Acute leukemia in infants (<1 year) is a distinct biological and clinical entity. The prognosis is generally poor and a large proportion of cases have chromosome rearrangements generating chimeric fusions of MLL with one of diverse partner genes. Studies of identical twin infants with leukemia and retrospective scrutiny of archived neonatal blood spots have revealed that MLL gene fusions arise antenatally, during pregnancy. These data, together with the very high concordance rate in monozygotic twins and the brief latency of disease, suggest that all essential steps in leukemogenesis may be completed before birth and that any genotoxic exposure is likely to be transplacental. MLL fusion genes are also common in secondary acute myeloid leukemia (usually French-American-British (FAB) M4/M5) associated with prior therapeutic exposure to topoisomerase-II inhibiting anthracyclines or epipodophyllotoxins. These observations have prompted speculation on possible exposure to topoisomerase-II inhibiting substances during pregnancy that might give rise to MLL fusions during fetal hematopoiesis. This view finds some support in the experimental demonstration that flavonoid chemicals can cleave the MLL gene. Preliminary epidemiological data have also implicated excess flavonoid exposure or other maternal chemical exposure during pregnancy. Many topoisomerase-II inhibiting chemicals contain quinone rings, the metabolism of which is critically regulated by the enzyme NQO1 or NAD(P)H:quinone oxidoreductase with pediatric acute lymphoblastic leukemias.

Background and Objectives. The enzyme NAD(P)H:quinone oxidoreductase (NQO1) detoxifies chemicals with quinone rings including benzene metabolites and flavonoids. Previous studies in Caucasian populations have provided evidence that a loss of functionality at nt 609 (C609T, Pro187Ser) is associated with increased risk of infant acute lymphoblastic leukemia (ALL) with MLL-AF4 fusion genes.

Design and Methods. We genotyped 103 infants (<18 months) with ALL or acute myeloid leukemia (AML) in Japan and 185 controls for the frequency of allelic variation at nt 609 and 465 in NQO1 using standardized polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodology.

Results. The C609T polymorphism is very common in Japan but we found no link with altered risk for infant ALL. However, a variant of another allele at nt 465 (C465T, Arg139Trp), also associated with diminished enzyme activity, was strongly associated (OR 6.36; CI 1.84-21.90; p=0.002) with infant ALL, especially in t(4;11)(q21;q23), MLL-AF4. No association was found between this allele and risk of infant AML with MLL gene fusions or infant ALL without MLL gene fusions. The same C465T allele has been linked recently, in an Oriental population, to sensitivity to benzene hematotoxicity.

Interpretation and Conclusions. These data endorse the notion that infant ALL with MLL fusion genes have a unique etiology possibly involving transplacental exposure to chemicals.

Key words: infant acute leukemia, NQO1, polymorphism, chemicals, in utero.

Haematologica 2005; 90:1511-1515
©2005 Ferrata Storti Foundation
with a diminished activity mainly due to increased alternative splicing events producing a truncated mRNA without exon 4. These data led to the prediction that if quinone-containing substances were relevant to the etiology of infant leukemia, i.e. via transplacental exposure, then there might be some significant association between \textit{NQO1} alleles and risk of diseases. This was found to be the case. In a UK-based study of 36 infants with \textit{MLL} allele positive leukemia, there was a highly significant association between the \textit{C609T} allele and risk, selective for \textit{MLL} fusion gene positive leukemia and most pronounced for infant acute lymphoblastic leukemia (ALL) with \textit{MLL-AF4} fusions (OR:8.63). The magnitude of this effect was surprising but was confirmed (for \textit{MLL-AF4} cases) in an independent US-based study of 39 patients (OR:10.82).

We sought to confirm these important genetic data in another ethnic group – the Japanese – and report the finding that there is again a very strong association with an \textit{NQO1} allele, but in this case with \textit{C465T} not \textit{C609T}.

\section*{Design and Methods}

\section*{Patient and control samples}

All infants with leukemia diagnosed before the age of 18 months who were registered by the Japan Infant Leukemia Study Group between December 1995 and December 1998 were included in this analysis. Diagnoses were made according to FAB classification. Detailed clinical data, treatment and outcome of some of these patients have been previously described. This study group covered approximately 80% of infant leukemias in Japan during the period considered. Informed consent was obtained from parents of each patient as appropriate according to institutional guidelines prior to initiation of therapy. Mononuclear cells obtained from patients’ bone marrow and/or peripheral blood at the time of diagnosis of acute leukemia were screened for the presence of \textit{MLL} gene rearrangement by Southern blotting and fluorescence \textit{in situ} hybridization (FISH). Karyotype analysis was carried out by conventional cytogenetics and translocation partners of \textit{MLL} were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) in cases with available samples. Controls consisted of umbilical cord blood samples obtained from healthy newborn Japanese infants after obtaining informed consent from their parents.

\section*{\textit{NQO1} genotyping}

Genotyping was performed by polymerase reaction restriction fragment length polymorphism (PCR-RFLP) analysis of DNA extracted from the patients’ blood samples and control umbilical cord blood samples. Twenty nmoles of the primers \textit{NQO1-609A}, 5’-CCTCTCTGTGCTTTCTGTATCC-3’ with \textit{NQO1-609B}, 5’-GATGGACTTGCCCAAGTGATG-3’ (for the \textit{nt 609} polymorphism) or \textit{NQO1 ex4g-1f}, 5’-CTAGCTTTACTCGGACCCACT-3’ with \textit{NQO1 ex4g-r}, 5’-GCAACAAGAGGGAAGCTCCATC-3’ (for the \textit{nt 465} 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 25 μL and subjected to PCR with 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) followed by an extension at 72°C for 10 min.

PCR products were digested with \textit{Hinfl} in the case of \textit{nt 609} polymorphism or with \textit{HpaI} in the case of the \textit{nt 465} polymorphism. Digested products were analyzed by electrophoresis in 3% agarose and viewed by ethidium bromide staining. Digestion of the PCR products for \textit{nt 609} polymorphism with \textit{Hinfl} yielded two bands for the homozygous wild-type (CC; 85 and 214 bp), four bands for heterozygotes (CT; 63, 85, 151, and 214 bp), and three bands for the homozygous variant (TT; 63, 85, and 151 bp) (Figure 1). Digestion of the PCR products for \textit{nt 465} polymorphism with \textit{HpaI} yielded two bands in the case of homozygous wild-type (CC; 111 and 353 bp), three bands for heterozygotes (CT; 63, 85, and 151 bp), and one band for the homozygous variant (TT; 464 bp).

\textbf{Statistical analysis}

For the statistical analysis of \textit{NQO1} nt 609 variant genotype, individuals with the homozygous and heterozygous variant genotypes were categorized as a group with low \textit{NQO1} activity, as described in a previous report, based on observations showing that the individuals with homozygous or heterozygous variant are deficient in \textit{NQO1} protein, primarily.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{\textit{C609T}} & \textbf{\textit{C465T}}} & \textbf{\textit{NQO1 genotyping}}
\hline
\textit{CC} (w/w) & 214 & 464 & (bp) \\
\textit{CT} (w/m) & 151 & 353 & \\
\textit{TT} (m/m) & 85 & 63 & \\
\hline
\end{tabular}
\caption{PCR-RFLP analysis of the \textit{NQO1} polymorphisms on a 3% agarose electrophoresis gel. The figure illustrates the results from five representative individuals. w: wild type; m: mutation or polymorphism. m/m of \textit{C465T} did not exist in any of the patients analyzed.}
\end{table}
Results

The study group consisted of 103 infants with ALL (72 cases) or AML (31 cases). Of these, 49 of the cases of ALL and 15 of the AML cases had MLL gene rearrangements involving different gene fusion partners (Table 1). We assayed DNA samples from these cases for the frequency of two allelic polymorphisms at two positions in the NQO1 gene - C609T (Pro187Ser) and C465T (Arg139Trp). Control population DNA was from cord blood. The allele frequency of C465T in cord blood was 0.34 which is considerably higher than that recorded for Caucasian populations (0.13–0.21).25,26 In contrast to previous reports,25,26 we found no association between the C609T allele and risk of infant ALL with MLL-AF4 or any other subgroup of infant leukemia analyzed (Table 2). A possible exception was found with MLL fusion gene-positive cases of AML. Only 15 cases were available for analysis but there was a suggestion that the low function allele might become statistically significant in a bigger series.

The frequency of the C465T allele in normal individuals was 0.019. In the case of this allele we found a striking and selective association with infant ALL, particularly for infant ALL with MLL-AF4 (OR 6.36, CI 1.84-21.90; p=0.002) (Table 3). The C465T allele was not associated with altered risk for infant AML with MLL gene fusions or for infant ALL or AML without MLL gene rearrangement enrolled in this study.

Discussion

In prior analyses of the association between NQO1 alleles and risk of infant ALL in UK and Caucasian US populations, a striking positive association was found in two independent studies between the C609T loss of function allele and risk of infant ALL with MLL-AF4.
No altered risk for infant ALL was found with the \( C465T \) allele of \( NQO1 \) in these populations.\(^{29-30} \) In a third study involving 50 Italian infants with acute leukemia, an increased risk was also observed among those inheriting the \( C609T \) of \( NQO1 \) but only for the subgroup (of 18 cases) without a \( MLL \) gene fusion.\(^{31} \) A very recent study of infant patients entered into BFM protocols in Germany and Austria also found no positive association between \( C609T \) and infant ALL with \( MLL-AF4 \) fusions.\(^{32} \) In the latter two negative studies, the \( C465T \) alleles were not assessed, understandably in terms of the prior lack of associations reported in Caucasian patients.\(^{33} \) The reason for these discrepant results is unknown. The \( C465T \) allele is also reported to encode diminished function although it has been less extensively evaluated than the \( C609T \) allele.\(^{34} \) The frequency of these two alleles varies markedly between different ethnic groups. The \( C609T \) allele is more common in Orientals than in Caucasians\(^{35} \) in normal individuals; however, in the current study of Japanese infant patients we found no association of this allele with risk of infant ALL or AML (with or without \( MLL \) gene fusions). In marked contrast, we found that the \( C465T \) allele, which is much less common in the Japanese population (~0.012), was strongly associated with an increased risk of infant ALL with \( MLL-AF4 \) (OR: 6.36; 95% CI 1.84-21.90). This implies that the \( C465T \) allele may be functionally more important in this ethnic population or genetic background than is the \( C609T \) allele, at least in relation to whatever exposure triggers \( MLL-AF4 \) fusions. We cannot rule out that the \( C609T \) allele also affects risk in Japanese populations; its high frequency in the normal population may preclude a clear demonstration of such an effect in a rare disease. The biological basis of the predominant impact of \( C465T \) over \( C609T \) in our study is unclear but its credibility is significantly endorsed by a recent report of \( NQO1 \) allele associations with benzene hematotoxicity in Chinese workers exposed to low levels of benzene.\(^{36} \) This study also found a significant positive association for \( C465T \) but not \( C609T \) suggesting a potent selective impact of the \( C465T \) allele on detoxification capacity in the setting of an oriental genetic background.

Our data from Japanese patients reinforce the idea that \( NQO1 \) enzyme function is probably involved in the exposure pathway that leads to infant leukemia and highlight the importance of assessing the impact of all functional allelic variations in different positions within a gene. The \( C465T \) allele effect could easily have been missed. As with prior \( NQO1 \) studies,\(^{37-38} \) the data also suggest that the \( NQO1 \) functional effect is selective for ALL rather than AML, though the number of patients with AML was small. The basis for this potential selectivity is unclear; to date no epidemiological studies have implicated transplacental exposure during pregnancy that might be specific for ALL.\(^{39} \) Nevertheless, the leukemic subtype selectivity endorses the credibility of the finding, particularly in the context of an inevitably numerically small cohort of patients.

\( NQO1 \) is an inducible enzyme that converts quinone to relatively stable hydroquinones bypassing the production of DNA-damaging semi-quinones and reactive oxygen species. The enzyme thus protects against the toxic and carcinogenic effects of quinones and related chemicals.\(^{40-41} \) This profile suggests that quinone-containing chemicals could well be relevant to the etiology of infant ALL; this would include benzene and its metabolites as well as flavonoid-containing substances. It should, however, be noted that \( NQO1 \) also exercises other functions, including an endogenous antioxidant activity (via reduction of \( \alpha \)-tocopherolquinone)\(^{42-43} \) and modulation of p53 activity\(^{44-45} \) so its precise contribution to the etiology of infant ALL remains to be determined.

ME-I, ME: performed the experiments, analyzed and interpreted the data, prepared all the figures and tables, and produced the final version of the paper to be published. EI: provided infant leukemia samples as the representative of the Japan Infant Leukemia Study Group. DK: performed some of the PCR and RFLP analyses. YS: provided RT-PCR data for some infant ALL samples. KI, HY: provided cord blood samples. SM: contributed to the conception and design of the study. MG: responsible for the conception and design of the study, drafting the article, interpretation of the data and production of the final version to be published. All authors reviewed and approved the final version. The authors declare that they have no potential conflicts of interest.

This study was supported by the Leukaemia Research Fund, UK and the Kay Kendall Leukaemia Fund, UK (ME-I, ME, DK and MG), a Grant-In-Aid for Cancer Research from the Ministry of Health and Labor of Japan, Japan Children’s Cancer Association and the Japan Leukemia Research Fund (EI, YS, KI, HY and SM).

We thank all the doctors participating in the Japan Infant Leukemia Study Group for providing samples and clinical information.

Manuscript received April 28, 2005. Accepted August 30, 2005.

### References

8. Eguchi M, Eguchi-Ishimae M, Greaves M. The role of the MLL gene in infant