RAPID COMMUNICATION

Multiple Tumor-Suppressor Gene 1 Inactivation Is the Most Frequent Genetic Alteration in T-Cell Acute Lymphoblastic Leukemia

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No constant genetic alteration has yet been unravelled in T-cell acute lymphoblastic leukemia (T-ALL), and, to date, the most frequent alteration, the SIL-TAL1 deletion, is found in approximately 20% of cases. Recently, two genes have been identified, the multiple tumor-suppressor gene 1 (MTSI) and multiple tumor-suppressor gene 2 (MTS2), whose products inhibit cell cycle progression. A characterization of the MTS locus organization allowed to determine the incidence of MTS1 and MTS2 inactivation in T-ALL. MTS1 and MTS2 configurations were determined by Southern blotting using 8 probes in 59 patients with T-ALL (40 children and 19 adults). Biallelic MTS1 inactivation by deletions and/or rearrangements was observed in 45 cases (76%). Monoallelic alterations were found in 6 cases (10%). The second MTS1 allele was studied in the 4 cases with biallelic material. A point mutation was found in 2 cases. The lack of MTS1 mRNA expression was observed by Northern blot analysis in a third case. A normal single-strand conformation polymorphism pattern of MTS1 exons 1α and 2 was found and MTS1 RNA was detected in the fourth case, but a rearrangement occurring 5′ to MTS1 exon 1 α deleting MTS1 exon 1β was documented. One case presented a complex rearrangement. Germline configuration for MTS1 and MTS2 was found in only 7 cases. The localization of the 17 breakpoints occurring in the MTS locus were determined. Ten of them (59%) are clustered in a 6-kb region located 5 kb downstream to the newly identified MTS1 exon 1β. No rearrangement disrupting MTS2 was detected and more rearrangements spared MTS2 than MTS1 (P < .01). MTS1 but not MTS2 RNA was detected by Northern blotting in the human thymus. These data strongly suggest that MTS1 is the functional target of rearrangements in T-ALL. MTS1 inactivation, observed in at least 80% of T-ALL, is the most consistent genetic defect found in this disease to date.

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THE INACTIVATION OF the multiple tumor-suppressor gene 1 (MTSI/p16INKA, CDKN2) and multiple tumor-suppressor gene 2 (MTS2/p15INKA) has frequently been shown in a large variety of human tumor cell lines. Both genes are located at chromosome 9p21, a region that has been shown by cytogenetical analysis to be deleted in approximately 10% of T-cell acute lymphoblastic leukemia (T-ALL). MTS1 contains four exons that generate two types of transcripts (here named MTS1α and MTS1β) initiated from distinct promoters. MTS1α includes exon 1α, exon 2, and exon 3 and encodes the p16INKA protein. MTS1β (exon 1β, exon 2, and exon 3) encodes a 132 amino acid polypeptide (Fp16 or p19ARF), which arises in major part from an alternative reading frame of the MTS1 gene and that is unrelated to p16INKA. MTS2 contains two exons, which encode the p15INKA protein. MTS2 exon 2 displays extensive sequence homology with MTS1 exon 2. p16INKA and p15INKA inhibit the activity of cyclin-dependent kinases (cdk) 4 and 6, which control progression through the G1 phase of the cell cycle. Ectopic expression of p19ARF in rodent fibroblasts induces G1 and G2 phase arrest. Studies based on primary tumor cells have shown that inactivation of MTS1 is predominantly found in a restricted number of tumor cell types (for a review see Hiramatsu et al), including acute lymphoblastic leukemia.

Since the initial studies describing the high incidence of MTS1 homozygous deletions in human ALL, a number of studies have confirmed that these events represent an important marker of this disease. The notion that MTS1 inactivation is more frequent in T-ALL than in B-lineage ALL found in our initial series has been subsequently confirmed by some but not all studies. Moreover, it was not clear from the available literature how frequently MTS1 was inactivated on the second allele in T-ALL with hemizygous MTS1 deletion or intragenic rearrangement.

The present study was therefore performed to establish a detailed restriction map of the MTS locus allowing a precise description of the structural events involving MTS genes, to determine the frequency of MTS1 homozygous deletions in a large single-center series of T-ALLs, to localize the chromosomal breakpoints that occur in the MTS locus in T-ALL, and to analyze T-ALL cases with hemizygous deletions or rearrangements.

Our results establish that MTS1 inactivation is the most consistent genetic defect found in T-ALL to date.

MATERIALS AND METHODS

Patients and cells. This single-center study is based on a cohort of patients referred to St Louis Hospital (Paris, France). From January 1985 to March 1995, the diagnosis of ALL was made in 655 patients, including 488 new cases. These 488 patients include 330 children (<16 years of age) and 158 adults. In more than 95%, bone marrow and/or peripheral blood samples were cryopreserved. Diagnosis of ALL was made according to standard procedures.

Immunophenotyping was performed by a combined approach of cytfluorometry and immunocytophony, as described. Sixty-six cases of T-ALL were diagnosed in children (20% of all pediatric ALL) and had the following characteristics: median age, 9 years; cases with white blood cell count (WBC) greater than 30 × 10^9/L, 82%; sex ratio (M/F), 4.7. Thirty-nine T-ALL cases were diagnosed in

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adults (25% of all adult cases) with the following characteristics: median age, 30 years; cases with WBC greater than $30 \times 10^9/L$, 73%; sex ratio (MF), 2.3.

Cryopreserved cells from 59 patients (40 children and 19 adults) with T-ALL were studied (Table 1). This series includes 43 patients randomly selected from our cell collection (January 1985 to December 1991) and 16 patients with T-ALL consecutively referred for diagnosis and/or therapy at our institution (from January 1992 to December 1994). Forty-seven cases were studied at presentation, 7 cases at relapse, and 5 cases both at presentation and at relapse.

The T-ALL-derived Molt 4 cell line, the B-lineage ALL-derived RS4.11 cell line (American Tissue and Cell Collection CRL 1873), and the epithelial-derived Hela cell line were used in some experiments.

**DNA probes.** *MTSl* exon 2 and exon 1 probes have previously been described. Other probes were designed for the work reported here. A *MTSf* exon 3 probe was obtained by the polymerase chain reaction (PCR) on genomic DNA using the following primers: 5'-CCGGAAATTCGACATCCCCGGATGAAA-3' and 5'-CCGGAAATTCGACATCCCCGGATGAAA-3'. A 290-bp DNA fragment from the *MTSf* exon 1 was amplified from complementary DNA obtained after reverse transcription of the Hela total RNA using the CDK22 (sense) and CDK23 (antisense) primers (CDK22, CTTGAGCTCGGCTGC4; CDK23, ATTCGGACGCCCCAGCC). C5.3, 2.3, and 2.7 sequence-tagged sites (STS) were amplified using previously described primers. PCR products were cloned in the plasmid PCR-Script SK(+) (Stratagene, La Jolla, CA) and used. The *MTSf* exon 3 probe was used to detect specific rearrangements. RNA integrity and loading were evaluated by dot-blotting on a Hybond N+ membrane (Amersham International plc, Buckinghamshire, UK) and successively hybridized with 32P-labeled oligonucleotides recognizing wild-type: (GCGATGCCTGAGGCCGTC) or mutated (GCGATGCCTGAGGCCGTC) alleles.

**Restriction map of the MTS locus (Fig 1A).** The map of the *MTS* locus (extending over approximately 55 kb) proposed by Kamb et al was corrected and greatly extended using various strategies. P1 clones spanning the locus were obtained by screening the Du Pont Merck Pharmaceutical Co Human Foreskin Fibroblast PL1 library 1 (Genome Systems, St Louis, MO) by the PCR using the following *MTSf*-specific primers: 5'-GGAAAATTGGAACTGGGGCCGTC and 5'-CCGGAAATTCGACATCCCCGGATGAAA-3'. MTSf-exon 3, C5.3, 2.7, and MTS2-exon 1 probes recognize unique sequences in genomic DNA. The MTSf-exon 2 probe recognizes both MTSf-exon 2 and MTS2-exon 2 sequences as shown. The 2.3 probe recognizes weakly repeated sequences in genomic DNA but a unique sequence in the *MTSf* locus (unpublished observations).

**Southern blot analysis of *MTS* loci.** Southern blots were prepared as described from polyA+-enriched RNA extracted from patient samples. In addition, a commercially available blot prepared from diverse human tissues (Clontech Lab Inc, Palo Alto, CA) was used. The *MTSf*-exon 3 probe was used to detect *MTSf* RNA. This probe detects all the *MTSf* species in the Hela cell line (unpublished observation). The *MTSf*-exon 1 probe was used to detect *MTS2* expression. This probe does not cross-hybridize with *MTSf* sequences. RNA integrity and loading were evaluated on ethidium bromide-stained gels and on stripped blots hybridized to a *ACTIN* or NADPH probe.

**RESULTS**

**Configuration of the *MTS* loci in T-ALL.** The configuration of the *MTS* locus was studied by Southern blotting analysis. Data were analyzed by comparison with the restriction map shown in Fig 1A. A representative experiment is shown in Fig 2 and all data are summarized in Table 1 and Fig 1B. All but 1 case (with a complex and not fully characterized rearrangement) were classified according to the configuration of *MTSl* and *MTS2*.

<table>
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<th>No.</th>
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<th>Cases With WBC Count &gt;30 \times 10^9/L</th>
<th>Male/Female Ratio</th>
<th>L1/L2 Ratio</th>
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<td>Total</td>
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**Abbreviations:** L1 and L2, cytological types of ALL according to the French-American-British classification. * For definition, see main text and Fig 1. In one adult case, the configuration was not determined.
A MTSl MTS2

Fig 1. Restriction map of the MTS locus and schematic representation of the configuration of this locus in T-ALL cells. (A) Restriction map of the MTS locus. Transcriptional orientations of MTS1 and MTS2 are shown by an arrow. B, BamHI; H, HindIII; K, Kpn I; N, Nsi I; R, EcoRI; S, SalI; SII, Sac II. A polymorphic BamHI site is shown as B-

A MTSl MTS2

configuration in 59 T-ALL samples and in the Molt4 cell line. Both chromosomes are schematized in each configuration type. A dashed line chromosome (type I11). Thus, monoallelic alterations of at least one of the three exons (exon 10, 2, and 3) that code for p16INK4a were found in 6 cases (10%).

In 7 cases, a germline configuration was detected for both copies of MTS1 and MTS2 (type IV configuration). Incidence of the different types of configuration is similar in the pediatric (40 cases) and adult (19 cases) series. Type I configuration was observed in 30 of 40 cases in children (75%) and 15 of 19 cases in adults (79%; Table 1).

Comparison of the random series (43 cases) with the consecutive series (16 cases) did not detect a statistically significant difference, although the proportion of type I configuration appeared to be slightly higher in the random series than in the consecutive series (35 of 43 cases and 10 of 16 cases, respectively; P = .18, not significant, by the χ2 test).

Age, cytological (French-American-British [FAB]) subtypes, and immunologic markers did not differ significantly in the three groups of patients (data not shown).

In the 5 cases with samples available at presentation and relapse, 4 were unmodified and 1 was a type II at presentation (T39p) and Ib at relapse (T39r).

Localization of the breakpoints in the MTS locus. In 34 samples with a biallelic MTS deletion, deletion analysis showed that the breakpoints fell outside the MTS locus (Fig 1).

In 15 samples with partial deletion of the MTS locus, plus the Molt4 cell line, the precise localization of 17 breakpoints (2 breakpoints in the sample T106 fell within the MTS locus) was obtained by Southern analysis (Fig 1B). One breakpoint occurred between exon 1α and exon 2 (T98), another between exon 2 and exon 3 (T81), and two breakpoints were localized immediately 5' to MTS1 exon 1α (T39p and T106). The 13 other breakpoints were localized between MTS1 exon 1α and MTS2 exon 1β, with 10 (59% of all breakpoints) being located between STS 2.3 and STS 2.7. No breakpoint disrupting MTS2 was observed.

Rearrangements were analyzed with respect to the alteration of the coding potentials of MTS1 and MTS2. Two rearrangements sparing MTS1 exon 1α, MTS1 exon 2, and MTS1 exon 3 (which collectively encode the p16INK4a protein from RNA initiated immediately 5' to exon 1α) were observed (T39p and T106), whereas 14 rearrangements sparing all MTS2 exons were detected (Fig 1B, P < .05 by the χ2 test). The contrast is even greater if the MTS1 exon 1β is
included in the analysis, because no rearrangement spares all MTS1 exons (Fig 1B).

SCCP and Northern blot analyses of T-ALLs with hemizygous alterations of MTS1. To detect mutations that may inactivate the second MTS1 allele, SCCP analysis of MTS1 exon 1 and exon 2 was performed in 4 cases with a type II configuration (T81, T94, T99r, and T109) and in 1 case with a type III configuration (T39p; see Fig 1B for the configuration of the MTS locus in these cases). Five cases with a normal (type IV) configuration were also studied as controls.

A normal MTS1 exon 1 SCCP pattern was found in all 10 cases. An abnormal and identical MTS1 exon 2 pattern was detected in 2 cases with a type II configuration (T99r and T109; Fig 3A). The MTS1 exon 2 nucleotide sequence was determined in these 2 cases and showed an identical mutation leading to a stop codon located at the beginning of the fourth ankyrin domain of p16INK4A (Fig 3B) and to a Gly124 to Arg124 substitution in the predicted aminoacid sequence of p19ARF. Several controls were performed to be sure that the same mutation was present in the tumor cells of the 2 patients: (1) molecular HLA DQ typing showed that the two DNA samples used came from 2 different patients (courtesy of Dr P. Loiseau, Laboratory of Histocompatibility, St Louis Hospital, Paris, France) and (2) SCCP and hybridization experiments were performed twice with consistent results. Results shown in Fig 3A and C provide good evidence against PCR contamination, because no germline sequences were detected in these cases that both display one MTS1 exon 2 copy on Southern blots (data not shown).

A normal SCCP pattern was thus found in cases T81, T94 (type II), and T39p (type III). Northern blot analysis of MTS1 RNA expression was performed on polyA+-enriched RNA in cases T81 and T39p (in which material was available) to search for transcriptional activity of the intact MTS1 allele. No significant expression was found in the T81 sample, whereas high levels of expression were detected in the T39p case (Fig 4).

MTS1 and MTS2 mRNA expression in the human thymus (Fig 5). The high frequency of MTS gene inactivation in T-ALL suggests that MTS1 and MTS2 may play an important role in thymocytes. MTS1 and MTS2 mRNA expression was studied by Northern blotting of thymic mRNA and various other human tissues. Clear expression was found in the thymus and spleen and strong expression of a small-sized MTS1 mRNA was found in the tests. A very faint signal was detected in peripheral blood lymphocytes, colon, small intestine, ovary, and prostate. By contrast, no MTS2 signal was found in the thymus. Faint MTS2 RNA signals were detected in other tissues and a small-sized RNA was found in the tests.

**DISCUSSION**

In this study, we have reported a Southern blot analysis of the MTS locus in 59 T-ALLs. An important finding of this work was the demonstration that both copies of MTS1 have been inactivated by deletions and/or rearrangements in 76% of cases (45 of 59 cases). The frequency of MTS1 homozygous deletions in T-ALL varies from 0% (0 of 11) to 83% (20 of 24) in previous studies. (Taking into account the present study, the average frequency of homozygous deletions is 54% [106 of 196 cases].) Reasons for large variations in the estimation of the MTS1 deletion
frequency in T-ALL are unclear. Identification of homozygous deletions in cases with admixture of neoplastic and normal cells is difficult and may have led to an underestimation of homozygous deletions in some studies. We have included dilution experiments in all our Southern blot studies and carefully compared the intensity of residual bands with the percentage of neoplastic cells. As shown in this study and others,\textsuperscript{18,20-22} MTS1 homozygous deletions with persistence of at least one MTS2 allele are not uncommon. To identify the restriction fragments, we have established a detailed restriction map of the locus and used a large panel of probes. With the exception of 1 case, we were able to precisely define the MTS configuration in all cases and are confident with our evaluation, which has been confirmed in selected cases by Western blot analyses of MTS1/\textsuperscript{p16\(^{INK4A}\)} protein expression (23 T-ALL cases tested; unpublished data).

Patient selection is another point that may explain variations among the results reported in the literature. Characteristics of the patients referred to our laboratory (see Materials and Methods) are similar to those of other large clinical series.\textsuperscript{28,29} The high frequency of inactivation is not only found in samples randomly selected from our cell collection, but also in consecutive cases. Because we have found a similar percentage of MTS1 homozygous deletions in adult and pediatric series (79\% and 75\%, respectively), age is unlikely to be a critical factor explaining differences among studies.

![Fig 3. Mutations in MTS1 exon2 in two cases with a monoallelic MTS1 structural alteration. (A) SSCP analysis. Arrow indicates the abnormal band found in T109 and T99r cases. The pattern observed in the other cases is normal. T94, T99r, and T109 are type II cases; T39p is a type III case; and T21, T96, and T104 are type IV cases. (B) Partial nucleotide sequence of MTS1 exon 2: 1, wild sequence; 2, mutated sequence in T109 and T99r. The mutated nucleotide is underlined leading to an in-frame stop codon (W110...stop 110). (C) PCR fragments corresponding to MTS1 exon 2 have been hybridized to a probe corresponding to the wild sequence (1) and the mutated sequence (2). Duplicate experiments are shown. PBL, peripheral leukocytes from an healthy donor; C, no DNA.]

![Fig 4. Northern blot analysis of MTS1 expression in selected T-ALL samples. Two different experiments are shown. The Hela and RS4;11 cell lines have been used as positive and negative controls, respectively. The size of transcripts are indicated by arrows. Hela RNA has been used undiluted (1) or diluted at 1/4 into water (1/4). Blots have been successively hybridized to a MTS1 exon3 (upper panel) or GAPDH (lower panel) probes. For the description of T39p and T81 cases, see main text.]

![Fig 5. Northern blot analysis of MTS1 and MTS2 mRNA expression in thymus and various other human tissues. Blots have been successively hybridized to a MTS1 exon 3, MTS2 exon 1, and \(\beta ACTIN\) probes.]
In addition to this report, two recent studies have analyzed the frequency of MTS1 homozygous deletions in series including more than 20 patients. A 39% incidence (22 of 56 cases) was found in the series from Ohnishi et al based on Japanese patients. In the work from Takouchi et al based on patients from the Multicenter ALL BFM 86 and 90 trials on childhood ALL of the German Berlin Frankfurter Munster (BFM) group, an incidence similar to that we have found was observed (17 of 22 cases [77%]). Whatever the actual figure, MTS1 inactivation is clearly the most prevalent molecular abnormality described to date in T-ALL, with the S1L-TAL1 deletion occurring in less than 20% of cases (10 of 59 cases in this series [17%]; unpublished data).

This work is the first attempt to determine whether chromosomal breakpoints occur at random or not within the MTS locus. Seventeen breakpoints (16 from T-ALL samples and 1 from the Molt4 cell line) have been localized. Interestingly, 10 of them are clustered in a 6-kb region, suggesting the presence in this region of sequences promoting the recombination. All breakpoints are located downstream of a newly identified MTS1 exon 1β-4 and upstream to MTS1 exon 3 and lead therefore to the disruption of the gene.

Although deletions and rearrangements are the most prevalent events inactivating MTS1 in T-ALL, other mechanisms occur in some cases. In 6 cases of this series, a monoallelic alteration of at least one of the MTS1 exons 1α, exon 2, and exon 3 was detected. In two of these cases, a point mutation introducing a stop codon at the beginning of the fourth ankyrin domain of the p16INK4a protein was found. This is a hot spot for nonsense mutations in nonhematopoietic tumors. Interestingly, deletion of the fourth ankyrin repeat abolishes p16INK4a activity completely. To date, MTS1 point mutations occurring in T-ALL have been described in only one study in which 4 mutations either in the first MTS1 intron (1 case) or in the MTS1 exon 2 (3 cases) were found. The status of the second MTS1 allele was not defined in this study. MTS1 transcriptional inactivation is another mechanism that has been suggested to occur in primary cells from lung carcinomas. One case from our series (T81) displays this phenotype. In this case, the SSCP analysis was normal, but only a barely detectable level of MTS1 mRNA was detected. Methylation of the CpG island located upstream to MTS1 exon 1α was analyzed and a nonmethylated pattern was found (unpublished data), showing that transcriptional inactivation is not due, in this case, to the acquired hypermethylation observed in other types of tumors.

The high frequency of MTS1 inactivation in T-ALL, the finding of point mutations and transcriptional inactivation in some cases with a monoallelic deletion, and the demonstration of the antioncogenic effect of the MTS1/p16INK4a protein make this gene the probable functional target of MTS locus alterations. Recently, a new MTS1 transcript (MTS1β) has been described. It includes MTS1 exon 1β, MTS1 exon 2, and MTS1 exon 3. This transcript has been shown to be expressed in many different tissues in mouse contrasting with the more restricted expression pattern of MTS1α. Its nucleotide sequence contains two open reading frames (ORF). The first ORF would encode a protein of 105 amino acids corresponding to 2.75 of the 4 ankyrin repeats of the p16INK4a. Its start codon is flanked by a sequence that is not a good match with the consensus Kozak sequence. The second ORF encodes for a product of approximately 22 kD (p19ARF). It is interesting that the ability to express this protein has been lost in all the T-ALL cases we have analyzed in which at least one structural event has been detected in the MTS3 locus (ie, types I, II, and III). It remains therefore unclear which protein(s) of p16INK4a and p19ARF is the functional target of MTS1 locus alterations.

Although MTS1 is one probable functional target of MTS locus alterations, it remains possible that genes other than MTS1 are targeted by this process. MTS2 located approximately 30 kb 5' to the MTS1-exon 1α (this study, Fig 1A) is an obvious candidate. The product of this gene, p15INK4b, shares identical biochemical properties with p16INK4a. However, several results suggest that MTS1 is more important than MTS2 for T-ALL oncogenesis. (1) MTS2 inactivation is clearly less frequent than that of MTS1; biallelic inactivation of MTS2 has been found in only 36 cases (61%) of this series and breakpoints spared MTS2 significantly more frequently than MTS1 (P < .01, by the χ2 test). (2) No MTS2 structural alteration was found by Southern blot analysis in this study. (3) Hyperphosphorylated forms of Retinoblastoma gene protein product are found in T-ALL irrespective of the presence or absence of an intact copy of MTS2 (23 T-ALL cases tested; unpublished data). (4) MTS1 mRNA is expressed in human thymus (albeit at a low level), whereas MTS2 mRNA is undetectable. Altogether, these data are consistent with the hypothesis of the predominant role of MTS1 in T-ALL cells that probably derived from thymocytes.

In conclusion, MTS1 is inactivated in a very high percentage of T-ALL. This defect, the most consistent acquired genetic defect found so far in this disease, could play a key role in the development of this human tumor and is an obvious target for future biologic therapies.

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