Rapid Communication

Mutations in the Wilms’ Tumor Gene WT1 in Leukemias

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The tissue-specific Wilms’ tumor gene WT1 is expressed in a range of acute leukemias and hematopoietic cell lines. Using single-strand conformational polymorphism analysis, we have found mutations in the WT1 gene in 4 of 35 acute leukemias. WT1 mutations are found in 15% of cases of acute myeloid leukemia, in which they are associated with a poor response to chemotherapy. The mutations comprise small insertions in exons 1 and 7 and a nonsense mutation in exon 9. All are predicted to produce a truncated WT1 protein with absence or disruption of the zinc finger region. These are the first mutations in the WT1 gene to be described in sporadic leukemia.

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The Wilms’ tumor gene WT1 encodes a zinc-finger transcription factor and was originally isolated by positional cloning from within chromosome 11p13, a locus involved in this childhood kidney cancer. WT1 is subject to alternative splicing, affecting the 17 amino acids of exon 5 (splice I) and 3 amino acids (KTS) at the end of exon 9 (splice II), and RNA editing to produce 8 potential isoforms. Splicing affects the DNA target sequence bound and the transactivational properties of the protein. Several genes have been proposed as targets for transcriptional activation or repression by WT1, including the PDGF-A chain, CSF-1, IGF-II, and WT1 itself.

WT1 mutations have been found in about 10% of Wilms’ tumors. Whereas most follow the original two-hit model for tumor-suppressor genes, a significant minority are heterozygous for WT1 mutation and produce proteins that may have dominant or dominant-negative effects. Germline WT1 mutations may also occur, causing maldevelopment of the urogenital system and predisposition to Wilms’ tumor in 4 of 35 acute leukemias and in hematopoietic cell lines.

Leukemia also occurs as a second primary tumor in Wilms’ tumor patients and hematopoietic malignancies are more common in relatives of children with Wilms’ tumor. We have previously reported a missense mutation in the remaining WT1 allele in a secondary leukemia from a patient who had a constitutional deletion of chromosome 11p12-14. On the basis of these findings, we hypothesized that WT1 mutations might have a role in leukemia. We have screened the WT1 gene for mutations by single-strand conformational polymorphism (SSCP) analysis and direct sequencing in 36 acute leukemias and have found four cases with WT1 mutations, all predicted to produce truncated protein. We also report preliminary investigations of the role of WT1 in normal hematopoiesis by analysis of its expression in normal bone marrow and in CD34+ progenitor cells.

Materials and Methods

Patient samples. Samples were taken from 11 children (<16 years of age) and 24 adults (median age, 35 years) with acute leukemia at diagnosis (n = 30) or at relapse (n = 5); 1 patient was analyzed at both presentation and relapse. There were 11 cases of acute lymphoblastic leukemia (ALL), 20 of acute myeloid leukemia (AML), 3 biphenotypic, and 1 null. All except 2 cases were diagnosed between 1990 and 1994. Bone marrow or peripheral blood with circulating blasts was collected into preservative-free heparin. The mononuclear cell fraction was isolated using Lymphoprep and the CD34+ cell line. DNA was extracted using the salting-out method or the RNA miniprep. Normal bone marrow from volunteer donors was treated in the same way.

Hematopoietic progenitor cell isolation. CD34+ cells were isolated from umbilical cord blood. The mononuclear cell fraction was recovered from the blood using Lymphoprep and CD34+ cells were separated using the MinIMACS Progenitor Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). RNA was isolated from unseparated cells and from both the CD34+ and CD34− fractions. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as described below.

SSCP. WT1 exons 1 to 10 were analyzed by SSCP as previously described, in which the SSCP conditions were optimized for the zinc-finger exons. Only the 3’ end of exon 1 was amplified because the exon is extremely GC rich. (Exon 1: primers c550 5’-CCGCCTCAC-TCCTAGAGCGGAGAGTCCCTG-3’ and JR1 5’TCTCTAGAGCGGAGAGTCCCTG-3’, 1.5 mmol/L magnesium and 1% formamide, 65°C; exon 7: c1552 and 945, 1.5 mmol/L magnesium, 50°C; exon 9: 5 and 9 and 3 and 93, 1.5 mmol/L magnesium, 60°C, SSCP gels were run at 40 W for 5 to 6 hours at 4°C.

Sequencing. Samples with altered SSCP bands were sequenced. Genomic PCR products from 100 μL reactions were purified from low melting point agarose using Wizard PCR Prep (Promega, Madison, WI). Sequencing was performed using Sequenase (Amersham, Little Chalfont, UK) and a modification of the “quick chill” method using the PCR oligos as primers. For the primer, template

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annealing step, 6.75 μL PCR product, 1 μL (2.5 pmol) primer, 1 μL (200 ng) alkaline denatured salmon testis DNA, and 1.25 μL dimethylsulfoxide (DMSO) were heated to 95°C for 3 minutes and snap-cooled on dry ice. The labeling reaction mix contained 1 μL MnSO₄ buffer, 1 μL 0.1 mM L-DTT, 2 μL diluted labeling mix, 1 μL (10 μCi) [α-³²P]dATP, and 2 μL diluted Sequenase per sample. Termination mixes were made to 10% DMSO and 2.5 μL was added to each tube (ACTG) in the usual way and placed in a microcentrifuge. The annealed DNA tubes were spun for 10 seconds to thaw and 7 μL of labeling mix was added. Aliquots of 3.5 μL were immediately pipetted onto the sides of the termination tubes and spun to mix (10 seconds). Tubes were transferred to a 37°C heating block and at 5 minutes were placed on ice, followed by the addition of 4 μL of stop solution. Two samples were processed simultaneously and increased throughput of samples was achieved by staggering the reactions. Products of sequencing reactions were run on standard gels and exposed to film for 1 to 10 days. RT-PCR products were sequenced in the same way.

**Subcloning.** Samples with insertions of several basepairs were subcloned and sequenced to confirm the results obtained from the direct sequencing. Purified PCR products were subcloned into a TA vector (pGEM-T Vector; Promega) and transfected into Escherichia coli JM 109 according to the manufacturer’s instructions. Sequencing of plasmid subclones was the same as for PCR products except that they were alkali denatured first.

**RNA analysis.** Northern blots were hybridized with a radioactively labeled 1.8-kb EcoR1 fragment of WT33 using standard methods.

For RT-PCR, 1 μg of RNA was reverse transcribed using Superscript (GIBCO BRL, Gaithersburg, MD) and 10 pmol of random hexamer primers according to the manufacturer’s instructions. A total of 0.5 μL of the 20 μL reaction was used for PCR. Amplification of a 602/611-bp PCR product was performed in 25 μL under standard conditions using 1.75 mM MgCl₂ and annealing at 59°C, using hexamer primers according to the manufacturer’s instructions. A 234-bp p-actin RT-PCR product was used as a control for the integrity of the cDNA and for the presence of DNA in the sample, because the primers will amplify a 329-bp product from RNA depending on the presence or absence of splice I. The two fragments are not seen as separate bands on a 1.5% gel. The cell line CCRF-CEM was used as a positive control for WT1 expression. A 234-bp β-actin PCR product was used as a control for the integrity of the cDNA and for the presence of DNA in the sample, because the primers will amplify a 329-bp product from DNA. Two microtiter of the actin product was mixed with 10 to 25 μL of the WT1 product and run on 1.5% agarose gel, alkaline blotted onto Zeta-Probe GT nylon membrane (BioRad, Hercules, CA), and hybridized with [³²P]dCTP–labeled WT1 cDNA probe and actin PCR product in a ratio of 10:1. RT-PCR products from the patients with mutations were purified and sequenced as above.

Poly A + RNA was isolated from total RNA from sample 58 using Oligotex–dT (Qiagen, Hilden, Germany) and amplified as above using primers B1 (in exon 1) and B908 5’-CACAGTCTTTGAACTGACAC 3’ (in exon 8). These primers amplify 827 bp or 776 bp from RNA depending on the presence or absence of splice I. The two fragments are not seen as separate bands on a 1.5% gel. The purified product was subcloned and sequenced as described above.

**Clinical details.** Case 1 (58) was a 33-year-old man with acute myeloblastic leukemia without maturation (French-American-British [FAB] type M1). Cytochemistry and immunophenotyping were compatible with this classification, although B-lymphoid markers were also detected. Dual-labeling procedures were not performed. Cytogenetic analysis did not show any clonal abnormality. He was treated with leukapheresis to achieve cytoreduction followed by combination chemotherapy with high-dose cytosine arabinoside, etoposide, and idarubicin to which he achieved a partial response. He proceeded to receive a mismatched bone marrow transplant, after which he was never clearly in remission. He died 6 months posttransplantation from bronchopneumonia.

Case 2 (132) was a 25-year-old woman who presented in 1986 with a morphologic diagnosis of ALL (FAB L2). Cytochemistry and immunophenotype (myeloid and T lymphoid) showed biphenotypic leukemia. Again, dual-labeling studies were not performed. Cytogenetics showed a hyperdiploid clone. After a brief partial response to ALL induction chemotherapy, treatment was changed to high-dose cytosine and etoposide, which eradicated circulating blasts. She died of infection during the pancytopenic phase.

Case 3 (232M) was an 18-year-old man who presented with typical features of acute promyelocytic leukemia with a severe coagulopathy. Immunophenotype was typical of AML(M3). Cytogenetic analysis was unsuccessful. He was treated immediately with all-trans retinoic acid followed by chemotherapy but died of intracerebral bleeding before any therapeutic response could be assessed.

Case 4 (216) was a 22-year-old woman with acute myeloblastic leukemia with maturation (FAB M2). Cytochemistry and immunophenotyping were compatible with this classification. Cytogenetic analysis did not show any clonal abnormality. Her leukemia was refractory to treatment with high-dose cytosine, etoposide, and idarubicin. There was a temporary response to the addition of cyclosporin as a drug-resistance modifier, but circulating blasts reappeared rapidly on count recovery. She underwent high-dose therapy but died of infection before response could be assessed.

**RESULTS**

**SSCP and sequence analysis.** All 10 exons of WT1 were analyzed by SSCP in 36 leukemic samples. Altered bands were seen in a number of samples in several exons. Five of these were shown to be mutations, with the remainder representing polymorphisms.

Altered bands were seen in three samples in exon 1. Two of these had a previously reported C to T polymorphism in codon Asn 130. The other sample (58) was found to have an insertion of a single A after codon Ser 121, causing a frameshift and termination codon after eight novel amino acids (Fig 1B and 2A). This is predicted to result in a protein of only 129 amino acids.

Exon 7 had a complex band pattern (Fig 3A) because there is a polymorphism at Arg 301 and one in the 3’ intron. Three samples were shown to have mutations. 132 has a heterozygous insertion of 5 bp, AAGTC, after codon Val 300, causing a frameshift and termination codon after eight novel amino acids (Fig 2A and 3B). 216 was found to have a heterozygous 4-bp insertion 3’ of the polymorphic site. The sequence GCCG or GGCC was inserted within codon Arg 302 (Fig 3C). This causes a frameshift and 14 novel amino acids are followed by a stop codon (Fig 2A). 58 has a heterozygous insertion of TCGG after the first base of codon Ala 314, causing a frameshift and the introduction of a termination codon after three novel amino acids (Figs 2A and 3D). The TCGG insertion is a direct repeat of the four preceding nucleotides. All three of these mutations would be predicted to cause the loss of the entire zinc finger region.

One sample (232M) had an altered band pattern in exon 9 (zinc finger 3; Fig 4A) and had a heterozygous nonsense mutation at codon Arg 390 (CGA to TGA), truncating the WT1 protein shortly after the second zinc finger (Fig 4B). This mutation has previously been reported in two Wilms’ tumor patients, in one of whom it was present as a germ-line mutation.

To determine whether the two mutations in sample 58 are on separate alleles, RT-PCR was attempted. However, only fragmented RNA could be obtained for this sample. A very
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small quantity of poly A+ RNA was isolated from a large amount of total RNA and successfully amplified from exon 1 to exon 8 of WT1. Of the seven subclones obtained, all had the exon 1 insertion of a single A but none had the exon 7 insertion (Fig 5A). This may be by chance or might suggest that only this allele is expressed. However, it does show that the two mutations are on different alleles, so this patient had no normal WT1 in his leukemic cells.

No altered bands were seen in exons 2, 4, 5, 6, or 10 in any of the 36 leukemic samples and 3 cell lines analyzed. Exon 8 consistently showed altered bands in some samples, but no sequence changes were found despite repeated sequencing (data not shown). Exon 3 showed a complex band pattern due to the presence of two polymorphic sites within the PCR product (data not shown). Both are in intronic sequences, but do not appear to introduce alternative splice donor or acceptor sites. The first is a c to t change 57 bp upstream of the start of exon 3. The second polymorphism was a g to a change 16 bp downstream of the end of exon 3, which has previously been reported. No other sequence changes were found in this exon.

RNA expression. RNA from the patients included in this study and others was used for RT-PCR (Fig 6A). 216, 132, and 232M all express WT1 at a relatively high level. The RNA from 58 was degraded. Overall, of the 57 patient samples analyzed, 18 of 25 (72%) ALLs, 19 of 25 (76%) AMLs, and 6 of 7 (86%) biphenotypic leukemias expressed WT1. Some samples were also analyzed by Northern blot. Of these, 1 of 3 (33%) ALLs, 9 of 13 (69%) AMLs, 1 of 2 (50%) biphenotypic leukemias, and 1 of 1 (100%) null leukemias expressed WT1. The numbers here are small, and the reduced percentages also reflect the lower level of sensitivity of Northern blotting compared with RT-PCR. 216, 132, and 232M all express WT1 mRNA at a level detectable by Northern blot (data not shown).

The RT-PCR products from the samples with mutations were sequenced to see if the mutant alleles were expressed (Fig 5). Sample 58 (discussed above) may only express the allele with the insertion in exon 1. Sample 132, which has an insertion in exon 7, was sequenced several times from different RT-PCR products from two different cDNAs but expressed only the mutant allele (Fig 5B). It is possible that the mutation is homozygous but appeared heterozygous at the DNA level because of contaminating normal cells that do not express WT1. 216 has a much more complex clonal evolution, because two alleles are expressed but neither of them carries the mutation (Fig 5C). Both DNA and RNA were reisolated from new aliquots of frozen cells with the same result, i.e., that the mutation was present in the DNA, although at a reduced intensity compared with the wild-type sequence, but that two normal alleles were expressed. Because normal bone marrow and lymphocytes express WT1 at only a very low level, the normal WT1 mRNA is more likely to originate from leukemic cells that express a high level of WT1 but do not have this mutation in zinc finger 3. The mutation has possibly occurred as a later event in a new clone that is present at a reduced level within the population of leukemic cells so that the mutant RNA is present at too low a level to be detected by RT-PCR. Alternatively, the mutant RNA may be degraded faster than the wild-type RNA or there may be some mechanism by which expression from the promoter of the mutant allele is reduced or completely prevented. Sample 232M, which has a nonsense mutation in exon 9, expressed both the mutant and wild-type alleles, although the mutant allele was of reduced intensity (Fig 5D). This could be due to clonal evolution or specific downregulation of the mutant mRNA. The mutation appears to be truly heterozygous in this patient.

WT1 expression in normal hematopoietic cells. To see if normal hematopoietic cells express WT1, a number of normal bone marrow samples were analyzed by RT-PCR. Several of these expressed WT1 at a level that can be seen on an ethidium bromide-stained gel, although others did not (Fig 6 and Pritchard-Jones et al). This is probably due to the presence of peripheral blood in the samples, which dilutes out the marrow cells. Earlier studies (K.P.-J. and L.K.-U., unpublished data) suggest that WT1 expression is mainly found in more immature myeloid and lymphoid leukemias, arguing for a role for the gene in early hematopoiesis. To investigate this possibility, CD34+ hematopoietic progenitor cells were isolated from human umbilical cord blood.

RNA from the unseparated cells and from the CD34+ and CD34- fractions was analyzed by RT-PCR (Fig 6B). There is detectable expression of WT1 in the CD34+ cells (lane 2).
and no detectable expression in the unseparated cells or the CD34− cells (lanes 1 and 3, respectively). Because CD34+ cells only account for about 0.1% to 0.5% of the mononuclear cell fraction of cord blood, the absence of detectable expression in the unseparated cells is not unexpected. However, WT1 clearly is expressed in early progenitor cells in normal hematopoiesis.

**DISCUSSION**

In this study, the tumor DNA from 35 patients with sporadic acute leukemia was analyzed for the presence of mutations in the Wilms’ tumor gene WT1. Five mutations were found in four patients, with one patient having two different mutations. Four of the mutations are insertions of 1 to 5 bp, one in exon 1 and the other three in exon 7. The fifth mutation is a nonsense mutation in exon 9. These are the first mutations in WT1 to be described in sporadic leukemias, extending our previous report of a point mutation in exon 9 in a secondary leukemia occurring in an individual with a germline WT1 deletion. All five mutations would be predicted to cause truncation of the WT1 protein, with the loss of all or part of the zinc finger region (Fig 7). In three cases,
Fig 3. Analysis of mutations in exon 7 of WT1. (A) SSCP of exon 7. Samples heterozygous for the A to G polymorphism are marked with asterisks. Samples 132, 216, and 58 are shown. (B) Direct sequencing of 132 PCR product. The arrow shows the position of the insertion that causes the superimposition of the normal and mutated sequences. (C) Sequencing of plasmid subclones from 216. The insertion of GCCG is indicated. (D) Direct sequencing of PCR product from 58. The arrow marks the position of the insertion and the two sequences are superimposed above this point.

only one allele is mutated, although mutation of the second allele cannot be entirely excluded because the remainder of the gene was not sequenced. The frequency of mutation detected in these samples is approximately 10%, which is comparable to that in Wilms' tumors. The true frequency of WT1 mutations in leukemia may be even higher, because SSCP detects only up to 90% of mutations and the promoter, the 5' and 3' untranslated regions (UTR) and the 5' end of exon 1 were not analyzed in this study.

WT1 has been shown to bind to DNA and can regulate expression from several genes, but it is unclear whether it functions as a transcriptional activator or repressor in vivo. The loss of the entire zinc-finger (ZF) region in samples 132, 216, and 58 would be expected to abolish DNA binding capacity, although protein-protein interactions may still be possible. 232M has a nonsense mutation in exon 9 and produces a protein containing only ZF1 and 2. This may exert a profound effect on the function of the mutant protein.
because the DNA-binding specificity may be different to that of the wild-type protein, so that, as well as the loss of normal function, a gain of function may occur. This mutation has previously been described as a homozygous mutation in a sporadic unilateral Wilms’ tumor\(^\text{16}\) and as a germline mutation in a child with bilateral Wilms’ tumors.\(^\text{17}\) In this latter case, the mutation remained heterozygous in one of the bilateral tumors, as in this case of leukemia.

Three of the mutations described in this study are apparently heterozygous; however, the presence of contaminating normal cells in the sample used for DNA analysis may mask true homozygosity in the tumor. It is also possible that the remaining allele may have a mutation in the promoter or the 3’ UTR, affecting the amount or stability of the mRNA. Truly heterozygous mutations may still be responsible for tumorigenesis if they act in a dominant or dominant-negative manner. Zinc finger mutations may cause binding to novel DNA targets. One heterozygous mutation found in a Wilms’ tumor, WT/AR, which causes an in-frame deletion of zinc-finger 3 and the loss of the KTS splice, has been proposed to act as a dominant mutation and has an oncogenic effect in conjunction with the viral oncogene E1A.\(^\text{44}\) Truncated proteins may bind to and sequester either the wild-type WTI or other proteins required for its normal function. Wang et al\(^\text{45}\) propose that the repressor domain of the protein (amino acids 85-124) interacts with and sequesters an unidentified nuclear factor that is required for the normal transcriptional repressor function of wild-type WTI protein. Similarly, Reddy et al\(^\text{46}\) show that the WTI protein can associate with itself both as full-length protein and as truncated protein containing the first 182 amino acids and that this association reduces the transcriptional activation activity of the wild-type protein. p53 has been suggested to interact with WTI. This interaction does not require an intact zinc finger region and may affect the functions of the two proteins in a dominant-negative fashion.\(^\text{47}\) The predicted protein products for samples 216 and 132, and both alleles for 58 retain at least the first 120 amino acids and could therefore interact with WTI or its protein partners.

From a mechanistic viewpoint, the mutations described here are interesting in that four of the five are small insertions. This type of mutation has only been described in a small number of Wilms’ tumors, with the majority of mutations being large deletions or point mutations. One insertion has been reported previously in exon 7 and is a direct repeat, similar to that in sample 58.\(^\text{33}\) Huff et al\(^\text{48}\) describe several

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**Fig 4.** Analysis of WTI exon 9. (A) SSCP of exon 9 showing sample 232M with altered bands. The arrow marks the position of the double-stranded control. (B) Direct sequencing of PCR product from 232M. The heterozygous C to T mutation is indicated.

**Fig 5.** RNA sequencing of mutants. (A) Sequencing of a single representative subclone of B1-B908 RT-PCR product from 58 mRNA. Left, sequence of part of exon 1. The arrow marks the position of the inserted A. Right, sequence of part of exon 7. The arrow marks the position at which the insertion of TCAG occurs in the other allele. (B) Direct sequencing of 132 B2-B4 RT-PCR product showing part of exon 7. The insertion AAGTC is marked; there is no superimposition of the normal sequence. (C) Direct sequencing of B2-B4 RT-PCR product from 216 showing part of exon 7. The arrow marks the position of the polymorphism G/A. There is no superimposition of the mutant sequence. (D) Direct sequencing of B2-B4 RT-PCR product from 232M showing part of exon 9. The arrow marks the heterozygous C to T point mutation.
deletions and one insertion in exon 1 in the vicinity of one or more copies of a deletion hotspot sequence, CCTG (CAGG), and close to dinucleotide and trinucleotide repeats. The two insertions we have found in exon 7 are imperfect repeats of the preceding 4 or 5 bases and are flanked by dinucleotide repeats. CCTG (CAGG) sequences can be found near the insertion sites in our cases in exons 1 and 7, as can TG and CGG repeats in exon 7 (Fig 2B). Small insertions at these presumed hotspots for genomic instability may be a novel and relatively common mechanism of mutation in the WT1 gene, particularly in hematopoietic cells.

Although no function has yet been found for WT1 in the hematopoietic system, it is likely to have an important role. It can be differentially regulated by a hematopoietic-specific enhancer that is activated by GATA-1.9 There is a specific alteration in splicing ratios to produce a relative excess of the +exon 5 isoform at splice 1 (J.R., manuscript submitted). The gene is expressed in the majority of acute leukemias and in a range of hematopoietic cell lines22 and appears to be downregulated during the induction of differentiation in culture.30,31 This supports the view that WT1 is expressed at a higher level in more immature cells. Several groups have analyzed the expression of WT1 in CD34+ hematopoietic progenitor cells. We have shown that WT1 expression can...
be detected in CD34+ cells. Inoue et al23 analyzed CD34+ subsets in normal bone marrow and found that the CD33+/lin− subset (which is believed to contain the most primitive stem cells) expresses 100 times more WT1 than the CD33+/lin+ fraction (which contains more differentiated precursors). The overall level of WT1 expression must be either very low or confined to a very small fraction of bone marrow cells, because other groups have not been able to show expression in CD34+ cells using different methodology.24 Although WT1 is not essential for hematopoiesis in the knock out mouse model,25 it may be that the presence of a mutated protein is more disruptive than its complete absence, as in the genitourinary system in humans, or that there is functional redundancy.

In this study, we have found mutations of WT1 in 3 of 20 (15%) cases of AML and 1 of 3 biphenotypic leukemias. This mutation frequency is equivalent to that found in sporadic Wilms’ tumors and suggests that WT1 may be equally important in both tumor types. These data also suggest that WT1 mutation may be associated with particularly aggressive forms of leukemia. Three of the four cases failed to achieve remission with standard induction chemotherapy and died of leukemia within 9 months of diagnosis; the remaining one died too early to assess response. For AML, the difference in remission rates is significant (Table 1). This finding is supported by that of Inoue et al,23 who found that high levels of WT1 expression at diagnosis in acute leukemia was significantly associated with a reduced remission rate and a poor prognosis. Because WT1 can negatively regulate its own transcription,13 it is possible that those leukemias with high levels of WT1 expression carried a WT1 mutation that causes a failure of this negative feedback. These results need to be confirmed in a larger sample but suggest that WT1 mutations may be more common in acute leukemias that are resistant to first line therapy and/or express mixed lineage markers. In this case, understanding the mechanisms by which mutant WT1 proteins interfere with normal hematopoietic differentiation may give important insights into the cellular pathways that are dysregulated in leukemia and suggest therapeutic targets.

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