticipation in familial leukemia (Horwitz et al. 1996*a*) is dynamic mutation of repetitive DNA sequences. The E2F-4 transcription factor interacts with the RB homologue p107 and is implicated in control of the cell cycle (Ginsberg et al. 1994); it has been mapped to 16q22.1

Table 1

Pairwise LOD Scores between Familial Leukemia and Chromosomes 9p21-22 and 21q22.1-22.2

	LOD Score at θ =								
Marker	.001	.01	.05	.1	.2	.3	.4	Z_{max}	θ_{max}
D9S126	−1.67	−.69	−.08	.11	.20	.16	.09	.20	.21
IFNA	−4.46	−2.50	−1.16	−.63	−.18	−.01	.04	.04	.40
UT7582	−∞	−5.10	−3.02	−2.13	−1.23	−.69	−.30	0	.5
D21S263	−2.21	−1.22	−.57	−.33	−.14	−.06	−.03	0	.5
IFNAR	−4.43	−2.46	−1.14	−.64	−.23	−.07	−.01	0	.5
D21S65	−5.20	−3.20	−1.83	−1.26	−.71	−.40	−.18	0	.5
D21S167	−4.57	−2.61	−1.28	−.77	−.34	−.15	−.06	0	.5

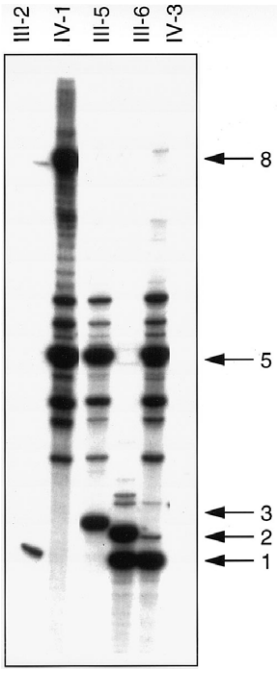

Figure 2 PCR analysis of *FRA16B* minisatellite repeat. Allele numbering is relative to additional alleles that have been identified in a CEPH control population (not shown).

and contains a polymorphic polyserine-encoding CAG repeat (Ginsberg et al. 1994). All individuals in this family appear to be homozygous for the most common allele of 13 repeats (not shown), excluding expansion of this repeat as a cause of leukemia here.

Since this family displays apparent autosomal dominant inheritance and, so far, all the repeat expansions with dominant inheritance are CAG triplets (La Spada et al. 1994; Sutherland and Richards 1995), we conducted RED analysis (fig. 3) to determine whether this family was transmitting an expanded CAG repeat (anywhere in the genome). The RED method is a general procedure based on the ligase chain reaction for detecting large expansions of repetitive DNA sequences (Schalling et al. 1993). There is no evidence for the presence of a large CAG repeat in individuals in this family. Note, however, that this does not completely exclude the possibility of pathological expansion of a short CAG repeat below the limit of RED detection, such as occurs in spinocerebellar ataxia (SCA) type 6 (Zhuchenko et al. 1997).

Discussion

Of the three candidate loci for leukemia in this family that we have proposed, we have excluded 9p21-22 and 21q22.1-22.2 by linkage analysis. We are unable to exclude 16q21-23.2 and, in fact, find evidence supporting linkage to this region. The highest two-point LOD score of 2.82 with D16S522 at $\theta = 0$ is below the generally accepted linkage criterion of a LOD score $\geqslant 3.0$. Nevertheless, we believe that this result is important for the following two reasons. First, linkage analysis here is vulnerable to a loss of information from missing individuals, incompletely informative markers, and the possibility of model misspecification resulting from complex inheritance (Horwitz 1997). To extract linkage information maximally, we examined the genotype data using nonparametric linkage analysis (Kruglyak et al. 1996). NPL is a computationally tractable method based on multipoint analysis of genotypic identity by descent. It has the advantages of greater power to detect significant linkage with incomplete pedigree data and is independent of inheritance model. The *P* value corresponding to the maximum NPL statistic is .00098. Notably, this is less than the conditional probability of linkage of .001 defined by the two-point LOD score standard of 3.0 and is nearly equal to the conditional probability of linkage ($10^{-3.01}$) corresponding to the theoretical maximum LOD score of $\sim$3.01 that can be generated from this pedigree structure of 11 meioses with missing individuals (as approximated from a two-point simulation study with a fully informative marker and nearly infinitely rare and polymorphic alleles). Second, familial leukemia is

Table 2

Pairwise LOD Scores between Familial Leukemia and Chromosome 16q21-23.2

	LOD Score at θ =								
Marker	0	.01	.05	.1	.2	.3	.4	Z_{max}	θ_{max}
D16S451	−3.00	−1.29	−.52	−.19	.06	.12	.08	.12	.30
D16S265	2.66	2.61	2.41	2.14	1.56	.94	.36	2.66	0
D16S186	.97	.96	.89	.79	.59	.38	.18	.97	0
D16S398	1.81	1.77	1.62	1.44	1.05	.66	.29	1.81	0
D16S301	.99	.98	.93	.86	.68	.48	.25	.99	0
D16S522	2.82	2.77	2.55	2.28	1.69	1.04	.41	2.82	0
D16S395	1.42	1.38	1.23	1.04	.68	.36	.13	1.42	0
D16S289	−2.79	−.51	.09	.26	.31	.25	.14	.32	.18

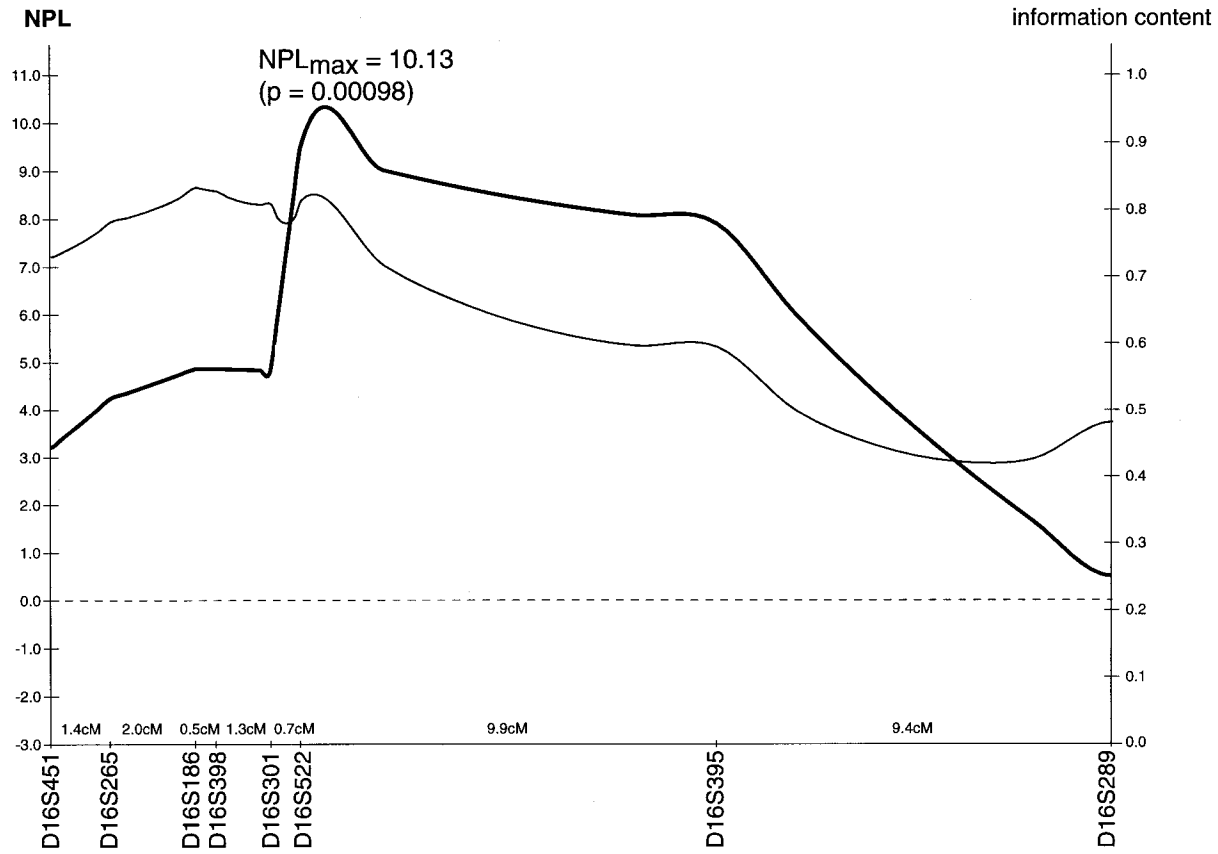

Figure 3 Nonparametric linkage analysis. Shown is the output of the GENEHUNTER program with NPL score (thick line) on the left ordinate and the information content (thin line) on the right ordinate. The NPL_{max} occurs at a map position of 5.99 cM with a corresponding *P* value of .00098. The indicated intermarker distances from the corresponding genetic maps (Shen et al. 1994; Kozman et al. 1995) were used in the analysis.

exceedingly rare, and the families are generally small and ascertained after most affected individuals have died (Horwitz et al. 1996*a;* Horwitz 1997), limiting opportunities for further sampling. Definitive linkage results may not be obtainable in a single study. The identification of reasonable candidate regions will facilitate the study of additional small families that are ascertained in the future.

There are clinical differences between some of the reported leukemia families (Horwitz 1997). The exclusion of linkage to 21q22.1-22.2, a locus to which inherited AML is mapped in another family (Ho et al. 1996), demonstrates that familial leukemia is a genetically heterogeneous disease. Evidence for other loci for familial leukemia is limited. Two brothers (Blattner et al. 1978) with acute lymphocytic leukemia (ALL) shared two HLA haplotypes, and, in a different family (Kato et al. 1983), two first cousins (whose parents were half siblings of one another) with ALL shared a common HLA haplotype, thereby suggesting the possibility of linkage to chromosome 6p. However, in a family with multiple siblings with chronic lymphocytic leukemia (CLL) (Schweitzer et al. 1973) and a multigenerational AML family (Gunz et al. 1978), HLA types differed among affected individuals. Three siblings developing CLL had a constitutional deletion of the short arm of chromosome 22 (Fitzgerald and Hamer 1969). Two brothers with childhood myelodysplasia with monosomy 7, a disorder that appears to be autosomal recessive (Horwitz 1997), had constitutional inversion of chromosome 1p22q23 (Paul et al. 1987). And, as noted, a father-daughter pair with AML and ALL apparently coinherited a 16q22 fragile site (Ferro et al. 1994).

We were led to test for instability of repetitive DNA sequence tracts, because we had previously observed anticipation in the form of a declining intergenerational age at onset in the inheritance of familial leukemia (Horwitz et al. 1996*a*). A preliminary mutational analysis excludes minisatellite repeat amplification at the *FRA16B* fragile site and CAG trinucleotide repeat amplification in the polyserine coding region of the E2F-4 transcription factor as responsible for leukemia in this family. Negative RED analysis further excludes large CAG repeat expansion as causative. Dynamic mutation of repetitive sequence elements is the only documented molecular mechanism for anticipation in inherited neu-

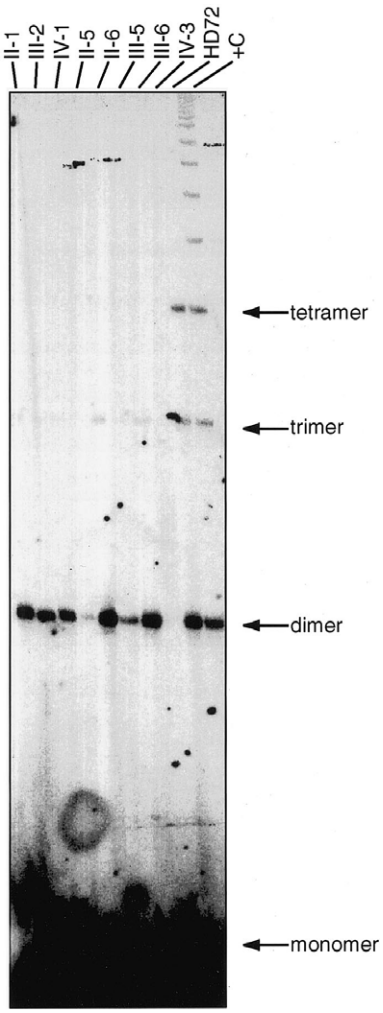

Figure 4 RED analysis for CAG triplet repeat expansion. The monomer is $(CTG)_{15}$. The positive controls HD72 and +C are from a Huntington disease patient with 72 CAG triplet repeats and a large CAG repeat–positive individual identified in a screen of patients with neurological diseases (authors' unpublished data), respectively. None of the affecteds and spouses in this family yield more than a trimer, indicating that the largest genomic repeat is $\leq$45 CAG triplets.

rodegenerative diseases (La Spada et al. 1994; Sutherland and Richards 1995; Buard and Jeffreys 1997), although we have proposed that anticipation in familial cancer might also result from polygenic inheritance secondary to a defect in a major gene responsible for DNA replication fidelity (Horwitz 1997).

There are at least three other candidate genes in this region. First, the proto-oncogene MAF (Nishizawa et al. 1989) maps to 16q22-23 (Yoshida et al. 1991) and has been implicated in the transcription of hematopoietic genes and the differentiation of leukemic cell lines (Igarashi et al. 1995; Francastel et al. 1997). Second, *CBFB* maps to 16q22.1 and is inverted to form a fusion transcript with smooth muscle myosin heavy chain in the erythroleukemic M4 AML subtype (Speck and Stacy 1995). It may be of relevance that the phenotype of mice with targeted interruption of *CBFB* includes CNS bleeding (Castilla et al. 1996; Wang et al. 1996); in the presently studied family, three individuals in three generations died of hemorrhagic stroke at young ages (Horwitz et al. 1996*b*). It is interesting that a gene encoding another subunit of the hematopoietic CBF transcriptional complex, *CBFA,* maps to the region on 21q22.1-22.2 (Speck and Stacy 1995), to which the other family with AML predisposition has been linked (Ho et al. 1996), although, thus far, there is no evidence of mutation of *CBFA* in that family (Legare et al. 1995). Moreover, the two known *CBFA* homologues, *AML2* and *AML3,* map to 1p and 6p, respectively (Levanon et al. 1994; Wijmenga et al. 1995), where, as discussed above, there is suggestive support for localization of familial myelodysplasia and familial ALL. Third, SCA4 has been mapped to 16q22.1 (Flanigan et al. 1996). Like the other spinocerebellar ataxias, SCA4 also demonstrates anticipation (Flanigan et al. 1996). It is clinically differentiated from the other types, however, by the additional presence of an axonal neuropathy, and the diagnosis can be clinically confused with Friedreich ataxia (Flanigan et al. 1996). Of note, the grandson of patient II-3 carries the diagnosis of Friedreich ataxia (Horwitz et al. 1996*b*), but the results of molecular genetic testing for this disorder have not been available for confirmation. The possibility that this family may also transmit SCA4, then, could be a significant observation, especially in light of the overlap of spinocerebellar ataxia and leukemia in yet another family (Li et al. 1981).

Acknowledgments

We thank Ellen Wijsman for discussion, Norman Doggett for a revised physical map of chromosome 16, Archie Coonrod for technical assistance, and Mark Matsushita, Ted Holzman, and Ming Lee for help with computer systems. M.H. was supported by a Damon Runyon—Walter Winchell Cancer Research Foundation fellowship, the Markey Foundation, and Public Health Service grant NICHD HD0108-03; K.F.B. was supported by Public Health Service grant T32 HL07312; W.H.R and J.W. were supported by Public Health Service grant R37 CA16448; R.I.R. was supported by the National Health and Medical Research Council of Australia and the Women's and Children's Hospital Research Foundation and thanks Shelley Richards for support and encouragement.

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