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PHARMACOGENOMICS OF ACUTE LEUKEMIA

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■ **Abstract** Over the past four decades, treatment of acute leukemia in children has made remarkable progress, from this disease being lethal to now achieving cure rates of 80% for acute lymphoblastic leukemia and 45% for acute myeloid leukemia. This progress is largely owed to the optimization of existing treatment modalities rather than the discovery of new agents. However, the annual number of patients with leukemia who experience relapse after initial therapy remains greater than that of new cases of most childhood cancers. The aim of pharmacogenetics is to develop strategies to personalize medications and tailor treatment regimens to individual patients, with the goal of enhancing efficacy and safety through better understanding of the person's genetic makeup. In this review, we summarize recent pharmacogenomic studies related to the treatment of pediatric acute leukemia. These include work using candidate-gene approaches, as well as genome-wide studies using haplotype mapping and gene expression profiling. These strategies illustrate the promise of pharmacogenomics to further advance the treatment of human cancers, with childhood leukemia serving as a paradigm.

CHILDHOOD ACUTE LEUKEMIA: CURRENT DIAGNOSIS AND TREATMENT

The incidence of cancer in children between 0 and 14 years of age is 1 in 7000. This measure is remarkably uniform across many countries of North America, Asia, Western Europe, and Australia. The most common type of childhood malignancy is acute lymphoblastic leukemia (ALL) that accounts for approximately 25%. Acute myeloid leukemia (AML) accounts for approximately 5%, whereas chronic leukemia is extremely rare in children. Four decades ago, childhood acute leukemia was practically incurable, whereas today the cure rates for ALL and AML are

approximately 80% and 45%, respectively (1, 2). Despite this progress, cancer is still the leading cause of death by disease in U.S. children aged 1 to 15 years, and leukemia causes the highest proportion of these deaths (33%).

The improved rate of cure can be attributed in large part to better knowledge regarding prognostic features (i.e., identification of patients at high risk of treatment failure), treatment intensification and combination chemotherapy (especially for patients with high-risk prognostic features), improved central nervous system (CNS) treatment, and better supportive care (e.g., antibiotics, antifungals). Over the past 20 years, empirical strategies were used mostly to improve the efficacy of combination chemotherapy (2). However, increasing evidence shows that deeper insight into the pathogenesis and mechanisms of drug resistance hold great promise for further improving cure rates (3).

Leukemia is a heterogeneous disease caused by nonrandom chromosomal translocations that produce aberrant gene fusions or inappropriate expression of oncogenes, and the prognosis for cure differs significantly among these genetic subtypes (2, 4). The primary cause of treatment failure is de novo or acquired somatic mutations that render leukemic cells resistant to drug therapy (5). Inherited interindividual differences in the metabolism and disposition of medications are caused by genetic polymorphisms [e.g., single-nucleotide polymorphism (SNP)] that affect gene expression or function in both normal and cancer cells. These polymorphisms alter drug-metabolizing enzymes, drug transporters, or drug targets that can, in turn, profoundly influence the efficacy and toxicity of antileukemic agents (6, 7).

DEFINITION OF AND STATE-OF-THE-ART TECHNOLOGY IN PHARMACOGENOMICS

Single-Nucleotide Polymorphism

The Human Genome Project and The SNP Consortium have unveiled many types of sequence variations within the entire 2.9-Gb human genetic code. Common variations include SNPs, insertions and deletions of nucleotides or entire genes, gene copy number, and variation in repetitive sequences.

The most common inherited sequence variations are SNPs, which occur in exons, introns, promoters, enhancers, and intergenic regions. Only a small portion ($\sim$60,000) of the known SNPs are located within coding regions of genes, and only about 30,000 of those result in amino acid changes in expressed proteins. The remaining ($\sim$30,000) SNPs that are located within the coding regions but do not alter the encoded protein are referred to as synonymous SNPs (8). More than 1.4 million SNPs were identified in the initial sequencing of the human genome, and it is now estimated that $\sim$7 million SNPs exist in humans with a minor allele frequency of $>$5% (plus another $\sim$4 million SNPs with a frequency of 1%–5%) (9–12). The main public SNP database is dbSNP (http://www.ncbi.nih.gov/SNP/). This database contains more than 10 million SNPs; however, approximately 15%

to 17% of those are false positives. Approximately 80% of all SNPs have a minor allele frequency of <10% (13).

Haplotypes are combinations of SNPs in the same region of DNA (usually <50 kb apart form each other) that are typically inherited together. Genome-wide haplotypes can be constructed by linkage disequilibrium (LD) analysis (14). Recent studies have suggested that the human genome is organized in blocks (~50 kb in size) of haplotypes with high LD (regions with a high level of concomitant inheritance) that are separated by regions of low LD (regions with a low level of concomitant inheritance) (11, 15). Therefore, SNPs that are in strong LD with a disease phenotype or a drug-response phenotype can point to the position on the chromosome where a susceptibility gene is located, even though the SNPs themselves may not cause the phenotype (16).

Modern genotyping methods have greatly increased throughput and reduced costs of obtaining relevant genotype information from genomic DNA. Older gel electrophoresis–based methods are PCR coupled with restriction fragment length polymorphism analysis (PCR-RFLP), multiplex PCR, oligonucleotide ligation assay, and mini-sequencing. Typical high-throughput methods use fluorescent dye–based technologies, including oligonucleotide ligation assay, pyro-sequencing, single-base extension with fluorescence detection, homogeneous solution hybridization such as TaqMan® or molecular-beacon genotyping assays. Rolling-circle amplification and Invader® assays can be used to genotype directly from genomic DNA without PCR amplification. Lastly, DNA chip–based microarray and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry genotyping technologies have been developed more recently, and innovations are still emerging (17–21). Currently, the greatest challenge is not genotyping but rather how best to use these methods to elucidate genotype-phenotype (i.e., drug response) relationships, many of which are complex and are multigenic traits (10, 22–24).

Gene Expression Profiling

Gene expression or mRNA analysis is facilitated by techniques that make it feasible to simultaneously analyze the expression of tens of thousands of genes in a single experiment. This technology is already proving to be an invaluable strategy for revealing complex cellular mechanisms (25). Four basic types of arrays exist. One consists of cDNA (200–600 nucleotides long) spotted on nylon membranes. This platform is easily accessible to any laboratory capable of analyzing isotopic blot hybridization. Radioactively labeled cDNA probes are generated by reverse transcription using total RNA or poly-A RNA. Most suppliers offer application-targeted arrays up to a density of several thousands of genes on one membrane. The second type of array, cDNA microarray, is a glass microscope slide robotically printed with 0.6 to 2.4-kb cDNA fragments. Parallel, simultaneous hybridization of test and reference probes on a single slide by red and green labeling with either Cye3- or Cye5-dUTP fluorescence dye is characteristic of this platform. Specialized core facilities typically handle large numbers of samples for high-throughput gene expression profiling and monitoring via cDNA microarrays. Analysis of

complete cDNA libraries or tens of thousands of specific genes per slide is thus possible. Protocols and equipment lists for making and running cDNA microarrays are publicly available (26). The third type of array is an oligonucleotide array (i.e., Affymetrix GeneChip®), where sets of 25-mer oligonucleotides represent a single full-length gene or cDNA. The selection of probes is biased toward the 3′ end in unique regions of each gene. A gene probe set consists of 11 to 16 probe pairs. Each pair consists of a perfect match and a mismatch oligonucleotide; the latter oligonucleotide serves as an internal control and contains a center homomismatch. This complex platform was primarily designed for assessing differential gene expression, but it is also capable of detecting and locating gene polymorphisms and variants. The oligonucleotides are synthesized in situ using photolithography and solid-phase DNA synthesis techniques, which require knowledge of the complete cDNA sequence. An amplified and fluorescently tagged fragmented cRNA probe is hybridized to the 4-cm^2 chip containing hundreds of thousands of oligonucleotides (27). Recently, the fourth type of array, ink-jet oligonucleotide microarrays (e.g., 60-mers), were introduced to reliably detect transcript ratios at one copy per cell in complex biological samples (28).

Pharmacogenomics

Pharmacogenomics comprises the study of inherited variability in drug targets or target pathways, variation in genes encoding drug-metabolizing enzymes or drug transporters, and genetic polymorphisms in genes encoding proteins that indirectly influence drug response. It also encompasses how these variations interact to produce inherited drug-response phenotypes (23, 29). Understanding how genetic differences influence drug response and using this knowledge to prospectively individualize the selection of medications and their doses to enhance efficacy and safety is the ultimate goal of pharmacogenomics. Traditionally, pharmacogenetic studies have focused on single-gene candidates chosen on the basis of our knowledge about the medication's pharmacokinetics, the variability of response observed in patients who received the medication, or both. Family studies were initially used to assess the inherited nature of interindividual differences in drug disposition or effect and then to elucidate the genetic basis of monogenic traits (6). Pharmacogenomics can be viewed as a broader strategy to elucidate the entirety of pharmacologically relevant genes, including the effects of genetic variation in single genes, the interaction among genes in biological and pharmacological pathways, the phenotype emerging from these variations, and the effect of the phenotype on drug response. A key feature is genome-wide, large-scale, high-throughput genomic analyses (i.e., DNA sequence variations, gene expression analysis, etc.) coupled with defining their relevance to specific drug responses as the target phenotype (23, 29). Pharmacogenomics has the potential to identify the right drug in the optimal dose for each patient (30), but it will never explain all of the differences in drug response, because many non-genetic factors influence drug response (e.g., patient's age, sex, race, disease state, organ function, and environmental factors).

Bioinformatics

Pharmacogenetics by its nature is a clear example of translational research that requires integration of diverse disciplines such as biology, medicine, pharmacology, genetics, and the fairly new discipline, bioinformatics. Bioinformatics, short for biomedical informatics, was boosted by the Human Genome Project (31, 32) and has been instrumental in the evolution of pharmacogenetics to pharmacogenomics. This discipline includes generation, maintenance, and mining of typically large and diverse public and private databases. Particularly challenging for pharmacogenomics is the integration of genomic, biological, pharmacological, and clinical data to elucidate the genetic basis of complex polygenic drug response traits (33).

Chemosensitivity and Genomics

Malignant cells carry the same germline genetic polymorphisms as do normal cells; however, the genetic instability of malignant cells leads to a relatively high frequency of additional mutations. For example, duplication of entire chromosomes is not uncommon in ALL cells, and this can alter the concordance of germline genotypes and cancer cell phenotypes (33b). Some important sites of germline variations affecting cell growth include the replication apparatus, cell cycle proteins, cytoskeletal proteins, nucleic acids, and signal transduction elements. Most primary cancer cells retain expression patterns similar to their tissue of origin, although some genes appear to be more highly expressed in many different cancers (34). The histologic type or grade of tumor can also alter the pattern of gene expression, as compared to the tissue of origin. Evaluation of these findings should be carefully scrutinized because cancer cell lines cultured in vitro may display substantially different expression characteristics than primary cancer cells in vivo.

There is a paucity of literature describing comprehensive evaluations of genetic features that confer chemoresistance in clinical samples; however, multiple mechanisms of resistance are likely to be responsible for drug resistance within a tumor cell population. Much of the deficiency in our current understanding of resistance to cancer chemotherapy is due to the complexity of the multiple mechanisms involved. However, contemporary methods (e.g., gene expression profiling, proteomics) show promise to facilitate more extensive, yet logistically feasible, studies to elucidate the underlying systems biology influencing drug resistance.

GENETIC POLYMORPHISMS THAT INFLUENCE METABOLISM AND DISPOSITION OF ANTILEUKEMIC CHEMOTHERAPY

Essentially all of the major human drug-metabolizing enzymes that catalyze either the modification of functional groups on drug molecules (classified as phase I reactions) or the conjugation of drugs with endogenous substances to render them more

readily excreted (classified as phase II reactions) exhibit genetic polymorphisms, and many studies have shown that these polymorphisms influence drug disposition (30).

Phase I Enzymes

As stated above, phase I enzymes metabolize the functional part of drug molecules, leading to activation or inactivation of the molecule. Most drugs undergoing metabolism are substrates of members of the cytochrome P450 enzyme family (e.g., CYP3A4, CYP3A5). However, dehydrogenases, hydrolases, and esterases also function as phase I enzymes.

CYTOCHROME P450 ENZYMES Cytochrome P450 enzymes (CYPs), which are primarily located in the liver and gastrointestinal tract, catalyze the metabolism of many antileukemic agents (e.g., vincristine, glucocorticoids, anthracyclines, etoposide, and teniposide) and may activate, inactivate, and/or eliminate anticancer drugs or their metabolites. In humans, 18 CYP families comprising approximately 57 genes have been reported to be highly polymorphic; some of these polymorphisms alter function and result in different inherited drug metabolism phenotypes. The most abundant cytochrome P450 family is CYP3A, and the two key members of this family expressed in human tissues, CYP3A4 and CYP3A5, catalyze the metabolism of a substantial percentage of medications (6, 7, 35). Induction of CYP3A, which may occur after anticonvulsant therapy (a supportive care treatment), increases the systemic clearance of several antileukemic agents, and has been associated with lower efficacy of ALL chemotherapy (36).

Transcriptional control of CYP3A4 is of particular interest in studies of phenotypic variation because CYP3A4 activity is correlated with its mRNA expression. A promoter SNP at position –288G > A (*CYP3A4*1B*) was proposed to be related to decreased CYP3A4 activity, but this notion remains controversial (37, 38). The *CYP3A4*1B* genotype was also hypothesized to predispose to therapy-related leukemia (t-AML), associated with ALL chemotherapy that inhibits topoisomerase II, primarily epipodophyllotoxins (e.g., etoposide, teniposide). CYP3A generates potentially DNA-damaging epipodophyllotoxin catechol and quinone metabolites. In a population of 22 pediatric patients with t-AML and 57 control pediatric patients with ALL, a significant association was reported between the wild-type genotype and increased risk for t-AML (39). However, in a different population of 53 pediatric patients with t-AML and 224 control patients with ALL, using an RFLP procedure on the same PCR product, no association was found between the CYP3A4 genotype and increased risk of t-AML (40). This promoter SNP was not detected in Japanese or in Taiwanese patients (39, 41).

CYP3A5 is expressed in tissues other than liver (e.g., kidney, lung, leukocytes). A high level of active CYP3A5 is expressed in individuals with at least one *CYP3A5*1* allele. Loss of CYP3A5 expression was mainly conferred by a SNP at nucleotide 6986A > G (*CYP3A5*3*). This SNP produces a cryptic splice site and the insertion of exon 3B (670 bp), and splicing consequently introduces a stop

codon. The translated protein is prematurely terminated (amino acid 109 of 502), and enzyme activity is greatly reduced in homozygous *CYP3A5*3* individuals (37). The importance of this SNP in antileukemic therapy has not yet been defined, but because almost half of all anticancer drugs are CYP3A substrates (e.g., paclitaxel, docetaxel, etoposide, anthracyclines, vincristine, vinblastine, etc.), polymorphisms in CYP3A5 might affect the metabolic clearance and pharmacological effects of several antileukemic agents. No association was found between the *CYP3A5*3* genotype and increased risk of t-AML (40).

The *CYP1A1*2A* (6235T > C) SNP, which is located in the noncoding region of this gene, has been associated with a highly inducible phenotype, and this phenotype may contribute to an increased risk of ALL and a worse therapeutic outcome (42, 43). However, the increased risk of ALL was not confirmed in another population of children (44). The *CYP1A1*2B* (4889A > G) SNP , which is located in the heme-binding region of CYP1A1 is in LD with *CYP1A1*2A*. Two polymorphisms are found in the carcinogenic activating CYP2E1 enzyme *CYP2E1*3* (10023G > A) and *CYP2E1*5* (−1293G > C, −1053C > T); the latter is associated with increased transcription and both are hypothesized to contribute to the etiology and incidence of ALL (45). Neither the *CYP1A1*2A* nor the *CYP2E1*3* were associated with increased ALL incidence by themselves, but in combination with the GSTM1-null and GSTP1-null alleles, they were (46). Another study found an association of adult AML susceptibility with the nonsynonymous SNP *CYP1A1*4* (4887C > A), and risk was further increased by either the GSTT1-null or the *CYP1A1*2B* allele (47).

NAD(P)H: QUINONE OXIDOREDUCTASE NAD(P)H: quinone oxidoreductase (NQO1) converts carcinogenic quinones into less toxic hydroxyquinones and helps inactivate the antileukemic agent doxorubicin. The SNP *NQO1*2* (609C > T) causes a missense mutation associated with decreased quinone reductase activity; therefore, individuals environmentally exposed to benzene-like substances may be at higher genotoxic and leukemogenic risk. The frequency of *NQO1*2* is 25% in non-Hispanic Caucasians, 43% in Hispanics and approximately 44% in Asians. An increased incidence of this inactivating polymorphism was found in 56 patients with t-AML, compared with 48 with AMLs (49). Clonal hematopoiesis and telomere shortening may be increased by this polymorphism, and in part, could explain t-AML predisposition (50). In a comparison of 36 infants with MLL-positive acute leukemia with 100 healthy newborn controls, low NQO1 activity genotypes (heterozygous CT or homozygous TT) were associated with AML or ALL containing the MLL gene rearrangements (51). A Japanese study confirmed higher frequency of the inactivating genotype in therapy-related leukemia or myelodyplastic syndrome (MDS) in adults compared with healthy volunteers but did not confirm higher incidence of the genotype in de novo AML (41). The low-activity NQO1 variant was compared in 53 children with t-AML and 224 children with ALL; no association was found that indicates an increased risk of t-AML. Two European studies found no association between the NQO1 variant and the incidence of pediatric ALL or AML (52, 53). The *NQO1*2* variant was associated with shorter

event-free survival in children with ALL (43), but this finding remains to be independently verified. In adults with AML the *NQO1*2* genotype was not associated with shorter event-free survival (54).

Phase II Enzymes

Phase II enzymes conjugate drugs with endogenous substances so that the drug can be more readily excreted. Enzymes in this group that are known to metabolize chemotherapeutic agents include glutathione S-transferases (GSTs), thiopurine S-methyltransferase (TPMT), and UDP-glucuronosyl transferase (UGT). The group of phase II enzymes also includes sulfotransferase, *N*-acetyltransferases, and catechol O-methyltransferase, but these enzymes have not been studied in relation to acute leukemia.

GLUTATHIONE S-TRANSFERASE The GST family is subdivided into four major subfamilies: GSTA, GSTM, GSTP, GSTT. These enzymes conjugate glutathione to many endogenous and xenobiotic electrophiles (including ALL chemotherapeutic agents) and their potentially damaging oxidative metabolites (e.g., cyclophosphamide, anthracyclines, topoisomerase II inhibitors). Conjugation typically is an inactivating process. GST enzymes are highly polymorphic and have been associated with the risk of de novo cancer and therapy-related cancers following chemotherapy and with the efficacy and toxicity of cancer chemotherapy. GST-null genotypes are not uncommon; approximately 50% of most populations have a homozygous deletion of GSTM1, and about 25% have a homozygous deletion of GSTT1 (7).

A higher frequency of the double-null genotype for GSTM1 and GSTT1 was found in African American children with ALL compared to healthy African American children (55). This finding was not observed in Caucasian children (55) and was not confirmed for either ethnic population by a later, larger study (56). However, in both studies the number of African American children was small. A higher frequency of GSTM1-null, but not GSTT1-null, genotypes was found in children with ALL compared with healthy children (42). Contradicting results on the GSTM1-null frequency in ALL were reported from different pediatric populations in which neither the GSTM1-null nor the GSTT1-null genotype was associated with childhood ALL etiology (44, 55–58). The GSTM1-null, but not the GSTT1-null, genotype was more frequent in children with AML or MDS (particularly in FAB type M3, M4) than in healthy controls (59). The etiology of acute leukemia in adults was linked with the GSTT1-null genotype but not with the GSTM1-null genotype or the combination of the two. Additionally, the association appeared stronger for ALL than for AML (60). Frequencies of the GSTM1 and the GSTT1 deletion were similar in patients with de novo AML compared with healthy controls (41, 61, 62).

Neither the GST-null genotype nor the combination was associated with t-AML incidence in pediatric ALL or adult AML (41, 63). The combination of

the GSTM1- and GSTT1-null genotypes was overrepresented in adult patients with therapy related AML or MDS whose primary malignancy was breast cancer and not testicular cancer or Hodgkin's disease/lymphoma (61). Recently, it was reported that the GSTT1-null genotype was linked to favorable early response to prednisolone in childhood ALL (64). Neither null genotype nor the combination was predictive of childhood ALL treatment outcome (55, 56), but the GSTM1-null genotype may be protective of CNS relapse (55). GSTT1-null but not GSTM1-null (or the lower activity *GSTP1*B,* see below) was related to good prednisolone response (65). The null genotype for either GST was associated with reduced risk of relapse in one study that included matched ALL controls (66). In a small Japanese study of pediatric ALL, patients with the double-null genotype were at higher risk of early relapse (58). Both null genotypes predicted poor treatment response in adult AML (67). More recently, a larger study of 246 children with ALL analyzed 16 common SNPs in 13 different candidate genes and their relation to treatment outcome. In patients with high-risk ALL, the GSTM1 non-null genotype showed a greater risk of hematologic relapse, which was further increased in patients who had a (3R) genotype associated with high expression of the thymidylate synthase gene (TYMS); this was significant in a multivariate analysis that included known prognostic factors. In contrast, CNS relapse was associated with a polymorphism in the vitamin D receptor start site (68). This polymorphism may disable the regulatory function of the Vitamin D receptor upstream of CYP3A4 and ABCB1 in the blood-brain barrier and thus lower the levels of active drug in the CNS (68).

In children with AML treated with intensive multi-agent chemotherapy, the GSTT1-null genotype was associated with a higher risk of drug toxicity and death during remission. In these patients, the absence of detoxifying GST enzymes had a greater impact on host toxicity than on the antileukemic effect. In contrast, the GSTM1-null genotype was not associated with decreased overall survival, but the frequency of disease relapse may be higher (69). Similar results were reported in adults with AML (54).

The lower activity GSTP1 polymorphism at 1578A > G (*GSTP1*B*) causes the amino acid change Val105Ile and is associated with higher etoposide clearance in African Americans treated with steroids, possibly by inducing ABCB1-mediated drug efflux (70). The homozygous *GSTP1*B* variant may also be associated with a better treatment outcome (66). Furthermore, this group found that patients with this genotype and intermediate or high-risk ALL have a reduced incidence of CNS relapse (71). No association with acute leukemia incidence and the *GSTP1*B* genotype was detected in adults (60). The *GSTP1*B* allele was more frequent in pediatric patients with ALL than in healthy controls (72). In one study, this polymorphism was overrepresented in adult t-AML compared with de novo AML cases (73). More recently, this variant, but not the GSTM1-null or the GSTT1-null genotype, was associated with increased risk of ALL in children (46).

THIOPURINE S-METHYLTRANSFERASE Thiopurine S-methyltransferase (TPMT) catalyzes the S-methylation of the thiopurine agents azathioprine, mercaptopurine

(MP), and thioguanine. The genetic polymorphism of TPMT is one of the most completely developed pharmacogenomic examples. In 1980, polymorphic TPMT activity was first recognized in normal volunteers (74, 75),Then, family studies showed autosomal codominant inheritance of TPMT phenotypes, and the gene polymorphisms responsible for those inherited differences were discovered (76, 77, 78). Ultimately, the clinical application of TPMT as a molecular diagnostic was demonstrated in 1997 (79).

At least 17 TPMT alleles have been identified (82), yet three of these alleles (*TPMT*2*, *TPMT*3A*, and *TPMT*3C*) account for more than 90% of intermediate- or low-activity cases (80, 81). The mutant allele *TPMT*2* is defined by a single nucleotide transversion (238G > C) in the open reading frame, leading to the amino acid substitution Ala18Pro (78b). *TPMT*3A* contains two nucleotide transition SNPs (460G > A and 719A > G) in the open reading frame, leading to amino acid substitutions Ala154Thr and Tyr240Cys, whereas *TPMT*3C* contains only the 719A > G transition SNP (77, 78). All three variant alleles are associated with lower enzyme activity owing to enhanced rates of proteolysis of the mutant proteins (83, 84). A rapid and relatively inexpensive assay is available that uses PCR or PCR-RFLP to detect the three signature SNPs in these alleles; this assay identifies ~95% of all variant alleles. In Caucasian populations, *TPMT*3A* is the most common variant TPMT allele (3.2%–5.7% of TPMT alleles), whereas *TPMT*3C* has an allele frequency of 0.2%–0.8%, and *TPMT*2* represents 0.2%–0.5% of TPMT alleles (79, 81). Population studies in Caucasian, African, and Asian populations have demonstrated the broad utility of this approach (82, 85–88) and revealed that the frequency of these mutant TPMT alleles differs among various ethnic populations. Patients who have one wild-type TPMT allele (*TPMT*1* or *TPMT*1S*) and one nonfunctional variant allele (*TPMT*2*, *TPMT*3A*, or *TPMT*3C*) have intermediate activity, and individuals who inherit two nonfunctional variant alleles are TPMT deficient (79, 87). Although most studies in leukemia patients have used erythrocytes as a surrogate tissue for measuring TPMT activity, TPMT genotype also determines TPMT activity (89, 90), as would be expected.

UDP-GLUCURONOSYLTRANSFERASE The endogenous substrate for hepatic UDP-glucuronosyltransferase (UGT) is bilirubin, and the level of UGT expression differs widely among patients. A member of the large and complex UGT1 family is UGT1A1 that has at least 12 alternative forms of exon 1, each with its own promoter. Reduced expression of UGT1A1 and low glucuronidation with increased blood concentrations of unconjugated bilirubin (Gilbert's syndrome, mild chronic hyperbilirubinemia) were associated with mutations in the UGT1A promoter region; 7 TA repeats [$(TA)_7TAA$; *UGT1A1*28*] instead of 6 TA repeats [$(TA)_6TAA$; wild-type]. Despite the fact that this variant was associated with irinotecan dose-limiting toxicity, hyperbilirubinemia has no clinical consequence, and most antileukemic drugs are not metabolized by UGT1A1 (7, 91). Nevertheless, etoposide is glucuronidated by UGT1A1, and a more recent study found lower etoposide clearance associated with the low-activity *UGT1A1*28* variant (70).

Membrane Transporters

Membrane transporters play an important role in acquired and de novo drug resistance. Transporters are often classified by the direction in which they transport drugs (i.e., efflux, influx, or bidirectional).

P-GLYCOPROTEIN The multidrug resistance gene P-glycoprotein (MDR1, PgP, or ABCB1) produces an efflux pump and is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily. ABCB1 transports and may transport many frequently utilized antileukemic agents (e.g., glucocorticoids, anthracycline, taxane, epipodophyllotoxin, and vinca alkaloid classes). ABCB1, which is normally expressed in the apical membranes of the liver, intestines, and kidney and in the brain capillary endothelium, is also expressed in many tumors. Functionally, this protein reduces intestinal absorption and brain uptake, whereas it increases biliary, intestinal, and renal drug excretion. One study in relapsed or refractory pediatric AML suggested that the addition of cyclosporine, an ABCB1 inhibitor, is clinically feasible and may improve chemotherapy response rates (91). However, it is unclear if this effect is due to a decrease in systemic clearance of antileukemic agents secondary to ABCB1 inhibition, inhibition of efflux of drugs from tumor cells, or both. Also, no difference in response rate was detectable between ABCB1-positive and ABCB1-negative patients (92). An evaluation of matched patient samples for AML revealed no association of the ABCB1 polymorphism 2677G > T (Ala 893Ser) with relapsed or refractory AML, with gene-related clonal selection, or with enhanced ABCB1 expression (93). In T-lineage ALL, increased ABCB1 expression, but not ABCB1 activity, may be related to worse outcome (94).

The polymorphism at 3435C > T causes no amino acid change (synonymous SNP), but may cause lower mRNA level of ABCB1 (95). However, this SNP was related to the incidence or relapse in an adult AML population (97) but in a pediatric ALL population it was not (96). In contrast to the latter, another group found the homozygous variant TT genotype to be associated with higher childhood ALL incidences and the CC genotype with shorter event-free survival in children (98). More recently, the CT genotype and the TT genotype were related to reduced incidence of CNS relapse in children with intermediate- or high-risk ALL (71). This finding is likely related to the function of ABCB1 as an efflux pump in the CSF-blood barrier (99). In a large cohort of adult patients with AML, ABCB1 expression at diagnosis was found to be an independent prognostic factor for response after initial therapy (48).

MULTIDRUG RESISTANCE–ASSOCIATED PROTEINS The MRP family of ABC transporters (ABCC or MRP) consists of nine members, each encoded by different genes. Functionally, these proteins serve as efflux pumps in the outer cell membrane, transporting a wide variety of lipophilic substrates. They are also found in many tumors, the airways, and the gastrointestinal tract. There is some overlap

in the substrates for ABCB1 and ABCC1; however, the latter additionally transports cationic and neutral compounds conjugated to glutathione, glucuronate, or sulfate, as well as many multivalent organic anions. Drugs such as phenobarbital, rifampicin, cisplatin, or vinblastine may induce ABCB1, ABCC1, and ABCC3 expression. In model systems, resistance to methotrexate (MTX), etoposide, vincristine, and doxorubicin can be influenced by overexpression of either ABCC1 or ABCC2. ABCC3 preferentially transports glucuronide conjugates, rather than glutathione conjugates. Expressions of ABCC1–3 have been associated with resistance to epipodopyllotoxins and MTX in vitro. ABCC4 and ABCC5 are important transport mechanisms for MP, its metabolites and nucleoside antiviral agents (100–102). There is evidence that expression of proteins encoded by ABCC1 and ABCB1 may be important determinants of response to chemotherapy for AML (102). The level of expression of ABCC1 in children with T-lineage ALL was much higher than that in children with B-lineage ALL (103); this finding could be related to the worse treatment outcome in T-lineage ALL.

BREAST CANCER–RESISTANCE PROTEIN The breast cancer—resistance protein (ABCG2 or BCRP) is extensively reviewed by Schuetz & Krishnamurthy in this volume (103a). Reduced response to antileukemic therapy has been related to ABCG2 expression in children with AML, and increased expression of this protein at the time of relapse has been reported (104). In pediatric ALL, no association was found between the level of ABCG2 mRNA expression and early prednisolone response or relapse-free survival (105). In ALL containing the TEL-AML1 fusion protein, overexpression of ABCG2 was found at diagnosis (103) and has been associated with low intracellular levels of MTX polyglutamates (MTXPG) in this subtype of ALL (106).

REDUCED FOLATE CARRIER MTX enters cells primarily via the reduced folate carrier (RFC or SLC19A1); therefore, mutations in SLC19A1 in tumor cells can lead to impaired MTX transport and resistance (107, 108). A common genetic polymorphism, 80G > A in exon 1, was associated with altered folate levels (109), higher MTX plasma concentrations, and increased relapse rates in childhood ALL (110). However, the 80G > A SNP has not been associated with altered SLC19A1 function in model systems, leaving uncertainty about its effects on MTX disposition (111). A CATG insertion at position 191, which was identified in patients with ALL and in MTX-resistant ALL cell lines, was caused by alternative splicing and resulted in a frame shift with a nonsense sequence. A relation with the level of MTX uptake was not found (108, 111). Higher expression of SLC19A1 was related to better outcome in children with ALL treated with relatively small doses of MTX (112). Higher MTXPG levels were found in children with hyperdiploid ALL, which was linked to extra copies of chromosome 21, where the SLC19A1 gene is located (113). Of note, this effect was significant only in patients treated with low-dose MTX (LDMTX) and not in patients treated with high-dose MTX (HDMTX) (103, 113). In ALL containing the E2A-PBX1 translocation, MTX

treatment intensification improved outcome, and this may be related to lower expression of SLC19A1 in this subtype of ALL (103).

GENETIC ABERRATIONS IN ANTILEUKEMIC DRUG TARGETS/RESPONSE ELEMENTS

Some genes encode proteins that are targets of medications, and polymorphisms in these genes can hinder (or enhance) the effectiveness of antileukemic treatment.

Thiopurines

The thiopurine antimetabolites MP and thioguanine are analogues of the purine nucleosides hypoxanthine and guanine. These medications (individually or together) are key components of ALL treatment protocols worldwide.

Cellular uptake of MP may be achieved via nucleoside transporters (e.g., SLC23A, SLC29A), and phosphoribosylation, S-methylation, and oxidation are the three major pathways for metabolism of these prodrugs. Phosphoribosylation and S-methylation pathways function in leukemic blasts. Phosphoribosylation activates MP through the purine salvage pathway via TIMP (6-thioinosine-5′-monophosphate) and TXMP (6-thioxanthosine-5′-monophosphate) to thioguanine nucleotides (TGNs), which are considered the main active metabolites of MP (114). The first essential activation step is catalyzed by hypoxanthine-guanine phosphoribosyl transferase (HPRT). Cell lines such as 697 or WIL2S have low HPRT activity and are intrinsically resistant to MP (115). HPRT activity can vary 100- to 1000-fold among cell lines. In contrast, HPRT activity varies approximately 10-fold in primary ALL cells from patients. Neither MP resistance nor ALL relapse has been correlated with low HPRT activity (116).

TPMT catalyses the S-methylation of MP and MP metabolites in hematopoietic cells, and S-methylation is the main intracellular detoxifying pathway in hematopoietic tissues. TPMT activity and TGN concentrations in red blood cells are often measured as surrogate markers for therapeutic drug monitoring in patients. Patients with high TPMT activity may be at higher risk of ALL treatment failure and require higher doses of MP, depending on the other components of therapy (90, 117, 118). Patients with TPMT deficiency accumulate excessive cellular concentrations of TGNs (119–121), predisposing them to hematopoietic toxicity that can be fatal (122).

Generally, S-methylation is considered to be inactivating. However, the most abundant S-methylated MP metabolite, S-methylthioinosine-5′-monophosphate (^{me}TIMP), can inhibit phosphoribosyl pyrophosphate–amidotransferase, an essential enzyme in the de novo purine synthesis pathway. Xanthine oxidase oxidizes MP to its inactive metabolite 6-thiouric acid. The level of this enzyme can vary as much as 10-fold among individuals. Xanthine oxidase activity is also tissue specific, with the highest activity in the intestine and liver and very low levels in hematopoietic cells (123).

Thiopurine cytotoxicity is explained in part by inhibition of de novo purine synthesis, which is essential for generating new purines for DNA and RNA synthesis. However, incorporation of TGNs into DNA and RNA is generally considered to be the principal mechanism of MP cytotoxicity. Primarily, 2-deoxy-6-thioguanosine-5′-triphosphate (dG^STP) is incorporated into DNA and has a characteristic delayed cytotoxic effect (124). Recent studies have shown that the incorporation of dG^STP into duplex DNA results in subtle localized modification of the DNA structure (125). Moreover, chromatin structure alteration is most evident in the G2 phase of the cell cycle (124). Enzymes necessary for DNA replication are affected by MP incorporation into DNA, including DNA polymerase, DNA ligase I (126), topoisomerase II (127), and RNase H (115). Furthermore, postreplicative mismatch repair proteins may play a role in the mechanism of MP action (128).

Mismatch repair proteins can tag the dG^STP^*T mismatched pair among them, and futile attempts to repair this DNA mismatch is thought to trigger apoptosis. More recently, a novel protein complex containing HMGB1, HMG2, HSP70, ERp60, and GAPDH has been shown to bind preferentially to duplex DNA into which a dG^STP has been incorporated, and HMGB1 deficiency has been associated with MP resistance in model systems (129). Finally, experiments show that thiopurine treatment results in cell cycle arrest and interferes with as yet uncharacterized mechanisms that regulate cell cycle progression. However, the precise mechanism(s) of MP cytotoxicity and resistance are still only partially understood after 50 years of study.

Clinical interest in TPMT pharmacogenetics is based on numerous studies showing that TPMT genotype or phenotype identifies patients who are at risk of hematopoietic toxicity after thiopurine therapy. These studies have consistently shown that patients with TPMT deficiency are at very high risk of severe hematopoietic toxicity if treated with conventional doses of thiopurines (117, 119). Furthermore, studies have now shown that patients who are heterozygous at the TPMT gene locus are at intermediate risk of dose-limiting toxicity (121). For example, Relling et al. (114) showed that TPMT-deficient patients with ALL tolerated full doses of MP for only 7% of the scheduled weeks of therapy over the 2.5 years of treatment, whereas TPMT-heterozygous or TPMT-homozygous wild-type patients tolerated full doses for 65% and 84% of scheduled weeks, respectively. The percentage of weeks in which MP dosage had to be decreased to prevent toxicity in TPMT wild-type patients was 2%; in TPMT-heterozygous, 16%; and TPMT-homozygous, 76% (114). In a separate series of patients treated at centers other than St. Jude Children's Research Hospital, approximately 70% of those who experienced hematopoietic toxicity during treatment with combination chemotherapy containing thiopurines were found to be TPMT heterozygotes or TPMT deficient (120). As shown in Figure 1, the TPMT heterozygotes required a mean dosage reduction of ∼35%, whereas the TPMT-deficient patients required a mean dosage reduction of 90%. Once the thiopurine dosage was appropriately adjusted, these patients were generally able to tolerate full doses of their other chemotherapy, a situation more likely to be associated with therapeutic efficacy.

TPMT deficiency has also been linked to a higher risk of second malignancies among patients with ALL who have developed topoisomerase-II-inhibitor–induced AML (130–132) or radiation-induced brain tumors (133). Recently, TPMT genotype was analyzed in conjunction with early response measured as minimal residual disease (MRD) at day 78 following ALL induction and consolidation therapy that included a four-week cycle of oral MP (60 mg/m^2/day) (118). The authors found similar toxicity profiles in patients who were TPMT-heterozygous compared to wild type and concluded that no dose adjustments are necessary for the initial four-week period of MP at that dosage. This effect is likely due to the short duration of MP treatment and the modestly lower dosage compared with that administered in other protocols (i.e., 75 mg/m^2/d in St. Jude patients). Of note, TPMT-heterozygous patients had a 2.9-fold lower risk of positive MRD; this finding was consistent with higher systemic exposure to thiopurines owing to lower MP metabolism. It remains to be determined whether further MP dose escalation in TPMT-homozygous wild-type patients would yield greater efficacy in protocols that routinely use lower MP doses (50–60 mg/m^2/d).

In summary, prospective determination of a patient's TPMT activity or genotype permits patient-specific MP dosages that reduce the risk of acute toxicity from thiopurine medications and may identify those patients at higher risk of radiation-induced second malignancies.

Methotrexate

MTX, an antifolate, is one of the most widely used drugs for the treatment of childhood ALL. Physiologically, folates are essential cofactors in one-carbon transfer reactions in purine and pyrimidine synthesis (134), and the structural analog MTX inhibits specific steps in these pathways.

If LDMTX (20–180 mg/m^2) is given, the main mechanism by which it and most other antifolates and natural folates enter cells is via the SLC19A1. SLC19A1 is a bidirectional, temperature- and pH-dependant anion-exchange carrier with an influx K_m of 1 to 5 μM for MTX, thus saturating at low extracellular MTX concentrations of 25 to 50 μM. MTX has a two-fold higher affinity than folic acid to SLC19A1. If HDMTX ($>$1000 mg/m^2) is given, then high extracellular concentrations of MTX (i.e., $>$100 μM) can lead to intracellular accumulation of MTX by passive diffusion. The third influx transport mechanism for folates is via the folate receptor, which saturates at nanomolar concentrations. MTX has a two-fold lower affinity to the folate receptor (K_m in the nM range) compared with folic acid (135). Thus, the folate receptor is not a relevant influx mechanism of MTX into leukemia cells using conventional doses.

MTX is a substrate for intracellular polyglutamation via folylpolyglutamate synthetase (FPGS). FPGS adds as many as six glutamate moieties to MTX. Cleavage of MTXPG via γ-glutamyl hydrolase (GGH) to MTX is localized primarily in the lysosomes. A polymorphism for GGH 452C $>$ T (Thr127Ile) was recently found to be associated with lower GGH catalytic activity for long-chain

$MTXPG_{(4-6)}$. Using long-chain MTXPG, this SNP was predicted to have a 2.7-fold higher K_m and a 67% reduction in catalytic efficiency compared with wild type GGH but little effect on short-chain $MTXPG_{(2-3)}$. MTX and short-chain MTXPGs can efflux from cells by diffusion or via ABCC1 and ABCC3, whereas long-chain MTXPGs are not transported by ABCC1 and ABCC3 and thus are retained in the cells. Resistance to MTX may be due to decreased cellular uptake, defective activation to polyglutamates, elevated expression of its target enzymes, or enhanced degradation or cellular efflux (108).

DIHYDROFOLATE REDUCTASE The mechanism of action of MTX is attributed largely to its ability to inhibit dihydrofolate reductase (DHFR)-mediated regeneration of tetrahydrofolates from dihydrofolates. MTXPGs also inhibit enzymes involved in the synthesis of thymidylate and de novo purines, i.e., TYMS, glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase. Ultimately, DNA synthesis is impaired by the restriction of deoxythymidine triphosphates and purine nucleotides and by subsequent misincorporation of uracil (136).

Intracellular accumulation and retention of MTXPGs at concentrations exceeding the binding capacity of DHFR lead to complete inhibition of tetrahydrofolate generation by DHFR. HDMTX treatment produces higher levels of MTXPGs in leukemic cells from patients than does LDMTX treatment (137), and higher intracellular concentrations of MTXPGs are associated with greater antileukemic effects, i.e., longer event-free survival, decreased number of circulating blasts, and complete inhibition of de novo purine synthesis (138–140).

MTX resistance mechanisms include reduced uptake in ALL cells via the SLC19A1, alterations in the DHFR gene that result in lower enzyme affinity for MTX, gene amplification of the DHFR protein, reduced FPGS-catalyzed polyglutamation, and increased GGH-mediated hydrolytic cleavage. Failure to accumulate MTXPG is associated with enhanced resistance and poorer prognosis (138, 139). Comparing leukemic blasts isolated from children with those isolated from adults, investigators found that the children's cells accumulate more intracellular MTXPGs than do the adults' cells (141). Children who have hyperdiploid B-lineage ALL (approximately 15% of patients) accumulate the highest levels of MTXPGs compared with those who have nonhyperdiploid B-lineage ALL ($\sim$70% of patients) or T-lineage ALL ($\sim$15% of patients). T-lineage ALL cells accumulate the lowest levels of MTXPGs (137, 142); however, there is a 2.1- to 5.5-fold range of MTXPG accumulation within each of these pediatric ALL subtypes.

Increased DHFR expression can cause resistance to MTX (112, 138, 143, 144). A SNP in the 3′ untranslated region of DHFR (829T > C) has been associated with increased DHFR mRNA expression (145). Patients with a DHFR-homozygous or -heterozygous variant have a 2- to 11-fold higher level of DHFR transcript than do patients with the wild-type genotype, but it remains to be determined whether this polymorphism is associated with MTX responses.

5,10-METHYLENETETRAHYDROFOLATE REDUCTASE MTX exerts many of its anticancer and immunosuppressive effects via inhibition of folate-dependent synthesis of DNA, RNA, and proteins. One of the indirect enzymatic targets of MTX is 5,10-methylenetetrahydrofolate reductase (MTHFR). Approximately 10% of Caucasians express an homozygous MTHFR variant (677C > T) genotype that exhibits only 30% of the MTHFR activity observed in those that express the wild-type MTHFR; 40% of Caucasians are MTHFR heterozygotes and demonstrate 60% bioactivity when compared to wild type (146, 147). Polymorphisms in MTHFR (and thus alterations in folate homeostasis) have been associated with neural tube defects, vascular disease, and some cancers (148, 149). The MTHFR 677C > T polymorphism (150) was assessed in a study involving 220 patients with chronic myelogenous leukemia who were given a short course of MTX for immunosuppression following allogeneic bone marrow transplantation (151). Patients with the TT genotype exhibited greater MTX-related toxicity (gastrointestinal and thrombocytopenia) compared with those with the wild-type MTHFR genotype. Similarly, patients with breast cancer and the TT genotype were more susceptible to neutropenia after adjuvant therapy with a cyclophosphamide, MTX, and 5-flurouracil (152). Prospective studies of the MTHFR genotype and MTX toxicity are needed to further elucidate the importance of the former in determining MTX toxicity in different treatment regimens. The clinical importance of this polymorphism is likely to be dependent on the treatment regimen (with or without leucovorin rescue) and other determinants of folate levels (e.g., diet), which may differ substantially among world populations and socioeconomic conditions (153). Recently, in a cohort of children with ALL, carriers of either the MTHFR T677/A1298 haplotype or the MTHFD1 1958G > A variant in combination with the TYMS (3R) had a shorter event-free survival (154).

THYMIDYLATE SYNTHETASE MTX also inhibits TYMS. TYMS is essential in proliferating cells, and increased levels of TYMS have been found in patients with the homozygous triple-tandem-repeat variant of the 5′ UTR in the TYMS enhancer (3R). This variant is associated with increased risk of adverse events in children with ALL (155). In contrast, another matched case-control study did not confirm this finding; possibly, high-dose MTX had masked the effect of the TYMS polymorphism (156). Higher TYMS expression and the TYMS (3R) in conjunction with the unfavorable GSTM1 status are associated with the risk of hematologic relapse. The underlying gene-gene interaction may be that, with the unfavorable GSTM1 non-null, the MTX target TYMS genotype is more critical (67).

Glucocorticosteroids

Glucocorticosteroids play a crucial role in lymphoid malignancies and induce apoptosis by binding to and activating the glucocorticoid receptor (NR3C1), a nuclear steroid hormone receptor. This complex then transcriptionally activates downstream target genes. The impact of NR3C1 200G > A, 1220A > G, and the

*Bcl*I RFLP on disease outcome was analyzed, and the homozygous 1220A > G allele was associated with a worse prognosis in children with ALL (157). However, receptor expression at the mRNA or protein level was not found to be related to glucocorticoid resistance (158, 159).

Kinase Inhibitors

A striking example of how the genetic characteristics of a tumor can be exploited to develop an effective therapeutic strategy involves the drug imatinib. This agent inhibits the tyrosine kinase fusion protein BCR-ABL, which is produced as a result of a chromosomal translocation in malignant cells; in ALL, the presence of this translocation is noted by the Philadelphia chromosome–positive classification. BCR-ABL protein is detected in almost all patients with chronic myelogenous leukemia and in a small percent of adults and children with ALL (Philadelphia chromosome–positive ALL).

Early clinical trials of oral imatinib resulted in a 90% response rate; approximately two thirds of patients treated with imatinib entered complete remission with relatively little toxicity (160). However, it is not clear whether treatment with imatinib is curative or whether it merely suppresses tumor growth as long as the drug is administered. Mutations in the BCR-ABL gene have been associated with clinical relapse (161). It will be important to determine the frequency of such mutations and how this genetic mechanism of resistance can be prevented or overcome. A second-generation ABL kinase inhibitor, BMS-354825, was identified and predicted to be active against imatinib-resistant BCR-ABL variants. In preclinical studies using in vitro and in vivo models of chronic myelogenous leukemia, BMS-354825 was 100-fold more potent than imatinib and, importantly, the inhibitor retained potency against imatinib-resistant BCR-ABL variants that have the Met351Thr mutation in the kinase domain (162). Other mutations, as well as other mechanisms of resistance, will most likely herald a move away from single-agent imatinib therapy and toward the combination chemotherapy paradigm of cancer treatment.

GENE EXPRESSION ANALYSES TO IDENTIFY NEW GENETIC DETERMINANTS OF TREATMENT RESPONSE IN ACUTE LEUKEMIA

Molecular Markers for Leukemia Classification and Outcome Prediction

DNA microarrays, providing a global view of gene expression, have emerged as an important analytical tool in the field of pharmacogenomics. Several pioneering studies in pharmacogenomics of ALL have already been undertaken. Acute leukemias comprise a heterogeneous disease, with subtypes varying in treatment and outcome. Gene expression profiling for diagnosis was initially demonstrated using oligonucleotide microarrays in 38 acute leukemia samples. This study

identified 1100 genes that were differentially expressed between AML and ALL and used the 50 most strongly correlated genes as the classifier. This approach correctly identified 29 of 34 unknown samples. The self-organizing map algorithm independently generated classes that corresponded largely to AML, T-lineage ALL, and B-lineage ALL (163). Clinical distinction between AML and ALL is not difficult, because several diagnostic methods are available, but importantly, this study provided the first strong evidence that gene expression profiles in leukemia cells can be used for cancer diagnosis (164). A subsequent study that included ALL with MLL gene translocations found that these can be distinguished from AML and ALL that do not have an MLL gene rearrangement (165). Two leukemia studies, one in children and one in adults, identified additional subclasses of AML and ALL by using cDNA microarray and oligonucleotide arrays, respectively (166, 167). Major subclasses of AML [i.e., t(8, 21), t(15, 17), and inv(16)] were verified (168). Furthermore, when profiles from patients with AML with normal cytogenetics were compared with those from patients with poor-prognosis AML who presented with trisomy 8, the latter profile showed downregulation of genes involved in apoptosis (169). Genes that are characteristic of the major AML subgroups and those characteristic of B-lineage ALL, T-lineage ALL, and AML that were positive for MLL gene rearrangements were also detected (170). Prognostically important gene expression profiles have been identified in AML (171–173) and in AML containing FLT3 mutations (174). These studies revealed that AML subtypes consistently harbor known genetic alterations such as EVI overexpression and CEBPA mutations; additionally, they discovered other genetic alterations associated with these diseases (175, 176). Therapy-related AML was identified as a heterogeneous disease, and the following three subgroups showed distinct gene expression: 5/del(5q); loss of TAL1, GATA1, and EKLF. Finally, possible treatment options were proposed on the basis of the pathways that were altered (177).

Prognostically important ALL subtypes are determined by morphology, immunohistochemistry, cytogenetics, and molecular diagnostics. The first detailed array analysis in pediatric ALL was provided by Yeoh et al. (178); using oligonucleotide microarrays, they identified distinct gene signatures for these subgroups: T-lineage, TEL-AML1, hyperdiploid, E2A-PBX1, BCR-ABL, and MLL-rearranged ALL. Additionally, these investigators were able to predict in which patients treatment failure and relapse was likely to occur and in which therapy-induced AML might develop (178); these prognostic ALL subgroups were further validated (179–181). In 28 children with ALL, 10 relapses occurred, and 18 patients remained in continuous complete remission (CCR) and these three groups were predicted with a ~70%–90% accuracy (181). For TEL-AML1–positive ALL, the erythropoietin receptor was consistently more highly expressed, potentially providing the essential proliferation and survival signals in this subtype of ALL (182).

T-ALL with MLL gene rearrangements also showed altered expression of oncogenes (e.g., HOX11, TAL1, LYL1); these latter alterations, which affect the expression of the genes that encode the T-cell receptor, are characteristic of early

thymocyte differentiation. The HOX11 cluster was associated with a more favorable prognosis. This alteration was linked to increased expression of genes promoting proliferation activity and inhibition of expression of antiapoptotic genes, which may explain the good response to therapy (183). A study in adults with T-ALL found a gene expression pattern related to the degree of T-cell differentiation, but this alteration was not related to outcome. These authors also identified a different pattern related to response following induction therapy (good response versus refractory) and with long-term clinical outcome (CCR versus relapse). The TTK gene encodes a kinase and is typically expressed at higher levels in proliferating cells than in mature cells. In this study, TTK was expressed at lower levels in T-ALL cells of patients who ultimately experienced relapse compared with patients who remained in CCR (184).

In pediatric patients with B-lineage ALL, the level of expression of TTK was consistently lower in patients with positive MRD (day 33 and week 12) than in those who were MRD-negative (185). Gene expression profiling identified the HOX gene family as the primary marker for oncogenic transformation induced by MLL gene rearrangements in T-lineage ALL (favorable) and B-lineage ALL (unfavorable). FLT3 activation was found in B-lineage ALL but not in T-ALL, indicating that myeloid features may give rise to therapy-induced AML. Interestingly, MLL transformation reduced cell proliferation in both lineages, which may have therapeutic consequences (186). Overexpression of HOX genes was confirmed in ALL positive for MLL rearrangements; FLT3 activation was also found but was not indicative of outcome. Gene expression signatures were associated with outcome in ALL with MLL rearrangements that were independent from the fusion partner and other clinical factors (187). FLT3 inhibition by the selective inhibitor CEP-701 was evaluated in eight ALL cell lines and in primary ALL cells from 40 patients, and high potency was found in ALL cells overexpressing FLT3 (i.e., MLL rearranged, hyperdiploid) (188).

Despite gene expression being reflective of tissue-type, it may also be germline driven. In a cohort of 42 children treated for ALL, candidate genes were identified that showed different expression in ALL cells from children in whom irradiation-associated secondary brain tumors had developed compared to those who did not develop secondary tumors (189).

Molecular markers with differential expression related to subgroups and prognosis may reflect leukemogenesis. For example, the receptor tyrosine kinase FLT3 has the potential to serve as a target for novel molecular-based treatment for leukemias harboring rearranged MLL with FLT3 alterations (190). In most cases, the candidates identified in expression studies using relapse or treatment outcome as an endpoint have yet to be verified independently. Conflicting results are largely due to differences in treatment. However, a consistent finding in studies of acute leukemias and is that leukemia subtypes have distinct gene expression profiles, and the marker genes from those profiles give new insight into the pathobiology of the diseases and serve as potential new targets for therapeutic intervention. A few drugs targeting these molecular targets are already in early clinical trials.

Gene Expression to Elucidate Genetic Determinants of Anticancer Drug Resistance

Great progress has been made in identifying gene expression patterns that correlate with different leukemia subtypes. Identifying expression patterns that predict drug sensitivity within ALL subtypes has proven to be difficult because both the host genome and the tumor genome affect drug response (7).

Comparison of expression profiles in malignant cells and their normal cellular counterparts may allow for identification of new diagnostic or therapeutic targets. Evaluation of patient outcome, when compared with leukemia cell gene expression patterns in the same patient, may also reveal new prognostic features and therapeutic targets. Changes in gene expression in tumor cells after exposure to chemotherapy may also help elucidate pathways important in drug resistance (191) and serve as a tool in drug discovery (192, 193). Most studies attempt to identify a set of genes, including both chemosensitive and chemoresistant genes, that are predictive of treatment response to a specific drug (194).

Current limitations include the availability of suitable primary analysis of cancer cells for gene expression or proteomic studies. Also, currently available DNA chips do not detect all human genes and their many splice variants, nor do they discriminate wild-type transcripts from those containing functional SNPs.

Evaluation of gene expression may also be a very sensitive method of aiding early dose-finding studies of experimental anticancer compounds, which are not thought to work through traditional cytotoxic mechanisms.

The National Cancer Institute screened ~60,000 compounds against a panel of 60 human cancer cell lines, including four leukemia cell lines. The growth inhibition for 50% of the cells was related to the intrinsic gene expression. Dihydropyrimide dehydrogenase (DPYD) is essential for the catabolism of pyrimidines and fluorouracil (5FU). Thus, the expression of DPYD was inversely correlated with sensitivity to 5FU treatment, and high levels of DYPD resulted in decreased cellular levels of active 5FU metabolites. In a similar manner, asparaginase synthetase (ASNS) expression was inversely correlated with sensitivity to L-asparaginase treatment. This correlation was stronger in the subpanel of leukemia cell lines, which express lower levels of ASNS than do other cancer cells. This finding indicated the importance of L-asparaginase in leukemia treatment (195). Additionally, some cases of ALL may respond better than others; therefore, ASNS expression may serve as a marker to assess L-asparaginase efficiency. Furthermore, these results indicate that for a subset of compounds, microarrays may be a feasible method for predicting chemosensitivity (196).

Infants with ALL positive for MLL gene rearrangements have a poor prognosis, but primary cells from these patients are highly sensitive to cytosine arabinoside (Ara-C). In a study using quantitative RT-PCR of Ara-C-metabolizing genes, the level of expression of the Ara-C-activating enzyme, deoxycytidine kinase, was approximately two-fold lower and the equilibrative nucleoside transporter 1 was ~2.5-fold higher in infant ALL with rearranged MLL compared with that in

children with ALL without rearranged MLL (197). The effects of changes in Ara-C–induced gene expression over 48 h were evaluated in the T-ALL cell line CEM; the cells apoptosed and in the chronic myelogenous leukemia cell line K562, the cells differentiated. Notably, ASNS expression decreased in K562 cells; therefore, sequential treatment of Ara-C and L-asparaginase was tested and found to have synergistic effects (198).

Gene Expression Patterns Associated with Drug Resistance and Treatment Outcome

To better understand mechanisms underlying resistance to different antileukemic agents, investigators analyzed in vitro sensitivity and gene expression profiles of primary leukemic cells. This approach revealed genes associated with single-drug resistance to one of four structurally different and widely used antileukemic agents (i.e., prednisolone, vincristine, L-asparaginase, and daunorubicin). There was minor overlap among resistance genes of the four agents, supporting the concept of multiagent chemotherapy. Importantly, in addition to known risk factors (i.e., age, WBC, ALL subtype), these resistance patterns were related to treatment response. Furthermore, this gene profile was validated in an independent cohort of children with ALL treated with, for the most part, the same agents but on a different protocol (199). It was observed that the in vitro sensitivities of primary cells for different drugs were correlated. Using a large set of sensitivity data, investigators identified a cross-resistance gene profile. Additionally, they detected a new phenotype of discordant resistance to vincristine and L-asparaginase related to altered expression of a large proportion of protein biosynthesis genes. Importantly, compared with the single-drug resistance gene profile, the cross-resistance gene profile further discriminated patients who were at risk of relapse. The cross-resistance gene profile was also related to MP sensitivity, a drug not included in the original analysis; the single-drug resistance gene profile was not (200, 201).

A small study related initial resistance and later relapse (6–12 weeks) with gene expression at diagnosis and gene expression changes during treatment with the antileukemic agent imatinib (STI571) and proposed genes for predicting sensitivity to this agent (202). In a larger study of gene expression, gene expression changes in primary ALL cells 42 h after initial treatment discriminated among three treatment arms of the protocol: HDMTX + MP, LDMTX + MP, or MP alone. Nearly 86% of the genes whose expression changed after combination therapy of MTX and MP were not previously identified after single-agent treatment, indicating that the effects of combination therapy are not simply the sum of the effects of each single agent (191, 203).

Using a candidate gene approach, investigators related the expression of 82 genes involved in the purine pathway to the rate of de novo purine synthesis in ALL cells from children with different subtypes of ALL. De novo purine synthesis was significantly lower in ALL cells positive for the TEL-AML1 gene fusion, and a subset of 16 genes was identified that discriminated TEL-AML1–positive from

TEL-AML1–negative ALL cells. Of note, only two of the purine metabolism pathway genes were identified previously as characteristic for TEL-AML1–positive ALL (204). Similarly, 32 folate pathway genes were associated with higher levels of MTXPG in ALL cells from children after initial therapy with HDMTX alone. T-lineage, TEL-AML1, and E2A-PBX1 ALL cells accumulated lower levels of MTX than did those that were either hyperdiploid or lacking any of the above abnormalities. In summary, FPGS (MTXPG catabolism) expression was lower in T-lineage cells; ABCG2 (MTX efflux) expression was higher in TEL-AML1 cells; and SLC19A1 (MTX influx) expression was lower in E2A-PBX1 cells (102).

Intracellular TGN levels after in vivo MP administration were higher in patients treated with the single agent compared with those in patients treated with MP plus either HDMTX or LDMTX (205). Gene profiles identified for both groups revealed, among other genes, the expression of the SLC29A1 associated with TGN accumulation. Importantly, inhibition of this transporter caused an approximately 40% reduction of TGNs in ALL cells in vitro (206).

Using global gene expression analyses, investigators found a subset of five genes that are associated with differentiation of AML cells. On the basis of the expression of these five genes from an initial high-throughput screen of 1739 compounds, eight drugs were identified that reliably induced this signature after in vitro treatment (192, 193). This approach provides a powerful and systematic approach to compound discovery that will be extended to the investigation of other diseases.

Finally, global gene expression profiling was related to 16 different gene loci of well-characterized polymorphisms that are important in ALL. In this study, the promoter-repeat polymorphism (UGT1A1*28) and the GSTM1-null produced gene signatures significantly related to the germline genotype (207).

THE FUTURE OF PHARMACOGENOMICS

Clearly, pharmacogenomics has great potential to improve the use of antileukemic agents by reducing toxicity and enhancing efficacy through optimal treatment selection, dose individualization, and new drug discovery. The investigation of genetic determinants of efficacy and toxicology of drugs used in the care of patients with leukemia will also enhance the utility of those drugs. Information available at this time obviously represents a small fraction of the future potential for these approaches, pointing to the need for additional research. Moreover, most drug responses are influenced by multiple genes, so polygenetic studies and models will be required to more fully elucidate the genetic determinants of drug response (23). A major challenge will be to establish optimal treatment approaches by determining how genetic information can be used in conjunction with data on nongenetic causes of interpatient variability in drug response. In addition, proteomic approaches may augment this information by providing information on transcription and posttranslational modifications. Another beneficial

application of pharmacogenomics will be in the drug-development process. Genetic profiling and proteomic analysis of tumor and normal tissue should facilitate the discovery of new targets for anticancer drugs and of previously unrecognized genomic determinants of cancer drug resistance and host toxicity. Together, these approaches should facilitate further individualization and optimization of cancer treatment.

It is clear that genetic polymorphisms are important determinants of drug disposition and effects in children with ALL. A specific genotype may be important in determining the effects of a medication for one population or disease but not for another. Therefore, pharmacogenomic relations must be extensively validated for each therapeutic indication and in different racial and ethnic groups to ensure accurate association of genetic determinants of drug response and to guide translation of pharmacogenomics into widespread clinical practice. To this end, informed consent should be sought from all patients enrolled on clinical drug trials to obtain samples of their genomic DNA and to perform pharmacogenetic studies (29) so that knowledge can be generated as new findings emerge.

Future studies using pharmacogenetics approaches are necessary to elucidate additional mechanisms of de novo or acquired drug resistance and other important prognostic pharmacogenomic determinants. These results hold great promise for optimizing personalized treatment, for developing more effective antileukemic drugs, and for ultimately improving cure rates for patients with acute leukemia.

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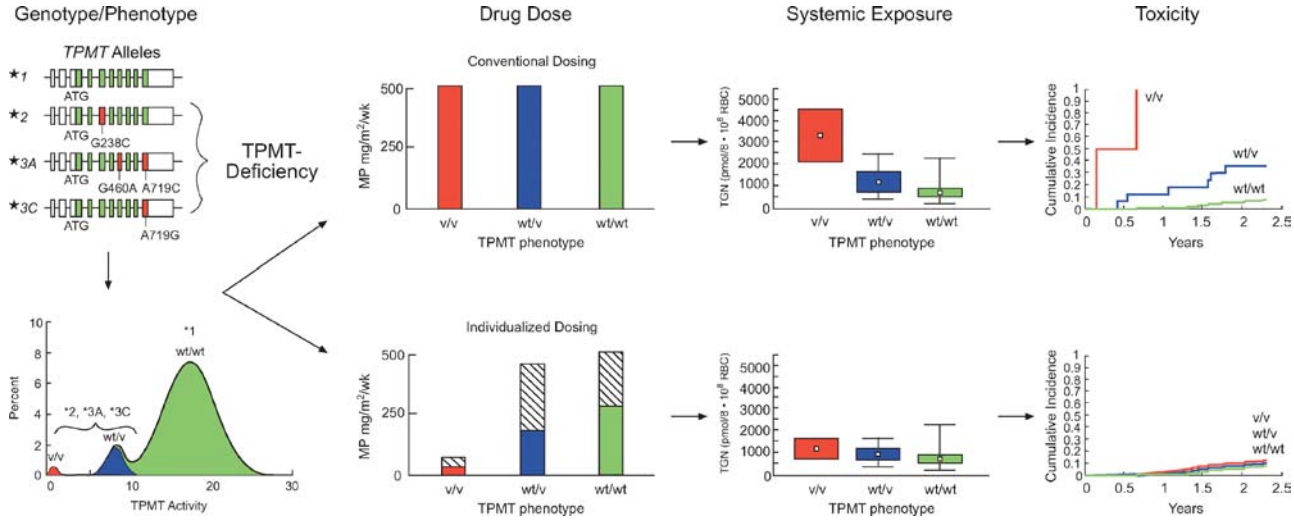

Figure 1 Genetic polymorphism of thiopurine methyltransferase (TPMT) and its role in determining toxicity to thiopurine medications. Depicted in the far left panels are the predominant TPMT variant alleles that cause autosomal codominant inheritance of TPMT activity in humans. The subsequent top three panels illustrate that, when uniform (conventional) dosages of thiopurine medications are given to all patients, TPMT-deficient (v/v) patients accumulate markedly (10-fold) higher cellular concentrations of the active thioguanine nucleotides (TGNs), and TPMT-heterozygous patients (wt/v) accumulate approximately two-fold higher concentrations of TGNs than do wild-type TPMT (wt/wt) patients. These higher levels of TGNs translate into a significantly higher frequency of toxicity (*far right panels*). The bottom three panels illustrate that, when genotype-specific dosages are administered, comparable cellular TGN concentrations are achieved, and all three TPMT phenotypes can be treated without acute toxicity. In the bottom panel depicting individualized drug dosing, the data are arranged by genotype group. The solid portion of each bar depicts the mean mercaptopurine [MP] doses that were tolerated in patients who presented with hematopoietic toxicity, whereas the striped portion depicts the mean dosage tolerated by all patients with that genotype. Figure reproduced with permission from Reference 132.

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