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Molecular biomarkers for the study of childhood leukemia

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Abstract

Various specific chromosome rearrangements, including t(8;21), t(15;17), and inv(16), are found in acute myeloid leukemia (AML) and in childhood acute lymphocytic leukemia (ALL), t(12;21) and t(1;19) are common. We sequenced the translocation breakpoints of 56 patients with childhood ALL or AML harboring t(12;21), t(8;21), t(15;17), inv(16), and t(1;19), and demonstrated, with the notable exception of t(1;19), that these rearrangements are commonly detected in the neonatal blood spots (Guthrie cards) of the cases. These findings show that most childhood leukemias begin before birth and that maternal and perinatal exposures such as chemical and infectious agents are likely to be critical. Indeed, we have reported that exposure to indoor pesticides during pregnancy and the first year of life raises leukemia risk, but that later exposures do not. We have also examined aberrant gene methylation in different cytogenetic subgroups and have found striking differences between them, suggesting that epigenetic events are also important in the development of some forms of childhood leukemia. Further, at least two studies now show that the inactivating NAD(P)H:quinone acceptor oxidoreductase (*NQO1*) *C609T* polymorphism is positively associated with leukemias arising in the first 1–2 years of life and polymorphisms in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene have been associated with adult and childhood ALL. Thus, low folate intake and compounds that are detoxified by NQO1 may be important in elevating leukemia risk in children. Finally, we are exploring the use of proteomics to subclassify leukemia, because cytogenetic analysis is costly and time-consuming. Several proteins have been identified that may serve as useful biomarkers for rapidly identifying different forms of childhood leukemia.

Keywords: Chromosome translocations; Neonatal blood spots; Cytogenetics; Proteomics; Leukemia; Children; Biomarkers; Molecular epidemiology; Pesticides

Introduction to childhood leukemia

Leukemia, the number one disease killer of children less than 14 years of age in the United States, represents around 31% of all cancer cases among children in this age group. The incidence of leukemia is 10-fold lower in children than adults, with approximately 3250 children and adolescents younger than 20 years of age diagnosed each year in the US, of which 2400 are acute lymphoblastic leukemia (ALL).

Leukemia is a heterogeneous disease, characterized by the dysregulated proliferation of blood precursor cells of myeloid or lymphoid origin. It can be classified as acute (low level of differentiation) or chronic (high level of differentiation) and can be further classified by cytogenetic subtype. For example, t(12;21) which generates the *TEL-AML1* fusion gene occurs in ~25% of patients with common ALL (cALL), a subtype of ALL with peak incidence between ages 2 and 5 (Romana et al., 1995). Translocation t(1;19) and high hyperdiploidy (>50 chromosomes) are also common in childhood ALL (Harrison, 2001), while various specific chromosome rearrangements, including t(8;21), t(15;17), and inv(16), are found in acute myeloid leukemia (AML) (Hall, 2001).

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Overview of biomarker types

Besides the various cellular and molecular characteristics discussed above, leukemias show further heterogeneity in response to therapy and subsequent risk of relapse. There is a clear need to develop techniques to delineate biomarkers of susceptibility and optimized therapy on an individual or subtype basis. This review outlines recent advances in molecular biomarkers of childhood leukemia. The term "genotype" will be discussed in two contexts. In the constitutive context, heritable germline susceptibility genes including rare mutations in high penetrance genes such as Breast Cancer 1 (*BRCA1*), retinoblastoma (*Rb*), Ataxia telangiectasia mutated (*ATM*), and/or common polymorphisms in low risk genes such as *MTHFR*, *NQO1*, glutathione S-transferase (*GST*) modulate an individual's risk of developing cancer. The tumor genotype describes mutations (at the gene or chromosomal level) that characterize the tumor, e.g., t(12;21), monosomy 7, or *Ras* mutation, and may evolve with disease progression. Epigenetic changes at the level of gene-specific methylation in leukemia will be discussed and preliminary data on the emerging field of proteomic biomarker discovery in leukemia will be outlined.

The Northern California Childhood Leukemia Study

Much of the research discussed in this review was based on samples from the Northern California Childhood Leukemia Study (NCCLS), a population-based case-control study begun at the University of California, Berkeley, in 1995 with the aim of investigating the relationship between environmental exposures and genetic risk factors for childhood leukemia (Fig. 1). To date, biological specimens (from peripheral blood, bone marrow and/or buccal cells), from over 800 cases occurring at 9 hospitals covering 35 counties, and a similar number of controls, have been processed and stored in our laboratories. Conventional cytogenetics and fluorescent in situ hybridization (FISH) analyses of NCCLS ALL cases show similar genotype proportions as those reported in the literature, with hyperdiploidy (29%) and t(12;21) (16%) being the most prevalent subtypes.

Gene and protein expression profiling

One approach to stratifying pediatric leukemia and discovering biomarkers is the use of gene expression profiling by microarray analysis to identify subtype discriminating genes. This typically involves statistical determination of patterns of gene expression against a background of global gene expression (~30,000 genes). A recent study demonstrated the ability of such an approach to diagnose pediatric ALL subtypes including *TEL-AML1* and hyperdiploidy, by virtue of unique gene expression profiles in less than 100 genes, with an overall accuracy of 97% (Ross et al., 2003). While this approach is very useful for gaining insight into the underlying biology of leukemia subtypes and developing targeted therapies, global gene expression analysis by microarray is not amenable to the clinical setting. Drawbacks include the need for RNA isolation from an enriched leukemic cell population to minimize background, inherent data complexity, and expense. RNA is also an inherently unstable molecule, making sample processing difficult in the typical hospital setting and gene expression may not reveal the true phenotype which is based on protein.

For these reasons, we and other groups have begun exploring the use of proteomics to subclassify leukemia. Our approach involves the use of the ProteinChip® system from Ciphergen, which combines affinity chromatography and mass spectrometry, allowing the rapid capture and identification of proteins based on their biochemical properties. Using this system, we initially analyzed crude cell lysates from 3 childhood leukemia cell lines: 697 which harbors t(1;19); REH which has t(12;21); and MV4;11 with t(4;11) (Gunn et al., 2004). Analysis of proteins retained on the array surface revealed 22 peaks using the Biomarker Wizard feature of the ProteinChip® software. Average peak intensities were compared among the 3 cell lines. 697 and REH, which are both acute lymphoblastic cell lines, had few differences between them. However, one protein of around 4 kDa was expressed in 697 but not in REH (Fig. 2, Peak A). This may be a marker of ALL cells harboring t(1;19). When the spectra of 697 and REH cells were compared with that of MV4;11, which is a biphenotypic myelomonocytic cell line, and the Kasumi AML cell line, several major differences were noted. One protein of 8.3 kDa was expressed at much higher levels in the ALL cell lines than in MV4;11 or Kasumi cells (Fig. 2, Peak B). This protein is currently being identified and may be a marker of ALL. Yet another protein of around 4 kDa was expressed at a >50-fold higher level in MV4;11 cells (Fig. 2, Peak C). This protein may serve as a useful biomarker for rapidly identifying mixed lineage leukemias harboring translocations involving the MLL gene at 11q23. We are currently attempting to identify the differentially expressed proteins and analyzing the cell lysates on additional chip surfaces. Based on these findings, we also propose to analyze the protein spectra of leukemic cells from bone marrow of childhood leukemia cases to further test the idea that different types of leukemia may be differentiated using ProteinChip® technology.

Chromosome translocations as biomarkers: backtracking childhood leukemia to birth

As discussed above, specific chromosome translocations characterize certain subtypes of childhood leukemia and themselves serve as markers of diagnosis, disease progres-

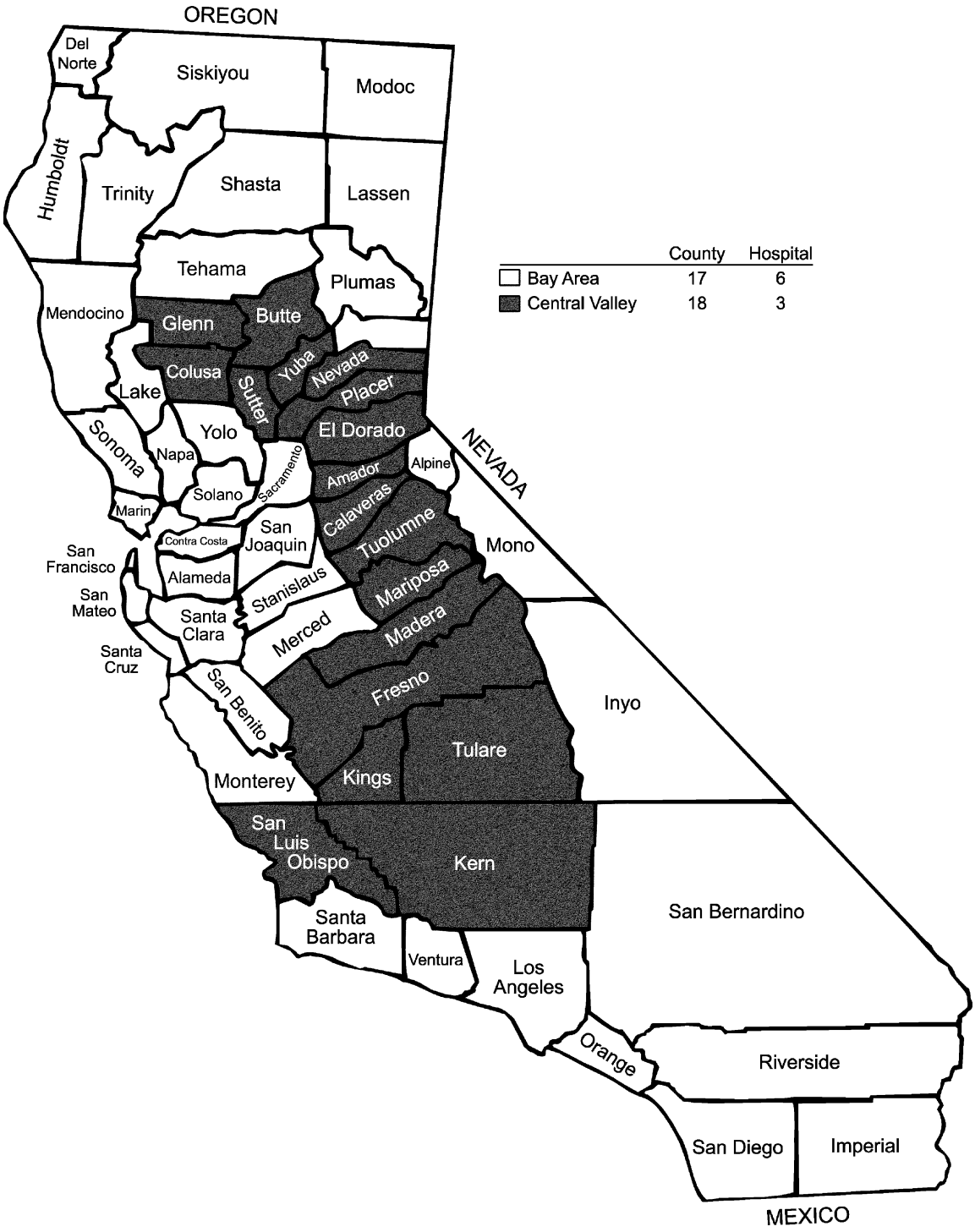

Fig. 1. Northern California Childhood Leukemia Study (NCCLS). NCCLS is a population-based case-control study begun at the University of California, Berkeley, in 1995 with the aim of investigating the relationship between environmental exposures and genetic risk factors for childhood leukemia. To date, biological specimens (from peripheral blood, bone marrow and/or buccal cells), from over 800 cases occurring at 9 hospitals covering 35 counties and a similar number of controls, have been processed and stored in our laboratories.

sion, and relapse. Indeed, detection of translocations by quantitative PCR is widely used to determine minimal residual disease and early pre-clinical relapse (Moppett et al., 2003). This is possible because the signature fusion gene sequence arising from a specific translocation is consistent across patients at the level of RNA, allowing the use of a generic assay for each translocation type. However, as the chromosome breaks occur for the most part randomly in very large (up to 160 Kb) introns, the fusion gene sequences at the genomic DNA level are clonotypic or patient-specific. This characteristic has been exploited in the process of backtracking where certain translocations in childhood

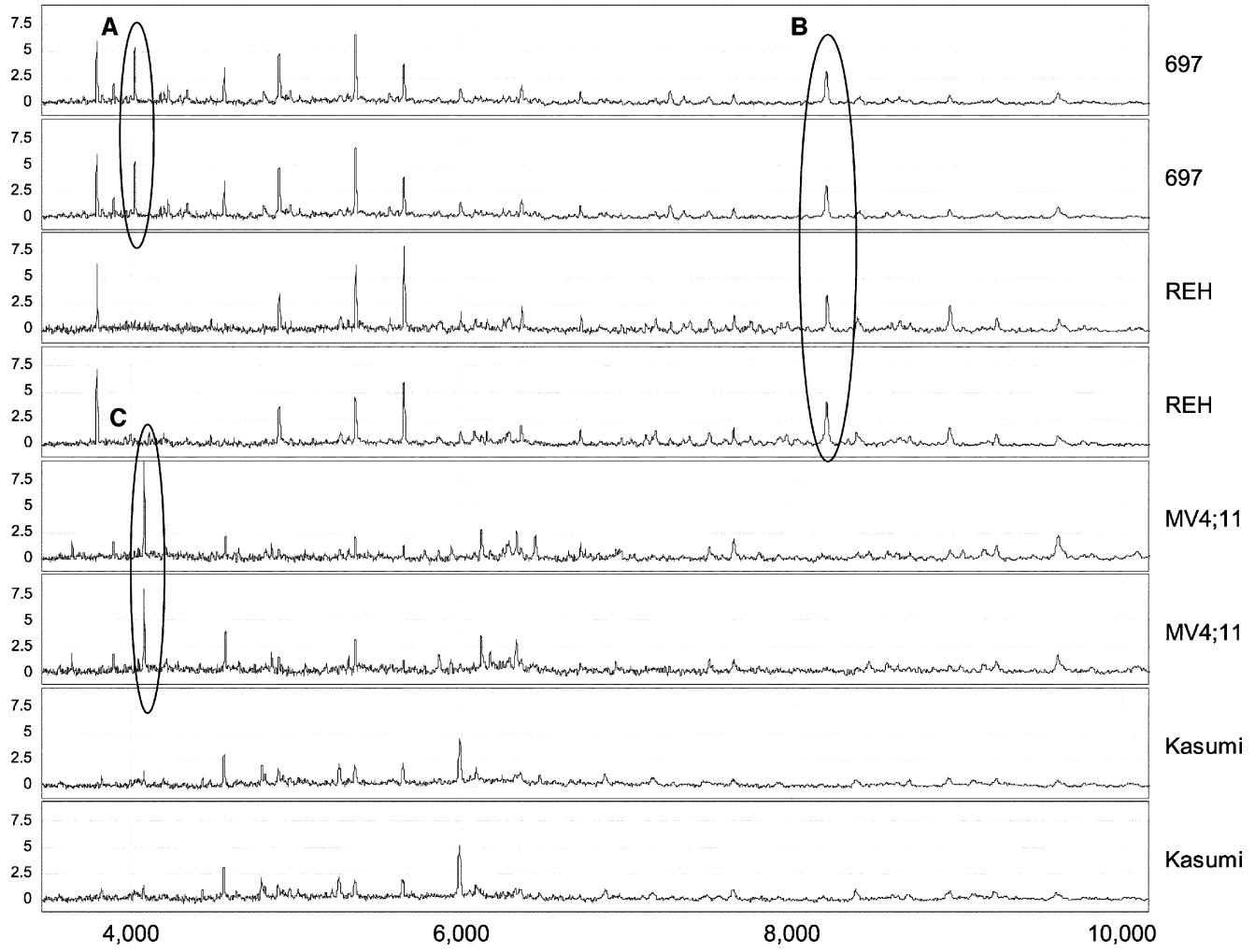

Fig. 2. Surface-enhanced laser desorption ionization time of flight (SELDI-TOF) mass spectrometry proteomics of childhood leukemia cell lines. The ProteinChip® system from Ciphergen, which combines affinity chromatography and mass spectrometry allowing the rapid capture and identification of proteins based on their biochemical properties, was used to determine proteins differentially expressed among leukemic cell lines. 22 peaks from proteins retained on the IMAC-Cu array surface were identified using the Biomarker Wizard feature of the ProteinChip® software. Resulting peaks for an 8.3-kDa protein which was expressed >10-fold more highly in 697 and REH, two acute lymphoblastic cell lines than in MV4;11, which is a biphenotypic myelomonocytic cell line, are shown. This peak is currently being identified.

leukemia were shown to arise in utero, preceding the disease by as long as 14 years. The process, illustrated in Fig. 3, involves sequencing genomic DNA from patients at diagnosis, developing clonotypic, highly-sensitive, nested PCR assays, and testing for the presence of the patient-specific fusion gene sequences on Guthrie cards, blood spots taken at birth for routine screening for metabolic disorders.

The first direct evidence for an in utero origin of specific translocations was for t(4;11) in children aged 5 months to 2 years (Gale et al., 1997). Following up on this pioneering work in Greaves' laboratory, we sequenced the translocation breakpoints of 56 patients with childhood ALL or AML harboring t(12;21), t(8;21), t(15;17), inv(16), and t(1;19), and demonstrated, with the notable exception of t(1;19), that these rearrangements are commonly detected in the neonatal blood spots (Guthrie cards) of the cases (see Table 1, McHale et al., 2003a, 2003b; Wiemels et al., 1999a, 2002b). Indirect support for a prenatal origin of several ALL cases lacking specific chromosomal translocations was provided by tracking clonotypic immunoglobulin heavy chain (*IgH*) and T cell receptor (*TCR*) rearrangements including cALL (Yagi et al., 2000), T-ALL (Fasching et al., 2000), and hyperdiploid ALL (Taub et al., 2002). Hyperdiploid ALL was also recently demonstrated to have an in utero origin by the identification of CD34(+)/CD19(+) B-lineage progenitor cells with triploid chromosomes in the stored cord blood of an individual who subsequently developed hyperdiploid ALL (Maia et al., 2004b). While t(12;21), characteristic of common acute lymphoblastic leukemia (c-ALL), has a peak incidence in children 2–5 years of age (Romana et al., 1995), other translocations of demonstrated prenatal origin such as t(8;21), t(15;17), and inv(16) characteristic of different AML subtypes occur more frequently in young adults (age 20–40) (Moorman et al., 2001). Indeed, in our

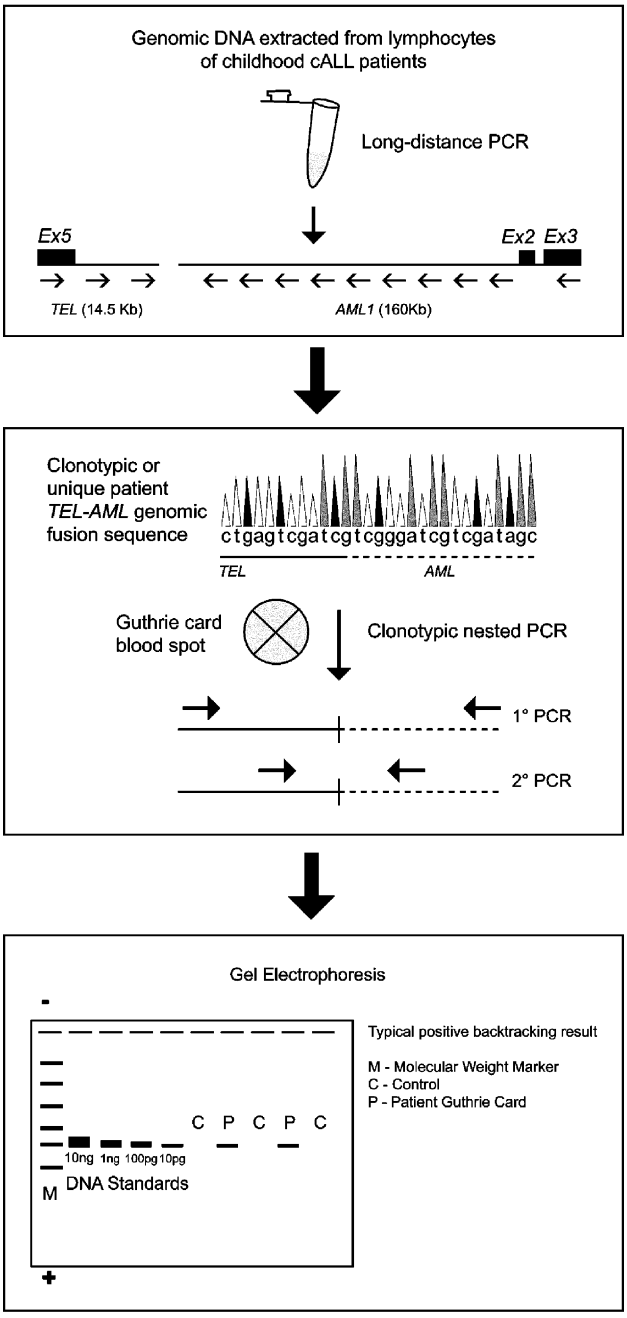

Fig. 3. Backtracking *TEL-AML1* rearrangement in newborn blood spots. Genomic DNA from each leukemic patient is amplified by long-distance PCR and the unique patient gene rearrangement is sequenced. A nested PCR assay is designed for the breakpoint junction and used to determine if the sequence is present Guthrie Card (heel-prick at birth) DNA.

studies, protracted postnatal latencies of over 10 years were demonstrated. The biological significance of such protracted natural histories in childhood leukemia has been reviewed (Maia et al., 2004a). It remains to be determined if leukemias presenting in young adulthood with these translocations also have a prenatal origin. We and others have recently reviewed the origins of chromosomal translocations in childhood leukemia (Greaves and Wiemels, 2003; McHale and Smith, 2004). Overall, these findings show that most childhood leukemias begin before birth and that maternal and perinatal exposures such as chemical and infectious agents are likely to be critical. Indeed, we have reported that exposure to indoor pesticides during pregnancy raises leukemia risk (Ma et al., 2002a).

Chromosome translocations in normal individuals

Translocations t(8;21) and t(12;21) have been shown to occur in the normal population (before birth) at a frequency that is 100-fold greater than the risk of developing the corresponding leukemia (Mori et al., 2002). Other translocations including t(9;22) and t(14;18) (Janz et al., 2003) have been reported in the literature and we have observed t(6;11), t(15;17) (unpublished findings), and del(1p) (Curry and Smith, 2003) in small numbers of normal individuals. The predictive value of these combined observations is unknown. The low concordance rate (10%) observed in c-ALL in twins (Greaves and Wiemels, 2003), and data from animal models (Bernardin et al., 2002; Castilla et al., 1999), imply that the presence of translocations alone is not sufficient to cause leukemia. Translocations may arise in cells with an environment non-permissive for neoplastic progression because of inappropriate lineage or differentiation stage, or lack of complementary mutations at the spontaneous or germline genotype level.

Using genetics to find environmental risk factors for childhood leukemia

Aside from benzene, radiation, and chemotherapy (Pedersen-Bjergaard, 2002), there is currently little direct evidence linking environmental exposure with leukemia. Most likely, gene–environment interactions modulate risk of leukemia development and progression. This is exemplified by Greaves' infectious hypothesis, which proposes that infectious exposures, subsequent to the in utero acquisition of a leukemia translocation, may result in highly dysregulated responses in susceptible individuals (those lacking important infection-driven modulation of the naive immune system in infancy). This could drive pre-leukemic

Table 1
Chromosome abnormalities arising in utero in childhood leukemia

Abnormality	Reference
t(4;11)	Gale et al., 1997
t(12;21)	McHale et al., 2003b; Wiemels et al., 1999a
t(8;21)	Wiemels et al., 2002b
t(15;17)	McHale et al., 2003a
inv(16)	McHale et al., 2003a
Hyperdiploidy	Maia et al., 2003; Panzer-Grumayer et al., 2002
t(1;19)[a]	Wiemels et al., 2002a

[a] Only rarely arises in utero.

clones to fully disseminated leukemia (Greaves, 1997). In support of this theory are the findings that lifestyle factors, such as exposure to multiple infections such as in daycare settings (Ma et al., 2002b), and genetic factors such as variant human leukocyte antigen (HLA) alleles that influence the immune system (Taylor et al., 2002), may reduce risk.

In light of the difficulties in assessing risks from specific exposures, genotype association studies in the case-control setting may be useful for the ascertainment of potential gene–environment interactions. Single nucleotide polymorphisms (SNPs) are common DNA sequence variations among individuals, which may underline differences in response to exposures. Genotype association studies include genotyping of an individual candidate gene, e.g., *NQO1*, or a candidate pathway, e.g., folate metabolism in the members of a study population. DNA pools, compiled from the DNA of many individuals, reduce the genotyping effort and can be used to analyze candidate pathways, e.g., cell cycle, DNA repair, apoptosis, or for genome scanning, but are quite costly ($2 million) and require large amounts of sample material (50 μg DNA per person). New methods involving multiplex analysis of hundreds or thousands of SNPs are becoming available from several companies and will soon be widely employed.

NQO1 and leukemia risk

NQO1 (formerly DT-diaphorase, diaphorase 4; EC 1.6.99.2) is a cytosolic flavoenzyme (homodimer of 273 residues) which catalyzes the obligatory two-electron reduction of quinones to hydroquinones, thus protecting against oxidative stress and toxicity of quinones and other electrophiles. *NQO1* is highly inducible. With regard to the functional genomics of *NQO1*, the homozygous ^{609}C → T mutation (^{187}Pro → Ser) is associated with lack of activity and protein. *NQO1* 609CC has high activity, 609CT has low to intermediate activity, while 609TT has no activity. The homozygous mutation rates among Caucasian/African-American, Hispanic, and Asian populations are ~5%, 15%, and 20%, respectively.

Table 2 summarizes studies examining the effect of the inactivating *NQO1* C609T polymorphism on leukemia risk. A 2.4-fold increased risk of benzene-induced hematoxicity was shown in individuals lacking *NQO1* activity in a case-control study of occupational benzene exposure in Shanghai, China (Rothman et al., 1997). Adult individuals with inactivating mutation were shown to be at increased risk of developing therapy-related acute myeloid leukemia (t-AML) (OR 1.4–1.6), probably through increased vulnerability to chemotherapeutic carcinogens (Larson et al., 1999). Similarly, an increased risk of de novo adult AML was demonstrated in a large case-control study (Smith et al., 2001). Three studies have shown that the inactivating *NQO1 C609T* polymorphism is positively associated with childhood leukemias, especially those arising in the first 1–2 years of life, particularly those with t(4;11) (Krajinovic et al., 2002; Smith et al., 2002; Wiemels et al., 2001).

Table 2
NQO1 C609T and leukemia risk

NQO1 study	OR (95% CI)
Benzene hematoxicity (Rothman et al., 1997)	2.4 (1.0–5.7)
Therapy-related AML (Larson et al., 1999)	1.4–1.6
Adult de novo AML (Smith et al., 2001)	1.5 (1.1–1.9)
Childhood ALL (Krajinovic et al., 2002)	1.7 (1.2–2.4)
Infant leukemia (Wiemels et al., 1999b)	2.5 (1.1–6.0)
Infant leukemia (Smith et al., 2002)	2.5 (1.1–5.7)
Infant t(4;11) (Smith et al., 2002)	7.7 (1.7–∞)

These data suggest that compounds, which are substrates for *NQO1* or cause oxidative stress, are causative factors in producing leukemia. Such compounds include benzene, phenol, hydroquinone, quinones, azo, and nitro compounds (the main source of these compounds is diet) (Smith, 1996, 1999). Others, potentially metabolized by *NQO1*, include dietary flavonoids, which are topoisomerase II inhibitors and have been linked with infant leukemia (Ross, 1998). Oxidative stress may also be generated by inflammation and low antioxidant intake, and the effects of low NQO1 activity may be indicative of their role in the progression of leukemia.

Folate metabolism and leukemia risk

Folate is critical in DNA synthesis and repair and in methylation processes. It is particularly important in cells with high turnover rates and thus having the greatest requirement for DNA synthesis. Deficiencies in folate contribute to DNA damage and impair DNA synthesis. Folate or one-carbon metabolism is channeled into two major pathways: purine and thymidine synthesis for DNA formation and repair, and synthesis of methionine and S-adenosylmethionine for protein and polyamine synthesis and methylation reactions.

Several functional polymorphisms have been identified in the folate pathway. The folate-metabolizing gene 5,10-methylenetetrahydrofolate reductase (*MTHFR*) shunts methyl groups between the DNA synthesis branch and the methylation branch. The C677T low function variant shunts one-carbon groups towards thymidine and purine synthesis, potentially protecting against DNA strand breaks and translocation formation.

An association between polymorphic variants at positions C677T and A1298C in *MTHFR* and a decreased risk of adult ALL was reported by our group (Skibola et al., 1999, 2002). To confirm the role of the folate pathway in leukemia susceptibility, polymorphisms were examined in 3 additional genes involved in folate metabolism (Skibola

et al., 2002). Thymidylate synthase (*TS*) plays a critical role in maintaining a balanced supply of deoxynucleotides required for DNA synthesis. *TS* is polymorphic at a unique tandem repeat sequence site in the 5′ untranslated region, containing either 2 (2R) or 3 (3R) 28-bp repeats (Kaneda et al., 1987). Heterozygotes (2R3R) exhibited a 2.8-fold reduction in ALL risk while the *TS* 3R3R genotype conferred an even greater level of protection (Skibola et al., 2002). Cytosolic serine hydroxymethyltransferase (*SHMT1*) catalyzes 2 reactions in the folate pathway and may play an important role in maintaining one-carbon unit homeostasis (Girgis et al., 1998; Stover and Schirch, 1991). A C1420T polymorphism, resulting in reduced plasma and red blood cell folate thereby mimicking folate deficiency, exists in the *SHMT1* gene (Heil et al., 2001). Individuals heterozygotic for *SHMT1* C1420T exhibited a 2.1-fold decrease in ALL risk, whereas homozygotes had a further reduction (3.3-fold) in risk (Skibola et al., 2002). Overall, this research revealed an association between alterations in the folate metabolic pathway affecting thymidylate synthesis and ALL that may underscore the widespread importance of compromised DNA fidelity and insufficient folate pools in the pathogenesis of adult ALL. Low intake of folic acid and folate cofactors may exacerbate ALL risk in individuals with "high-risk" genotypes. A combination of unfavorable genotypes, diet, and vitamin B status may conceivably be the key factor in susceptibility to adult ALL that may also hold true for certain subtypes of childhood ALL.

Two studies have addressed the role of *MTHFR* polymorphisms in modulating childhood ALL risk (Krajinovic et al., 2004; Wiemels et al., 2001). Wiemels et al. examined the role of MTHFR polymorphisms in molecularly defined subtypes of childhood ALL and showed a significant association between C677T and *MLL* translocations as well as hyperdiploidy (Wiemels et al., 2001). The association between *MTHFR* and childhood ALL was confirmed in a case-control study (270 ALL patients and 300 controls) and furthermore showed a potential gene–environment link between the protective effect of the *MTHFR* variant and folate intake. Indeed, maternal folate supplementation in pregnancy was recently shown to protect against childhood ALL in a case-control study (83 cases and 166 controls) of children in Australia (Thompson et al., 2001).

Folate and fragile histidine locus (*FHIT*) gene methylation

Besides a crucial role in DNA synthesis, folate is involved in the formation of S-adenosylmethionine, a universal methyl donor critical for DNA and protein methylation throughout the body. Aberrant DNA methylation, including global hypomethylation and gene-specific hypermethylation at cytosine-guanine (CpG) islands in gene promoters, may result in dysregulated gene expression.

We examined aberrant gene methylation in different cytogenetic subgroups of our NCCLS population and found striking differences between them, suggesting that epigenetic events are also important in the development of some forms of childhood leukemia. The *FHIT* gene encodes a tumor suppressor gene with proapoptotic function whose aberrant expression was previously reported in leukemia (Peters et al., 1999; Yang et al., 1999). *FHIT* is hypermethylated in a promoter CpG island in several cancer types (Tanaka et al., 1998; Zochbauer-Muller et al., 2001). Our study demonstrated aberrant methylation of *FHIT* as an important mechanism for inactivation of this tumor suppressor gene in hematological malignancies (Zheng et al., 2004). *FHIT* methylation was more common in pediatric ALL (27.4%) than AML (5.9%) and was strongly associated with the hyperdiploid subtype (55.4%) and inversely associated with chromosomal translocations.

Need for a childhood leukemia consortium based on the Interlymph Consortium model

The studies to date on genetic risk for childhood leukemia have all been quite small and even our study of over 800 cases and the British study of over 1000 cases are too small to effectively study subtypes or gene–environment interactions. A consortium needs to be built that will allow thousands of cases to be studied along with similar numbers of controls. A suitable model could be the Interlymph Consortium, which is an international collaboration of 17 non-Hodgkin lymphoma (NHL) studies in 12 countries with over 14,000 cases and extensive environmental data. The scale of this study overcomes limitations of smaller molecular cancer epidemiology studies and enhances power for analyses of gene–environment interactions. Further, it allows the study of NHL subtypes based on molecular and genetic criteria. A similar approach should be taken for childhood leukemia allowing us to determine if specific constitutive genotypes are related to certain subtypes of the disease.

Conclusions

In summary, it has been shown that most childhood leukemias harboring translocations begin before birth. These translocations are present at birth at 100 times the leukemia rate, showing that additional changes are also critical and that screening for translocations at birth is not going to be a suitably predictive biomarker for the disease. It does, however, tell us that the prenatal period is critical for the initiation of childhood leukemia and that exposures during pregnancy should be further examined by epidemiologists. Indeed, in our own study, preliminary findings show that indoor pesticide exposure during

pregnancy raises risk of childhood leukemia. The exact nature of the pesticides responsible or other chemical components in the commercial mixtures used are being investigated further.

With regard to genetic susceptibility, studies to date have shown that the *NQO1* C609T polymorphism raises risk of leukemia especially in infants. Polymorphisms in the folate metabolizing pathway may also be involved, showing that folate and perhaps other dietary components are important risk factors. International collaboration is needed to produce large studies so that these associations can be definitively examined in the case-control setting.

Acknowledgments

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