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# A Lack of a Functional NAD(P)H:Quinone Oxidoreductase Allele Is Selectively Associated with Pediatric Leukemias That Have *MLL* Fusions[1]

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## ABSTRACT

**Rearrangements and fusion of the *MLL* gene with various alternative partner genes occur in ~80% of infant leukemias and are acquired during fetal hemopoiesis *in utero*. Similar *MLL* gene recombinants also occur in topoisomerase II-inhibiting drug-induced leukemias. These data have led to the suggestion that some infant leukemia may arise via transplacental fetal exposures during pregnancy to substances that form cleavable complexes with topoisomerase II and induce illegitimate recombination of the *MLL* gene. A structural feature shared by many topoisomerase II-inhibiting drugs and other chemicals is the quinone moiety. We assayed, by PCR-RFLP, for a polymorphism in an enzyme that detoxifies quinones, NAD(P)H:quinone oxidoreductase (NQO1), in a series ($n = 36$) of infant leukemias with *MLL* rearrangements *versus* unselected cord blood controls ($n = 100$). *MLL*-rearranged leukemias were more likely to have genotypes with low NQO1 function (heterozygous CT or homozygous TT at nucleotide 609) than controls (odds ratio, 2.5; $P = 0.015$). In contrast, no significant allele bias was seen in other groups of pediatric leukemias with *TEL-AML1* fusions ($n = 50$) or hyperdiploidy ($n = 29$). In the subset of infant leukemias that had *MLL-AF4* fusion genes ($n = 21$), the bias increase in low or null function *NQO1* genotypes was more pronounced (odds ratio, 8.12; $P = 0.00013$). These data support the idea of a novel causal mechanism in infant leukemia involving genotoxic exposure *in utero* and modulation of impact on a selective target gene by an inherited allele encoding a rate-limiting step in a carcinogen detoxification pathway.**

## INTRODUCTION

Pediatric acute leukemia is a diverse cancer in terms of its underlying biology and clinical response (1, 2). Infants with ALL[4] or AML usually have acquired *MLL* gene fusions as their major consistent genetic abnormality (3, 4). In contrast, the most common leukemia in children, B-cell precursor or cALL in the 2–5-year age peak of disease incidence (2), have other genetic abnormalities, most frequently *TEL-AML1* gene fusion or hyperdiploidy (5–7). These subsets have been postulated to have distinct etiologies (8), although both can be initiated *in utero* (9, 10). The risk of leukemia in children, in common with cancer in general, may be a composite of the complex interplay between inherited predisposition, exogenous exposures, and chance events. Each subtype of leukemia may then be expected to have distinct or preferential causal networks. Constitutive predisposition can operate via highly penetrant mutant genes like *p53* in Li-Fraumeni syndrome or, more commonly, via low penetrance genetic polymorphisms that indirectly modulate risk to more modest levels, such as cytochrome P-450s or glutathione transferases (11). The latter are most often reported in the context of cancers linked to particular genotoxic exposures (12). Very few such associations have been reported in childhood leukemia (13, 14). Preliminary evidence suggests that particular HLA DQ/DP haplotypes may be associated with increased risk of cALL, which accords with the postulated role of infection in this biological subset of leukemia (15).

*MLL* gene rearrangements are common in secondary acute leukemias (usually myeloblastic M4/M5) associated with prior exposure to epidophyllotoxin or anthracycline drugs that inhibit topoisomerase II (16, 17). Breaks in the *MLL* gene occur within a ~10-kb cluster region at the 3′ end of which is a functional topoisomerase II binding site (18). In secondary leukemias, breaks in the *MLL* gene occur more often in the 3′ side of the BCR within a few kb of the topoisomerase II site (19). *De novo* ALL or AML with *MLL* gene breaks were reported to have more common 5′ breaks (19), but subsequent studies have reported either preferential 3′ breaks in infant ALL with *MLL-AF4* fusions (20) or little or no bias in breakpoint distribution in infant cases (21). These discrepancies can be accounted for, at least in part, by differences in the definition of 5′ and 3′ regions of the BCR. These data, coupled with the prenatal origin of *MLL* gene fusions (9, 22), have suggested a plausible etiological mechanism for infant acute leukemia involving transplacental exposure to substances that form cleavable complexes with topoisomerase II-inhibiting substances (8, 23). A number of candidate substances have been identified and provide the focus for ongoing epidemiological case/control studies (4). Some prior epidemiological associations reported for infant leukemia are also in accord with this suggested mechanism (24, 25).

The potential exposures of the pregnant mother and fetus to dietary, medical, or environmental chemicals that interact with topoisomerase II may be orders of magnitude lower in functional dose than those of chemotherapy drugs used in the treatment of cancer, although in some cases the chemicals involved in the former are as biologically active in the role of topoisomerase II inhibitors as the chemotherapeutics (26). We anticipated that interindividual differences in metabolism of these chemicals might play an important role in response to such lower doses and modulate the risk of pediatric leukemias with *MLL* gene fusions but not that of other subtypes. Many topoisomerase II-inhibiting compounds are quinone-containing substances (27–30). The metabolism of quinones, as exemplified by benzene detoxification, is critically controlled by the enzyme NQO1 (or DT-diaphorase, EC 1.6.99.2; Ref. 31). NQO1 converts toxic benzoquinones to hydroquinones in an obligate two-electron reduction. This reaction is in competition with one-electron reduction reactions by cytochromes P-450, producing the semiquinone, which generate free radicals and reactive oxygen species via redox cycling.

Two polymorphic variants of NQO1 have been identified: a C→T

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[4] The abbreviations used are: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; cALL, common ALL; BCR, breakpoint cluster region; NQO1, NAD(P)H:quinone oxidoreductase; nt, nucleotide; OR, odds ratio; CI, confidence interval.

change at nt 609 yields a proline to serine substitution (32), and a T→C substitution at nt 464 results in a tryptophan replacement of arginine (33, 34). The first of these (*C609T*) effectively inactivates the enzyme due to decreased catalytic activity and stability of NQO1 protein (35, 36); the second (*T464C*) has not yet been completely characterized. We have analyzed these polymorphisms in three subgroups of infant and childhood leukemias with the hypothesis that: (*a*) *MLL* leukemia patients and/or their mothers will show a higher prevalence of low NQO1-inducing genotypes, reflecting a reduced ability to detoxify carcinogens that promote *MLL* translocations; and (*b*) other groups of childhood leukemias, including those cALLs with *TEL-AML1* translocations and those exhibiting hyperdiploidy, that have not been epidemiologically associated with chemical exposure should not demonstrate a bias in allele frequency.

## MATERIALS AND METHODS

**Patient and Control Samples.** All of the patients (<15 years old) were enrolled in the United Kingdom Childhood Cancer Epidemiology Study (UKCCS). Patient samples taken at the time of diagnosis of acute leukemia were screened and classified for common molecular subgroups of pediatric leukemia by banded karyotyping and by fluorescence *in situ* hybridization (for hyperdiploidy) and reverse transcription-PCR (for *MLL* fusions and *TEL-AML1* fusions). Remission blood samples were also obtained. Controls consisted of umbilical cord blood samples obtained from healthy new-born infants. A small proportion (<10%) of patients was from minority ethnic groups in the United Kingdom (i.e., Asian, Afro-Caribbean black, and Oriental). No attempt was made to screen out such individuals, and we have no evidence that they are disproportionately represented in any leukemia subgroup.

***NQO1* Genotyping.** Genotyping was performed by PCR-RFLP analysis of DNA extracted from patient blood samples and controls. Infant (<24 months) leukemic patient samples used were taken either at diagnosis of leukemia ($n = 16$) or, when in remission, within 3 months of diagnosis ($n = 20$). All of the other patient DNA samples for genotyping were from blood taken during remission. Twenty nmoles of the primers NQO1609A, CCTCTCTGTGCTTTCTGTATCC with NQO1609B, GATGGACTTGCCCAAGTGATG (for the nt 609 polymorphism) or NQO1464A, CTGGTCTTACCTCAATGATGTC with NQO1464B, CCTGCATCAGTACAGACCACC (for the nt 464 polymorphism) were mixed with 60 ng of DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 pmol of each dNTP, and 1.25 units Taq polymerase in a total volume of 50 μl and subjected to 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) in an MJ Research thermal cycler (Watertown, MA) followed by an extension at 72°C for 7 min. PCR products were checked on agarose gels. The remainder of the PCR reaction was digested with *Hin*F1 in the case of nt 609 polymorphism or with *Msp*I in the case of the nt 464 polymorphism. Digestion with *Hin*FI yielded two bands for the homozygous wild-type bp 609 (84 and 214 bp), four bands for heterozygotes (65, 84, 149, and 214 bp), and three bands for homozygous variant (65, 84, and 149 bp). Digestion of the second PCR reaction with *Msp*I yielded two bands in the case of homozygous wild type (62 and 144 bp), three bands for heterozygotes (62, 144, and 209 bp), and one band for homozygous variants (209 bp). Digestion products were analyzed by electrophoresis in 0.7% agarose with 2% Synergel (Diversified Biotech) and viewed by ethidium bromide staining/UV *trans*-illumination (Fig. 1).

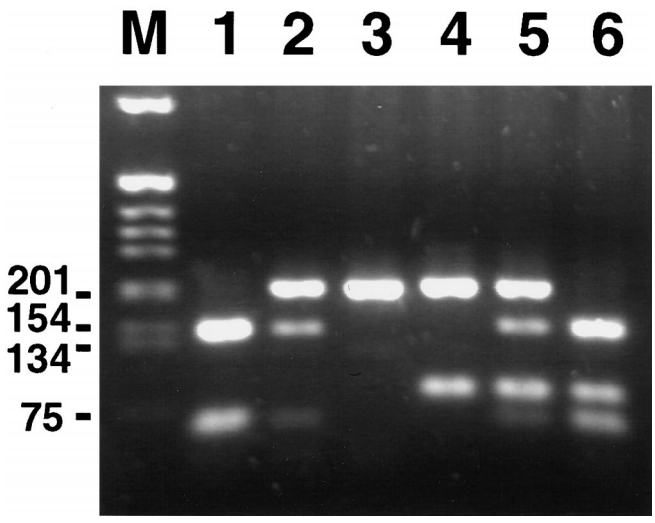


Fig. 1. PCR-RFLP analysis of NQO1 polymorphisms in six individuals analyzed on a 0.7% agarose/2% Synergel electrophoresis gel. *Lane 1*, Marker (Life Technologies, Inc., 1 kb). *Lanes 1–3*, nt 464 PCR reactions digested with *Msp*I; *Lanes 4–6*, nt 609 PCR reactions digested with *Hin*FI. *Lanes 1* and *4*, homozygote normal individuals for the respective polymorphisms; *Lanes 3* and *6*, homozygote variant individuals for the respective polymorphisms; *Lanes 2* and *5*, heterozygote individuals for the polymorphisms.

Table 1 *Subclassification of infant leukemia cases*[a] *with* MLL *gene fusions*

| | Total *n* | Fusion partner[b] | | |
|---|---|---|---|---|
| | | *AF4* | *ENL* | *AF9* |
| ALL | 30 | 21 | 4 | 5 |
| AML | 6 | 1 | 1 | 4 |

[a] All of the patients were <18 months of age.
[b] Detected by reverse transcription-PCR using primers specific for *AF4*, *ENL*, *AF9*, and *AF6* (58); screening did not include primers for other potential, but less frequent, partner genes. No cases of *MLL-AF6* fusion were detected in this series.

**Statistical Analysis.** Studies in humans and cell lines have determined that individuals harboring the *NQO1 bp 609 variant* genotype in the homozygous or heterozygous state are deficient in NQO1 protein, primarily because of decreased protein stability (35, 36). Heterozygous individuals have significantly lower NQO1 protein in saliva samples than homozygous wild-type individuals ($P < 0.01$; Ref. 36), and, therefore, we grouped heterozygous individuals with homozygous variant individuals for statistical analysis as "low NQO1." Homozygous wild-type genotypes at bp 609 were considered "high-*NQO1*" genotypes. Two-by-two tables were constructed, and ORs were computed; 95% CIs and *P*s were derived by exact methods. The statistical package EGRET was used for the calculations.

## RESULTS

Of the 36 cases of *MLL* fusion gene-positive infant leukemias available, 30 were classified morphologically and by immunophenotype as ALL and 6 as AML. *MLL* fusion gene partners were as shown in Table 1. As anticipated, most of the cases were *MLL-AF4*-positive. Two hundred fifteen individuals were typed for *NQO1* genotypes and are presented in Table 1. A 17% allele frequency was found for controls for the nt 609 variant allele, which is intermediate to the 13–25% allele frequency found in comparable studies in Caucasians in Europe and North America (34, 37–39). In the largest group to date, a 16% allele frequency was found among 575 individuals (34). A 5.5% allele frequency was found for controls at the nt 464 variant, which is similar to that found in Canadian Caucasians (5%; Ref. 34). Representative PCR gel results are illustrated in Fig. 1.

Patients with *MLL* translocations were found to harbor 2.5 times increased frequency of low *NQO1* genotypes [heterozygous CT or homozygous TT at nt 609; OR, 2.5; 95% CI, 1.08–5.96; $P = 0.015$] than controls (Table 1). The allele frequency of the variant nt 609 allele in $MLL^+$ leukemias was also significantly different (32% *versus* 17%; two-tailed $P = 0.011$). In addition, there was a significant "dose response" effect, homozygous TT having a higher risk of leukemia than heterozygous CT patients (test for trend, $P = 0.0076$). $MLL^+$ infant leukemias did not demonstrate an allele bias compared with controls for nt 464 variants (OR = 1.12), nor did *TEL-AML1* or hyperdiploid leukemias.

When *NQO1* genotypes were analyzed for the major molecular subtype of *MLL* gene rearranged ALL, *i.e.*, those with *MLL-AF4* fusions, a more pronounced bias toward low-function genotypes (as nt 609) was evident [OR, 8.12; 95% CI, 2.29–31.48 (Table 2)]. Other subgroups of these infant leukemias, *i.e.*, AML or ALL with *MLL-*

Table 2 *NQO1 nt 609 polymorphism in subgroups of pediatric leukemia*

| Category | *n* | *NQO1 609*[a] CC[b] | CT | TT | *NQO1 609 T* allele freq. | Low *NQO1*[c] OR (CI) | *P* |
|---|---|---|---|---|---|---|---|
| Controls | 100 | 67 | 32 | 1 | 0.17 | 1.0[d] | |
| *MLL*$^+$ total[e] | 36 | 16 | 17 | 3 | 0.32 | 2.54 (1.08–5.96) | 0.015 |
| *MLL/AF4* subset | 21 | 4 | 15 | 2 | 0.45 | 8.63 (2.45–33.22)[f] | <0.0001[f] |
| *TEL-AML1*$^+$ | 50 | 28 | 20 | 1 | 0.22 | 1.52 (0.71–3.25) | 0.16 |
| Hyperdiploid | 29 | 20 | 9 | 0 | 0.16 | 0.91 (0.33–2.38) | 0.52 |

[a] bp corresponding to the major functional *NQO1* polymorphism.
[b] CC, homozygous functional allele; CT, heterozygous allele; TT, homozygous nonfunctional allele.
[c] Low *NQO1* is defined as homozygous variant or heterozygous at the nt 609 polymorphism. ORs compare the ratio of low *NQO1* patients in each category to controls.
[d] Reference group.
[e] All of the cases with *MLL* gene rearranged (*i.e.*, with *AF4, AF9,* or *ENL* partners).
[f] We have considered the possibility that these elevated risks may be an artifact of including patients studied only in remission, *i.e.*, some impact of *NQO1* alleles on clinical response rather than incidence of disease. Although we cannot rule out some effect of selection, it is unlikely to explain our results. A total of 85–95% of patients with *MLL* gene fusions do enter remission on current therapeutic protocols, although some 50% may relapse by 12 months (59). Our remission samples were collected after the achievement of remission and generally within 3 months. In the case of the critical subset of patients with *MLL/AF4* fusions, 12 were genotyped at diagnosis and 9 in remission and for the diagnosis samples only. The elevated OR holds for both: 4.06 (CI, 1.006–17.5; $P = 0.029$).

*ENL* or *MLL-AF9* fusions had too few cases for a separate analysis of *NQO1* allele frequency.

In contrast to *MLL*$^+$ infant leukemias, childhood ALLs with *TEL-AML1* translocations or hyperdiploidy did not demonstrate increased frequencies of *NQO1 variant* alleles. *TEL-AML1* leukemias had a slightly higher odds of a low *NQO1 nt 609* alleles (OR, 1.5), but the increase was not significant (Table 2).

## DISCUSSION

NQO1 is a cytoplasmic, ubiquitously expressed enzyme that catalyzes an obligate two-electron reduction of a wide variety of quinones using NADH or NADPH as the reducing cofactor. NQO1 is generally known for its detoxifying properties, effective with such chemicals as menadione (40), benzene (41), and benzo(*a*)pyrene quinone (42). NQO1 is also known to be involved in the recycling of membrane antioxidants ubiquinone and vitamin E (43, 44). The simplest quinone, benzoquinone, is a metabolite of benzene produced in the bone marrow from hydroquinone via myeloperoxidase (45) and is also derived from arbutin, a glycoside conjugate of hydroquinone, which is common in the food supply (46). Studies suggest that the balance between myeloperoxidase and NQO1 levels in the bone marrow determines the level of toxic effects of hydroquinone and may ultimately determine the leukemogenicity of benzene (47, 48). NQO1 strongly inhibited a class of DNA adducts induced by hydroquinone (49), which suggests a potential mechanism for its protection of the hemopoietic system in benzene-exposed individuals (41). NQO1 has a wide substrate specificity and may play a similar role in detoxifying other environmentally derived quinones and quinone-imines.

Two polymorphic variants in NQO1 have been found, a C→T substitution at nt 609 and a T→C substitution at nt 464. The nt 609 polymorphism has been recently associated with specific leukemogenic changes, including clonal abnormalities in chromosomes 5 and/or 7, in therapy-related leukemias (50). This polymorphism is effectively completely inactivating, whereas the nt 464 polymorphism has not been characterized in mammalian cells. A higher prevalence of low NQO1-inducing genotypes (*C609T*), as we describe here for pediatric leukemia with *MLL* gene fusions, therefore, reflects a reduced ability to detoxify quinone-based carcinogens. Although the allele bias that we describe is statistically significant, especially for the small series of cases ($n = 21$) with *MLL-AF4* gene fusions, it will be important to confirm this association in an independent series of patients. The prevalence of the *C609T* polymorphism varies among different ethnic groups. The highest reported allele frequency of the variant is approximately 40% in Asian populations (37, 41). It may be significant in this context that infant leukemia is more frequent in Oriental than in Western countries (analysis of data from Ref. 51).[5] The lack of association of nt 464 variant alleles with *MLL*$^+$ leukemias may indicate a low functional phenotype of this polymorphism.

Felix *et al.* (52) recently reported that an excess risk of secondary leukemias (both with and without *MLL* gene fusion) was associated with a reduced likelihood of inheritance of the *CYP3A4-V* allele of cytochrome P-450 *CYP3A4* gene, which metabolizes epidophyllotoxins (and other chemicals) to quinone metabolites. However, no such association was observed in *de novo* leukemias with *MLL* gene fusions, most of which were in infant cases. Taken at face value, these data seem to conflict with our own but need not necessarily do so. Firstly, cytochrome P-450 enzymes may be more critical or dose-limiting in the context of high-dose chemotherapeutic exposures generating genotoxic metabolites. Secondly, *CYP3A4* is not expressed in fetal development (53, 54) and, therefore, cannot contribute to the risk of *MLL* gene fusions in the context of infant leukemia. NQO1 is expressed in fetal liver.[6]

There was no significant bias of allele frequency in other subsets of pediatric ALL with alternative acquired molecular abnormalities, hyperdiploidy, or *TEL-AML1* fusion genes. The latter subtypes are members of the common (c) variant of childhood ALL in which an abnormal response to infection is postulated to be a major etiological factor (8). We, therefore, demonstrate a unique and hitherto undescribed link between an inherited genetic polymorphism and a specific acquired genetic abnormality in a cancer subtype. We hypothesize that the link is associated with suspected patterns of chemical exposure during pregnancy. The hypothesis is that substances that form cleavable complexes with topoisomerase II are prime candidates for the induction of *MLL* gene fusions (8, 23). This idea was prompted by the observation that *MLL* gene fusions characteristic of infant leukemia are also common in secondary leukemias associated with prior exposure to therapeutic drugs including epidophyllotoxins or anthracyclines, which operate via topoisomerase II inhibition (16, 17).

Candidate substances that might generate infant leukemia with *MLL* gene fusions and that would be metabolized to quinones in the fetal liver include dietary flavonoids, podophyllin toxins, and benzene. Ongoing case/control studies are assessing via maternal, questionnaire-based data, exposure patterns during pregnancy. Epidemiological studies have implicated a number of different maternal exposures during pregnancy that are associated with infant leukemia, although cases were not analyzed for *MLL* gene status. These include

[5] Freda E. Alexander, unpublished observations.
[6] Joseph Wiemels and Mel Greaves, unpublished observations.

diets rich in flavonoids (25), pesticides, marijuana, alcohol (55), and benzene or gasoline exposures (reviewed in Ref. 24). Some of these, if causally relevant, could operate via the quinone metabolic pathway. Benzene metabolites (in gasoline and in tobacco marijuana smoke, for example) as well as flavonoids are oxidized by peroxidases to yield semiquinones and quinones (31, 56). In the case of benzene metabolites, the oxidized products interact with topoisomerase II (57). A NQO1-deficient individual may be less able to cope with the quinone assault. Unoxidized flavonoids are excellent topoisomerase II inhibitors (26), and, therefore, the role of NQO1 is less clear.

It should be remembered that the ultimate mechanism for *MLL* breakage leading to translocation is unknown and could involve topoisomerase II inhibition or, alternatively, the formation of reactive metabolites or reactive oxygen species. Indeed, many topoisomerase II-inhibiting drugs form reactive oxygen species with great facility as do benzene metabolites (31) and flavonoids (56). The 3′ end of the *MLL* BCR has been shown to be a DNase-hypersensitive site and liable to breaks by a variety of apoptotic-inducing stimuli, which indicates that other mechanisms not involving topoisomerase II may play a role. The ultimate mechanism of NQO1-modified attack on the *MLL* gene in infant leukemia, therefore, remains to be uncovered. The objective of additional studies lies in uncovering the genetic-environmental interactions and pathway leading to the development of a highly malignant and clinically intractable leukemia in infants. Prevention is the long-term goal.

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