Microsatellite Instability and p53 Mutations in Therapy-Related Leukemia Suggest Mutator Phenotype

By Dina Ben-Yehuda, Svetlana Krichevsky, Offer Caspi, Deborah Rund, Aaron Polliack, Dvora Abeliovich, Orly Zelig, Vered Yahalom, Ora Paltiel, Reuven Or, Tamar Peretz, Susana Ben-Neriah, Orly Yehuda, and Eliezer A. Rachmilewitz

During the last decade the frequency of therapy-related acute leukemia (t-leuk) and myelodysplastic syndrome (t-MDS) has been increasingly observed. Over the past 15 years, we treated 56 patients with t-leuk who had received prior chemotherapy (39%), radiotherapy (11%), or both (45%). The drugs received included alkylating agents and topoisomerase II inhibitors. The primary tumors included hematological malignancies (49%) and solid tumors such as breast or ovarian cancer. The median age at diagnosis of the primary tumor was relatively young (43 years ± 18). Twelve patients had more than one primary tumor and 31 patients had a family history of malignancy. Karyotypic abnormalities were found in 91% of the patients. Prognosis was uniformly poor, with an overall median survival of 10 months. Twelve of the 18 patients examined (67%) had a multidrug resistance phenotype. P53 genes of the leukemic cells, as well as the original tumors, were analyzed in 21 patients using polymerase chain reaction (PCR) with single-stranded conformation polymorphism analysis followed by sequencing. P53 mutations were identified in 38% of these patients, a relatively high prevalence compared with other forms of MDS or de novo acute myeloid leukemia. Mutations were nongermline and restricted to the leukemic cells. We identified different p53 mutations in the various primary tumors of individual patients. The presence of a mutator phenotype was assessed by PCR analysis of microsatellites in eight loci (one trinucleotide repeat sequence, four dinucleotide, and three mononucleotide repeat sequences). Microsatellite instability in two to seven loci were found in 15 of 16 (94%) of the patients. This instability is compatible with a mutator phenotype, which predisposes the patients to the development of malignancies including t-leuk.

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THE DEVELOPMENT and widespread use of combination chemotherapy, as well as the combination of chemoradiotherapy has generally improved the outcome of many patients with cancer. However, impressive advances in the cure rate and survival have been accompanied by a distressing increase in the number of cases of therapy-related leukemia (t-leuk) and myelodysplastic syndromes (t-MDS). Initial reports of AML following chemotherapy with alkylating agents were published in the 1970s, and since then, the incidence of t-AML and t-MDS in patients treated for another malignancy has been estimated to range between 2% and 10% depending on a variety of factors including age, dose, duration, and the type of chemotherapy employed, as well as the use of combined chemoradiotherapy. T-leuk represents approximately 10% to 15% of all AML. When considering the number of patients exposed to chemotherapy and radiotherapy each year, it is critical to recognize the potential causes of therapy-related bone marrow (BM) damage to develop strategies to prevent it. Moreover, both t-leuk and t-MDS are much more resistant to treatment than the primary cancer and efforts to restore normal hematopoiesis have been largely unsuccessful.

Two different types of t-leuk have emerged: one type appears following therapy with alkylating agents, increases in frequency with age, and often presents with prior MDS, with poor response to chemotherapy. This type shows typical chromosomal changes including monosomy 7, (−7) monosomy 5 (−5), or loss of various portions of the long arms of these chromosomes (5q− and 7q−). The second class of t-leuk seems to be related to therapy with cytostatic drugs targeting DNA-topoisomerase II, often presenting with overt acute leukemia and responds more favorably to chemotherapy. This type of t-leuk shows balanced chromosomal aberrations, primarily translocations involving chromosome bands 11q23 and 21q22.

The common endpoint of treating leukemic cells with alkylating agents, topoisomerase II inhibitors, and radiation is DNA damage. The tumor suppressor protein p53, which recognizes DNA breaks, directs cells with damaged DNA along the path of apoptosis. P53 also plays a central role as a key regulator in the cell cycle and has appropriately been termed “the guardian of the genome.” The p53 gene, which consists of 11 exons, 10 introns, and two promoters, is located on chromosome 17 p13.1. Chromosome 17 has been cited as the third most frequently involved chromosome in secondary MDS and acute leukemia. Despite the fact that p53 mutations are infrequently encountered in de novo AML or MDS, we decided to study the role of this gene in patients with t-leuk, because of its role in cell cycle regulation following DNA damage.

MATERIALS AND METHODS

Patients. We studied 56 patients with t-leuk and t-MDS diagnosed at Hadassah University Hospital from 1980 to 1994. The types of leukemia and MDS were diagnosed according to the French-American-British (FAB) classification, using standard techniques including immunohistochemistry, immunophenotyping, cytogenetics, and molecular biology. For cytogenetic analysis, BM aspirates were processed according to the method of Michaeli et al using conditioned medium derived from the human bladder carcinoma cell line 5637. G-banding was performed according to Klinger.

Rhodamine 123 dye exclusion. Peripheral blood or BM mononuclear cells were separated on Ficoll gradients. Wright’s stained slides of the mononuclear cell layer were inspected to verify the cell composition (% lymphocytes or blasts). In cases where the blast composi-
tion did not exceed 90%, the analysis was performed on gated blast populations determined by forward and side scatter characteristics. The cells were stained with rhodamine 123 (150 ng/mL; Sigma, St Louis, MO), with and without preincubation with 10 μmol/L verapamill as described by Chaudhary. Analysis of stained cells was performed by fluorescence-activated cell sorter (FACS) (FACStar Plus; Becton Dickinson, Mountain View, CA). Cells were counterstained with propidium iodide to allow exclusion of dead cells by gating. The presence of an MDR1 phenotype was assessed by the presence of a population of cells that demonstrated increased fluorescence with the addition of verapamill (>5 to 10,000 cells analyzed).

DNA isolation. Blood and BM samples from patients with t-leuk and t-MDS were collected in EDTA. Fresh tumor tissue and blocks of paraffin-embedded tumor and BM biopsies were obtained from the same patients. Genomic DNA from phenol-chloroform extraction following proteinase K treatment.

DNA from paraffin-embedded tissue was prepared using thick sections from the block that were placed in a microfuge tube and deparaffinized with xylene. After drying, the pellets of tissue were resuspended in 48 μL of DNA lysis buffer (50 mmol/L Tris-HCl pH 7.5, 0.1 mol/L NaCl, 1 mmol/L EDTA). A total of 2 μL of 10% sodium dodecyl sulfate (SDS) and 2 μL (20 μg/μL) of Proteinase K were added, and the samples were incubated overnight at 37° C. The DNA solution was purified using Wizard miniprep DNA purification system (Promega, Madison, WI).

PCR of exons 4-8, and 10 of the p53 gene. A total of 0.5 μg of DNA was amplified. The 20 μL polymerase chain reaction (PCR) contained 1× PCR buffer (10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1% Triton X 100, 0.2 mg/mL bovine serum albumin [BSA] or gelatin, 0.5 mmol/L deoxyribonucleoside triphosphates [dNTPs] mix), 10 μmol/L of each primer, and 1 U of Taq polymerase (Appligene; Oncor Inc, Gaithersburg, MD), in addition to 0.5 μCi P32-deoxyctydine 5'-triphosphate (dCTP) (Amersharn International plc, Buckinghamshire, UK). Forty cycles of PCR were performed, with denaturation at 98°C for the first cycle and then 92°C for 1 minute. Annealing temperatures were set at 53° C to 58° C (for various primers) and extension was set at 72° C for 30 seconds.

Single-stranded conformation polymorphism (SSCP). Amplified PCR fragments were analyzed by SSCP electrophoresis with 6% polyacrylamide gels, with and without preincubation with 1 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO). With and without preincubation with 10 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO). With and without preincubation with 10 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO). With and without preincubation with 10 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO). With and without preincubation with 10 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO). With and without preincubation with 10 μg/mL deoxyribonuclease I (DNase I; Sigma, St Louis, MO).

Sequencing of SSCP fragments. Abnormal individual SSCP fragments were cut directly from the dried gel and eluted in distilled water. A 5-μL aliquot of supernatant containing the single-stranded DNA was used for another round of PCR amplification with the corresponding primers, in a 20 μL reaction. PCR products were analyzed on 12% polyacrylamide gels with 7 mol/L urea. Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite). Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite).

Sequencing was performed on preheated (68°C for 10 minutes) PCR products with DNA Sequencing Kit Version 2.0 (USB, Cleveland, OH) and analyzed on polyacrylamide gels with 7 mol/L urea. All samples were sequenced with sense and antisense primers.

Analysis of microsatellite instability. We examined the extracted DNA for genetic alterations at eight separate microsatellites, localized to the following chromosomes: 17p (p53 gene, a dinucleotide repeat),14p (D14S426, a dinucleotide repeat retrieved from the DMB database; F primer: CCCAAAATATCTACCCAAAT, R primer: GAGTGGCAACCACTCTTGT), 13p (RB gene, a dinucleotide repeat),15 19p (DMI gene, a trinucleotide repeat),22 BAT 25 and BAT 40 are randomly chosen microsatellite markers consisting of an (A)n repeat,23 and a (T)n repeat in the eighth intron of the cystic fibrosis transmembrane conductance regulator (CFTR gene) on chromosome 7q.24 Amplified fragments were analyzed on 12% polyacrylamide gels with visualization of bands using ethidium bromide (pS3 microsatellite), or by P32 radiolabeled PCR (RB gene). The D14S426, the GABBR3, the myotonic dystrophy (DMI) genes, and the mononucleotide repeats were visualized using silver staining technique as described.25 The protocols for the analysis of these loci were as previously described.25 25

RESULTS

Patients. A markedly increased number of patients with t-leuk were observed during the past 5 years; 37 patients were diagnosed between 1990 and 1994, compared with only 19 patients seen between 1980 and 1989.

Fig 1. A markedly increased number of cases of t-leuk were observed during the past 5 years; 37 patients were diagnosed between 1990 and 1994 compared with only 19 patients seen between 1980 and 1989.

The median age at diagnosis of the primary tumor was generally relatively young. For example, the median age at diagnosis of breast carcinoma and ovarian carcinoma was 43 and 48 years, respectively, whereas in the general Israeli population, the median age at diagnosis is 60 years for breast and 63 for ovarian carcinoma. Twelve patients had more than one primary tumor and three had more than two. More than half of the cases (31 patients) had a family history of malignant disease. Primary tumors were treated with chemo-
therapy (52%), radiotherapy (7%), or both modalities (41%). Drugs included alkylating agents (60%) and topoisomerase II inhibitors, either alone (15%) or in combination with alkylating agents (25%).

The time interval between completion of the primary therapy and the diagnosis of the t-leuk or t-MDS was relatively short. Half of the patients developed t-leuk or t-MDS within 2 years of therapy, while the frequency decreased markedly approaching the fifth year posttreatment (Fig 2). The types of t-leuk included AML (54%), MDS in transformation (38%), and ALL (8%). Of the 46 patients analyzed, 42 had an abnormal karyotype (91%) (Fig 3 and Table 2), and in 21 cases (46%) the abnormality was of chromosomes 5 and/or 7.

Ten patients underwent allogeneic BM transplantation and one autologous transplant. All the other patients were treated with either single agent or combination chemotherapy. The median survival was 26 months for the transplanted patients, compared with 8 months in the remaining cases.

*Multidrug resistance analysis.* Eighteen patients were assessed for the presence of rhodamine 123 efflux, a standard assay for activity of the MDR1 protein (Fig 4). Twelve patients (67%) were positive for rhodamine efflux. Of the six who were negative, all but one had balanced cytogenetic aberrations. Among them, three had acute promyelocytic leukemia, with a typical t(15;17) translocation as the sole chromosomal abnormality, and two had a t(9;11) translocation following treatment with etoposide. All the MDR1 positive patients, except for one, had deletions or absence of chromosomes 5 or 7, or trisomy 8.

*p53 mutations.* We analyzed 68 samples from 21 patients and found p53 mutations in the t-leuk cells in eight cases (38%). The mutations were nongermline and restricted to the t-leuk cells. The mutations were not identified in the patients’ normal tissues, ie, fibroblasts or normal BM cells (collected in complete remission). We analyzed the DNA of the primary tumors in these patients and found different p53 mutations in each of their primary tumors. Only one of the mutations was a nonsense mutation, while all the others were missense mutations (Fig 5A and B).

*Microsatellite instability.* The number of repeated sequences in the DNA obtained from the t-leuk or t-MDS cells, as well as the primary tumors, were compared with normal tissue obtained from the same patient. Table 3 details the results for each patient. We found evidence for microsatellite instability at the p53 locus in all eight patients who had a p53 mutation and in only one of the 13 patients without a p53 mutation. Sixteen of these patients were analyzed for microsatellite instability at other loci. Fifteen of these (94%) had microsatellite instability at two to seven loci (Fig 6a and b, Table 3). Two patients had instability in two loci, four patients in three loci,
Fig 3. Chromosomal changes in 42 patients with t-leuk/MDS. Each box represents one aberration; some patients had more than one aberration. The upper histogram demonstrates the locations of gain or loss of part or all of each chromosome. The lower histogram demonstrates locations of breakpoints.

one in four loci, three in five loci, three in six, and two in seven loci. Such instability in the t-leuk and t-MDS patients suggests the presence of a mutator phenotype.

DISCUSSION

Accumulating evidence links secondary leukemia and MDS to therapeutic irradiation and cytotoxic chemotherapy. Both cytotoxic drugs, irrespective of their mode of action, and ionizing irradiation, are known to cause DNA damage. This association has accelerated efforts to define the role of p53 gene, which normally directs cells with DNA damage to apoptosis. To address this issue, radiation and several cytotoxic agents have been investigated recently with respect to their p53-inducing capacity in cultured cells. These

Fig 4. Rhodamine dye exclusion of leukemic cells. Peripheral blood blasts were stained with rhodamine 123 without (A, C) and with (B, E) verapamil preincubation. Histogram D depicts superimposition of histograms C and E. There is a marked (nearly one log) increase of mean cell fluorescence with addition of verapamil compared with that without the addition of verapamil.
Fig 5. Identification of p53 mutations. Mutations were identified in genomic DNA, using the PCR/SSCP method. An example of SSCP of one patient with t-APL is shown in (A). The patient’s leukemic cells showed an abnormal pattern in exon 8 (a), which became normal in remission (b). Positive control (c) and normal DNA (d and e) are shown. Before the APL, this patient had colon and breast cancer. (B) Demonstrates a different mutation in another exon (exon 10), found in the colon cancer of the same patient (f). The breast cancer had a mutation in exon 8 (not shown). Positive control (g) and normal DNA (d and e) are shown. (C) Demonstrates sequencing of the PCR products of the breast cancer mutation of this patient, a missense mutation in exon 8, codon 282 (CGG to TGG) resulting in an arginine to tryptophan change.

Studies found that alkylating agents, drugs that bind to topoisomerase II, antimetabolites, as well as energy-rich radiation, induced p53 overexpression. The accumulation of wild-type p53 arrests cell cycle progression until initial DNA damage is repaired, but if the repair fails, p53 may then proceed to trigger apoptosis.

We studied 56 patients with t-leuk or t-MDS and found a significant increase in the number of cases of t-leuk or t-MDS during the last 5 years compared with the prior decade. We also found a short latent period between the treatment of the primary tumor and the development and diagnosis of t-leuk or t-MDS. Dose intensification of cytotoxic drugs in recent years and the increased usage of etoposide may contribute to these observations.

Because the patients studied were selected according to the diagnosis of t-leuk or t-MDS, they did not comprise a

Table 3. Microsatellite Instability Analysis

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Abbreviations: ND, not done due to insufficient DNA; +, instability was found; -, no instability.
MUTATOR PHENOTYPE IN THERAPY-RELATED LEUKEMIA

Fig 6. (a) Microsatellite instability (MIN) of the p53 gene. Analysis of DNA from paired normal (N) and neoplastic cells (L), showing MIN. Genomic DNA was amplified using primers from a microsatellite in the first intron/exon junction of the p53 gene. A different banding pattern is evident on comparison of paired samples from individual patients. Panel A demonstrates fibroblast DNA (NI from a patient with rhabdomyosarcoma. His tumor DNA showed the same germline pattern. Analysis of BM cells from t-MDS and frank t-leuk (L,L) of this patient show different patterns. Panel B demonstrates normal DNA (NI and DNA from bilateral metachronous breast cancers of one patient (1L,L), which were found to have different p53 mutations, one in exon 8 and one in exon 10. Panel C demonstrates normal cryopreserved BM (NI and etoposide-induced t-leuk (L) in a patient after treatment for NHL. (M) is a marker (PhiX174, Haelll digest). (b) Microsatellite instability (MIN) at other loci: Analysis of DNA from paired normal (N) and leukemic cells (L) from nine patients, showing MIN. DNA from the breast cancer of patient 5 is also demonstrated (T). Genomic DNA was amplified using primers from a microsatellite in the p53 gene (1), the Rb gene (2,3), the D14S426 locus (4), the DMI gene (5), the GABRB3 gene (6, 7), the BAT40 (8), and the BAT25 (9). A different banding pattern is evident on comparison of paired samples from individual patients.

homogenous group with regard to the primary type of tumor or the treatment given. This was particularly striking in the cases diagnosed during the last 5 years (Fig 2). The most prominent features of this group were: young age at the time of diagnosis of the primary malignancy, more than one primary tumor, and a family history of each cancer. Each of these characteristics suggests a genetic susceptibility to cancer. Most patients received heavy treatment with multiple agents for their primary malignancies, and in most cases, t-leuk was characterized by cytogenetic aberrations (including del (17p) or -17 in 13% of the patients), MDR1 overexpression and a poor therapeutic outcome.

The fact that p53 has been shown to accumulate in cultured cells treated with chemoradiotherapy prompted us to study the P53 gene in these patients. We found a high frequency of mutations in this gene (38%). This is in contrast to patients with primary AML and MDS in whom the frequency of p53 mutations was found to be low, 13 of 204 AML patients (6%) and 12 of 258 MDS patients (5%). Despite the suggestive evidence for the familial nature of t-leuk and t-MDS in our patients, including the high frequency of p53 lesions found, all the mutations encountered were restricted to the leukemic cells and were non-germline in nature. Furthermore, different mutations were found in a variety of primary tumors in individual patients. Taken together, these data suggest that these patients have a predisposition to acquire mutations, known as a mutator phenotype.

The concept that oncogenesis involves a mutator phenotype has recently been considered by several investigators. The first set of genes to be studied were those that participate in mismatch repair and are responsible for correcting replication errors. Five human genes involved in DNA mismatch repair, MSH2, MLH1, PMS1, PMS2, and GTBP have recently been cloned and characterized. Germline mutations in these genes cause heritable susceptibility to colorectal and related cancers. In our series of patients, mismatch repair genes could not be studied directly because of insufficient amounts of DNA and RNA. Therefore, we looked for microsatellite instability as an indicator of a mutator phenotype. Each microsatellite contains multiple repetitive nucleotide sequences that are relatively constant in normal cells. Variation in the repeat number and the microsatellite's length, due to slippage during DNA replication, may be caused by a variety of factors. Microsatellite variations reflect genomic instability and have been observed in certain tumors. In some of these cancers, they occur concomitantly with mutations in mismatch repair genes. The latter appear to be an early event in the development of cancer, as they have been demonstrated in diploid colon cancer cells. It was suggested that the mutations in "stability" genes generate a series of secondary mutations
Throughout the genome. Some of these involve oncogenes that alter the regulatory mechanisms of the cell, which may lead to the development of tumors. It is, therefore, significant that we found microsatellite instability in up to 94% of the patients with t-leuk or t-MDS, suggesting a mutator phenotype in these patients. The fact that we found microsatellite instability at the p53 locus in all the cases who had a p53 mutation, but in only one of the patients who lacked a p53 mutation, is an intriguing finding. This raises the question of the biological role of microsatellite instability of a given gene on the generation of mutations in that gene.

Our suggested hypothesis for the development of t-leuk or t-MDS is as follows: patients who have a mutator phenotype because of germline inherited mutations in a stability gene, are likely to develop cancer at an early age. The treatment given for the primary malignancy appears to accelerate DNA instability in many additional cancer-associated genes (eg, oncogenes and tumor suppressor genes), contributing to the development of secondary malignancies. An alternative hypothesis is that the treatment given for the primary disease causes acquired mutations in DNA stability genes. Thus, identification of the genes involved in the mutator phenotype would allow us to distinguish between these two possibilities.

The ability to identify patients who have preexisting abnormalities of these genes could enable the development of strategies to prevent DNA damage to the BM. These could include cryopreservation of uninvolved autologous marrow before the administration of chemotherapy in specific cases. As a first step in this direction, we are currently studying the mismatch repair genes in the increasing number of newly diagnosed patients with t-leuk or t-MDS.

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References

MUTATOR PHENOTYPE IN THERAPY-RELATED LEUKEMIA


Microsatellite instability and p53 mutations in therapy-related leukemia suggest mutator phenotype

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