Advances in Brief

p53 Mutations in Leukemia and Myelodysplastic Syndrome after Ovarian Cancer[1]

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Abstract

Purpose: **Although *p53* mutations occur in alkylating agent-related leukemias, their frequency and spectrum in leukemias after ovarian cancer have not been addressed. The purpose of this study was to examine *p53* mutations in leukemias after ovarian cancer, for which treatment with platinum analogues was widely used.**

Experimental Design: **Adequate leukemic or dysplastic cells were available in 17 of 82 cases of leukemia or myelodysplastic syndrome that occurred in a multicenter, population-based cohort of 23,170 women with ovarian cancer. Eleven of the 17 received platinum compounds and other alkylating agents with or without DNA topoisomerase II inhibitors and/or radiation. Six received other alkylating agents, in one case, with radiation. Genomic DNA was extracted and *p53* exons 5, 6, 7, and 8 were amplified by PCR. Mutations and loss of heterozygosity were analyzed on the WAVE instrument (Transgenomic) followed by selected analysis by sequencing.**

Results: **Eleven *p53* mutations involving all four exons studied and one polymorphism were identified. Genomic DNA analyses were consistent with loss of heterozygosity for four of the mutations. The 11 mutations occurred in 9 cases, such that 6 of 11 leukemias after platinum-based regimens (55%) and 3 of 6 leukemias after other treatments (50%) contained *p53* mutations. Two leukemias that occurred after treatment with platinum analogues contained two mutations. Among eight mutations in leukemias after treatment with platinum analogues, there were four G-to-A transitions and one G-to-C transversion.**

Conclusions: p53 **mutations are common in leukemia and myelodysplastic syndrome after multiagent therapy for ovarian cancer. The propensity for G-to-A transitions may reflect specific DNA damage in leukemias after treatment with platinum analogues.**

Introduction

Leukemias comprise a small fraction of all second cancers, although they represent the major carcinogenic complication of chemotherapy (reviewed in Ref. 1). Since the first observations of alkylating agent-related leukemia and MDS[3] (2), leukemia has become increasingly common after effective chemotherapy (reviewed in Ref. 3). Chemotherapy is implicated in AML of several morphological subtypes, MDS, ALL, and even chronic myelogenous leukemia (reviewed in Ref. 1). Therapeutic radiation also has been linked to an increased risk of leukemia (4, 5).

Two broad classes of cytotoxic drugs are associated with leukemia: alkylating agents and DNA topoisomerase II inhibitors (1, 3, 6). Germ-line mutations in the conserved region of the *p53* gene, a critical element in the cellular DNA damage response pathway, have been associated with leukemias after alkylating-agent treatment (7–9), suggesting that certain individuals are genetically predisposed. Complex numerical and structural karyotypic abnormalities in alkylating-agent-related leukemias associated with germ-line *p53* mutations, typically including loss of chromosomes 5, 7, and 17p, suggest genomic instability, which accompanies loss of wild-type p53 (7–9). In a recent study, *p53* mutations were observed in the leukemic cells in 21 of 77 unselected cases (27%) of treatment-related AML

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[3] The abbreviations used are: MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; LOH, loss of heterozygosity; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts.

and treatment-related MDS (10). Alkylating-agent exposure was documented in 19 of the 21 cases with *p53* mutations, indicating that *p53* mutations are common molecular alterations in AML and MDS after alkylating-agent treatment (10). In contrast, in DNA topoisomerase II inhibitor-related leukemias, *p53* mutations generally are absent, suggesting that the p53 component of the DNA damage response pathway is intact (7).

The classic alkylating agents used as anticancer drugs include nitrogen mustard and its analogues (11, 12). These agents form covalent bonds with bases in the DNA and can cause interstrand cross-links. Resultant *O*-6-methylguanine residues are carcinogenic (11, 12). Although not a classic alkylating agent, cisplatin, which has been in clinical use since 1969, forms intrastrand *N*-7-alkyl adducts on adjacent deoxyguanosines or deoxyguanosine and deoxyadenosine (11, 13, 14). Monoadducts and interstrand cross-links can also be formed by cisplatin (11, 13, 14). The platinum analogues cisplatin and carboplatin typically are used in combination with other leukemogenic drugs. In the early 1990s, it was suggested that cisplatin administered with DNA topoisomerase II inhibitors could be leukemogenic (13, 15). AML with the t(8;21) translocation was observed after osteosarcoma treatment with cisplatin and the DNA topoisomerase II inhibitor doxorubicin (15). Cases of AML with translocations involving chromosome band 11q23 or 21q21, recurrent translocations in leukemias related to DNA topoisomerase II inhibitor, were reported after treatment of germ-cell tumors with cisplatin and etoposide (16–18). Characteristic of alkylating-agent-related leukemias, other leukemias that occurred after regimens including platinum analogues contained monosomies of chromosomes 5 and 7 (8, 9, 19).

Although platinum analogues are instrumental in ovarian-cancer treatment (20), they are linked with significantly increased, dose-dependent risks of leukemia (21). In a large, case-control study of women with ovarian cancer who received cisplatin- or carboplatin-containing regimens, a 4-fold risk of leukemia was observed (trend for cumulative platinum dose, $P < 0.001$; Ref. 21). A similar, highly significant dose-response relationship was found between the cumulative cisplatin dose used in testicular cancer treatment and leukemia risk (22). However, few studies have described the molecular features of leukemias after successful treatment of ovarian cancer, and data on specific *p53* mutations that incorporate cumulative doses of cytotoxic drugs and radiation are sparse. The goal of the present study was to determine the frequency and spectrum of mutations in conserved exons of the *p53* gene in cases of leukemia after ovarian-cancer treatment.

Patients and Methods

Study Population. All available tissue samples were requested from five of seven population-based cancer registries that participated previously in an international case-control investigation of leukemia after ovarian cancer (21). The case-control investigation was conducted within a population-based cohort that included 28,971 women with ovarian cancer in which 96 cases of leukemia were observed (21). Of the 96, 82 were reported to the five cancer registries participating in the present study (Ontario, Sweden, New Jersey, Iowa, Finland) and occurred within the cohort of 23,170 women with ovarian cancer in these registries. Tissue samples were obtained from 27 (33%) of the 82 cases. There were sufficient archived bone marrow aspirate specimens or, in one case, peripheral blood with evidence of leukemia or MDS for *p53* mutation analysis in 17 cases. Ten cases in which materials were available were excluded because of insufficient amount or poor quality of DNA. Molecular studies are difficult on tissues preserved in fixatives other than formalin or alcohol. Bone marrow core biopsies are unusable because decalcification is incompatible with molecular studies. Therefore, several available tissue samples could not be used for the present study. Data on specific cytogenetic aberrations were not available because the diagnoses of leukemia and MDS in the population-based cohort occurred in many different institutions, which were often local hospitals where cytogenetic analyses were not performed routinely.

DNA Preparation. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks of bone marrow aspirates. 15–20-μm thick sections were first cut from a control blank block and then from the blocks containing tissue. The sections were deparaffinized with xylenes followed by 100% ethanol. Deparaffinized sections were incubated at 55°C for 1–3 h in 200 μL of a solution containing 288 μg proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) and ×1 PCR buffer (PE Biosystems, Foster City, CA). To deactivate the proteinase K, the mixture was incubated at 100°C for 10 min, followed by microcentrifugation at 13,500*g* for 10 min. The supernatant containing the DNA was removed to a clean, 1.5-ml microcentrifuge tube and used directly for PCR.

For genomic DNA extraction from bone marrow or peripheral blood smears on glass slides, 50 to 100 μL of Cell Lysis Solution (Gentra Systems, Inc., Minneapolis, MN) containing one-tenth volume of 14.4 mg/ml proteinase K solution (Boehringer Mannheim Biochemicals) were added directly to the slides, causing solubilization and lysis of the cells. The solution was transferred to a 1.5-ml microcentrifuge tube and incubated at 55°C for 1–3 h. One-third volume of Protein Precipitation Solution (Gentra Systems) was added and the mixture was vortex-mixed and microcentrifuged at 13,500*g* for 10 min. The supernatant was transferred to a clean 1.5-ml microcentrifuge tube. After the addition of an equal volume of isopropanol, the mixture was incubated at −20°C from 20 min to overnight and microcentrifuged at 13,500*g* for 20 min. The supernatant was removed, and the DNA pellet was washed with 70% ethanol, dried, and, depending on the pellet size, resuspended in 30–100 μL of 10 mM Tris/0.1 mM EDTA solution for use in PCR.

PCR Amplification of *p53* Exons 5–8. Exons 5, 6, 7, and 8 of the *p53* gene and flanking intronic sequences were amplified in separate PCRs. The 50-μl reaction mixtures contained 1 μl of DNA, sense and antisense primers at 0.2 μM each, 0.2 mM each dNTP, 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer; Foster City, CA), 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.4. A master mixture of all reagents except AmpliTaq Gold DNA polymerase and DNA was made in advance and stored at −20°C for up to 2 months. Similar primers have been used previously (23). The sense and antisense primers for the *p53* exons were as follows: *p53* exon 5, 5′-TTCCTCTTCCTACAGTACTC-3′ and 5′-GCAACCAGCCCTGTCGTCTC-3′; *p53* exon 6, 5′-ACCATGAGCGCTGCT-

CAGAT-3′ and 5′-AGTTGCAAACCAGACGTCAG-3′ *p53* exon 7, 5′-GTGTTGTCTCCTAGGTTCGC-3′ and 5′-CAAGTGGCTCCTGACCTGGA-3′; and *p53* exon 8, 5′-CCTATCCTGAGTAGTGGTAA-3′ and 5′-TGAATCTGAGGCATAACTGC-3′. Underlined nucleotides in the exon 6 and 7 primers are mismatches with the *p53* genomic sequence. Expected PCR-product sizes were 238, 236, 139, and 212 bp for *p53* exons 5, 6, 7, and 8, respectively. Amplification was performed in a Perkin-Elmer 9600 thermal cycler (Emeryville, CA). The three-step PCR consisted of an initial cycle at 95°C for 11 min, 55°C for 30 s, and 72°C for 1 min, followed by 44 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, a final extension at 72°C for 3 min and a hold at 4°C. Samples that did not amplify with 1.25 units of AmpliTaq Gold were amplified again with 2.5 units of AmpliTaq Gold and an additional 10 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for a total of 55 cycles. For amplification of exon 7, 2.5 units of AmpliTaq Gold were used and the cycling included 94°C for 9 min followed by 45 cycles of 94°C for 75 s, 58°C for 75 s, and 72°C for 30 s (increase elongation time by 1 s/cycle) followed by 72°C for 10 min and a hold at 4°C.

Genomic DNA samples with known *p53* mutations in exons 5, 6, 7, and 8 were used as positive controls (24–26). The positive control DNA for exon 5 was from ALL cells with a TGC-to-TTC transversion at codon 141 (24). The positive control DNA for exon 6 was from ALL cells with a 2-bp (TA) deletion at codons 214–215 (25). The positive control DNA for exon 7 was from ALL cells with a TTT-to-TGT transversion at codon 270 (25). The positive control DNA for exon 8 was from the RD cell line with a CGG-to-TGG transition at codon 248 (26). DNA from peripheral blood mononuclear cells of a normal subject was used as a negative control.

WAVE Detection System for *p53* Mutations. The presence of a mutation was detected with the WAVE System (Transgenomic, Omaha, NE). The method is based on ion-pair, reverse-phase high-performance liquid chromatography and temperature-modulated heteroduplex analysis. Sequence variations create mismatched heteroduplexes during reannealing of PCR products containing wild-type and mutant sequences. The difference in melting temperature between heteroduplexes and homoduplexes allows for their separation by ion-pair, reverse-phase high-performance liquid chromatography and identification of the presence of mutations. PCR products with mutant sequences were detected with the protocol specified by the manufacturer. First, 5 μL of the PCR product were run under nondenaturing conditions to determine DNA concentrations. PCR products then were mixed 1:1 by concentration with PCR products from negative control DNA to assure detection of mutant sequences in the presence of LOH. The mixtures were denatured at 95°C for 5 min followed by a 45-min ramp to 25°C and incubation at 25°C for 30 s. The denatured, reannealed samples were run on the WAVE instrument with a melting temperature of 63°C and the regular gradient for *p53* exon 5, 62°C and the regular gradient for *p53* exon 6, 61°C and a gradient shifted by −3° for *p53* exon 7, and 63°C and the regular gradient for *p53* exon 8. WAVEMaker software was used to calculate the regular gradient for each PCR product on the basis of the length and melting temperature of each DNA fragment.

Sequence Confirmation of Mutations. The specific sequence alteration in each PCR product with a mutant sequence or polymorphism detected by WAVE analysis was identified by direct sequencing. The PCR product was directly sequenced first. If the mutation was not identified by direct sequencing, the heteroduplex peak from WAVE analysis containing 50% mutant DNA and 50% normal DNA was collected and the collected peak was sequenced. The eluted peak containing the heteroduplex DNA was collected in a fraction collector according to the WAVE System protocol. Mutations in collected peaks were confirmed by direct sequencing in both directions with the Big Dye Terminator Kit (PE Applied Biosystems) and the Prism ABI 310 instrument (PE Applied Biosystems).

A base substitution in the sequence and absence or near absence of the normal sequence was interpreted as evidence of LOH, although the unlikely presence of the same mutation in both *p53* alleles was not excluded. Detection of a heterozygous pattern by direct sequencing could have been attributable to either heterozygosity in the leukemic cells or the presence of nonleukemic cells in the specimen. Therefore, in cases with substantial nonleukemic cells, as in MDS, LOH in the leukemic cells could not be excluded by the heterozygous pattern.

Analysis of Sensitivity. The sensitivity of mutation detection by WAVE analysis for the primers and conditions used was evaluated by serial dilution of DNA samples with known *p53* mutations into normal DNA.

Statistical Analyses. Data analyses were performed by Wilcoxon's rank-sum test. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

The *p53* gene was examined in secondary leukemia or secondary MDS specimens from 17 women with primary ovarian cancer. Clinicopathological data including demographic features, all therapy for ovarian cancer, latency and type of leukemia are summarized in Table 1. Cisplatin and/or carboplatin were administered in 11 cases (patients 1, 2, 3, 5, 6, 7, 8, 10, 12, 14, 17) in combination with other alkylating agents in four cases, or in combination with both other alkylating agents and DNA topoisomerase II inhibitors in the other seven. Patient 3 received both cisplatin and carboplatin. Table 1 shows the cumulative platinum analogue doses expressed in total milligrams in accordance with the underlying case-control study from which these patients were derived (21). The median cumulative cisplatin dose administered to the nine patients who received cisplatin (patients 1, 2, 3, 5, 6, 8, 10, 12, 14) was 710 mg (range, 422–910 mg). The median cumulative carboplatin dose administered to the three patients who received carboplatin (patients 3, 7, 17) was 2660 mg (range, 1710–3550 mg), and the median cumulative carboplatin dose expressed as a cisplatin-equivalent dose (27) was 665.0 mg (range 427.5–887.5 mg). In six patients, therapy included alkylating agents but not platinum analogues (patients 4, 9, 11, 13, 15, 16). Three patients also received radiation (patients 5, 8, 11), and the radiation dose to total active bone marrow is shown in Table 1.

Eight of the 17 patients presented with AML, one with chronic myelogenous leukemia and eight with MDS. Latencies between the diagnosis of ovarian cancer and the diagnosis of

Table 1 Clinicopathological features and secondary leukemias in patients with primary ovarian cancer

Patient	Age at OV[a] diagnosis (yrs)	mo/yr at OV diagnosis	OV stage	Chemotherapy	CDDP dose (mg)	Radiation dose to TABM (Gy)	Interval between OV diagnosis and leukemia/MDS (yrs)	Morphology of leukemia/MDS	Vital status after leukemia/ MDS (living or dead)	Interval between leukemia/MDS and last known vital status (yrs)
1	54	10/88	III	CPM, **CDDP**[b] (12 cycles)/5-FU, MTX (9 cycles)	710	None	3.2	RAEB	Dead	0.2
2	61	09/88	IV	**CDDP**, ADR, CPM (12 cycles)/IFOS (8 cycles)	880	None	4.7	RAEBT	Dead	1.0
3	69	01/87	IV	CPM, **CDDP** (8 cycles)/CPM (22 cycles)/ **CBDCA**, CPM (6 cycles)	650, 1710[c]	None	5.3	AML, NOS	Dead	<0.1
4	67	01/84	IV	CHLB (12 mos)	None	None	5.1	AML, NOS	Dead	0.1
5	67	03/85	II	**CDDP**, ADR, CPM (10 cycles)/HMM, MTX, 5-FU (10 mos)/VP-16, **CDDP** (2 cycles)/ MITC, VLB (18 mos)	630	8.9	5.1	AML, FAB M2	Dead	<0.1
6	57	05/89	III	**CDDP**, ADR, CPM (7 cycles)	840	None	1.3	AML, FAB M4	Dead	0.1
7	51	12/89	IV	**CBDCA**, CPM (6 cycles)	3550[c]	None	2.8	CML	Living	2.5
8	63	02/80	II	**CDDP**, ADR, CPM (6 cycles)	422	19.0	7.0	AML, NOS	Dead	0.1
9	72	02/81	II	CPM (15 cycles)	None	None	1.9	AML, NOS	Dead	0.1
10	45	02/82	IV	HMM, CPM, ADR, **CDDP** (8 cycles)/LPAM (15 cycles)	546	None	2.5	RARS	Dead	1.0
11	35	07/83	I	LPAM (18 cycles)	None	13.4	4.8	RAEB	Dead	1.1
12	67	10/84	IV	CPM, **CDDP** (7 cycles)/ADR, CPM (2 cycles)/LPAM (13 cycles)	881	None	2.6	MDS, NOS	Dead	0.8
13	76	05/85	III	CHLB (23 mos)	None	None	4.0	MDS, NOS	Dead	1.8
14	67	05/89	IV	CPM, **CDDP** (9 cycles)/LPAM (6 cycles)	910	None	1.3	RAEB	Dead	0.4
15	72	04/81	III	LPAM (19 cycles)	None	None	7.7	AML, FAB M1	Dead	<0.1
16	69	01/85	I	LPAM (15 cycles)	None	None	1.7	AML, NOS	Dead	<0.1
17	57	07/81	III	ADR, CPM (4 cycles)/LPAM, HMM (11 cycles)/**CBDCA** (5 cycles)	2660[c]	None	7.8	MDS, NOS	Dead	0.6

[a] OV, ovarian carcinoma; CDDP, cisplatin; TABM, radiation dose to total active bone marrow; CPM, cyclophosphamide; 5-FU, 5-fluorouracil; MTX, methotrexate; ARD, doxorubicin; IFOS, ifosfamide; RAEBT, RAEB in transformation; CBDCA, carboplatin; NOS, not otherwise specified; CHLB, chlorambucil; HMM, hexamethylmelamine (altretamine); VP-16, etoposide; MITC, mitomycin C; VLB, vinblastine; LPAM, melphalan; FAB, French-American-British morphology; FAB M2, acute differentiated myelogenous leukemia; FAB M4, acute myelomonocytic leukemia; FAB M1, acute undifferentiated myelogenous leukemia.

[b] Bold text indicates platinum analogues.

[c] CBDCA dose (mg).

leukemia or MDS ranged from 1.3 to 7.8 years (median, 4.0 years). Sixteen of the 17 patients are deceased. The median duration of survival from time of diagnosis of secondary leukemia or secondary MDS to death or end of study was 0.2 year (range, <0.1 to 2.5 years).

Eleven *p53* mutations and one known *p53* exon 6 polymorphism were detected in 9 of the 17 cases studied (Table 2). Serial-dilution experiments showed that the presence of 3.25% mutant allele in a wild-type background was detectable by WAVE analysis with the primers and conditions used for *p53* exons 5, 6, 7, and 8, indicating that somatic-mutation detection was feasible in specimens containing a significant proportion of nonleukemic cells.

Three mutations involved exon 5. The codon 141 TGC-to-TAC transition in the AML of patient 3 was associated with LOH (Table 2; Fig. 1) and would result in a Cys-to-Tyr substitution. The French-American-British M2 AML of patient 5 contained both an exon 5 codon 143 GTG-to-ATG transition that would result in a Val-to-Met substitution and an exon 8 codon 274 GTT-to-GCT transition that would change Val to Ala; LOH was not detected at either of these codons. The codon 134 TTT-to-CTT transition in the AML of patient 16 would result in a Phe-to-Leu change and was not associated with LOH.

One mutation and one polymorphism were identified in exon 6. In patient 10 with RARS, analysis of DNA samples from a paraffin-embedded bone marrow aspirate and from a bone marrow smear, both from the time of MDS diagnosis, indicated a codon 220 TAT-to-TGT transition that would result in a Tyr-to-Cys substitution. LOH was detected in one of the specimens (bone marrow smear slide) containing both normal and dysplastic cells (Table 2; Fig. 2). In the RAEB of patient 14, WAVE positivity identified an exon 6 sequence alteration. Direct sequencing was consistent with the known CGA-to-CGG polymorphism involving codon 213 (28), although a new, silent mutation was not excluded because DNA from normal tissue from the patient was not available for comparison. In the RAEB of the same patient, there also was an exon 7 codon 236 TAC-to-TGC transition that would result in a Tyr-to-Cys substitution with suggested LOH (Table 2).

In addition to the *p53* codon 236 mutation in the RAEB of patient 14, there were three other mutations involving exon 7. The RAEB of patient 1 contained both an exon 7 codon 248 CGG-to-CCG transversion that would change Arg to Pro (Table 2; Fig. 3, *A* and *B*) and an exon 8 codon 273 CGT-to-CAT transition that would change Arg to His (Table 2; Fig. 4, *A* and *B*). LOH was suggested at codon 248, but not at codon 273. Alternatively, if both *p53* alleles were present in the affected cells, the same exon 7 mutation may have been present on both alleles (see "Discussion"). The AML of patient 15 contained the same codon 248 CGG-to-CCG transversion with suggested LOH (Table 2). The AML of patient 4 contained a silent codon 236 TAC-to-TAT transition without LOH (Table 2).

One other *p53* exon 8 mutation was identified in addition to the codon 274 mutation in the AML of patient 5 and the codon 273 mutation in the RAEB of patient 1. The MDS of patient 12 contained a codon 275 TGT-to-TAT transition that was associated with LOH and would change Cys to Tyr (Table 2).

In two of the 17 leukemias (patients 15, 16), not all four exons were examined either because there was no amplification or because there was insufficient DNA for additional PCR analyses.

Specific genotoxic exposures relative to the observed *p53* mutations are summarized in Table 3. Six of 11 leukemias after platinum-based therapy contained *p53* mutations; in two of these six cases (patients 1, 5), there were two mutations. In the leukemias of patients exposed to any platinum analogue, the mutations were predominantly transitions; seven transitions and only one transversion were observed. Four of these seven transitions were G-to-A substitutions.

The median ages at primary cancer diagnosis in cases with and without *p53* mutations of 67.0 years (range, 45–72 years) and 59.0 years (range, 35 to 76 years), respectively, were not significantly different (rank-sum test, $P > 0.05$). In patients whose leukemias harbored *p53* mutations, the median latency from ovarian-cancer diagnosis to leukemia diagnosis was 3.2 years (range, 1.3 to 7.7 years), compared with 4.4 years (range, 1.3 to 7.8 years) in cases without *p53* mutations (rank-sum test, $P > 0.05$).

Discussion

We investigated the frequency of *p53* mutations in all available cases of leukemia and MDS after primary ovarian cancer within five of seven population-based cancer registries participating in an epidemiological study of treatment-related leukemia (21). The percentages of cases in which material was available (33%) and in which the material was adequate (21%) is comparable with proportions collected for other molecular studies undertaken in these or similar population-based cancer registries (29, 30). Because the retrospective availability and adequacy of archived materials is unlikely related to the occurrence of selected molecular alterations, but rather reflective of hospital protocol and storage practices, the results should not be biased.

Eleven confirmed mutations and one known polymorphism occurred in 9 of 17 cases, indicating that *p53* mutations are common in this setting. Previous therapy included a platinum analogue in six of the nine cases with mutations and other alkylating agents without a platinum analogue in the other three. *p53* mutations were present in 6 of 11 leukemias after platinum-based regimens. In two of these six cases, there were two mutations; in one case, there was a polymorphism as well as a mutation.

We and others previously observed associations between germ-line *p53* mutations and leukemias after alkylating-agent treatment (7–9). The types of malignancies with which germ-line *p53* mutations are associated generally portend the types of malignancies where somatic *p53* mutations will be found. Indeed, Christiansen *et al.* (10) recently demonstrated that *p53* mutations are common in leukemias after alkylating-agent treatment. Of 21 secondary leukemias with *p53* mutations, 17 occurred after exposure to a classic alkylating agent, and two occurred after exposure to cisplatin (10). The purpose of the present study was to examine *p53* mutations in leukemias after primary ovarian cancer, for which platinum analogues and other alkylating agents have been widely used. The results demonstrate prevalent *p53* mutations in leukemias after treatment with platinum analogues, as well as after other treatments. The high frequency of *p53* mutations in leukemia and MDS after ovarian-

Table 2 *p53* mutations in cases of leukemia and MDS after ovarian-cancer treatment

Patient No.	PET[a] or slide	Tissue source	Status at sampling	Blasts in sample (%)	*p53* exon 5	*p53* exon 6	*p53* exon 7	*p53* exon 8
1	Slide	BM aspirate	RAEB	16	Negative	Negative	QNS	273 het CGT-CAT, Arg-His
	Slide	BM aspirate	RAEB	15	Negative	Negative	248 homo CGG-CCG, Arg-Pro	273 het CGT-CAT, Arg-His
	Slide	BM aspirate	RAEB	11	Negative	Negative	QNS	273 het CGT-CAT, Arg-His
2	PET	BM aspirate	RAEBT	25	Negative	Negative	Negative	Negative
3	Slide	PB	AML	23	141 homo TGC-TAC, Cys-Tyr	Negative	Negative	Negative
4	PET	BM aspirate	AML	50	Negative	Negative	236 het TAC-TAT, Tyr-Tyr	Negative
5	PET	BM aspirate	AML	40	143 het GTG-ATG, Val-Met	Negative	Negative	274 het GTT-GCT, Val-Ala
6	PET	BM aspirate	AML	>90	Negative	Negative	Negative	Negative
7	PET	BM aspirate	CML		Negative	Negative	Negative	Negative
	PET	BM aspirate	CML/AML	30–40	Negative	Negative	Negative	Negative
8	Slide	BM aspirate	AML	30	Negative	Negative	Negative	Negative
9	Slide	BM aspirate	Pre-AML	10–15	Negative	No amplification	Negative	Negative
	Slide	BM aspirate	AML	23	Negative	Negative	Negative	Negative
10	PET	BM aspirate	RARS	NA	Negative	220 het TAT-TGT, Tyr-Cys	Negative	Negative
	Slide	BM aspirate	RARS	NA	Negative	220 homo TAT-TGT, Tyr-Cys	Negative	Negative
11	Slide	BM aspirate	RAEBT	17–24	Negative	Negative	Negative	Negative
	Slide	BM aspirate		43	Negative	Negative	Negative	Negative
	Slide	BM aspirate		27	Negative	Negative	No amplification	QNS
	Slide	BM aspirate		6–17	Negative	Negative	Negative	Negative
12	PET	BM aspirate	MDS	10	Negative	Negative	Negative	275 homo TGT-TAT, Cys-Tyr
13	Slide	BM aspirate	MDS	42	Negative	Negative	Negative	Negative
14	PET	BM aspirate	MDS	10	Negative	213 het CGA-CGG, Arg-Arg	236 homo TAC-TGC, Tyr-Cys	Negative
15	PET	BM aspirate	AML	NA	No amplification	No amplification	248 homo CGG-CCG, Arg-Pro	No amplification
16	PET	BM aspirate	AML	NA	134 het TTT-CTT, Phe-Leu	No amplification	No amplification	Negative
17	Slide	BM aspirate	MDS	15–20	Negative	Negative	Negative	Negative

[a] PET, paraffin-embedded tissue; BM, bone marrow; QNS, insufficient DNA for PCR; RAEBT, RAEB in transformation; PB, peripheral blood smear; CML, chronic myelogenous leukemia; NA, not available.

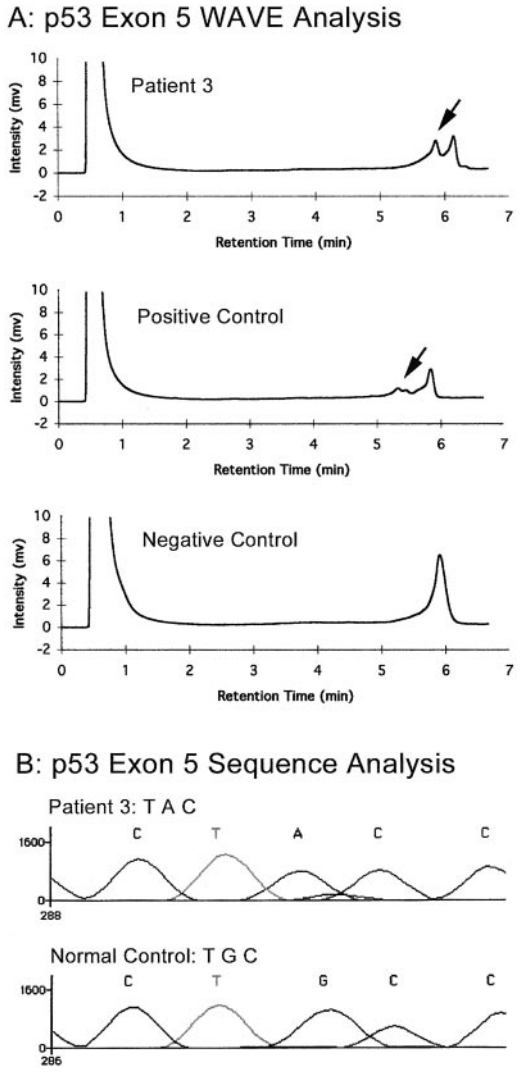

Fig. 1 Detection of *p53* exon 5 mutation in AML of patient 3 using the WAVE system (Transgenomic). *A, p53* exon 5 PCR product from AML DNA of patient 3 was mixed with an equal amount of wild-type exon 5 PCR product and analyzed on the WAVE instrument (*top panel*). *Arrows* indicate heteroduplex peaks in the AML specimen (*top panel*) and in the positive control (*middle panel*). Under the partially denaturing temperature conditions of the assay, heteroduplexes are denatured and eluted from the column before homoduplexes. There was no heteroduplex peak in the negative control (*bottom panel*). *B,* direct sequencing of *p53* exon 5 PCR product confirmed a codon 141 TGC-to-TAC transition with associated LOH in AML of patient 3 (*top panel*). The *bottom panel* shows the normal sequence.

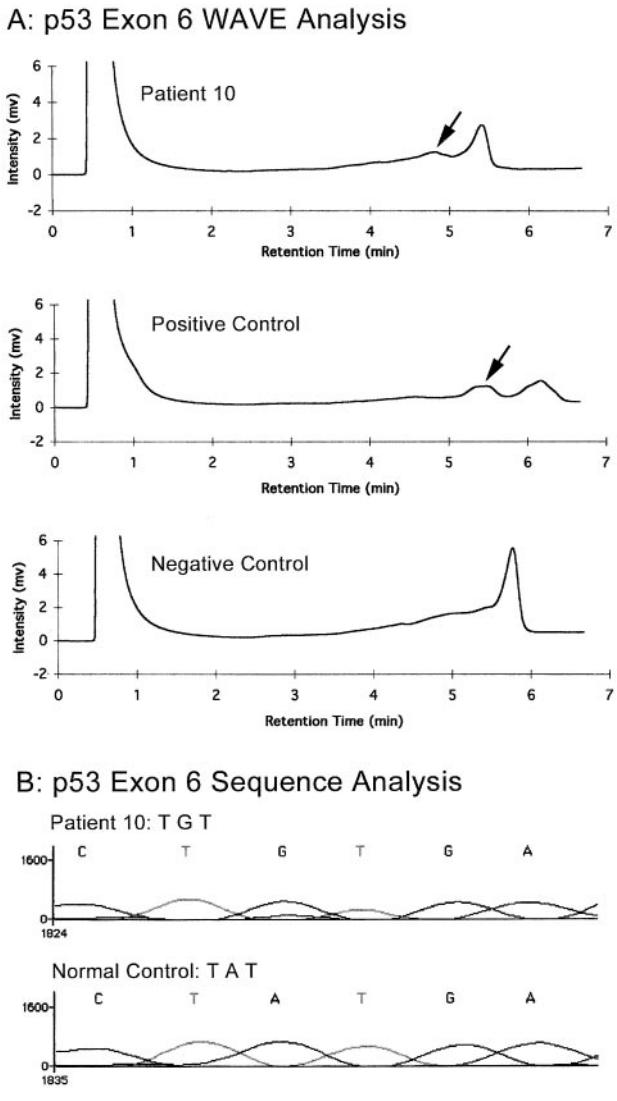

Fig. 2 Detection of *p53* exon 6 mutation in RARS of patient 10. *A, p53* exon 6 PCR product from RARS DNA of patient 10 was mixed with an equal amount of wild-type exon 6 PCR product and analyzed on the WAVE instrument (*top panel*). *Arrows* indicate heteroduplex peaks in the RARS specimen (*top panel*) and in the positive control (*middle panel*). There was no heteroduplex peak in the negative control (*bottom panel*). *B,* direct sequencing indicated that the *p53* exon 6 mutation in the RARS of patient 10 was a codon 220 TAT-to-TGT transition with LOH. Normal sequence is shown in the *bottom panel*.

cancer treatment is in contrast to lower reported frequencies of *p53* mutations in cases of primary MDS (31, 32). Sugimoto *et al.* (32) detected only three *p53* mutations in 50 cases of primary MDS or MDS-derived leukemia. Jonveaux *et al.* (31) identified five *p53* mutations among 135 cases of primary and 16 cases of secondary MDS.

The trend toward transition-type *p53* mutations, especially G-to-A transitions, in the leukemias after platinum analogues is noteworthy (Table 3). Although this observation may suggest that platinum analogues cause specific damage to the DNA, we remain circumspect given the low number of cases and the use of combination chemotherapy. Others have suggested that cisplatin preferentially induces mutations at G residues (10, 33). Consistent with these observations (10, 33), five of eight mutations in leukemias after treatment with platinum analogues in the present study (63%) involved G residues, including four G-to-A transitions and one G-to-C transversion. The other three mutations in leukemias after treatment with platinum analogues in the present study included two A-to-G transitions and one T-to-C transition. It has also been observed that cisplatin-

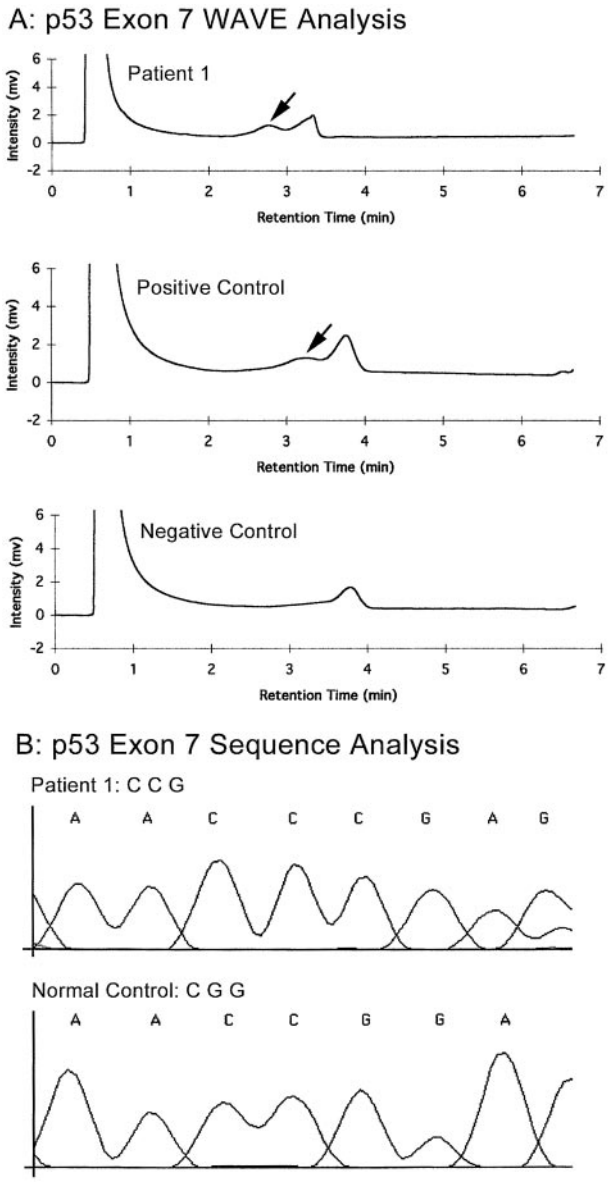

Fig. 3 Detection of *p53* exon 7 mutation in RAEB of patient 1 by WAVE analysis. *A*, *p53* exon 7 PCR product from RAEB DNA of patient 1 was mixed with an equal amount of wild-type exon 7 PCR product and analyzed on the WAVE instrument (*top panel*). *Arrows* indicate heteroduplex peaks in the RAEB specimen (*top panel*) and in the positive control (*middle panel*). A heteroduplex peak is absent in the negative control (*bottom panel*). *B*, direct sequencing of the exon 7 PCR product from the RAEB DNA of patient 1 revealed a codon 248 CGG-to-CCG transversion with LOH (*top panel*). The *bottom panel* shows the normal sequence.

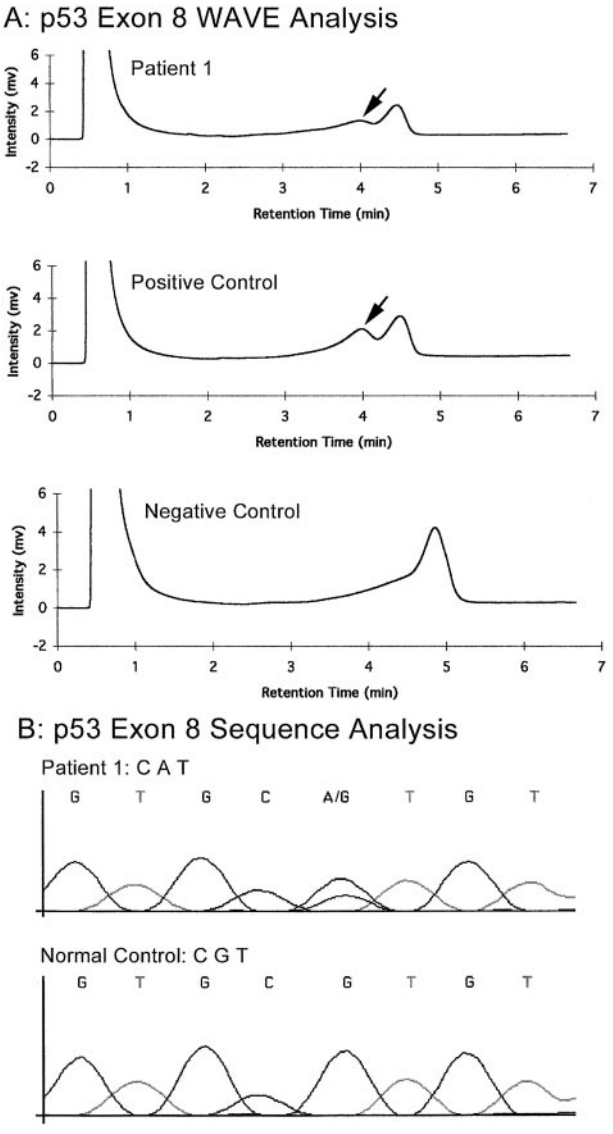

Fig. 4 Detection of *p53* exon 8 mutation in RAEB of patient 1 by WAVE analysis. *A*, *p53* exon 8 PCR product from RAEB DNA of patient 1 was mixed with an equal amount of wild-type exon 8 PCR product and analyzed on the WAVE instrument (*top panel*). *Arrows* indicate heteroduplex peaks in the RAEB specimen (*top panel*) and in the positive control (*middle panel*). Heteroduplex peak is absent in the negative control (*bottom panel*). *B*, LOH was not detected with the *p53* exon 8 codon 273 CGT-to-CAT transition in the RAEB of patient 1 as indicated by the normal G peak in addition to the mutant A peak in the sequence. The normal sequence is shown in the *bottom panel*. Apparent LOH in exon 7, but not in exon 8, may indicate the presence of the exon 7 mutation on both alleles (compare with Fig. 3).

induced mutations in the *aprt* gene in Chinese hamster ovary cells preferentially occurred at or proximal to 5′-AGG-3′ and 5′-GAG-3′ sequences (34). Seven of the eight *p53* mutations in leukemias after treatment with platinum analogues in the present study were proximal to the 5′-AGG-3′ or 5′-GAG-3′ sequences in either the sense or antisense strand of the DNA (Fig. 5). The two *p53* mutations described by Christiansen *et al.* (10) in leukemias after treatment with cisplatin were G-to-A transitions at 5′-AGG-3′ sequences. This mutation spectrum appears to be distinct from the preferred mutations at A:T base pairs suggested by Christiansen *et al.* (10) in leukemias after various cyclophosphamide-, cyclophosphamide-busulfan-, or chlorambucil-based treatments. Taken together with the study by Christiansen *et al.* (10), these results may suggest that the types of

Table 3 Exposures and *p53* mutations in cases of leukemia and MDS after ovarian cancer treatment

Exposure	Patients with mutations (*n*)	Patient no.	Exon	Codon	Mutation	Mutation type (*n*)	Amino acid change	LOH	Mutation frequency[a]
Platinum analogue, other alkylating agent	3	1 (2)[b]	7	248	C**G**G-C**C**G	Transversion	Arg-Pro	Yes	3/4
			8	273	C**G**T-C**A**T	Transition	Arg-His	No[c]	
		3	5	141	T**G**C-T**A**C	Transition	Cys-Tyr	Yes	
		14	6	213	CG**A**-CG**G**	Transition/Polymorphism	Arg-Arg (silent)	No	
			7	236	T**A**C-T**G**C	Transition	Tyr-Cys	Yes[c]	
Platinum analogue, other alkylating agent, DNA topoisomerase II inhibitor	2	10	6	220	T**A**T-T**G**T	Transition	Tyr-Cys	Yes	2/5
		12	8	275	T**G**T-T**A**T	Transition	Cys-Tyr	Yes	
Platinum analogue, other alkylating agent, DNA topoisomerase II inhibitor, radiation	1	5 (2)[b]	5	143	**G**TG-**A**TG	Transition	Val-Met	No	1/2
			8	274	G**T**T-G**C**T	Transition	Val-Ala	No	
Other alkylating agent	3	4	7	236	TA**C**-TA**T**	Transition	Tyr-Tyr (silent)	No	3/5
		15	7	248	C**G**G-C**C**G	Transversion	Arg-Pro	Yes	
		16	5	134	**T**TT-**C**TT	Transition	Phe-Leu	No	
Other alkylating agent, radiation	0	NA							0/1
Any platinum analogue	6	1, 3, 5, 10, 12, 14		See above		Transversion (1) Transition (7)	See above		6/11
No platinum analogue	3	4, 15, 16		See above		Transversion (1) Transition (2)	See above		3/6

[a] Mutation frequency indicates fraction of total patients studied in specific categories whose leukemic cells showed *p53* mutations.

[b] (2), two mutations in the same leukemic specimen.

[c] Suggestion of LOH at one codon but not another in the same leukemia may indicate the presence of the same mutation with suggested LOH on both alleles, or mixtures of leukemic and normal cells confounding LOH detection.

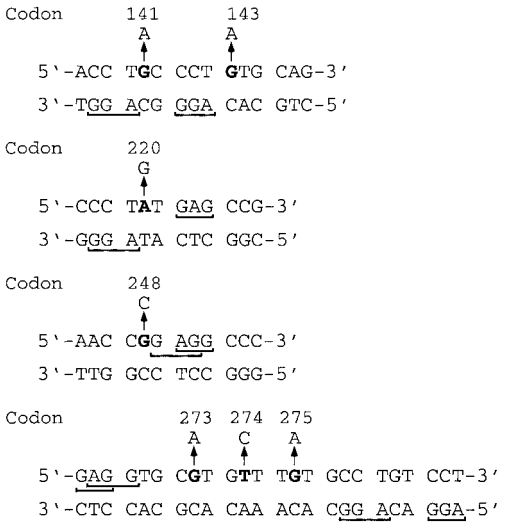

Fig. 5 5′-AGG-3′ and 5′-GAG-3′ sequences (34) proximal to identified *p53* mutations in leukemias after treatment with platinum analogues (*brackets*). Codons containing *p53* mutations are as indicated. *Arrows* show specific single base-pair changes in the coding strands. Mutations were verified by sequencing the noncoding strand (not shown).

p53 mutations in treatment-related leukemias are the result of specific drug exposures; however, the significance of the sequence specificity with respect to the formation of specific platinum adducts and their processing remains to be determined.

No archival germ-line tissues were available for analysis of constitutional DNA samples in this retrospective study, and the somatic *versus* germ-line origin of the observed *p53* mutations could not be assessed. It is likely, however, that they were of somatic origin. The ages of the women at primary cancer diagnosis generally were older than that of patients with the Li-Fraumeni syndrome; ovarian cancer also does not occur in classic Li-Fraumeni families with germ-line *p53* mutations (35, 36). In the leukemias in this study, the codon 248 CGG-to-CCG transversion that changes Arg to Pro, comprised 2 of the 11 mutations. Germ-line codon 248 mutations are prevalent in the Li-Fraumeni syndrome, but typically these are CGG-to-TGG (Arg-to-Trp) or CGG-to-CAG (Arg-to-Gln) transitions (35, 36). The codon 220 TAT-to-TGT transition observed in the leukemia of patient 10, however, has been reported in Li-Fraumeni syndrome (36).

Different types of *p53* mutations also were observed in other cases of leukemia after multimodality, alkylating-agent-containing regimens for sarcomas where the mutations were of germ-line origin (7–9, 37). The mutations in these cases included a 2-bp TA deletion at *p53* codon 209 (7), a CGA-to-TGA nonsense mutation at codon 306 (37), and 1- and 4-bp deletions, suggesting replication errors in regions of mononucleotide C repeats at codons 250 and 299–301 (8, 9). In contrast, in the present study, the mutations were all single-bp changes and either missense or, in one case, silent. The somatic *versus* germ-line origin of the frequent *p53* mutations in leukemias after primary ovarian cancer warrants future investigation including correlation with chemotherapy and radiotherapy exposures.

The sequence changes and resultant amino-acid substitutions in p53 associated with 70 cases of ovarian cancer have been studied previously (38). The five most frequent *p53* mutation sites in ovarian cancer were codons 273, 248, 282, 245, and 175; CGT-to-CAT transitions at codon 273 leading to Arg-to-His substitutions were most common (38). This same codon 273 transition represented only 1 of the 11 mutations in the leukemias in this series. The *p53* codon 248 mutations observed in ovarian cancer (38) differed from the *p53* codon 248 CGG-to-CCG transversion detected in two leukemias in the present study.

The results of genomic DNA analysis were consistent with LOH for 4 of the 11 identified mutations. The RAEB of patient 1 contained both exon 7 codon 248, and exon 8 codon 273 mutations, with LOH suggested for the exon 7 but not the exon 8 mutation. If both *p53* alleles were present, as suggested by the analysis of exon 8, the same exon 7 mutation may have been present on both alleles. Chromosomal reduplication of the allele containing the exon 7 mutation, followed by mutation of exon 8 on one allele, may be one explanation for this finding. Alternatively, mixtures of leukemic and normal cells in the dysplastic marrow may have confounded LOH detection for the exon 8 mutation, although this seems less likely because the amplification of exon 7 and exon 8 should have been affected equally by the presence of the normal cells. Similar explanations may apply to the RAEB of patient 14, in which there was an exon 7 codon 236 mutation with suggested LOH, but heterozygosity at codon 213 in exon 6. It has been proposed that RNA-based studies may be more sensitive than genomic-DNA approaches for LOH detection because mutant *p53* mRNA is generally more stable and present at high levels compared with very low-level *p53* mRNA expression in normal hematopoietic cells (10). RNA was unavailable for comparison in this study.

Cooperating mutations affecting proliferation and differentiation are believed essential to the pathogenesis of AML (39). The predicted tumor suppressor genes at critical regions of chromosomes 5q and 7q where deletions are observed in many alkylating-agent-related leukemias are yet to be identified (39). Specific, well-characterized molecular fusions involving *MLL*, and other balanced translocations, are considered primary alterations in DNA topoisomerase II inhibitor-related cases (reviewed in Refs. 40, 41). The nature of the mutations cooperating with these hallmark alterations, including *p53* mutations, are of major interest. *RAS* mutations are features of alkylating agent-related but not DNA topoisomerase II inhibitor-related cases (42, 43). Germ-line mutations in the *NF1* tumor suppressor gene, the product of which is a GTPase protein that accelerates GTP hydrolysis on Ras proteins (44), predispose to MDS with monosomy 7 after alkylating-agent treatment (45, 46). *p53* mutations are also features of alkylating agent-related cases (7, 10), but not of cases after DNA topoisomerase II inhibitors (7) as described above. Segmental jumping translocations are chromosomal abnormalities in which multiple copies of various oncogenes are dispersed throughout the genome and extrachromosomally (37, 47). Gene amplification potential accompanies loss of wild-type p53 (48), and *p53* mutations and *MLL* segmental jumping translocations are strongly correlated and occur after alkylating-agent treatment (49). The *p53* mutations may be of germ-line origin (37). Noteworthy is the recent finding that

internal tandem duplications in the FLT3 tyrosine kinase, which confer a myeloproliferative phenotype (50) and are common in *de novo* AML (39), are infrequent in treatment-related cases (51). Besides mutations in oncogenes and tumor suppressor genes, the observation of microsatellite instability has suggested that a mutator phenotype coupled with the relevant exposures may be of importance (52). In addition, genetic polymorphisms affecting the CYP3A4, NQO1, and GST drug-metabolizing enzymes confer susceptibility (53–55).

The WAVE system (Transgenomic; Ref. 56) provides a new approach for *p53*-mutation screening. Mutations and LOH can be analyzed with this automated scanning method, which resolves hetero- and homoduplexes using temperature-modulated liquid chromatography column separation (56). The method is gel-free (56) and, therefore, more rapid than single-strand conformation polymorphism analysis (7, 24–26, 37, 57–61), constant denaturant gel electrophoresis (62), or conformation-sensitive gel electrophoresis (53, 63). The sensitivity of the WAVE system for detection of mutations in other genes has been reported to approach 100% (64, 65). The WAVE system (56) proved a practical approach for rapid analysis of unknown *p53* mutations for this study.

A high frequency of *p53* mutations was observed in leukemia and MDS after primary ovarian-cancer treatment with regimens containing platinum analogues as well as other regimens. The acquisition of *p53* mutations in the setting of platinum-based chemotherapy has been reported in other tumor systems including an osteosarcoma cell line (66). Additional studies have suggested that resistance to chemotherapy and γ-radiation may exist in part because of the presence of mutant p53 or deleted p53 function (67–69). Both cisplatin and etoposide, as well as γ-radiation, induce wild-type p53 to activate downstream signals that initiate cell-cycle arrest and/or apoptosis. In the presence of mutant p53, these biological endpoints are not achieved and the cells become resistant (67–69). Experimental systems to investigate the relevance of *p53* mutations and alterations in activation of the downstream targets of p53 to the emergence of the leukemic clone in treatment-related leukemias should also be developed.

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