Low NAD(P)H:quinone oxidoreductase 1 activity is associated with increased risk of acute leukemia in adults

Martyn T. Smith, Yunxia Wang, Eleanor Kane, Sara Rollinson, Joseph L. Wiemels, Eve Roman, Philippa Roddam, Raymond Cartwright, and Gareth Morgan

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme that detoxifies quinones and reduces oxidative stress. A cysteine-to-threonine substitution (C → T) at position 609 in the NQO1 gene results in a lowering of NQO1 activity. Individuals homozygous for this mutation have no NQO1 activity, and heterozygotes have low intermediate activity compared with people with the wild type.

DNA samples from 493 adult de novo acute leukemia patients and 838 matched controls were genotyped for NQO1 C609T. The majority of cases were diagnosed as acute myeloid leukemia (AML) (n = 420); 67 as acute lymphoblastic leukemia (ALL); and 6 as other forms of acute leukemia. The frequency of cases with low or null NQO1 activity (heterozygote + homozygous mutant) was significantly higher among total acute leukemia case subjects compared with their matched controls (odds ratio [OR] = 1.49; 95% confidence interval [CI], 1.17-1.89). Both ALL (OR = 1.93; 95% CI, 0.96-3.87) and AML case subjects (OR = 1.47; 95% CI, 1.13-1.90) exhibited a higher frequency of low or null NQO1 genotypes than controls. For de novo AML, the most significant effect of low or null NQO1 activity was observed among the 88 cases harboring translocations and inversions (OR = 2.39; 95% CI, 1.34-4.27) and was especially high for those harboring inv(16) (OR = 8.13; 95% CI, 1.43-46.42). These findings were confirmed in a second group of 217 de novo AML cases with known cytogenetics. Thus, inheritance of NQO1 C609T confers an increased risk of de novo acute leukemia in adults, implicating quinones and related compounds that generate oxidative stress in producing acute leukemia.

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Patients and methods

Case-control study population and sample collection

This study was based on subjects participating in a population-based case-control study of adult acute leukemia conducted by the Leukaemia Research Fund’s Centre for Clinical Epidemiology in the United Kingdom. Details of the study are published elsewhere. Briefly, patients recruited into the study were aged 16 to 69 years and had received a new diagnosis of acute leukemia between April 1, 1991, and December 31, 1996, while resident in parts of the north and southwest of England. All diagnoses were pathologically confirmed, and cytogenetics were obtained from cytogenetic laboratories. For each case subject, 2 controls matched on gender, year of birth, and leukemia type were identified. The control group was derived from the same general population. The study population and sample collection procedures have been reported in detail elsewhere.15

For each tumor sample, DNA was extracted from fresh-frozen tissue and stored at −80°C for later analysis. Genotyping was by use of RFLP analysis.16 DNA samples were amplified by polymerase chain reaction (PCR) and then digested with restriction enzymes. The RFLP patterns were scored as wild type, heterozygote, or homozygote for the C609T mutation. For optimal reproducibility, a duplicate PCR-RFLP analysis was performed on all samples.

The frequency of cases with low or null NQO1 activity (heterozygote + homozygous mutant) was significantly higher among total acute leukemia case subjects compared with their matched controls (odds ratio [OR] = 1.49; 95% confidence interval [CI], 1.17-1.89). Both ALL (OR = 1.93; 95% CI, 0.96-3.87) and AML case subjects (OR = 1.47; 95% CI, 1.13-1.90) exhibited a higher frequency of low or null NQO1 genotypes than controls. For de novo AML, the most significant effect of low or null NQO1 activity was observed among the 88 cases harboring translocations and inversions (OR = 2.39; 95% CI, 1.34-4.27) and was especially high for those harboring inv(16) (OR = 8.13; 95% CI, 1.43-46.42). These findings were confirmed in a second group of 217 de novo AML cases with known cytogenetics. Thus, inheritance of NQO1 C609T confers an increased risk of de novo acute leukemia in adults, implicating quinones and related compounds that generate oxidative stress in producing acute leukemia.

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birth, and race were randomly selected from persons registered with the same local physician as the case patient. Case patients were considered ineligible if they had a diagnosis of myelodysplastic syndrome or chronic myeloid leukemia in the 6 months prior to diagnosis of acute leukemia, or a malignancy within 2 years. Control patients ineligible under these criteria were replaced. Case and control subjects were asked, with the physician’s permission, to be interviewed. If a control patient’s physician or the control patient refused permission to be interviewed, the control selection process continued until 2 eligible persons had agreed to be interviewed or there were no further suitable persons to approach. After the interview, all subjects were invited to provide a blood sample. Blood was collected by phlebotomy, and DNA was isolated by means of sodium dodecyl sulfate/protease K treatment, followed by a phenol/chloroform extraction and ethanol precipitation. For case material, DNA was extracted from peripheral blood obtained at presentation or during remission following treatment. For the United Kingdom Medical Research Council (MRC) case series, all samples were obtained at presentation.

Information collected from medical notes and cytogenetic laboratory permitted further diagnostic classification. Acute leukemia was defined as de novo if the patient had no history of chemotherapy or radiotherapy and had no prior diagnoses of myelodysplastic syndrome, chronic myeloid leukemia, or chronic myeloproliferative disorder. Cytogenetic abnormalities among acute myeloid leukemia (AML) cases were hierarchically classified into one of the following groups: normal; reciprocal translocations/inversions associated with good prognosis, that is, t(15;17), t(8;21) or inv(16); and other cytogenetic abnormalities not otherwise classified.

Cytogenetically characterized case series

The NQO1 C609T polymorphism was also examined in blood samples obtained from patients entered into the MRC AML treatment trials.16,17 Persons of interest were those aged 16 to 69 years with a diagnosis of de novo AML. Of the 3045 such patients recruited since 1988, cytogenetic data were available for 82%; 781 patients had one of the defined cytogenetic abnormalities: that is, the balanced translocations/inversions t(15;17), t(8;21), and inv(16); partial or complete deletion of chromosomes 5 or 7, that is −5/5q−/−7/7q−; and other cytogenetic abnormalities not otherwise classified.

Analysis of NQO1 genotype

Laboratory personnel were blinded to case-control status (DNA was isolated in Leids and sent encoded to Berkeley for analysis). NQO1 alleles were analyzed as previously described. Briefly, DNA from study subjects was PCR-amplified with sense primer NQO1 F: 5′-AAG CCA ACC CCA ACT TCT T-3′, and antisense primer DT-2: 5′-TCT CCT CAT GTA CCT CT-3′, amplifying a 304-base pair (bp) region including the NQO1 polymorphism. The PCR reaction mixture consisted of 0.1 to 0.5 µg DNA, 25 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 pmol of each dNTP, 5% dimethyl sulfoxide (DMSO), and 0.25 units Taq polymerase in a total volume of 50 µL. This was 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 on agarose.

If the DNA did not amplify by regular PCR, a nested PCR was applied. The DNA was first PCR-amplified with the sense primer NQO1 454A: 5′-GAG CAG CTA GCT CTT AAC TGA T-3′, and antisense primer NQO1 454B: 5′-GGA AAT CCA GCC TAA GGA AT-3′. The master mix contained 0.1 µg DNA (± 10 ng), 25 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 pmol of each dNTP, and 0.25 U Taq polymerase in a total volume of 50 µL and was subjected to 35 cycles (94°C for 30 seconds, 58°C for 30 seconds). A second nested PCR using 1 µL of the first PCR product was performed with the same reagents but with primers NQO1F and DT-2 (see above). This reaction was also subjected to 35 cycles (94°C for 30 seconds, 58°C for 30 seconds). The second PCR product was electrophoresed in 2% agarose.

Hinfl 10 × digestion buffer (1.5 µL) was added to 25 µL of the PCR product to adjust pH and salt concentration, followed by 10 units of Hinfl enzyme (Boehringer Mannheim, Indianapolis, IN). The mixture was incubated at 37°C for at least 2 hours. The digestion product was electrophoresed in 4% agarose and visualized by staining with ethidium bromide. The 304-bp PCR product contained one nonpolymorphic Hinfl site as well as the polymorphic site. A 33-bp fragment was excised from the nonpolymorphic Hinfl site independent of genotype. The polymorphism also introduces a second Hinfl restriction site, which after digestion with Hinfl resulted in 3 different combinations of bands: only one band of 271 bp corresponding to the genotype of homozygotes for the wild-type allele; 3 bands, 271 bp, 151 bp, and 120 bp in length, corresponding to the genotype of heterozygotes; and 2 bands, 151 bp and 120 bp in length, corresponding to the genotype of homozygotes for the mutant allele. Both positive and negative control samples were included in the analysis at all times.

Data analysis

For the case-control study, odds ratios and 95% confidence intervals were estimated by means of conditional logistic regression. The analysis was restricted to white case subjects and their individually matched white controls. Socioeconomic status was adjusted for using an area-based deprivation indicator that was created by linking to the 1991 United Kingdom census and coding the Townsend score of the address at diagnosis. The likelihood ratio test was used to test for interaction between NQO1 and other factors such as gender; age (as both a continuous variable and in the categories younger than 40 years, 40 to 54 years, and 55 years and older); and smoking status at 2 years before diagnosis (never/ever smoked). Subgroup analyses were conducted for AML and acute lymphoblastic leukemia (ALL), by French-American-British (FAB) group or immunophenotype, and by cytogenetics. For the case series, associations within specific cytogenetic subgroups were examined by means of Pearson’s χ2 test. All analyses were conducted by means of the statistical package Stata (Stata, College Station, TX).

Results

In the control population, the NQO1 C609T genotype was distributed as follows: 67% wild-type (CC), 29% heterozygotes (CT), and 4% homozygous mutants (TT). The mutant allele frequency was 0.188, which is consistent with previous reports in whites and the Hardy-Weinberg formula, as well as the first report of null NQO1 activity in 4% of the British population. Acute leukemia cases had the following distribution: 58% CC; 38% CT; 4% TT.

Effect of the NQO1 C609T polymorphism on risk of de novo acute leukemia

Being homozygous or homozgyous for the mutant NQO1 C609T allele was associated with a 49% increased risk of acute leukemia in 490 case patients, compared with 836 matched controls who were successfully genotyped (OR = 1.49; 95% CI, 1.17-1.89) (Table 1). This increased risk was higher for ALL than for AML, although the difference is not statistically significant. Subcategorization of the AMLs according to the FAB subtypes and of the ALLs by immunophenotype revealed no significant differences among groups (Table 1).

Effect of the NQO1 C609T polymorphism on risk of de novo acute myeloid leukemias with differing cytogenetics

Table 2 shows, for the case-control study, odds ratios for de novo AMLs with specific cytogenetics, relative to their matched controls. There was a significant association of low or null NQO1 activity with AML of a normal karyotype (OR = 1.71; 95% CI,
1.09-2.69] and for those AMLs harboring specific translocations and inversions (OR = 2.39; 95% CI, 1.34-4.27). The highest and most significant association was found for AML with inv(16) (OR = 8.13; 95% CI, 1.43-46.42). Risks were increased, although not significantly, for AMLs with t(15;17), t(8;21), or loss or partial deletion of chromosomes 5 and 7. Conversely, acute myeloid leukemias with other cytogenetic abnormalities were not associated with the mutant NQO1 C609T allele.

Table 3 presents the distribution of NQO1 within the MRC case series excluding the 58 patients previously analyzed in the case-control study. Within the case series, the distribution of NQO1 among cytogenetic groups showed evidence of heterogeneity (χ² = 11.04, P = .01). As in the case-control study, a greater proportion of patients with inv(16) (66%) than with wild type (34%) had low or null NQO1 activity. Among t(15;17) cases, low or null activity occurred in 50% of cases, greater than the 33% observed in the control population of the case-control study. For the remaining 2 cytogenetic subgroups of t(8;21) and 5q–/7q–, the occurrence of NQO1 appeared to be no different from the control population. The distribution of NQO1 within each cytogenetic subgroup changed little when the 58 cases from the case-control study were included in the analysis of the case series, and the test for heterogeneity remained significant (χ² = 8.51, P = .04) (data not shown).

Effect of age, sex, and smoking on risk associated with NQO1 C609T polymorphism

Stratification by sex, age, or smoking status had no effect on the analysis, so the effect of NQO1 on the risk of acute leukemia is similar for males and females, for any age group, and for smokers and nonsmokers.

Discussion

In a large case-control study of more than 1300 white adults, we report here that an inactivating C609T polymorphism in the NQO1 gene is associated with a significant excess of de novo acute leukemia, with increased risks for both ALL and AML. This builds upon earlier findings that the NQO1 C609T polymorphism is associated with an enhanced risk of therapy-related leukemia12 and infant leukemia with MLL gene rearrangements.13 The NQO1 C609T polymorphism has also been shown to be associated with a greater risk of benzene-induced hematotoxicity and leukemia.21

We subcategorized as many of the AML cases in our study as possible according to their clinically established cytogenetics. The strongest effect of the NQO1 C609T polymorphism was observed for AML cases harboring translocations or inversions, with inv(16) cases exhibiting the highest odds ratio of 8.13. We were concerned

Table 1. Number of case and control subjects, adjusted odds ratios, and 95% confidence intervals by diagnosis for the NQO1 enzyme, using wild type as the reference

<table>
<thead>
<tr>
<th>Diagnosis*</th>
<th>Wild type†</th>
<th>Heterozygote + Homozygote†</th>
<th>OR‡</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>Case</td>
<td>Control</td>
<td>Case</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Acute leukemia*</td>
<td>285</td>
<td>562</td>
<td>205</td>
<td>274</td>
</tr>
<tr>
<td>AML</td>
<td>244</td>
<td>484</td>
<td>175</td>
<td>235</td>
</tr>
<tr>
<td>AML M0</td>
<td>14</td>
<td>27</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>AML M1</td>
<td>47</td>
<td>86</td>
<td>28</td>
<td>42</td>
</tr>
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<td>AML M2</td>
<td>49</td>
<td>110</td>
<td>47</td>
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<td>AML M3</td>
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<td>19</td>
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<td>40</td>
<td>77</td>
<td>31</td>
<td>43</td>
</tr>
<tr>
<td>AML M5</td>
<td>23</td>
<td>51</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>AML M6</td>
<td>12</td>
<td>24</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>AML M7</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>ALL</td>
<td>36</td>
<td>73</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>ALL B</td>
<td>22</td>
<td>50</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>ALL T</td>
<td>8</td>
<td>13</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

NQO1 indicates NAD(P)H:quinone oxidoreductase 1; OR, odds ratio; CI, confidence interval; AML, acute myeloid leukemia; and ALL, acute lymphoblastic leukemia.

*Four cases had a diagnosis of acute biphenotypic leukemia, and 2 cases had a diagnosis of unspecified acute leukemia.
†Wild type showed high activity of NQO1 enzyme; heterozygote, low activity; and homozygote, no activity. Samples for 3 cases and 2 controls would not amplify.
‡Odds ratios were estimated by means of conditional logistic regression adjusted for deprivation.

Table 2. Number of de novo acute myeloid leukemia case and control subjects, adjusted odds ratios, and 95% confidence intervals by cytogenetics for the NQO1 enzyme, using wild type as the reference

<table>
<thead>
<tr>
<th>Cytogenetics*</th>
<th>Wild type</th>
<th>Heterozygote + Homozygote</th>
<th>OR†</th>
<th>95% CI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>Control</td>
<td>Case</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>83</td>
<td>177</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>Translocations:</td>
<td>46</td>
<td>106</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>28</td>
<td>51</td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>12</td>
<td>33</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>inv(16)</td>
<td>6</td>
<td>22</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>5q–/7q–</td>
<td>11</td>
<td>21</td>
<td>11</td>
<td>16</td>
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<tr>
<td>Other abnormality</td>
<td>53</td>
<td>90</td>
<td>28</td>
<td>48</td>
</tr>
<tr>
<td>Failed/not tested</td>
<td>29</td>
<td>49</td>
<td>29</td>
<td>49</td>
</tr>
</tbody>
</table>

NQO1 indicates NAD(P)H:quinone oxidoreductase 1.
*Translocations include t(15;17), t(8;21), or inv(16); 5q–/7q– includes 5q–/7q–; other abnormality includes other clonal abnormalities not classified elsewhere.
†Odds ratio were estimated by means of conditional logistic regression, adjusted for deprivation.
that this might be a chance finding, because it was based on a relatively small number of cases following subclassification by cytogenetics. However, performing an unmatched analysis on the case-control study data, using all control subjects and stratifying on the matching variables, still resulted in increased odds ratios within the same cytogenetic subgroups as were observed with a matched analysis. In particular, low activity of NQO1 remained associated with inv(16) (OR = 4.16; 95% CI, 1.54-11.24). Furthermore, analysis of the additional MRC cases of AML confirmed our findings for the cytogenetic subgroups in the case-control study, especially the high frequency of inv(16) cases with low or null activity at NQO1. Our finding is also consistent with the Larson et al study, which reported that the frequency of heterozygotes with low NQO1 activity in a small group of 10 cases with inv(16) or t(15;17) was 70%, twice the expected rate of 34%.

Thus, it seems likely that low NQO1 activity confers a significantly increased risk of contracting AML with inv(16). The question then becomes why cases with inv(16) should have the highest risk. The obvious explanation for the strong association between AML cases with inv(16) and low or null NQO1 activity is that certain substrates that are normally detoxified by NQO1 are highly effective at causing inv(16). However, it is of interest that the NQO1 gene is located on chromosome 16q22.1, one of the breakpoints for the inv(16) rearrangement. It is possible that one copy of the NQO1 gene is disrupted by the rearrangement, with the result that heterozygotes would have null NQO1 activity in leukemic cells with the inv(16). This loss of activity could be strongly associated with the production of secondary genetic changes caused by exposure to NQO1 substrates after an inv(16) has arisen, leading to a leukemic clone.

Although the risk for inv(16) is the highest for all the cytogenetic changes we analyzed, it should be noted that other classified cytogenetic subgroups of AML also had odds ratios of 1.46 or greater and that none of these differ significantly from one another as confidence intervals overlap (Table 2). Some of these increased odds ratios were not significantly elevated, however. For example, the increased risk for AML harboring alterations in chromosomes 5 and 7 had an odds ratio of 1.57 but was not significantly elevated, although our upper confidence limit certainly does not refute an excess of such cases. This is somewhat at odds with the earlier findings of Larson et al, in which the greatest risk associated with low NQO1 activity was for leukemias with alterations in chromosomes 5 or 7. There are many differences, however, between the leukemia cases studied here and those in the Larson et al study. More than half of the cases in the Larson et al study in Chicago were therapy-related AMLs, whereas we chose to examine only de novo leukemia cases. Some recent publications have indicated that therapy-related AMLs may be pathologically distinct from the de novo group. A much higher incidence of micrornas presented instability and abnormalities of the mismatch repair pathway has been reported in t-AML, and although subsequent studies have not corroborated these findings, it remains a distinct possibility that risk factors important in these types of leukemia will be different. The source of DNA was also different in the 2 studies. The Larson et al study used lymphoblastoid cell lines, which may result in bias through analysis of a selected subgroup of patients who have transformable lymphocytes. The current study looked at patient material directly, which more accurately reflects the group as a whole. A slight concern is the potential for contamination of the material with leukemic blast DNA that could have a different genotype than the normal host DNA. However, it is highly unlikely that the NQO1 C609T polymorphism arises from a novel mutation in the leukemic cells or is selected for in tumor progression. At most, 1 or 2 cases may have been misclassified as a result of a different genotype in the leukemic blast cells. This would not significantly affect the data or alter the conclusions made.

By inference, our data suggest that environmental agents that are normally detoxified by NQO1 are risk factors for producing ALL and AML with chromosomal translocations and inversions. Chromosome translocations and inversions most probably arise as a result of DNA double-strand breaks followed by erroneous repair. Thus, agents that cause double-strand breaks, inhibit DNA repair, and are normally detoxified by NQO1 are candidate environmental agents responsible for leukemia. Interestingly, the phenolic metabolites of the established leukemogen benzene accumulate in the bone marrow. These metabolites—phenol, hydroquinone, catechol, and trihydroxybenzene—can cause double-strand DNA breaks and inhibit DNA repair and topoisomerase II and are normally maintained in their reduced state by NQO1. This suggests that benzene exposure from gasoline, cigarette smoking, and air pollution may be a risk factor for some forms of leukemia in the general population. Although smoking has been associated with an increased risk of acute leukemia in this and other studies, there was no evidence of interaction between NQO1 and smoking in our data. Perhaps a more important source of phenol, hydroquinone, and catechol may be the diet and the intestinal breakdown of excess dietary protein. These dietary sources outweigh those derived from environmental benzene exposure, and we have recently proposed that phenols derived mainly from diet are potentially important risk factors for acute leukemia. There are many other compounds that are substrates for NQO1, including quinones, quinone-epoxides, quinone-imines, naphthoquinones, methylene blue, azo, and nitro compounds, and these may be involved in leukemia induction. Others, potentially metabolized by NQO1, include dietary flavonoids, which are topoisomerase II inhibitors and have been linked with infant leukemia. NQO1 also protects cells from the effects of chronic oxidative stress by maintaining antioxidant forms of ubiquinone and vitamin E. Thus, agents that induce chronic oxidative stress through inflammation or other mechanisms may also play a role in producing acute leukemia.

One puzzling aspect of our finding of an association between null or low NQO1 activity and adult acute leukemia is that NQO1 protein expression in peripheral blood cells and bone

Table 3. Number of de novo acute myeloid leukemia case subjects aged 16 to 69 on United Kingdom Medical Research Council trial, not in analysis of adult case-control study, with cytogenetic abnormality by NQO1

<table>
<thead>
<tr>
<th>Cytogenetics*</th>
<th>Wild type</th>
<th>Heterozygote + Homozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>54.4</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>48</td>
<td>50.0</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>26</td>
<td>65.0</td>
</tr>
<tr>
<td>inv(16)</td>
<td>11</td>
<td>34.4</td>
</tr>
<tr>
<td>5q−/7q−</td>
<td>33</td>
<td>67.4</td>
</tr>
</tbody>
</table>

Cytogenetic abnormality was assessed by Pearson's chi-square and P = .01. NQO1 indicates NAD(P)H:quinone oxidoreductase 1.*Cases were classified hierarchically as follows: t(15;17); t(8;21); inv(16); and 5q−/7q− (which includes −5/5q−/−7/7q−).
marrow progenitors is normally very low, but is highly inducible.2,35 Aside from its inducibility, the presence of NQO1 in other cells such as the bone marrow stroma and/or liver hepatocytes, where it is highly expressed, may be important in protecting against leukemogenesis.

In summary, we report that null or low NQO1 activity caused by inheritance of one or more mutant C609T alleles is associated with increased risk of de novo acute leukemia in adults. Further work is likely to elucidate a number of other low-penetrance genes that are associated with acute leukemia, and this will provide further clues to its potential etiology in the general population.

Acknowledgments

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