

Prevalence of the Inactivating $^{609}\text{C}\rightarrow\text{T}$ Polymorphism in the NAD(P)H:Quinone Oxidoreductase (NQO1) Gene in Patients With Primary and Therapy-Related Myeloid Leukemia

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NAD(P)H:quinone oxidoreductase (NQO1) converts benzene-derived quinones to less toxic hydroquinones and has been implicated in benzene-associated hematotoxicity. A point mutation in codon 187 (Pro to Ser) results in complete loss of enzyme activity in homozygous subjects, whereas those with 2 wild-type alleles have normal activity. The frequency of homozygosity for the mutant allele among Caucasians and African Americans is 4% to 5% but is higher in Hispanics and Asians. Using an unambiguous polymerase chain reaction (PCR) method, we assayed nonmalignant lymphoblastoid cell lines derived from 104 patients with myeloid leukemias; 56 had therapy-related acute myeloid leukemia (t-AML), 30 had a primary myelodysplastic syndrome (MDS), 9 had AML de novo, and 9 had chronic myelogenous leukemia (CML). All patients had their leukemia cells karyotyped. Eleven percent of the t-AML patients were homozygous and 41% were heterozygous for the NQO1 polymorphism; these proportions were significantly higher than those expected in a population of the same ethnic mix ($P = .036$). Of the 45 leukemia patients who had clonal abnormalities of chromosomes 5 and/or 7, 7 (16%) were homozygous for the inactivating polymorphism, 17 (38%) were heterozygous, and 21 (47%) had 2 wild-type alleles for NQO1. Thus, NQO1 mutations were significantly increased compared with the expected proportions: 5%, 34%, and 61%, respectively ($P =$

.002). An abnormal chromosome no. 5 or 7 was observed in 7 of 8 (88%) homozygotes, 17 of 45 (38%) heterozygotes, and 21 of 51 (41%) patients with 2 wild-type alleles. Among 33 patients with balanced translocations [14 involving bands 11q23 or 21q22, 10 with inv(16) or t(15;17), and 9 with t(9;22)], there were no homozygotes, 15 (45%) heterozygotes, and 18 (55%) with 2 wild-type alleles. Whereas fewer than 3 homozygotes were expected among the 56 t-AML patients, 6 were observed; 19 heterozygotes were expected, but 23 were observed. The gene frequency for the inactivating polymorphism (0.31) was increased approximately 1.4-fold among the 56 t-AML patients. This increase was observed within each of the following overlapping cohorts of t-AML patients: the 43 who had received an alkylating agent, the 27 who had received a topoisomerase II inhibitor, and the 37 who had received any radiotherapy. Thus, the frequency of an inactivating polymorphism in NQO1 appears to be increased in this cohort of myeloid leukemias, especially among those with t-AML or an abnormality of chromosomes 5 and/or 7. Homozygotes and heterozygotes (who are at risk for treatment-induced mutation or loss of the remaining wild-type allele in their hematopoietic stem cells) may be particularly vulnerable to leukemogenic changes induced by carcinogens.

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THE WIDESPREAD USE of intensive combination chemotherapy regimens and megavoltage radiation therapy has resulted in steadily improving long-term survival among patients in whom cancer had previously been fatal. This therapeutic success has led to the survival of large numbers of patients who formerly were destined to die within a few years. One of the most serious consequences of cancer therapy is the development of a second cancer, especially myeloid leukemia. Therapy-related acute myeloid leukemia (t-AML) is a neoplastic hematopoietic disorder arising in most cases from a multipotent stem cell and, in a few cases, from a lineage-committed progenitor.¹ The term therapy-related leukemia is descriptive and is based on a patient's history of exposure to cytotoxic agents. Although a causal relationship is implied, the mechanism of this remains to be proven. This term may ultimately be too restrictive, because the leukemias that develop after exposure to benzene or to atomic bomb irradiation are very similar or identical to the therapy-related leukemia syndrome.²

It has been known for many years that benzene causes hematotoxicity and is also associated with AML.²⁻⁷ Many clinical reports suggest that individuals vary greatly in their susceptibility to adverse health outcomes from benzene exposure. One explanation for this diversity is interindividual variation in metabolic activation and detoxification of benzene in humans.^{8,9}

Benzene is metabolized by the hepatic enzyme cytochrome P4502E1 (CYP2E1) to benzene oxide, which spontaneously forms phenol and is itself further metabolized by CYP2E1 to

hydroquinone.⁹ Hydroquinone and related hydroxy metabolites are converted in the bone marrow by myeloperoxidase to benzoquinones.^{10,11} These latter compounds are potent hematotoxins and genotoxins that can be converted by the enzyme NAD(P)H:quinone oxidoreductase (NQO1) to less toxic hydroxy metabolites. It has recently been shown in a case-control study of benzene-poisoned workers in Shanghai, China that lack of NQO1 enzyme activity was associated with benzene poisoning, leading to hematotoxicity.⁹

NQO1 encodes an enzyme also known as DT-diaphorase.

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This enzyme is a dimeric flavin adenine dinucleotide (FAD)-containing cytosolic protein that catalyzes the 2 electron reduction of a variety of quinone compounds.¹² The reduction of the quinone moiety to a hydroquinone prevents the generation of free radicals and reactive oxygen species, thus protecting cells from oxidative damage. However, the NQO1 enzyme also functions as a mechanism for the reduction and ultimate activation of certain chemotherapeutic drugs and of environmental carcinogens such as nitroaromatic compounds, heterocyclic amines, and possibly cigarette smoke condensate.¹³⁻¹⁷

The NQO1 gene locus maps to chromosome band 16q23. A polymorphism exists due to a C→T substitution at nucleotide 609 in the cDNA, giving rise to a missense mutation in codon 187 (proline-serine).¹⁶⁻¹⁹ Among Northern Europeans and Caucasian Americans, the gene frequency is 0.79 for the wild-type allele and 0.21 for the mutated allele.^{17,20-22} The frequency of the mutated allele is known to be slightly higher among African Americans and considerably higher among Hispanics and Asians.^{9,17,22} NQO1 enzyme activity is normal in individuals with 2 wild-type alleles. It is variably reduced in individuals who are heterozygotes for the polymorphism.¹⁹ The NQO1 protein and activity are absent in those who are homozygous for the point mutation.²⁰

We have been interested in trying to understand whether the development of therapy-related leukemia is a stochastic process (occurring by chance) or whether it is idiosyncratic, ie, whether certain individuals are at greater risk.^{1,23-25} The majority of t-AML cases are associated with alkylating agents or radiotherapy and are characterized by a median latency of 5 to 7 years, trilineage hematopoietic dysplasia, and the loss or deletion of chromosomes 5 and/or 7.^{1,26} Similar features are thought to characterize benzene-associated AML.^{5,27} A smaller proportion of cases is associated with exposure to topoisomerase-II-inhibiting drugs, suggesting a different mechanism of leukemogenesis.^{1,28,29} These cases are characterized by a shorter latency, a monocytic phenotype, and balanced translocations involving the *MLL* gene at chromosome band 11q23 or the *AML1* gene at band 21q22.

Our hypothesis is that the frequency of the 609C→T base substitution that results in an inactivating polymorphism in the NQO1 gene differs between different subgroups of patients with AML and will be greatest in those patients who develop t-AML after chemotherapy and in those with abnormalities of chromosomes 5 or 7.

MATERIALS AND METHODS

Patients with various myeloid leukemias gave informed consent for collection of blood and bone marrow specimens for this research. To perform this study, karyotypes were prepared by cytogenetic analysis on pretreatment bone marrow cells from leukemia patients using previously described methods.³⁰ Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines were established from peripheral blood B lymphocytes from the same leukemia patients and provided the DNA from nonmalignant cells.³¹ Polymerase chain reaction (PCR) primers were used to amplify DNA from exon 6 of the NQO1 gene. Restriction enzyme digestion with *HinfI* gave 3 possible patterns of bands: a 271-bp band in those patients who were wild-type homozygotes; 3 bands of 271, 151, and 120 bp in heterozygotes; and 2 bands of 151 and 120 bp in patients who were homozygous for the mutation.

DNA was isolated using conventional methods. The DNA was

PCR-amplified by the method developed by Eickelmann et al,³² with the following modifications. The sense primer NQO1 F (5'-AAG CCC AGA CCA ACT TCT-3') and antisense primer DT-2 (5'-TCT CCT CAT CCT GTA CCT CT-3') amplified a 304-bp region, including the NQO1 polymorphism, using a hot start protocol. DNA (1.5 μ L; 0.1 to 0.5 μ g), 25 pmol of each primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 pmol of each dNTP, 5% dimethyl sulfoxide (DMSO), and 2.5 U Taq polymerase in a total volume of 50 μ L were subjected to 40 cycles (94°C for 50 seconds, 52°C for 50 seconds, and 72°C for 30 seconds) followed by an extension at 72°C for 10 minutes. The PCR products were electrophoresed in 2% agarose.

A nested PCR method was used if regular PCR failed. The DNA was first PCR amplified with the sense primer NQO1 454A (5'-GAG ACG CTA GCT CTG AAC TGA T-3') and antisense primer NQO1 454B (5'-GGA AAT CCA GGC TAA GGA AT-3'). DNA (0.1 μ L; 10 ng/ μ L), 25 pmol of each primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 pmol of each dNTP, and 2.5 U Taq polymerase in a total volume of 50 μ L were subjected to 35 cycles (94°C for 30 seconds and 58°C for 30 seconds). A second nested PCR using 1 μ L of the first PCR product was performed with the same reagents and primers NQO1 F and DT-2 as described above.

PCR products were ethanol precipitated and digested with 10 U *HinfI* enzyme at 37°C for 2 hours or overnight. The digestion was stopped by heating to 65°C for 5 minutes.

The polymorphism was detected on an 8% polyacrylamide gel. Undigested DNA was 304 bp. One *HinfI* restriction site cuts a 33-bp fragment in all samples, acting as a digestion control. The restriction site polymorphism is a second *HinfI* site and results in three different combinations of bands: only one band of 271 bp corresponding to the genotype of homozygotes for the wild-type allele (C/C); three bands with 271, 151, and 120 bp in length corresponding to the genotype of heterozygotes (C/T); and two bands with 151 and 120 bp in length, corresponding to the genotype of homozygotes for the mutant allele (T/T).

Statistical considerations. It should be kept in mind that this is a selected series of patients. That is, the 104 patients who were studied all had myeloid leukemia, were referred for evaluation to the University of Chicago, provided a sample of blood or bone marrow, were successfully karyotyped, and had an EBV-transformed lymphoblastoid cell line successfully generated. Cell lines were selected for analysis for the NQO1 polymorphism based on the knowledge of the clinical diagnosis and the karyotype. Prospective unselected studies will be needed to validate the observations described herein.

The following allele frequencies for the NQO1 polymorphism within different ethnic groups were used: Caucasian, 0.21; African American, 0.23; Hispanic, 0.39; and Asian, 0.45.^{9,17,20-22} The expected values for the control group that we used for calculating statistical significance were taken from the literature involving different (although ethnically similar) populations. Conclusions based on prevalence estimates drawn from such external populations are less reliable. The χ^2 distribution was used to test for statistical significance between the observed frequencies of the NQO1 polymorphism and the frequencies expected in a population with the same ethnic mix.

RESULTS

We studied 104 patients with myeloid leukemias. Their characteristics are shown in Table 1. Fifty-six had developed t-AML after treatment with cytotoxic drugs or radiotherapy. The other 48 patients had primary myelodysplastic syndrome (MDS; 30 patients), AML de novo (9 patients), or chronic myelogenous leukemia (CML; 9 patients).

The frequencies with which the NQO1 polymorphism was detected in 48 patients with primary MDS, AML de novo, or CML are compared with that for the 56 patients with t-AML in

Table 1. Characteristics of 104 Patients With Myeloid Leukemias

	No. (%) of Patients		
	Total	Primary Leukemia	t-AML
Sex			
Male	52 (50)	26 (54)	26 (46)
Female	52 (50)	22 (46)	30 (54)
Race			
Caucasian	82 (79)	36 (75)	46 (82)
African American	17 (16)	8 (17)	9 (16)
Hispanic	3 (3)	2 (4)	1 (2)
Asian	2 (2)	2 (4)	0
Leukemia			
t-AML	56		
Primary MDS	30		
AML de novo	9		
CML	9		

Table 2. Also shown are the expected frequencies of NQO1 polymorphism for a population with the same racial and ethnic composition as these 104 patients. Thus, we would expect that approximately 61% of our entire cohort would have 2 wild-type alleles, 34% would be heterozygotes, and 5% would be homozygous for the mutation. We found that the frequency of homozygous mutants was 4% among the primary leukemia patients and 11% among those with t-AML. Heterozygotes were also more common among both types of leukemia patients than expected in the general population. The frequency of the NQO1 polymorphism was significantly increased among all 104 patients ($P = .050$) and among the 56 patients with t-AML ($P = .036$) compared with the frequency expected. The allele frequency for the polymorphism observed among the t-AML patients was increased approximately 1.4-fold over that expected.

The median age at the time of first exposure to cytotoxic therapy for the 27 t-AML patients with wild-type alleles was 48 years (range, 11 to 73 years). The median age for the 23 t-AML patients with heterozygosity was 51 years (range, 7 to 82 years) and for the 6 t-AML patients who were homozygous for the NQO1 polymorphism was 52 years (range, 32 to 69 years).

Table 2. Frequency of the NQO1 Polymorphism in Primary and Therapy-Related Myeloid Leukemia

Diagnosis	No. of Patients	No. (%) of Patients With			<i>P</i>
		Wild-type Alleles	Heterozygous Mutation	Homozygous Mutation	
Primary MDS, AML de novo, or CML					
Observed	48	24 (50)	22 (46)	2 (4)	.27
Expected*		28.56	16.72	2.72	
t-AML					
Observed	56	27 (48)	23 (41)	6 (11)	.036
Expected		34.42	18.93	2.66	
Total					
Observed	104	51 (49)	45 (43)	8 (8)	.050
Expected		62.98	35.65	5.38	

*Calculated from the frequency of the polymorphism reported within different ethnic groups and the racial composition of the observed group (see Materials and Methods).

Table 3. Specific Clonal Cytogenetic Rearrangements and the NQO1 Polymorphism

Cytogenetics	No. of Patients	No. (%) of Patients With		
		Wild-type Alleles	Heterozygous Mutation	Homozygous Mutation
Abnormal no. 5	13	8 (62)	2 (15)	3 (23)
Abnormal no. 7	16	4 (25)	9 (56)	3 (19)
Abnormal no. 5 & 7	16	9 (56)	6 (38)	1 (6)
t(11q23) or t(21q22)	14	10 (71)	4 (29)	0
inv(16) or t(15;17)	10	3 (30)	7 (70)	0
t(9;22)	9	5 (56)	4 (44)	0
Other abnormality	14	6 (43)	8 (57)	0
Normal	15	8 (53)	6 (40)	1 (5)
Total numbers	107*	53 (50)	46 (43)	8 (7)

*Three of the 104 patients had complex karyotypes with 2 recurring abnormalities, and each is included twice in this table. Two had wild-type alleles for NQO1: an Hispanic man with t-AML had del(5q) and t(3;21), and a Caucasian woman with AML after MDS had abnormalities of no. 5 and 7 and t(11;16)(q23;p13). One patient was heterozygous for the NQO1 polymorphism: a Caucasian man with AML de novo had inv(16) and t(11;21)(q13;q22).

In Table 3 are shown the specific cytogenetic rearrangements detected in the leukemia cells from these patients and the observed frequency of NQO1 polymorphism within each subgroup. Of the 13 patients with an abnormality of chromosome 5, 23% were homozygous for the mutation. Of the 16 with an abnormality of chromosome 7, 19% were homozygous, 56% were heterozygous, and only 25% had 2 wild-type alleles. An additional 16 patients had abnormalities of both chromosomes 5 and 7, and 1 (6%) of these was homozygous and 6 (38%) were heterozygous. There were no homozygous mutants among the 33 patients with a balanced translocation involving bands 11q23, or 21q22, or an inv(16), t(15;17), or t(9;22). Fourteen patients had other clonal abnormalities, and 15 patients had a normal karyotype.

In Table 4, the 45 patients with clonal abnormalities of chromosomes 5 or 7 or both are examined more closely. Thirty-six (80%) were Caucasian, 8 (18%) African American, and 1 (2%) was Hispanic. Forty-seven percent were observed to have 2 wild-type alleles, whereas 61% were expected. Thirty-eight percent were heterozygotes, whereas 34% were expected. Sixteen percent had 2 mutant alleles and, therefore, likely had no enzyme activity; only 5% were expected ($P = .002$). The mutant allele frequency was calculated to be 0.34 among these 45 patients, and this was increased approximately 1.6-fold over what was expected. In contrast, among the 33 leukemia patients

Table 4. Frequency of the NQO1 Polymorphism in 45 Primary and Therapy-Related Myeloid Leukemia Patients With Clonal Abnormalities of Chromosomes 5 and/or 7

NQO1 Genes	No. Observed (%)	No. Expected (%)	<i>P</i>
2 Wild-type alleles	21 (47)	27.58 (61)	.002
Heterozygous	17 (38)	15.25 (34)	
Homozygous	7 (16)	2.16 (5)	

The observed frequency of the mutant allele (0.34) was increased approximately 1.6-fold within this group of 45 patients ($P = .002$). Among 33 patients with balanced translocations, the mutant allele frequency was 0.23, approximately what would be expected.

Table 5. The Frequency of the NQO1 Polymorphism in 56 t-AML Patients According to Their Primary Cytotoxic Treatment

	No. of Patients*	No. of Patients With					
		Wild-Type Alleles		Heterozygous Mutation		Homozygous Mutation	
		Observed	Expected	Observed	Expected	Observed	Expected
Alkylating agents	43	21	26.23	17	14.62	5	2.06
Topo II inhibitors	27	13	16.47	11	9.18	3	1.30
Anti-metabolites	17	7	10.37	9	5.78	1	0.82
Anti-tubulin drugs	30	15	18.30	12	10.20	3	1.44
Radiotherapy	37	18	22.57	16	12.58	3	1.78
Totals	56	27	34.42	23	18.93	6	2.66

*Patients are listed more than once if they received more than one class of drugs or radiotherapy.

with balanced translocations, the mutant allele frequency was 0.23, or approximately what would be expected in the general population.

We also analyzed our data to see if the frequency of NQO1 polymorphism correlated with the primary treatment that had been received by these 56 t-AML patients. This was made difficult by the fact that most of our patients had received multiple chemotherapy agents in various combinations or together with radiation therapy. We grouped patients according to their exposures to alkylating agents, topoisomerase II inhibitors, antimetabolites, antitubulin drugs (principally vinca alkaloids), and radiotherapy (Table 5). In most categories, the frequency of the NQO1 polymorphism was higher than expected but not markedly so. For example, among the 43 patients who had received an alkylating agent, the frequencies of both heterozygous and homozygous mutations were increased over the number expected.

DISCUSSION

Our hypothesis postulated that heterozygosity and homozygosity for the base pair substitution in codon 187 of NQO1 were associated with a functional decrease in the amount of quinone reductase activity and that these individuals would have a markedly increased susceptibility to the genotoxic and leukemogenic effects of cytotoxic therapy. This hypothesis is supported by epidemiological data that have associated the ⁶⁰⁹C→T mutation of NQO1 with benzene-induced hematotoxicity.⁹ Occupational benzene poisoning is itself strongly associated with the subsequent development of AML and the related MDS.²⁻⁷ Because benzene-associated leukemia has clinical, morphologic, and cytogenetic features similar to the myeloid leukemias that follow exposure to alkylating agents, we focused on the frequency of NQO1 polymorphism in our patients who had AML with abnormalities of chromosomes 5 and/or 7 and compared them with other patients with myeloid leukemias characterized by balanced translocations or normal karyotypes. It is reasonable to assume that the mechanism of leukemogenesis between these two categories (ie, loss or deletion of no. 5 or 7 and balanced translocations) is considerably different.^{1,28,29}

Although typically thought of as a detoxification mechanism, NQO1 activity is also a well-documented component of pathways for mutagen and carcinogen activation.¹²⁻¹⁴ NQO1 is an inducible enzyme and is increased, for example, by cigarette smoking. Lung cancer in Mexican Americans and African Americans has been associated with the wild-type genotype of the NQO1 polymorphism.^{16,17} In this situation, functional

NQO1 probably results in the activation of potential lung carcinogens.

In summary, the frequency of an inactivating polymorphism in NQO1 appears to be increased in a cohort of myeloid leukemia patients with abnormalities of chromosomes 5 and/or 7, but not in those with balanced translocations, other clonal abnormalities, or normal karyotypes. Most of the former group of patients had therapy-related AML. The mutant allele frequency was approximately 1.6-fold higher than expected among patients with abnormalities in chromosomes 5 and/or 7 and 1.4-fold higher than expected among all patients with t-AML. Thus, individuals who are homozygous for the inactivating allele of NQO1 and thereby completely lack enzyme activity may be particularly vulnerable to leukemogenic changes induced by carcinogens. Heterozygotes may share this increased leukemogenic risk through two mechanisms. NQO1 enzyme activity may be variably reduced in heterozygotes and then further depleted by the oxidative stress of cytotoxic drugs, or these individuals may experience a treatment-induced mutation or loss of the remaining wild-type allele in one of their hematopoietic stem cells. Further studies on a large population of patients are required to confirm these findings.

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