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Mutational Analysis of the Candidate Tumor Suppressor Genes *TEL* and *KIP1* in Childhood Acute Lymphoblastic Leukemia[1]

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ABSTRACT

We have shown previously that loss of heterozygosity at chromosome band 12p13 is among the most frequent genetic abnormalities identified in acute lymphoblastic leukemia (ALL) of childhood. Two known genes map within the critically deleted region of 12p: *TEL*, the gene encoding a new member of the ETS family of transcription factors, which is rearranged in a variety of hematological malignancies; and *KIP1*, the gene encoding the cyclin-dependent kinase inhibitor p27. Both genes are, therefore, excellent candidate tumor suppressor genes. In this report, we determined the exon organization of the *TEL* gene and performed mutational analysis of *TEL* and *KIP1* in 33 childhood ALL patients known to have loss of heterozygosity at this locus. No mutations in either *TEL* or *KIP1* were found; this suggests that neither *TEL* nor *KIP1* is the critical 12p tumor suppressor gene in childhood ALL.

INTRODUCTION

Malignant transformation through loss of function has emerged as an important mechanism of oncogenesis, which has become better understood as increasing numbers of tumor suppressor genes have been identified. Perhaps the most extensively characterized tumor suppressor is the retinoblastoma susceptibility gene product, RB, identified through linkage analysis of families with hereditary retinoblastoma (1, 2). Similar strategies have been used to clone other putative tumor suppressors, such as BRCA1, associated with familial breast cancer, and MSH2, associated with hereditary nonpolyposis colon cancer (3–5). Several new tumor suppressor genes, however, have been identified by virtue of their localization within regions of the genome commonly deleted in tumors. For example, the genes encoding the cdk[4] inhibitors p15 and p16 map within a region of chromosome band 9p21 initially noted to be deleted in malignant melanoma, but these genes were subsequently shown to be deleted in a wide variety of human tumors (6–10).

We recently identified a new putative tumor suppressor locus on chromosome band 12p12–p13 in childhood ALL (11, 12). Microsatellite analysis showed that 17–33% of children with ALL have LOH at this locus, representing one of the most common genetic abnormalities associated with childhood cancer. Mapping of the critical region of 12p LOH identified two candidate genes: *TEL*, a new ETS-related gene rearranged in several types of leukemia, and *KIP1*, the gene encoding the cdk inhibitor p27. This finding was supported by fluorescence *in situ* hybridization studies, which mapped similarly the critical region of 12p deletion in hematological malignancy to a region that includes *TEL* and *KIP1* (13).

TEL is a new member of the ETS family of transcription factors, which was initially identified because of its fusion to the platelet-derived growth factor receptor β in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation (14). We and others (15–19) have shown subsequently that several additional translocations result in the formation of chimeric *TEL* transcripts. These fusions include *TEL-ABL* in t(9;12) ALL and AML (15, 16), *MN1-TEL* in t(12;22) AML (17), and *TEL-AML1* in t(12;21) ALL (18, 19). In some instances, both alleles of the *TEL* gene are involved, one by chromosomal translocation and the other by deletion; this leads to complete loss of TEL function in the leukemic cells. The function of the normal TEL protein is not known, but other members of the ETS family, including ETS-1 and PU.1, have been shown to be essential for normal hematopoietic development (20, 21).

p27 is similarly an excellent candidate tumor suppressor in that other members of the cdk inhibitor family, including p15 and p16, have been implicated in the pathogenesis of malignancy through loss of function (6, 7, 9, 10). Loss of p27 function might be expected to accelerate the G_1-S transition of the cell cycle because of a decreased ability to inhibit the activity of cyclin D/cdk4 or cyclin E/cdk2 complexes (22, 23). Loss of function mutations in other cell cycle regulatory proteins, such as RB and p53, have also been identified in a broad range of malignancies (24, 25). *KIP1* mutations have been reported recently in non-Hodgkin's lymphoma; however, preliminary screening of other tumors has yet to reveal mutations, although acute leukemias have not been analyzed extensively (26, 27).

In this report, we describe the mutational analysis of the candidate genes *TEL* and *KIP1* in childhood ALL patients known to have LOH at this locus. In accordance with Knudson's two-hit hypothesis (28), if either *TEL* or *KIP1* is the critical tumor suppressor gene on 12p, then mutations affecting the residual allele should be identifiable in tumors in which one allele is lost through chromosomal deletion.

MATERIALS AND METHODS

Cloning *TEL* Intron/Exon Boundaries. ECORI restriction fragments from a P1 phage genomic clone containing the 3′ end of the *TEL* gene were cloned into pBluescript KS+, and the resulting subclones were screened via hybridization to *TEL* exonic oligonucleotide probes using standard methods (29). Clones containing the intron/exon boundaries for exons 4–8 were obtained in this manner and partially sequenced using a Sequenase 2.0 kit (United States Biochemical Corp.). A cosmid containing exon 3 was obtained by subcloning the YAC 964c10 into sCos, with subsequent screening of the cosmid library with an exon 3 probe. The 5′ boundary of exon 2 was obtained by partial sequencing of the Los Alamos National Laboratory cosmid

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[4] The abbreviations used are: cdk, cyclin-dependent kinase; YAC, yeast artificial chromosome; ALL, acute lymphoblastic leukemia; LOH, loss of heterozygosity; AML, acute myeloid leukemia; SSCP, single-strand conformation polymorphism; BFM, Berlin-Frankfurt-Münster; oligo(dT), oligodeoxythymidylic acid.

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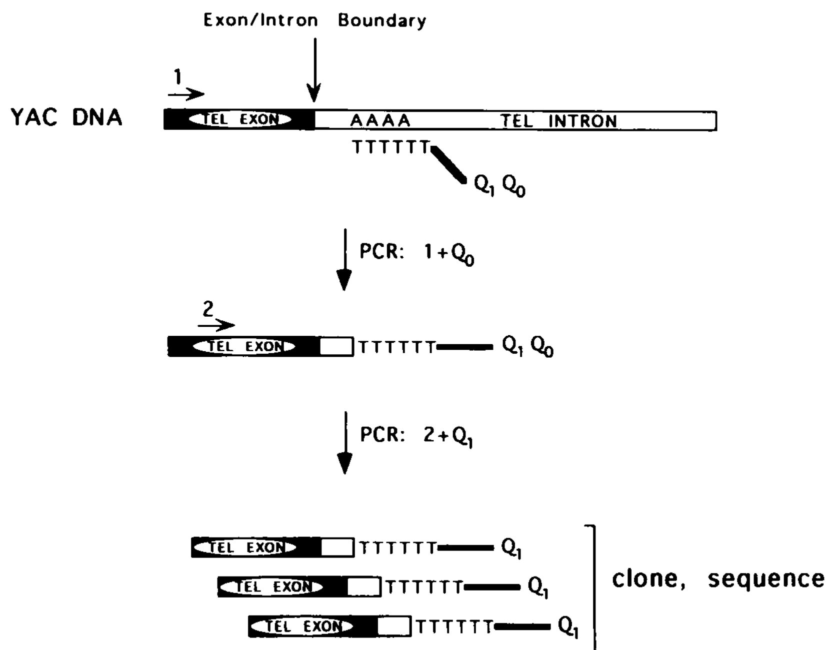

Fig. 1. Cloning exon/intron boundaries using anchored PCR. The anchored PCR strategy to obtain the 3′ boundaries of *TEL* exons 1 and 2 is shown. Genomic DNA derived from YAC 964c10 known to contain the *TEL* gene was used as a template in an extension reaction with primer Q_T, which contains oligo(dT) in contiguity with unique priming sequences Q_0 and Q_1. The oligo(dT) portion of the primer anneals to A-rich sequences within the intron. In the first round of PCR, *TEL*-specific primer 1 and primer Q_0 are used. A second round of amplification is then performed, using nested *TEL*-specific primer 2 and primer Q_1. The resultant PCR products are cloned and sequenced.

LL12NCO1N50F4 (provided by K. Montgomery), which had been shown previously to contain exon 2 (30).

To obtain the 3′ boundaries of exons 1 and 2, an anchored PCR strategy was used as illustrated in Fig. 1. Ten ng of YAC 964c10 DNA were used as a template in an extension reaction with 20 μM oligonucleotide Q_T (94°C for 3′, 45°C for 1′, and 72°C for 5′). Primer Q_T (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3′) contains oligo(dT) in contiguity with unique 5′ priming sequences as described previously (31). Primer 5′UT (625 μM; derived from the *TEL* 5′ untranslated region) and primer Q_o (125 μM; 5′-CCAGTGAGCAGAGTGACG-3′; identical to the unique priming sequences within primer Q_T) were then added, and 30 cycles of PCR were performed (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). A second round of amplification was then performed using nested primers 5′C12 and Q_1(5′-GAGGACTCGAGCTCAAGC-3′). The resultant PCR products were then cloned and sequenced using standard methods. In a similar manner, the 3′ boundary of exon 2 was obtained using the exon 2-specific primers 106 and 127 in the first and second rounds of amplification, respectively. The sequence of the oligonucleotide primers used in this study are shown in Tables 1 and 2.

Direct Sequencing. Genomic DNA was prepared from the bone marrow of patients with ALL using standard methods (32) after informed consent was obtained. DNA (20–100 ng) was used in a series of PCR reactions to amplify each of the eight *TEL* exons and both *KIP1* exons. The primer combinations used in these experiments are shown in Table 3. Forty cycles of PCR (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min) were performed using an M. J. Research, Inc. (Watertown, MA) thermal cycler as described previously (33). For some exons, a second round of amplification was necessary to obtain adequate DNA for direct sequencing.

PCR products were used directly in a sequencing reaction using a Sequenase PCR Product Sequencing Kit (United States Biochemical Corp.) according to the manufacturer's directions. The sequencing primers used in this analysis are shown in Table 3. In general, we were most successful using *TEL* or *KIP1* sequencing primers internal to those used in the PCR reaction.

SSCP Analysis. The PCR-SSCP analysis was performed using a modification of a previously reported method (34). Each 20-μl PCR reaction contained 25 ng of DNA, 10 pmol of each of the primers, 2 nmol of each of the four deoxyribonucleotide triphosphates, 0.5 units *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, MD), 3 μCi [α-^{32}P]dCTP, and 1.5 mM $MgCl_2$. The oligonucleotide primers used for each exon were as follows: exon 1, 5′UT and 0RA; exon 2, 0F and 1RB.2; exon 3, 191F and 2R; exon 4, 2FA and 3R.2; 5′-half of exon 5 (5a), 3FB and 750R; 3′-half of exon 5 (5b), 701 and 4RB; exon 6, 4FA and 5R; exon 7, 5FA and 6R.2; and exon 8, 6FA and 1404R. The

Table 1 *TEL oligonucleotide primer sequences*[a]

Exon	Primer	Primer sequence
1	5′UT	GTGGAGCCTTTCTGGGTTGG
	5′C12	TGATCTCTCTCGCTGTGAGA
	0R	GCTGCATCTCCTTCCCTCGC
	0RA	CCCCAAATTAATTTCCTGCG
	0RB	AACAGCCTGCTCTGAGGAGC
2	0F	CCCTCTTCCTGCCCTTAT
	106	CGGAATTCTGCTTCCTCGACGCCACTTC
	127	CGGAATTCTGTTCCAGTGCCTCGAGCGC
	1R.2	AACATGTCATAGTCAGGGAA
	1RB.2	GAGAAACAAGGAACAGGAAC
3	1FA.2	GATGTGGAGATTCATTTTGTTAAC
	191F	TGCAGCCAATTTACTGGAGC
	200	TTTACTGGAGCAGGGATGAC
	1029R	GCGAAAGTCCTCTTTGGTCAG
	2R	CTAAGAGACTTTCCAAGTTG
	2R.2	CTGTTGGGATTTCTTGGGCC
4	2FA	AGGTGCGCTCCAATTGTATC
	2FB	CTCCGTAGATCGTTCTTGGA
	3R.2	ATCGTGAGCTTCAGGGAACC
5	3FA	AGGGAGTTTCCTGTCCTGCC
	3FB	GGTCTGTGATTGTCTTTCCC
	541	CCTCCCACCATTGAACTGTT
	701	AGAACAACCACCAGGAGTCC
	750R	ATTATTCTCCATGGGAGACA
	4R	CTAATTGGGAATGGTGCGAG
	4RB	TGGCTGCAAAGATCACAGGG
6	4FA	ATTCTTCTGGTTAGTGCC
	4FB	CAACAAGAAACATTTTGAACAGTG
	1150R	TTTCCCCACAGTCGAGCCAG
	5R	AAGACAGACGATTATCCTCC
7	5FA	CCGCAGTGCCTTTTCTGAGG
	5FA.2	ACAGGCAGCAGCTGAAGAGC
	6R	AGAGATCTTAACAGTCTCCC
	6R.2	TTGGGTATCAGATAGGCAGG
8	6FA	CCAGCTGTATAAGATGATGG
	6FB	TCTTTATATACAGGCTAGAGTTCA
	1404R	TGAGGTGGACTGTTGGTTCC

[a] *TEL* oligonucleotide primer sequences used for PCR and direct sequencing are shown. Sequences are in the 5′ to 3′ orientation. See Table 3 for primer combinations used.

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Table 2 *KIP1 oligonucleotide primer sequences*[a]

Exon	Primer	Sequence
1	KIP5'F	TCGTGCAGACCCGGGAG
	KIP156F	GGAAGAGGCGAGCCAGCG
	KIP309F	GCAGGAGAGCCAGGAT
	KIP255R	GGGCAAGCTGCCCTTCTCC
	KIP394R	CCACCAAATGCGTGTCCT
	KIPRIIR	CGGAATTCTGTCTGAAGGCCCCAAACAC
2	KIPIF	GCCAACTTCTGCCAGCCATT
	KIP3'UR	CCAGTAAGATCAGGTATC
	KIP3'R	CGGAATTCTGTAATTTGCCAGCAACCA

[a] *KIP1* oligonucleotide primer sequences used for PCR and direct sequencing are shown. Primer sequences are in the 5' to 3' orientation. See Table 3 for primer combinations used.

Table 3 *Primer combinations for PCR amplification and sequencing of TEL and KIP1*[a]

Exon	PCR		Sequencing
	Sense	Antisense	
TEL			
1	5'UT	0R	0RB
	5'UT[b]	0R[b]	
2	0F	1R.2	1R.2
			1RB.2
3	1FA.2	2R.2	200
	1FA.2[b]	2R[b]	1029R
			2R
4	2FA	3R.2	2FB
5	3FA	4R	541
	3FB[b]	4RB[b]	701
			750R
6	4FA	5R	4FB
			1150R
7	5FA.2	6R.2	6R
	5FA[b]	6R[b]	
8	6FA	1404R	6FB
KIP1			
1	KIP5'F	KIPRIIR	KIP156F
			KIP255R
			KIP309F
			KIP394R
			KIPRIIR
2	KIPIF	KIP3'R	KIPIF
			KIP3'UR

[a] Oligonucletide primer combinations used for PCR amplification and direct sequencing of *TEL* and *KIP1*. Sense and antisense primers used for PCR are shown.
[b] Primers used for a second round of PCR amplification, which was required for some exons.

sequence of the oligonucleotide primers is shown in Table 1. Thirty-five cycles of denaturation for 40 s at 94°C, annealing for 30 s at 60°C (58°C for exons 3, 5a, 6, and 8), and extension for 40 s at 72°C were performed in a programmable thermal cycler (M. J. Research, Inc.). After amplification, PCR samples were diluted 10-fold in loading buffer containing 20 mM EDTA, 96% formamide, and 0.05% each of bromophenol blue and xylene cyanol. The products were heated to 95°C for 5 min and chilled on ice. One μl of the dilutions was then electrophoresed at 450 V for 20 h at room temperature through HydroLink mutation detection electrophoresis gels (J. T. Baker, Inc., Phillipsburg, NJ). The gel was then dried and subjected to autoradiography using Kodak XAR film at −80°C. Samples with altered migration by SSCP were reamplified as described above, gel purified by electroelution, and the double-stranded DNA then sequenced directly using the double-stranded DNA Cycle Sequencing System (GIBCO-BRL). All amplified exons were sequenced in both the sense and antisense directions.

RESULTS

Exon Organization of the *TEL* Gene. To facilitate the mutational analysis of the *TEL* gene from genomic DNA, the intron-exon boundaries were first elucidated. As detailed in "Materials and Methods," several strategies were used to clone intronic sequence flanking each of the *TEL* coding exons. Two of the intron/exon boundaries were cloned using a novel anchored PCR approach. We have previously used anchored PCR to identify unknown fusion partners for chimeric RNA transcripts in patients with chromosomal translocations disrupting the *TEL* gene (14, 15, 18). In the course of those experiments, we noted that the oligo(dT)-containing primer intended to anneal to the poly(A) tail of the message would, on occasion, anneal promiscuously to stretches of A-rich sequence within unspliced or incompletely spliced RNA. This resulted in the unexpected PCR selection of small fragments that included *TEL* exonic and intronic sequences. Therefore, we attempted to exploit this phenomenon to clone normal *TEL* intron-exon boundaries by PCR using YACs containing the *TEL* gene as starting material. The PCR product obtained using a *TEL* exonic primer in conjunction with the oligo(dT)-containing primer Q_T was cloned and sequenced, revealing *TEL* coding sequence followed by a splice acceptor site and intronic sequence. This strategy is illustrated in Fig. 1. Such anchored PCR experiments, combined with sequence analysis of *TEL* cosmid and P1 clones, established the exon organization of the *TEL* gene.

As shown in Fig. 2, the 1356-nucleotide *TEL* coding sequence is distributed over eight exons. It is curious that exon 5 is 546 nucleotides in length, unusually long for an internal mammalian exon (35). The amino terminal helix-loop-helix domain is encoded by exons 3 and 4, and the carboxy terminal DNA-binding domain is encoded by exons 6 and 7. The size of the introns separating the coding exons has not been determined precisely, but the work of others suggests that the *TEL* gene spans at least 150 kb (17).

Mutational Analysis of the *TEL* Gene. Direct sequence analysis was performed on the nine childhood ALL patients from the Dana-Farber Cancer Institute shown previously to have LOH at the *TEL/KIP1* locus (11). The oligonucleotide primer combinations used to amplify each of the *TEL* exons from genomic DNA are shown in Table 3. No mutations were found within the coding region or splice donor/acceptor sequences in these patients. As shown in Table 4, a silent coding polymorphism was identified in exon 3 (nucleotide 282,

Table 4 *TEL coding polymorphisms*[a]

Exon	Nucleotide	Base change[b]	Amino acid	Patient
3	282	G→A	Thr→Thr	DFCI-6,[c] DFCI-8, BFM-24, BFM-29
5	626	T→C	Leu→Pro	BFM-73
6	1099	C→A	Arg→Arg	BFM-89

[a] Coding polymorphisms detected in childhood ALL patients with *TEL* LOH.
[b] All nucleotide base changes were present in both the leukemic and normal cells of each patient.
[c] DFCI, Dana-Farber Cancer Institute patients.

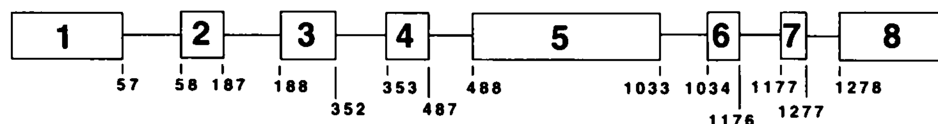

Fig. 2. Exon organization of the *TEL* gene. The eight coding *TEL* exons are shown. Numbers indicate the nucleotide positions following the numbering in GenBank accession no. U11732. The helix-loop-helix domain is encoded by exons 3 and 4, and the putative DNA binding domain is encoded by exons 6 and 7. The size of the intervening introns is not known.

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G→A) in two of the patients (patients 6 and 8 in Ref. 11). Of note, the G→A polymorphism ablates an *Afl*III restriction endonuclease site. Mutational analysis within the 5′ and 3′ noncoding regions of the *TEL* gene was not performed.

The *TEL* mutational analysis was next extended to an additional group of 24 childhood ALL patients from the BFM group of the ongoing multicenter trial ALL-BFM 90, who were shown previously to have *TEL* LOH (12). For these patients, a SSCP approach was used. In some of the patients, aberrant SSCP bands were seen both in leukemic samples and in normal tissue derived from the same patient. This result is most consistent with polymorphism but would also be seen in the case of germline mutations. To pursue this further, *TEL* exons that resulted in shifted SSCP bands were subjected to direct DNA sequence analysis. As summarized in Table 4, several nucleotide base changes were detected. Two silent coding polymorphisms were found (nucleotide 282 G→A in 2 patients; nucleotide 1099 C→A in one patient). In addition, two intronic polymophisms were detected (a one-nucleotide insertion in intron 1 in one patient, and an A→G transversion in intron 4 in two patients). Finally, a single patient (BFM-73) showed a T→C transversion at nucleotide 626, resulting in a leucine→proline amino acid change, detectable in both the leukemic and normal cells from this patient. To determine whether this nucleotide change represented a polymorphism present in the general population, 81 normal individuals were screened by SSCP using primers that flank *TEL* nucleotide 626 in exon 5. One of 81 normal individuals showed a shifted band with identical migration to that seen in patient BFM-73. This suggests that the leucine→proline substitution is more likely to be a rare polymorphism rather than a *bona fide* mutation. We conclude from these studies that mutations within the *TEL* coding sequence are uncommon.

Mutational Analysis of the *KIP1* Gene. The *KIP1* gene has been reported previously to contain two coding exons encoding a 198-amino acid polypeptide (27, 36). Direct sequencing of *KIP1* in the nine Dana-Farber Cancer Institute patients with *KIP1* LOH demonstrated no *KIP1* mutations within the coding region or splice donor/acceptor sites. A previously described coding polymorphism at codon 109 [GTC (valine)→GGC (glycine); Refs. 27, 36, and 37] was identified in two patients (patients 3 and 5 in Ref. 11). SSCP analysis of the 24 BFM patients with *KIP1* LOH is reported elsewhere and revealed no *KIP1* mutations (36).

DISCUSSION

The frequent finding of *TEL/KIP1* LOH in childhood ALL is suggestive of a tumor suppressor gene at or near this locus on chromosome band 12p13. In a classical tumor suppressor model, mutations should be present in the residual allele of a candidate tumor suppressor gene; this would lead to a complete loss of function in the affected cells. We did not detect mutations in either *TEL* or *KIP1* in the leukemic cells of childhood ALL patients with 12p LOH. These data suggest that neither *TEL* nor *KIP1* is a tumor suppressor gene.

Several caveats must be mentioned in the interpretation of these results: (*a*) we evaluated only the coding region of *TEL* and *KIP1* in this analysis. Promoter mutations, for example, might lead to decreased expression of *TEL* or *KIP1* but would not be detected in our analysis. Such noncoding loss-of-function mutations have been observed in other tumor suppressor genes such as *RB* (38). Unfortunately, RNA was not available from the patients studied; (*b*) small intragenic deletions of either candidate gene might not be detected using these methods. Because small numbers of normal cells are present in the patient samples, it is conceivable that if homozygous deletions of a small portion of the gene were present in the leukemic cells, then the only PCR products generated in these patients would be derived from normal cells. The presence of large homozygous deletions has been excluded by previous microsatellite mapping (11, 12); (*c*) the interpretation of these data assumes that both alleles of a tumor suppressor gene must be affected to reveal a phenotype. To our knowledge, there are no reported examples of hemizygous deletion of tumor suppressor genes being implicated in the pathogenesis of human cancer. However, a decrease in gene dosage could potentially result in diminished protein expression. A slight diminution in the expression of a cdk inhibitor, for example, might affect cell cycle progression, because the activity of these proteins is in part determined by their level of expression relative to other regulators of the G_1-S transition (22, 23); and (*d*) structural rearrangements of either *TEL* or *KIP1* might be missed by our analysis. That is, translocations occurring within the introns of either gene would not be detected by this exon-by-exon analysis. Whether *TEL* or *KIP1* rearrangements accompany LOH frequently is under investigation.

The precise genomic distance that constitutes the critically deleted region of 12p in childhood ALL is not known, but the region probably spans several megabases of DNA (11). In addition to *TEL* and *KIP1*, therefore, there are undoubtedly additional genes within this region that are potential candidate tumor suppressor genes. The refinement of the 12p physical map, the characterization of new transcription units in the region, and the analysis of additional patients should lead to the identification of the 12p tumor suppressor, a gene that is likely to be important in the pathogenesis of childhood ALL.

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