RAPID COMMUNICATION

Association of Germline p53 Mutation With MLL Segmental Jumping Translocation in Treatment-Related Leukemia


Segmental jumping translocations are chromosomal abnormalities in treatment-related leukemias characterized by multiple copies of the ABL and/or MLL oncogenes dispersed throughout the genome and extrachromosomally. Because gene amplification potential accompanies loss of wild-type p53, we examined the p53 gene in a case of treatment-related acute myeloid leukemia (t-AML) with MLL segmental jumping translocation. The child was diagnosed with ganglioneuroma and embryonal rhabdomyosarcoma (ERMS) at 2 years of age. Therapy for ERMS included alkylating agents, DNA topoisomerase I and DNA topoisomerase II inhibitors, and local radiation. t-AML was diagnosed at 4 years of age. The complex karyotype of the t-AML showed structural and numerical abnormalities. Fluorescence in situ hybridization analysis showed multiple copies of the MLL gene, consistent with segmental jumping translocation. A genomic region including CD3ε, MLL, and a segment of band 11q24 was unarranged and amplified by Southern blot analysis. There was no family history of a cancer predisposing syndrome, but single-strand conformation polymorphism (SSCP) analysis detected identical band shifts in the leukemia, ganglioneuroma, ERMS, and normal tissues, consistent with a germline p53 mutation, and there was loss of heterozygosity in the ERMS and the t-AML. Sequencing showed a CGA→TGA nonsense mutation at codon 306 in exon 8. The results of this analysis indicate that loss of wild-type p53 may be associated with genomic instability after DNA-damaging chemotherapy and radiation, manifest as a complex karyotype and gene amplification in some cases of t-AML.

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MATERIALS AND METHODS

The Institutional Review Boards at the Children’s Hospital of Buffalo and the Children’s Hospital of Philadelphia approved this research and the parents gave informed consent. Karyotype, FISH, and Southern blot analyses were performed on the t-AML of patient RUPN 84 and on the cell line 2L1, which was derived from the marrow of patient RUPN 84 at diagnosis of t-AML.

Characterization of the cell line 2L1. The cell line 2L1 was continuously passaged for 14 months in Iscove’s modified Minimum Essential Medium (MEM; Life Technologies, Inc, Gaithersburg, MD) containing 20% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL.

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penicillin, and 100 µg/mL streptomycin. The diagnostic marrow and the 2L1 cells expressed CD33, CD13, CD15, CD11b, and CD38; however, the 2L1 cells were also strongly positive (>90%) for CD2, CD10, and CD56. The karyotypes of the 2L1 cells and of the diagnostic marrow of patient RUPN 84 were the same (see Results).

Cytogenetic analysis. Bone marrow of patient RUPN 84 at diagnosis of t-AML was cultured for 24 hours with and without mitotrexate synchronization and processed by conventional cytogenetic methods. Methaphase chromosomes were trypsin/Wright stain banded, and karyotypes were described according to ISCN guidelines.

FISH analysis. Probes used for FISH analysis included a YAC clone containing MLL (Oncor, Gaithersburg, MD), a CD3ε probe for analysis of the region centromeric to the MLL gene (a kind gift from Dr Thomas Shows, Roswell Park Cancer Institute, Buffalo, NY), and the more telomeric cosmid, C11q7q24, from chromosome band 11q24. The probes were labeled with digoxigenin, and metaphase spreads from the marrow of patient RUPN 84 at diagnosis of t-AML and from the cell line 2L1 were examined by FISH analysis using standard methods.

Southern blot analysis of the MLL gene. Genomic DNAs from the diagnostic marrow of patient RUPN 84, from the cell line 2L1, and from control peripheral blood mononuclear cells were examined by Southern blot analysis. BamHI-, HindIII-, SstI, EcoRl-, and Bgl II-digested DNAs were hybridized with a 0.7-kb cDNA probe from the MLL bcr. BamHI- and HindIII-digested DNAs were also hybridized with a 2.2-kb genomic fragment of the SCL gene at chromosome band 1p33-p13p4. To assess equivalence in loading, HindIII-digested samples were simultaneously hybridized with the MLL bcr cDNA probe and the SCL genomic probe.

BamHI-, HindIII-, and EcoRl-digested DNAs were hybridized with polymerase chain reaction (PCR)-generated fragments from MLL exon 3, exon 25, and exon 34. The sense and antisense PCR primers used to amplify MLL exon 3 were 5’-GGT ACG TCT TTC GTG TGG C-3’ and 5’-GGG CAA GCC CTA GCT GGC CTA GCG C-3’, respectively. The sense and antisense PCR primers used to amplify MLL exon 25 were 5’-GTC AGT GCT A TC TCC TCG CG-3’ and 5’-GGT ACG TCT TTC GTG TGG C-3’, respectively. The sense and antisense PCR primers used to amplify MLL exon 34 were 5’-CAG AGA CAG AGT TGA GGT CTC G-3’ and 5’-CAG AAG TGA ACT CTC GAG TGG C-3’, respectively. BamHI-, HindIII-, and SstI-digested DNAs were hybridized with a 1.3-kb cDNA probe from the CD3ε gene, which is centromeric to the MLL gene at chromosome band 11q23 (a kind gift from Dr Thomas Shows). Southern blot analysis also was performed on BamHI- and HindIII-digested DNAs using a 1.5-kb nonreiterated genomic fragment, C11q15SS, from the cosmid C11q7q24, which maps to chromosome band 11q24.

Signal intensity on the autoradiographs was quantitated using a Molecular Dynamics computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The MLL, CD3ε, and band q24 chromosome 11 signals were normalised by comparison with the signal from hybridization with the SLC probe from chromosome band 1p33-p1p4.

p53 single-strand conformation polymorphism (SSCP) analysis. Genomic DNAs from the marrow of patient RUPN 84 at diagnosis of t-AML and from the cell line 2L1 were screened by PCR/SSCP analysis. The oligonucleotide primers have been reported. PCR fragments containing p53 exons 5 and 6 or exons 7 and 8 and incorporating [α-32P]dCTP were amplified using 100 ng genomic DNA as template. Aliquots of the products containing exons 5 and 6 or exons 7 and 8 were digested with Aap I or Dra I, respectively, to reduce the fragment sizes and separate the exons. Aliquots of the digested products were diluted with loading buffer, denatured by heating at 90°C for 5 minutes and electrophoresed at 4°C in nondenaturing polyacrylamide at constant power as previously described.

Characterization of p53 mutation suggested by SSCP. Fresh aliquots of genomic DNA from the marrow of patient RUPN 84 at diagnosis of t-AML were amplified in 3 separate PCR reactions with the same SSCP primers encompassing exons 7 and 8. The 100 µL PCR reaction mixtures contained 1 µg genomic DNA, 2.5 U AmpliTag DNA polymerase, 200 µmol/L of each dNTP, PCR reaction buffer at 1X final concentration (Perkin Elmer, Norwalk, CT), and 100 pmol of each primer. After initial denaturation at 94°C for 9 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes were used, followed by a final elongation at 72°C for 7 minutes. Products of 3 separate PCR reactions were subcloned into pBluescript II SK+ vector (Stratagene, La Jolla, CA). Six separate subclones from the 3 independent PCR reactions were sequenced by automated methods.

Extraction of genomic DNAs from paraffin-embedded tissues for SSCP analysis of p53 exon 8. Genomic DNAs were extracted from formalin-fixed paraffin-embedded tissue blocks of the ERMS and ganglioneuroma of patient RUPN 84. After thorough cleaning of the microtome and installation of a new disposable knife, 15- to 20-µm sections providing a 1 cm2 area were cut from a control blank block and from the blocks containing tissue. Microdissection was performed to isolate the tumor from the surrounding normal tissue. The sections were deparaffinized with a 1:1 mixture of xylene and ethanol. Deparaffinized sections were incubated at 55°C for 1 to 3 hours in 100 µL of a solution containing 6 µg proteinase K, 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, and 0.01% gelatin. The solutions were then incubated at 100°C for 10 minutes, followed by microcentrifugation at 14,000 rpm for 10 minutes. The supernatant containing the DNA was removed to a clean microcentrifuge tube and diluted with ∆H2O to a final volume of 200 µL.

SSCP analysis of p53 exon 8 in genomic DNAs from paraffin-embedded tissues. A new PCR/SSCP sense primer beginning at the Dra I site in p53 intron 7 was designed to amplify a 245-bp product containing p53 exon 8 when used with the same intron 8 antisense primer as described above. The sense primer was homologous to positions 13937-13954 of the p53 genomic sequence. One hundred nanograms of genomic DNA from the marrow of patient RUPN 84 at diagnosis of t-AML and 2 µL of genomic DNAs prepared from the paraffin-embedded tissues were amplified in 10 µL reactions incorporating [α-32P]dCTP using exactly the same PCR conditions as described above for SSCP. Blank blocks processed in the same manner as the paraffin-embedded tissues and ∆H2O were negative controls. One microliter of each PCR reaction was diluted with 9 µL of loading buffer and denatured by heating at 90°C for 5 minutes. Two microliters of each heat-denatured sample (1/50 of the initial PCR reaction) was electrophoresed at 4°C in nondenaturing polyacrylamide at constant power for 5 hours.

RESULTS

Case history. Patient RUPN 84 was diagnosed simultaneously with ganglioneuroma and nonmetastatic nasopharyngeal ERMS at 2 years of age. Treatment for ERMS included multiagent chemotherapy and local radiation. Cumulative chemotherapy doses for ERMS were vincristine (40 mg/m2), actinomycin D (17.5 mg/m2), ifosfamide (7,300 mg/m2), and cyclophosphamide (8,600 mg/m2). Additional therapy doses for locally recurrent ERMS were etoposide (1,200 mg/m2), doxorubicin (300 mg/m2), and carboplatinum (3,810 mg/m2). The child was diagnosed with t-AML at 4 years of age after 10 months off therapy for ERMS. There was no family history of a cancer-predisposing syndrome. The diagnostic marrow contained 60% blasts of the FAB M5 monoblastic subtype that expressed CD33, CD13, CD15, CD11b, and CD38. The complex karyotype of the t-AML was 45,X,Y, der(5)↓(5;11)(5pter--5q12::11q23→11qter),del(11)(pter--q12::q24--q12::q13--qter), t(17. Remission induction with daunomycin, cytosine arabinoside, and...
thioguanine, and teniposide was unsuccessful. The patient underwent HLA-matched allogeneic marrow transplantation but died from his disease.

Evidence for MLL gene amplification in the t-AML of patient RUPN 84. Consistent with segmental jumping translocation, 5 or 6 signals consistently were identified on hybridization of the MLL YAC probe with a total of 20 metaphase spreads prepared from the diagnostic marrow of patient RUPN 84. In the example shown in Fig 1, the MLL YAC probe detected signals on the der(5) chromosome, on the normal chromosome 11, and at distinct centromeric and telomeric regions on the der(11) chromosome. The telomeric and centromeric signals from the der(11) chromosome were consistently more intense than the MLL signal from the normal chromosome 11, suggesting that the centromeric and telomeric regions on the der(11) chromosome contained multiple copies of the MLL gene. In addition, at least 5 discrete signals were observed in the nearby interphase nucleus (Fig 1). Similar results were obtained by FISH analysis with CD3e and 11q24 probes, indicating that the amplified genomic region extended centromeric and telomeric of the MLL gene. FISH analysis of the cell line 2L1 derived from the leukemia showed the same results (data not shown).

Consistent with the FISH analysis, Southern blot analysis suggested that there were multiple copies of the MLL gene in the t-AML of patient RUPN 84 and in the cell line 2L1 (Fig 2). The ratio of MLL signal intensities in the t-AML and in the cell line 2L1 were 4.3:1 compared with the peripheral blood mononuclear cell (PBMC) control when normalized for equal loading by hybridization with the SCL probe. These results suggest approximately 8 to 9 copies of the MLL gene in the t-AML of patient RUPN 84 and in the cell line 2L1 derived from the leukemia (Fig 2). Although the patient received DNA topo-isomerase II-targeted chemotherapy and had monoclonal leukemia, the MLL gene was not rearranged by Southern blot analysis. The results of Southern blot analysis with multiple restriction digests used in combination with MLL exon 3, exon 25, and exon 34 probes and a CD3e probe showed similar unarranged, amplified patterns, whereas hybridization with an 11q24 probe showed the unarranged pattern but less amplification (2.0:1), again indicating that the amplified region extended both centromeric and telomeric of the MLL gene (data not shown).

Association of MLL gene amplification with p53 mutation. SSCP analysis of genomic DNAs from the marrow of patient RUPN 84 at diagnosis of t-AML and from the cell line 2L1 detected identical band shift patterns and LOH in the region of p53 exon 8 (Fig 3). Sequencing of 6 individual genomic subclones from 3 independent PCR reactions performed on the marrow DNA identified a CGA→TGA nonsense mutation at codon 306 that created a premature termination codon and would foreshorten the predicted protein. SSCP analysis of p53 exon 8 in DNA prepared from paraffin-embedded tissues showed the same band shift pattern in the normal tissues and in the sarcoma and ganglioneuroma, indicating that the p53 mutation was of germline origin. There was LOH in the sarcoma but not in the ganglioneuroma. As predicted with a truncated protein, p53 immunostaining was negative in the ganglioneuroma and in the sarcoma (data not shown).

DISCUSSION

We used FISH and Southern blot analysis to determine MLL gene copy number in a t-AML with a complex karyotype and monoblastic features and detected MLL gene amplification consistent with the jumping translocations of chromosomal segments containing MLL or ABL that were first discovered by Tanaka et al in 1997. Detailed molecular analyses demonstrated that the amplified MLL gene was not rearranged. The prior history of early onset ERMS and DNA damaging chemotherapy and radiation, and the central role of wild type p53 in maintaining genomic stability and ploidy, led to the investigation of the p53 gene and detection of the germline codon 306 mutation and LOH in the ERMS and the t-AML.

This case brings the total number of treatment-related leukemias with segmental jumping translocations that have been described to four. With additional detailed FISH and molecular analyses, the incidence of segmental jumping translocations in treatment-related leukemias should become apparent, because the abnormalities are not detected by karyotype alone. Consistent with the FISH analyses of Tanaka et al, we determined that there was intrachromosomal amplification of the specific segment containing MLL and that the segment containing MLL had moved to at least one other chromosome. Although the t-AML was monoblastic, Southern blot analysis
showed that the amplified \textit{MLL} gene, including regions 5' and 3' of the breakpoint cluster region, was not rearranged. The results of FISH and Southern blot analyses with CD3e and 11q24 probes indicate that the amplified, unrearranged genomic region extended centromeric and telomeric of the \textit{MLL} gene. Disruption of the breakpoint cluster region of the \textit{MLL} gene by chromosomal translocation specifically is associated with the development of leukemia,\textsuperscript{16} but the role of \textit{MLL} gene amplification in leukemogenesis currently is unknown.

The karyotype of the t-AML that we examined and the karyotypes of the other three leukemias with segmental jumping translocations were complex\textsuperscript{3} and suggest genomic instability. In this regard, the t-AML was similar to an alkylating agent-induced leukemia in which we previously detected a germline 2-bp deletion at p53 codon 209 that was inherited from the father.\textsuperscript{17} In the latter case, FISH analysis was not performed and it is not known whether there was a segmental jumping translocation, but the karyotype was 45, XY, hsr(2)(q22), −5, der(7)del(7)(q11.23)hsr(7)(q11.23), der(12)t(12;19)(p11.2;q12), der(17)t(5;17)(p12;p11.2), −19, +mar1.\textsuperscript{17}

As was true for the child in the present study, the patient was diagnosed at an early age (1 year and 10 months) with primary ERMS, and there was not a family history of the Li-Fraumeni syndrome.\textsuperscript{17} In a study of patients with RMS without family histories of the Li-Fraumeni syndrome, Diller et al\textsuperscript{6} detected germline p53 mutations in 3 of 13 children diagnosed before 3 years of age, but found no germline p53 mutations in 20 older children. These observations suggest that germline p53 mutations may predispose a fraction of young children undergoing therapy for RMS to t-AML, because the defective G1 cell cycle
checkpoint that accompanies loss of wild-type p53 brings about genomic instability with DNA damaging chemotherapy and radiation.

Wild-type p53 blocks cell cycle progression in late G1 in the presence of DNA damage caused by certain anticancer drugs and radiation and, depending on the level of the damage, either mediates apoptosis or permits DNA repair and cell cycle re-entry. p53-dependent apoptosis is responsible, in part, for the cytotoxic activity of anticancer drugs and γ-radiation, while cells deficient in wild-type p53 are resistant to the induction of apoptosis by these agents.

p53 mutant cells lose the ability to inhibit cell growth after DNA-damaging chemotherapy and γ-radiation. Thus, p53 was a candidate gene to examine in a t-AML with gene amplification because wild-type p53 maintains genomic stability and ploidy, whereas altered cell cycle arrest, gene amplification potential and aneuploidy occur with loss of wild-type p53. Furthermore, heterozygosity for mutant p53 does not result in gene amplification in experimental systems. In the leukemia in this study, p53 SSCP analysis showed both mutation and LOH and the karyotype showed chromosome 17 monosomy, which explains the LOH.
The child in the present study and 3 patients with t-AML with ABL and MLL segmental jumping translocations reported on by Tanaka et al. received heterogeneous chemotherapy and, in some cases, radiation. Our own observations suggest that young children with RMS and germline p53 mutations may be at increased risk for t-AML resulting from genomic instability on exposure to genotoxic agents.17 There is insufficient information to recommend treatment changes based on the current knowledge, but systematic study of p53 mutations and prior therapy in a larger cohort of patients with this form of t-AML may inform the rational design of individualized primary cancer treatment for at-risk individuals.

The results of this analysis establish that the pathogenesis of the gene amplification in treatment-related leukemias with segmental jumping translocations involves loss of wild-type p53. Just as gene amplification potential accompanies loss of wild-type p53 in Li-Fraumeni fibroblasts in vitro, germline p53 mutations with LOH may be associated with MLL gene amplification in t-AML. In vitro studies also indicate that there are alternative pathways that allow gene amplification when p53 is wild-type.2 The frequency of mutant p53 in treatment-related leukemias with segmental jumping translocations remains to be determined. Hartwell15 suggested that genomic instability is a genetic trait. The demonstration of a germline p53 mutation in association with the complex karyotype and MLL gene amplification in the t-AML in the present study proves that this is indeed the case. Future studies will explore the roles of the specific genetic changes resulting from the instability in the genesis of leukemia.

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