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Genetic Polymorphisms and the Treatment Outcome in Standard-Risk Pediatric Acute Lymphoblastic Leukemia

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Genetic Polymorphisms and the Treatment Outcome in Standard-Risk

Pediatric Acute Lymphoblastic Leukemia

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A Dissertation

Presented to

The Faculty of Natural Sciences and Mathematics

University of Denver

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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By

Qi Wei

June 2011

Advisor: Phillip B. Danielson

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Title: GENETIC POLYMORPHISMS AND THE TREATMENT OUTCOME IN
STANDARD-RISK PEDIATRIC ACUTE LYMPHOBLASTIC LUEKMIA
Advisor: Phillip B. Danielson
Degree Date: June 2011

Abstract

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and
accounts for 30 - 35% of all cancers in children. Significant improvement in the treatment
of pediatric ALL has been achieved in recent years. Only 50 years ago, the disease was
uniformly fatal with an Overall Survival (OS) rate < 5%. Modern-day, multi-drug
chemotherapy is associated with an overall survival rate over 80%. Standard-risk ALL
comprises the majority of ALL with an overall survival approaching 90%. Despite this
success, children who relapse from this disease accounts for the majority of cancer-
related deaths in children. The backbone of treatment protocols have incorporated
somatic but not host genetic features in the treatment regimens. The current study
examined 12 genetic polymorphisms affecting the pharmacodynamics of antileukemic
drugs in an attempt to identify biologic markers related to the risk of disease relapse.

In the current research program, 125 standard-risk ALL patients who were treated
at The Children's Hospital were enrolled in a retrospective study. Statistical analysis was
performed to evaluate the association between genetic polymorphisms and risk of disease
relapse in this study cohort. The *GSTM1* null genotype was associated with a decreased
risk of disease relapse (HR = 0.394, 95% CI = 0.127 - 1.224, *P* = 0.107). A combination
analysis of the *GSTM1* and *GSTT1* genotypes revealed a stronger association between the

both the *GSTM1* and *GSTT1* normal genotype and an increased risk of leukemia relapse (HR = 2.73, 95% CI = 0.9 – 7.9, *P* = 0.063), compared with patients having either the *GSTM1* or *GSTT1* null genotype. A "drug exposure" model was used in this study. The risk of relapse in individual with a low or intermediate "drug exposure" genotype increased 2.4-fold (HR = 2.39, 95% CI = 0.8 – 6.9, *P* = 0.107) compared with the high "drug exposure" genotype. No significant associations with relapse were observed for the *CYP1A1*, *CYP2B6*, *CYP3A4*, *CYP3A5*, *MTHFR C667T*, *MTHFR A1298G*, or *TYMS* polymorphisms. The findings from this single institution study suggest that polymorphisms within genes of the GST superfamily may influence the treatment outcome in standard-risk ALL. They also point to the need of prospective, large multi-institutional studies to validate these findings prior to clinical implementation.

Acknowledgements

First and foremost I would like to express my deepest gratitude to my advisor, Dr. Phillip Danielson. He has been a great source of inspiration. Throughout the ten years of my studies, he was always there when I needed guidance. His immense knowledge of and insights into the field have been a great resource as I studied at University of Denver. His energy and enthusiasm is contagious and will always stay with me.

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Chapter 1

Introduction

The successful treatment of childhood acute lymphoblastic leukemia represents one of the great medical accomplishments of the 20th century. Only five decades ago this disease was uniformly fatal. Since the introduction of multi-drug chemotherapy, the cure rate has risen to over 80%. In fact, the most recent results from the patients with ALL enrolled in Children's Oncology Group (COG) protocols showed an improvement in the 5-year overall survival rate from 83.6% to 90.3% over the last decade (Friedrich 2009). This success reflects the use of risk-directed therapy in which clinical variables related to the patient (*e.g.*, age and leukocyte count at initial diagnosis) and biological variables specific to the leukemic cell population (*e.g.*, DNA ploidy, *ETV6/RUNX1* chromosomal translocation and Philadelphia chromosome) were used to stratify treatment (Sather 1986, Crist 1986, Seeger 1998, Heerema 2000). Unfortunately, ALL continues to be the leading cause of cancer-associated death in children despite improved treatment regimens. Moreover, the cure rate in patients who experience disease relapse has not improved over the past decade. Due to drug toxicity, simple increases in chemotherapeutic drug doses to decrease the relapse rate do not represent a viable solution. As seen in the most recent

COG protocols, the therapeutic limits of these toxic drugs have already been maximized (Landier 2008). Additionally, the long-term consequences of relapsed ALL include secondary malignancies, growth retardation, and cognitive impairments. More than two-thirds of ALL patients reported at least one adverse late effect, and half of those reported an event that was severe or life-threatening.

In order to eradicate the leukemic cells, cytotoxic drugs typically have to be used at maximum dose intensity. These drugs are then administered as frequently as possible to discourage tumor regrowth. One of the greatest challenges of leukemia treatment, therefore, is to adjust the drug dose to achieve the desired therapeutic outcome while limiting toxicity. Drug dosages have been administered based on the patient's body surface area or weight for decades. This practice, however, is not based on solid research studies and has not taken into consideration the patient's renal and hepatic functions, bone marrow reserve, or concurrent medical conditions (Sawyer 2001). Great efforts have been focused on dosage adjustment, based on renal function, to attain a desired drug concentration in plasma or area under the concentration-time curve (AUC) – an important factor in determining the tissue exposure to the drug. The successes of this approach were exemplified by methotrexate (MTX) dose adjustment for pediatric ALL therapy. Relling (1999a) demonstrated that the therapeutic outcome was related to achieving a target MTX plasma concentration during high-dose therapy. Dose adjustment based on monitoring of steady-state MTX levels improved treatment outcome. On the other hand, genes encoding drug-metabolizing enzymes can influence the efficacy and toxicity of chemotherapy

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(Evans 1999). Despite efforts to anticipate drug complications, however, chemotherapeutic agents have variable pharmacokinetics and toxicity in individual patients. The causes of this variability are not always clear but are thought to be related to interindividual differences in drug metabolism and drug interactions.

Treatment outcome is dependent not only on chemotherapy used, but also on the underlying biology of the host. Thus, numerous important biologic and therapeutic questions remain to be answered in order to achieve the goal of curing every child with ALL. With the completion of Human Genome Project, comprehensive studies of genetic polymorphisms in drug-metabolizing enzyme genes and drug-target genes have become possible. This information may make it possible to better customize drug dosage levels and drug combinations for individual patients. Insights into the role of host genetic variations will likely further improve the treatment outcome by integrating pharmacodynamic and pharmacogenomic studies to individualizing treatment regimen for children with acute lymphoblastic leukemia.

1.1. Hypotheses and specific aims

1.1.1. Specific Aim 1

To determine the genotypes of *CYP1A1, CYP2B6, CYP3A4, CYP3A5, GSTT1, GSTP1, TYMS*, and *MTHFR* genes from patient DNA samples.

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1.1.2. Specific Aim 2

To identify the genetic polymorphisms of those genes that influence the treatment outcome in standard-risk ALL patients.

Hypothesis 1: Variations in the drug-metabolism genes, individually or in combination, will be associated with the treatment outcome in standard-risk ALL patients.

Hypothesis 2: Variations in the drug-target genes, individually or in combination, will be associated with the treatment outcome in standard-risk ALL patients.

1.1.3. Specific Aim 3:

To identify the possible association between the host genetic variation and *ETV6/RUNX1* fusion transcript status in standard-risk ALL patients.

Hypothesis: Host genetic variations (*i.e.*, drug-metabolism and drug-target gene polymorphisms), individually or in combination, will be associated with *ETV6/RUNX1* fusion transcript status.

These specific aims, taken together, represent a first step toward understanding the relationship between host genetic variations and treatment outcome in this clinically defined "homogeneous" group of patients.

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1.2. Significance

There is no doubt that host variation in the genes that encode drug-metabolizing enzymes and drug targets has the potential to alter drug serum concentration levels and molecular target engagement. The ultimate goal of pharmacogenetic studies is the individualization of drug dosage to each patient so as to achieve the maximum treatment efficacy while limiting toxicity. This study is significant for many reasons. **First**, this study will identify host genetic features that may affect the efficacy and toxicity of anti-leukemic chemotherapy. **Second**, this study will obtain pertinent information regarding the potential association between genetic polymorphisms (alone or in combinations) and disease relapse in patients treated for pediatric standard-risk ALL. **Third**, this study may help to further refine risk-classification for pediatric ALL patients. This study represents a unique opportunity to provide data to further optimize treatment regimens for individual patients regardless of their presenting characteristics.

Given the potential for genetic variation to influence patient response to drug treatment regimens, this work is particularly important in terms of its potential for improving the therapeutic outcome for children with lymphoblastic leukemia. Futhermore, this will be the first study conducted using a "homogeneous" cohort.

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1.3. Outcome Measure(s)

The primary outcome is to identify whether or not there exists an association between polymorphisms in the *CYP1A1, CYP3A4/5, CYP2B6, GST, TYMS,* and/or *MTHFR* genes and with treatment outcome (*i.e.,* relapse *vs.* no relapse) for standard-risk ALL. This study should be considered preliminary in nature due to the limited number of patients.

The secondary outcome measures are to: 1) identify the frequencies of individual polymorphisms in the *CYP1A1, CYP3A4/5, CYP2B6, GST, TYMS,* and *MTHFR* genes in standard-risk ALL patients who carry the *ETV6/RUNX1* fusion transcript; 2) explore the potential effects of combinations of genetic polymorphisms (or gene-gene interactions) on the treatment outcome of standard-risk ALL.

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Chapter 2

Background

2.1.   Acute Lymphoblastic Leukemia

Leukemia or **leukaemia** (Greek leukos $\lambda\varepsilon\upsilon\kappa\acute{o}\varsigma$, "white"; aima $\alpha\acute{\iota}\mu\alpha$, "blood") literally means "white blood" and is a cancer of the blood or bone marrow characterized by an abnormal increase of blood cells, usually leukocytes (white blood cells). It is part of the group of diseases categorized as hematological neoplasms. Depend on their progression, leukemias are usually divided into two major groups based on their clinical behavior. These are: 1) Acute Leukemia, which progresses quickly with many immature white cells; and 2) Chronic Leukemia, which progresses slowly with more mature white cells (Pui 2006).

Normally, the bone marrow contains self-renewing hematopoietic stem cells which develop into different types of mature blood cells including B lymphocytes, T lymphocytes, natural killer cells, various types of granulocytes, red blood cells, and platelets (Figure 2.1). ALL results from the clonal expansion of lymphoid cells arrested at an early stage of differentiation. ALL accounts for 75% of childhood leukemia cases with

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an incidence of 3-4 children per 100,000 individuals. Thus, between 2500 and 3000 children in the United States are diagnosed annually. ALL is the most common cancer in children representing about 25-35% of all childhood cancers and its diagnosis peaks between the ages of 2 and 5 years (Xie 2003). The incidence of the disease for this age group is approximately 4-fold higher than that for infants and is close to 10-fold higher than that for adolescents (ages 16 – 21 years) (Ries 2010). If leukemia is left untreated, it is uniformly fatal. A long list of often conflicting epidemiologic and environmental studies has attempted to elucidate the etiology of ALL. Two parallel infection-based hypothesis have been postulated based on an abnormal responses to infection; a peak in ALL incidence at 2-5 years of age; increased prevalence of the disease in the developed countries; and the presence of some geographical case clustering.

Kinlen (2004) predicts that excess cases of childhood leukemia result from exposure to common but low-pathologic infections after population-mixing with carriers occurs. This hypothesis was supported by a U.S study (Wartenberg 2004) through US SEER data. However, other studies have shown higher incidence of ALL in urban or high-density regions (Li 1998, Hjalmars 1999, Adelman 2005). Greaves (2006) suggested a delayed-infection hypothesis based on a two-hit model involving a delayed and dysregulated immune response to common infectious pathogens which leads to the transformation of pre-leukemic cells into acute lymphoblastic leukemia. So far, there is little evidence to support any role of viral transformation as a cause. Furthermore, it does not appear that

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there is a single cause for childhood ALL – for most patients, a combination of factors

appears necessary. Possible genetic causes of the disease will be discussed later.
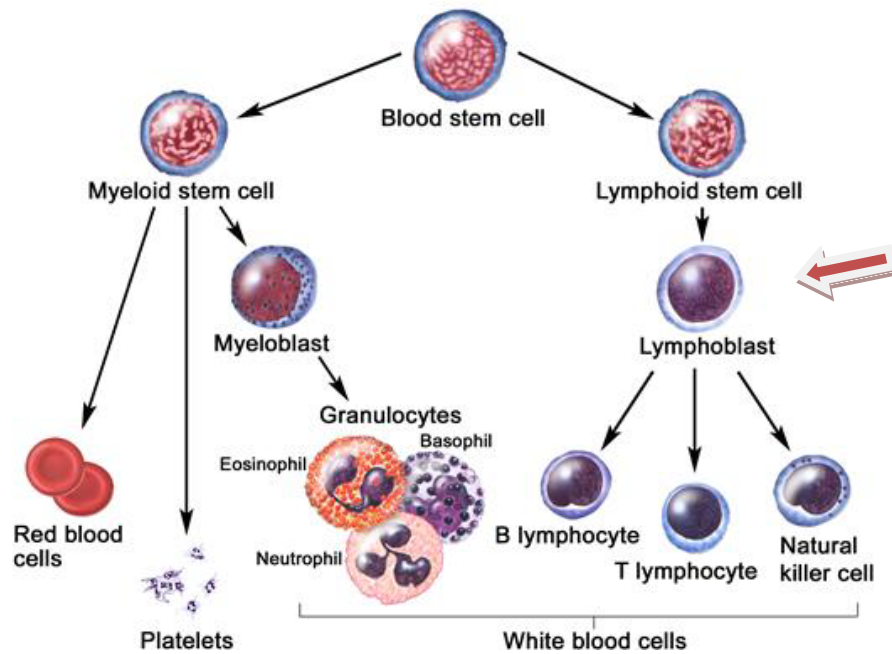
Figure 2.1 Blood cell development. Several steps are needed for a blood stem cell to
become a red blood cell, platelet, or white blood cell.
http://www.cancer.gov/cancertopics/pdq/treatment/childALL/HealthProfessional/ last
accessed Nov. 20, 2010

There are conflicting reports of factors that pose an increased risk for ALL,

including parental occupation; maternal reproductive history; parental exposure to

pesticides; and even exposures to high levels of residential electric and magnetic fields

(Ahlbom 2000, Buffler 2005). Recurrent genetic abnormalities have also been observed

in ALL patients, including chromosomal translocations that deregulate gene expression;

chromosome copy number variations; and gene-specific mutations. The precise biological

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and genetic mechanisms that lead to the development of ALL, however, remain unknown. Based on phenotypic observation and experimental models, however, it likely involves genes that control lymphoid cells differentiation, resulting in a clonal neoplastic disorder of the hematopoietic system. Recently, studies suggest that the etiology of ALL may be linked to a variety of genetic lesions in blood progenitor cells that are committed to differentiation or in some cases to lesions that arise in a hematopoietic stem cell (HSC) that has multi-lineage differentiation capacity The cellular microenvironment also appears to have an impact on leukemic cell transformation (Armstrong 2005, Wang 2005, Barabe 2007).

2.2.   Clinical Features

The clinical presentation of ALL is often acute, although a small percentage of cases evolve slowly over several months (Pui 1998 and 2006). The common symptoms include fever, fatigue, bone or joint pain and bleeding. These presenting symptoms correlate with the uncontrolled growth of the malignant cell population invading the bone marrow, lymphoid organs, and extramedullary (outside of the bone marrow) sites. Anemia, neutropenia, thrombocytopenia, lymphadenopathy, splenomegaly, and hepatosplenomegaly are common clinical features. Bone pain is common among younger children with ALL, whose first symptom is often the onset of a limp or a refusal to walk. These result from the infiltration of periosrteum by leukemic cells, bone infarction, or expansion of the marrow cavity. Findings at initial physical examination are usually

unremarkable. Asymptomatic lymphadenopathy and hepatosplenomegaly occur in more than half of patients. The most common affected extramedullary site is the central nervous system (CNS). Although CNS involvement at diagnosis is relatively uncommon, when symptoms do occur, the clinical features can include headache, nausea and vomiting, lethargy, and irritability.   The common clinical features of ALL are presented in **Table 2.1.** Other symptoms include:

- Fatigue

- Paleness resulting from anemia caused by insufficient numbers of red blood cells.

- Recurrent minor infections due to insufficient numbers of healthy mature white blood cells to fight off infection.

- Fever without a known cause

- Bruising, poor healing of minor cuts, uncontrolled bleeding due to insufficient numbers of platelets (thrombocytopenia).

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Table. 2.1 Clinical and Laboratory Features in Childhood ALL

Features	% of Patients*
Clinical Presentations	
Fever	55
Bleeding	45
Bone and joint pain	30
Splenomegaly	60
Hepatomegaly	70
Lymphadenopathy	50
Abdominal pain	10
Laboratory Features	
WBC count (X 10^3/μl)	
< 10	50
10 - 49	30
>50	20
Hemoglobin (g/dL)	
< 7	43
7 - 11	45
>11	12
Platelet count (X 10^3/μl)	
< 20	30
20 - 99	50
>100	20

*The percentages are approximations

2.3. Diagnosis

Several procedures can be employed to help confirm the diagnosis of ALL. These include a complete morphologic, immunologic, and genetic examination of the leukemic cells. This is essential to establish the diagnosis of acute lymphoblastic leukemia. The cellular hallmark of ALL, is the presence of lymphoblasts, a relatively undifferentiated cell with diffusely distributed nuclear chromatin, one or more nucleoli, and a basophillic cytoplasm. Accurate diagnosis and classification are the foundation for the successful treatment and biologic study of childhood acute leukemia. Modern classification of leukemia is based on the incorporation of morphologic findings, immunophenotype, and genetic abnormalies, in an attempt to define homogeneous disease subtypes that are clinically and biologically relevant.

2.3.1. Laboratory:

Clinical laboratory findings are usually unremarkable for ALL. The most common laboratory findings in ALL include anemia, thrombocytopenia, neutropenia, and leukocytosis. Other laboratory abnormalities include increased serum levels of uric acid, potassium, phosphorus, calcium, and lactate dehydrogenase (LDH). The degree of abnormality reflects the leukemic cell burden and cell lysis. Coagulation studies including prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen level, and D-dimer level are used to assess the degree of disseminated intravascular coagulation (Pui 2006).

2.3.2 Pathology:

2.3.2.1. Morphology

The morphologic diagnosis of leukemia consists of two broad steps. First, is establishing a general diagnosis of leukemia and second is classifying the leukemic process. For most patients with ALL, examination of the peripheral blood smear is normally sufficient to reveal leukemic lymphoblasts. Definitive diagnosis, however, usually requires examination of the bone marrow. According to the WHO, at least 25% blast cells are required to render a final diagnosis of acute leukemia. More than three fourths of patients, however, have more than 50% lymphoblasts in the bone marrow at initial presentation.

The lymphoblasts in ALL are relatively uniform in appearance with round to oval to indented nuclei under a light microscope. The classic morphologic characteristics of ALL were established by the 1976 French-American-British (FAB) system, based on the microscopic features of the leukemia cells using Wright-Giemsa-stained smears. The FAB classification system groups ALL into three morphologic sub-groups designated L1, L2, and L3 (Bennett 1981). In the most common subtype L1, the lymphoblasts appear small in size, with scant cytoplasm, homogeneous nuclear chromatin and indistinct or absent nucleoli. The less common L2 sub-type presents with large cell size, more heterogeneous nuclear chromatin, irregular/clefting nuclear shape and prominent nucleoli. In practice, the majority of ALLs show a morphologic spectrum between the L1

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and L2 subtypes, making the distinction between these categories somewhat arbitrary. The L3 sub-type is rarely seen. It consists of large blasts, regular nuclear features, with an abundant deeply basophillic cytoplasm, finely stippled chromatin, and often prominent nucleoli. Subsequent studies involving the immunophenotype and molecular biology of Burkitt lymphoma, revealed the L3 subtype was the leukemic phase of non-Hodgkin's lymphoma with a mature B-cell immunophenotype (Hecht 2000). Up to 10% of lymphoblast cells may depart from the characteristics of each morphology sub-type. The FAB system lacks independent prognostic significance and thus is seldom used in current medical practice.
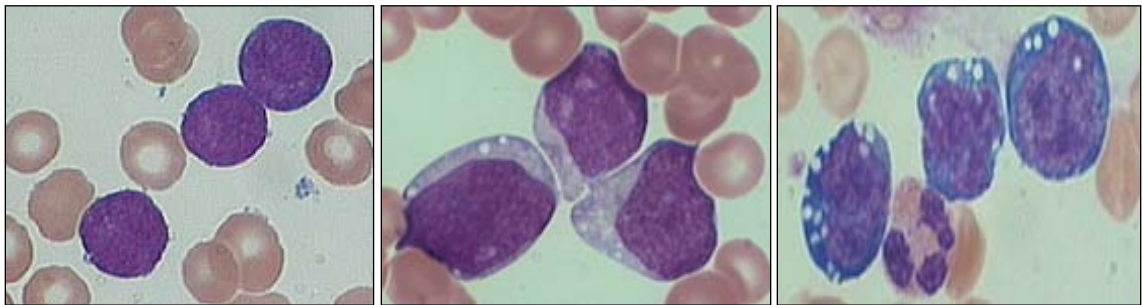
Figure 2.2 Morphological representation of lymphoblasts. L1, left; L2, middle; L3, right. *Source:* Dept. of Pathology, The Children's Hospital, Aurora, CO.

2.3.2.2. Immunophenotype

The diagnosis and treatment of ALL are depended on the recognition of a leukemic population, its lineage, and sometime its stage of maturation. Immunophenotypic studies are an essential part of the diagnostic workup of ALL; the

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results complement morphologic studies by establishing the leukemic cell lineage, determining the precise stage of differentiation, and often clonality. Unlike morphologic features, immunophenotyping divides acute leukemia into two broad, but clinically and biologically meaningful, categories: B-cell ALL (B-ALL) and T-cell ALL (T-ALL).

Several hundred monoclonal antibodies have now been assigned to over 300 cluster differentiation (CD) groupings by the International Workshops on Leukocyte Differentiation Antigens (Manson 2002). A panel of antibodies is used to establish the diagnosis of ALL by flow cytometry. This process includes include at least one marker that is highly lineage specific. For example, CD19 marks the B-lineage; CD7 marks the T-lineage; and CD33 marks myeloid lineage cells. For the B-lineage, the panel often also includes PAX-5 (B-cell specific activator protein), CD20, CD22, and CD79a. A large percentage of B-ALL cases also show expression of CD10 (common acute lymphoblastic leukemia antigen). Other markers should be included for a diagnosis of pre-B cell ALL. These include CD34 and terminal deoxynucleotidyl transferase (TdT). The pre-B-cell group accounts for 80% of ALL cases and is subdivided on the basis of cytoplasmic immunoglobulin (Ig) expression into early pre-B-ALL (which lacks the Ig expression), pre-B-ALL (with expression of cytoplasmic μ chains, but without Ig light chains), and transitional pre-B-ALL (with cytoplasmic and weak surface expression of μ chains, without Ig light chain expression) (Swerdlow 2008, Li 2003).

T-ALL is characterized by the expression of the T-lineage-associated antigens CD2, CD3, CD4, CD5, CD7, CD8 as well as CD10, CD34, HLA-DR, and TdT

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(Swerdlow, 2008). The current study is focused on B cell ALL, so T-ALL will not be discussed further.

2.4. Genetic Features

Despite relatively homogeneous morphologic and immunophenotypic features, ALL displays significant heterogeneity at the genetic level. The genetic characteristics define disease subsets with distinct biologic behavior and prognostic implications. They are used in the risk stratification for most modern treatment protocols. Molecular techniques have contributed greatly to our understanding of the pathogenesis and prognosis of ALL through the discovery of various common genetic alterations that occur in leukemic cells. The general underlying mechanisms of ALL are similar. They consist of aberrant proto-oncogene expression; chromosomal translocations that generate fusion transcripts encoding active kinases and altered transcription factors; and hyperdiploidy ($\geq$ 50 chromosomes per cell). These genetic alterations are linked to essential changes in cellular regulation and function that support the leukemic transformation of hematopoietic stem cells. These genetic changes enhance the cell's capacity for self renewal by altering the normal cell proliferation, blocking differentiation, and promoting resistance to programmed cell death (apoptosis).

2.4.1. Chromosomal abnormalities

ALL is associated with several chromosomal abnormalities with distinct biologic features that are critical for modern risk stratification. These abnormalities occur in 60 –

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80% cases of childhood ALL. The clinicopathologic and genetic features of the major genetic abnormalities seen in ALL are summarized in **Table 2.2**. Hyperdiploidy occurs in almost 30% of ALL cases with favorable prognosis. Leukemia cells with hyperdiploidy are more susceptible to the induction of apoptosis and the accumulation of high levels of chemotherapeutic agents or their metabolites. This may explain the favorable outcomes typically observed in these patients. Trisomy (extra copies of certain chromosomes) is another favorable prognostic factor observed in some ALL patients. Patients whose leukemic cells have extra copies of chromosome 4 and 10 appear have a particularly favorable outcome among hyperdiploid ALL patients. The presence of trisomies 4 and 10 has been incorporated into current treatment protocols. Approximately 1% of childhood ALL cases have less than 45 chromosomes, a condition termed hypodiploidy. These patients are at high risk for treatment failure. A significant, progressively worse outcome is seen for ALL patients with a decreasing numbers of chromosome. Patients with 24 – 46 chromosomes have the worst outcome.

2.4.2. Chromosomal translocations

Recurring chromosomal translocations can be detected in approximately 35 – 40% of childhood ALL patients. In some cases, this has prognostic significance (**Figure 2.3**). Currently, there is little evidence as to whether translocations are a product of errors in the DNA processing or are caused by external factors such as chemicals or viruses. However, most translocations are not sufficient to cause disease since they are more

common (perhaps by up to 100-fold) in the general population than their associated leukemias (Eguchi-Ishimae 2001, Mori 2002).

ETV6/RUNX1 [molecular counter part of t(12;21)] is most commonly observed genetic lesion in childhood B-ALL (22 – 25% of cases) but it is only rarely seen in T-cell ALL. This genetic lesion is thought to arise *in utero* in a B precursor cell during fetal hematopoiesis to generate a pre-leukemia clone. It has been suggested, therefore, that this translocation is an initiating event in B-ALL (Greaves 2006). Patients with *ETV6/RUNX1* are generally diagnosed with B-ALL between the ages of 2 – 9 and have excellent treatment outcome even in the case of relapsed patients. The *ETV6*/*RUNX1* fusion transcript probably inhibits the transcription of the normal *RUNX1* gene involved in the proliferation and differentiation of hematopoietic cells. This phenomenon has been clearly observed in clinical trials featuring intensive chemotherapy with asparaginase (Elspar®, Merck & CO., Inc). Interestingly, leukemic cells that express *ETV6/RUNX1* are highly sensitive to asparaginase *in vitro*.

BCR-ABL1 fusion transcripts [known as a Philadelphia chromosome, t(9;22)] are formed as a head-to-tail fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 with the Abelson gene (*ABL1*) on chromosome 9. *BCR-ABL1* encodes two distinct BCR-ABL1 oncoproteins, termed p210 and p190, that arise from two different splice sites in the BCR gene (Heisterkamp 1985). In chronic myeloid leukemia (CML), the *BCR-ABL1* fusion transcript (p210) originates in HSC, whereas the p190 fusion in pediatric ALL appears to arise in a lymphoid lineage precursor. It (p190) is

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observed in approximately 3% of childhood ALL cases and it confers an unfavorable prognosis, especially when associated with a high White Blood Cell (WBC) count at the time of diagnosis. Philadelphia-positive ALL has higher frequency in older patients, shows poor response to prednisone, and is associated with a higher level of residual disease after induction chemotherapy.

E2A-PBX1 fusion transcript [molecular counter part of t(1;19)] occurs in 3 – 6% of childhood ALL cases and exits as either a balanced or an unbalanced translocation. This rearrangement generates a fusion transcript that encodes a chimeric transcription factor from the amino-transactivation domain of E2A and the DNA-binding domain of PBX1 (Kamps 1990). E2A-PBX1 may contribute to leukemogenesis by binding and sequestering normal partners of the PBX protein, such as HOX proteins – thereby leading to uncontrolled cell-cycle progression (Aspland 2001). This is primarily observed in the pre-B ALL (*i.e.*, cytoplasmic Ig positive). Some studies suggested that patients with a balanced t(1;19) translacation may do worse than patients with an unbalanced translocation, but this remains a point of debate due to inconsistencies between studies on the subject.

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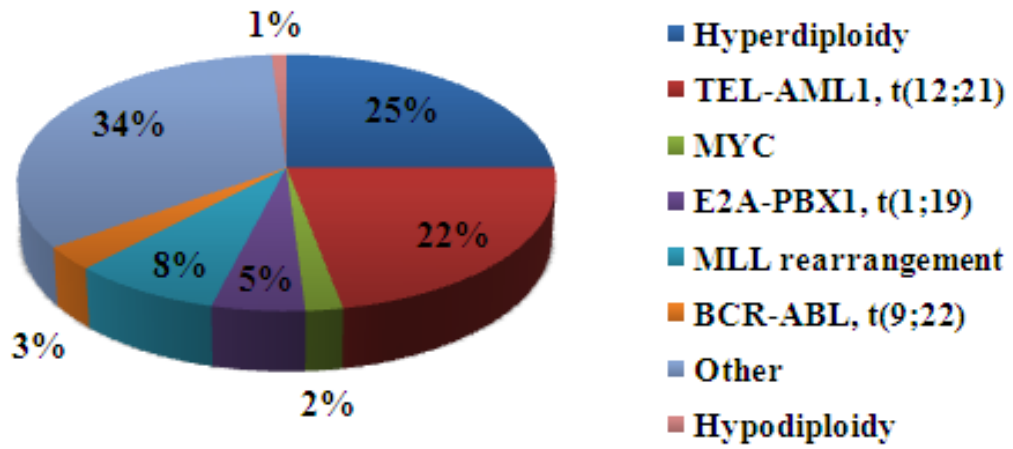
Figure 2.3 Distribution of chromosomal abnormalities in ALL.
Data were modified from Pui and Downing to exclude the T-cell genotypes (Pui and Downing, 2004)

Table 2.2 Cytogenetic subtypes of pre-B ALL and their clinical/pathological features

Cytogenetic Subgroup	Frequency (%)	Cytogenetic Abnormality	Immunopheno-typic Features	Pharmacologic Features	Prognostic Category
Hyperdiploid ALL	27-29	51 -65 chromosomes	NA	Higher sensitivity to MTX, 6-MP	Favorable
ALL with t(12;21)	22-25	t(12;21)(p13;q22)	Early pre-B ALL My$^+$	Higher sensitivity to asparaginase	Favorable
ALL with t(1;19)	3-6	t(1;19)(q23:p13)	Pre-B ALL CD9++ CD20- CD34-/dim+	NA	Unfavorable
Philadelphia + ALL	2-3	t(9;22)(q34;q11.2)	NA	NA	Unfavorable High-risk
ALL with t(v;11q23)	2-3	t(4;11)(q21;q23) t(9;11)(p13;q23)	Early pre-B ALL CD10- CD15+ CD65+	Higher sensitivity to cytarabine	Unfavorable High-risk
Hypodiploid ALL	5-6	< 46 chromosomes	NA	NA	Unfavorable High-risk

MTX, methotrexate; 6-MP, 6-mercaptopurine; My, myeloid antigens; Pre-B, precursor B; NA, not applicable.
Adapted from Mihaela, O. "Acute Lymphoblastic Leukemia" Hematol Oncol Clin N Am 23 (2009):655-74.

2.5. Prognostic Groups

The National Cancer Institute (NCI) classifies childhood ALL patients into different treatment regimen groups. Patients between 1- 10 years old who have a white blood cell count of less than 50,000/µl at diagnosis are classified as standard-risk ALL. The remaining patients are classified as high-risk ALL. The very high-risk group is defined by the presence of t(9;22) or hypodiploidy.

The prognosis of childhood ALL has improved dramatically over last five decades as a result of adapting therapy to the level of risk of disease relapse and continual reconfiguration/optimization of existing chemotherapeutic drugs.

2.6. Treatment

Leukemia is a systemic disease the treatment for which is primarily based on the use of chemotherapy. The backbone of contemporary multi-drug chemotherapeutic regimens consists of three treatment stages: induction, consolidation/intensification, and maintenance (**Figure 2.4**). Successful treatment of children with ALL requires the control of systemic disease as well as treatment of extramedullary disease – particularly in the central nervous system (CNS) because systemically administered anti-leukemic drugs do not cross the blood-brain barrier. CNS prophylaxis therapy, therefore, is generally administrated during each treatment stage. The general aim of therapy in ALL is to cure the patient of the disease which includes: 1) to induce a clinical and hematologic remission; 2) to maintain remission by systemic chemotherapy and prophylactic CNS

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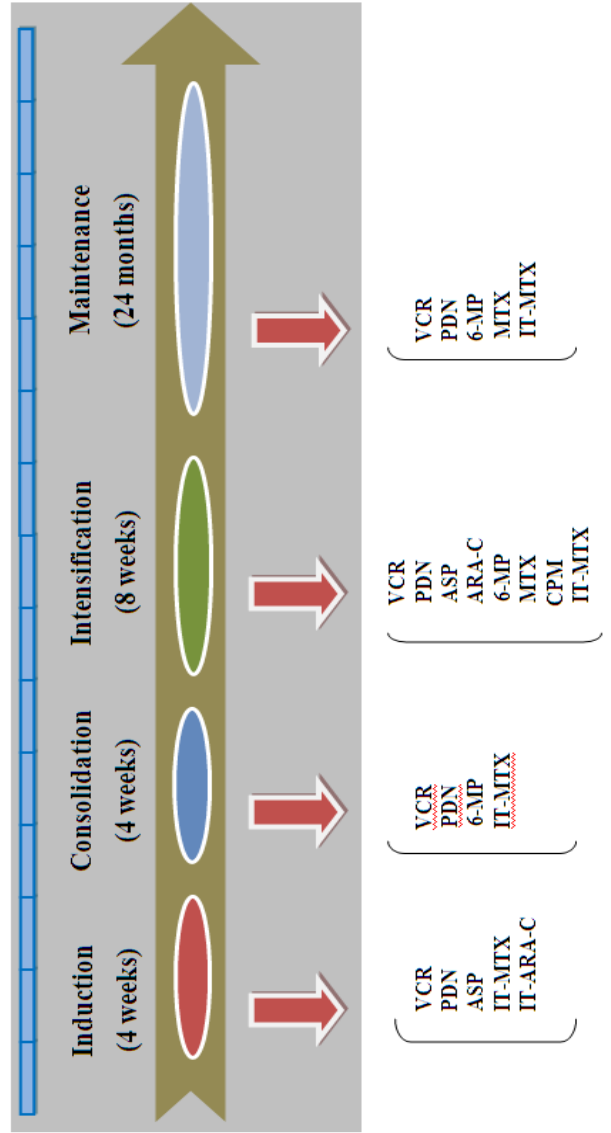
Figure 2.4 CCG-1952 Standard-risk ALL Therapy. VCR: Vincristine; PDN: Prednisone; ASP: Asparaginase; MTX: Methotrexate; CPM: Cyclophosphamide; 6-MP: 6-Mercaptopurine; ARA-C: Cytarabine; IT-MTX: Intrachecal Methotrexate; IT-ARA-C: Intrachecal Cytarabine.

The treatment protocol was obtained from the Center for Cancer and Blood Disorders, The Children's Hospital.

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therapy, and 3) to treat any complications arising from the therapy or the disease. The following section will discuss these approaches in greater detail.

2.6.1. Induction

The goal in this treatment stage is to eradicate the signs and symptoms of the disease and to restore normal hematopoiesis. Success of this stage is marked by "clinical remission" (CR). A patient in CR must have no morphologic evidence of leukemia (*i.e.*, <5% lymphoblasts) and a normal complete blood cell count (CBC). CR status also includes the absence of detectable CNS or extramedullary disease as evaluated by microscopic examination of the bone marrow and cerebrospinal fluid (CSF). The treatment regimen consists of a 3-drug (vincristine, steroids and asparaginase) or a more intense 4-drug (*i.e.*, adding anthracycline to the previous mix) treatment combination. For patients who are at standard-risk of treatment failure, the more intense 4-drug induction therapy appears not to be necessary. In general, more than 95% of ALL patients will achieve a complete remission within the first 4 weeks after diagnosis. Day-28 bone marrow is evaluated morphologically for CR and its minimal residual disease (MRD) status. Those cases that show a very slow response to induction therapy typically receive an additional 2 weeks of treatment after which, a bone marrow sample is evaluated. Patients with induction failure (< 3%) and a high-risk status receive an additional 4-drug therapy. The early institution of adequate CNS prophylaxis therapy is critical for

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eliminating CNS disease and preventing CNS relapse in patients without clinical CNS involvement at diagnosis. Intrathecal chemotherapy may be the most effective form of presymptomatic CNS therapy. Alternately, methotrexate alone, methotrexate in combination with cytarabine plus hydrocortisone and/or cranial radiation may be used. This regimen usually started at the beginning of induction, intensified during the consolidation stage, and often continued throughout the maintenance stage. The CNS relapse rate is less than 5% for the standard-risk ALL patients treated with this regimen.

2.6.2 Consolidation

Early studies demonstrated that disease remission alone is insufficient to cure ALL. A significant amount of additional therapy is required to eradicate all malignant lymphoblasts – thus reducing the risk of relapse. Although the importance of this treatment phase is very clear, there is little consensus on the best regimens and duration of treatment. The most commonly used strategies include high-dose methotrexate plus mercaptopurine and reinduction with the same chemotherapeutic agents that had been administered initially. The consolidation stage also includes systemic chemotherapy treatment in conjunction with additional CNS sanctuary therapy. The Children's Hospital of Denver belongs to the COG, and thus, its regimen consists of a combination of 6-mercaptopurine, vincristine, corticosteroids, and methotrexate with intrathecal therapy. This stage usually lasts 3 months. Afterwards, a delayed intensification phase (3 months) with asparaginase, cyclophosphamide, doxorubicin, and cytarabine is initiated. This approach has significantly improved the outcome in standard-risk ALL cases.

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2.6.3. Maintenance

For reasons that are still poorly understood, patients with ALL require continuation treatment to effectively prevent relapse of the disease. Although approximately two-thirds of ALL patients can be treated successfully with only 12 months of therapy, prospectively indentifying those patients with any certainty is impossible (Toyoda 2000). Therefore, all patients receive additional therapy. The last stage (maintenance) of treatment for childhood ALL includes low dose daily oral mercaptopurine and weekly intravenous methotrexate administration. Maintenance therapy is the longest stage for ALL patients. It generally continues for 2- 3 years from the point of complete remission. In some protocols, additional pulses of vincristine and corticosteroids may be added. A COG randomized trial demonstrated an improved outcome for patients receiving vincristine/prednisone pulses. A meta-analysis of data from multiple clinical trials showed increased event-free survival as well. The CCG-1952 protocol illustrates the common approach used to treat standard-risk ALL (**Table 2.3**).

2.7. Clinical Trial Results

The clinical trials from most large cooperative groups between 1980 and 1990 showed very similar results (**Table 2.4**). An improvement in ALL treatment outcome was obtained using very different treatment strategies, however, all of these shared a common approach toward treatment intensification.

Data from the Berlin-Frankfurt-Münster (BFM) group showed an improvement from a 66% successful rate in treating ALL patients in 1981 (ALL-BFM 81) to a 76% success rate in 1990 (ALL-BFM 90). These results demonstrated that delayed intensification is a crucial component of treatment. Other information obtained from BFM-90 study was that certain time-points during the post-induction minimal residual disease evaluation helped to identify more precisely those patients at high risk for relapse. The COG reported marked improvement during their two successive trials (1983-1988 and 1989-1995). Overall 10 year event-free survival (EFS) was 62% for the 1983-1988 study and 72% for the 1989-1995 study. Delayed intensification was deemed to be the most crucial factor responsible for the improved therapeutic outcome. The St. Jude Children's Research Hospital (SJCRH) reported that in their recent trial (Total XIII), early intensification of intrathecal therapy yielded a 5-year EFS of 79% (Pui 2004).

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Table 2.3 Treatment details for CCG 1952 (Standard-risk ALL)

Phase and drug	Dose	Schedule
Induction, 4 wk		
IT cytarabine	Age-adjusted[†]	Day 0
Vincristine	1.5 mg/m^2 (2 mg max)	Days 0, 7, 14, 21
Asparaginase[‡]	6000 U/m^2	M, W, F x 9 doses
Prednisone	40 mg/m^2/d	Days 0-27
IT MTX	Age-adjusted[†]	Days 7, 28
Consolidation, 4 wk		
Vincristine	1.5 mg/m^2 (2 mg max)	Day 0
Prednisone	Taper	Days 0-10
Mercaptopurine or	75 mg/m^2/d	Days 1-27
Thioguanine[§]	50 or 60 mg/m^2/d	Days 1-27
IT MTX or	Age-adjusted[†]	Days 7, 14, 21
ITT	Age-adjusted[†]	Days 7, 14, 21
IM no. 1, 8 wk		
Vincristine	1.5 mg/m^2 (2 mg max)	Days 0, 28
Prednisone	40 mg/m^2/d	Days 0-4, 28-32
Mercaptopurine or	75 mg/m^2/d	Days 0-49
Thioguanine[§]	50 or 60 mg/m^2/d	Days 0-49
MTX	20 mg/m^2/d	Weekly x 8 doses
DI no. 1, 8 wk		
Vincristine	1.5 mg/m^2 (2 mg max)	Days 0, 7, 14
Asparaginase[‡]	6000 U/m^2	M, W, F x 6 doses
Dexamethasone	10 mg/m^2/d	Days 0-6, 14-20
Doxorubicin	25 mg/m^2/d	Days 0, 7, 14
Cytarabine	75 mg/m^2/d	Days 28-31, 35-38
Cyclophosphamide	1000 mg/m^2	Day 28
Thioguanine	60 mg/m^2/d	Days 28-41
IT MTX or	Age-adjusted[†]	Days 0, 28, 35
ITT	Age-adjusted[†]	Days 0, 28, 35
IM no. 2, 8 wk		
As in "IM no. 1"	—	—
DI no. 2, 8 wk		
As in "DI no. 1"	—	—
Maintenance,[*] 12-wk cycles		
Vincristine	1.5 mg/m^2 (2 mg max)	Days 0, 28, 56
Prednisone	40 mg/m^2/d	Days 0-4, 28-32, 56-60
Mercaptopurine or	75 mg/m^2/d	Daily
Thioguanine[§]	50 or 60 mg/m^2/d	Daily
MTX (oral)	20 mg/m^2/dose	Weekly
IT MTX or	Age-adjusted[†]	Day 0
ITT	Age-adjusted[†]	Day 0

[*] Total duration of treatment for boys was 38 months; for girls, 26 months.

[†] Intrathecal; see Table 2 for dosing.

[‡] Asparaginase preparation: *E coli* (Elspar; Merck, Whitehouse Station, NJ); *Erwinia* asparaginase replaced Elspar following severe allergic reactions.

[§] The 100% targeted dose of thioguanine was changed from 60 to 50 mg/m^2/d in February 1998.

Source: Matloub Y, *et al.* (2006) Blood, Vol. 108: 1165-73.

Table 2.4 Results of Selected Clinical Trials in Children with ALL

Study Group	Duration	No. of Patients	5-Yr EFS (%)
ALL-BFM 90	1990-1995	2178	78±1.0
CCG-1800	1989-1995	5121	75±1.0
SJCRH XIII	1991-1998	412	79.4±2.3
NOPHO ALL-92	1992-1998	1143	77.6±1.4
COALL-92	1992-1997	538	76.9±1.9

Source: Pui and Downing (2004)

Treatment outcome for childhood acute lymphoblastic leukemia has improved dramatically over the past three decades. The 5-year survival rate increased from 53% in 1974-76 to 80% in 1992-99. **Figure 2.5** illustrates survival rates of successive CCG protocols (Hunger, 2010). These successes are in large part the result of new combinations and schedules of chemotherapy using establish agents rather than the result of new drug development. Despite this success, 25% of ALL patients ultimately relapse and at least 15% of patients die. Relapsed leukemia, on its own, represents the fourth most common malignancy in children. These numbers are greater than for children diagnosed with other pediatric malignancies including osteosarcoma, Ewing sarcoma and retinoblastoma. Thus, the overall prognosis for relapsed patients remains unsatisfactory despite recent improvements in treatment outcome.

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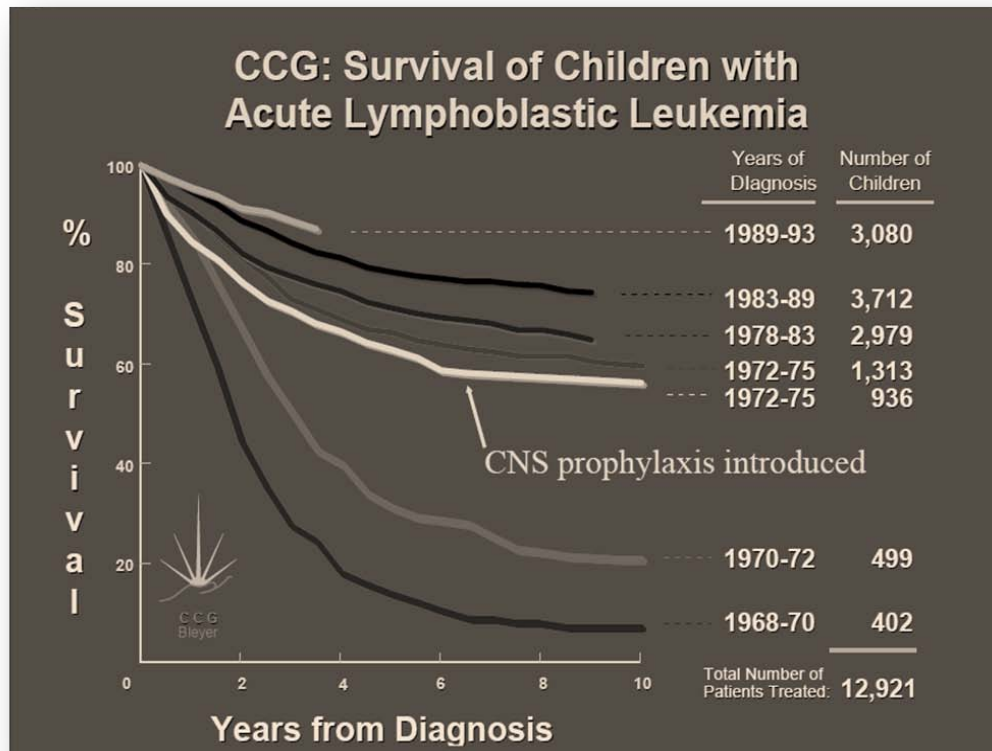
Figure 2.5 Survival curve for CCG treatment protocols
Source: Stephen Hunger, M.D. (personal communication)

ALL can reappear in the bone marrow, central nervous system, testis, and other

extramedullary "sanctuary" sites. Diagnosis is made by morphologic confirmation of

lymphoblasts at relapse sites. Medullary ALL relapse is defined as the presence of $\geq 25\%$

lymphoblasts in a bone marrow sample following the first complete remission. CNS

relapse is defined as the presence of mophologically distinct lymphoblasts on a CSF

cytospin with ≥ 5 blasts per microliter (µl). Testicular relapse is defined as the

histological findings of lymphoblastic infiltration in one or both testes. The

immunophenotypic features of relapsed ALL are largely similar to those seen in the

diagnostic samples. Treatment of relapsed ALL varies between the different cooperative

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groups. Typical treatment of a first relapse is often similar to the induction therapy employed in response to the initial diagnosis of ALL, and thus, it involves a combination of vincristine, a glucocorticoid, and asparaginase, plus an anthracycline, methotrexate, or cytarabine in varying doses and schedules. All patients who experience a second remission receive additional chemotherapy to maintain control of their disease. Hematopoietic stem cell transplantation (HSCT) is often offered to all patients with early bone marrow relapse. Fundamentally, both additional chemotherapy and hematopoietic cell transplantation are risky approaches and neither is highly effective for curing most ALL patients with bone marrow relapse.

A recent Children's Cancer Group study of 9,585 children with newly diagnosed ALL between 1988 and 2002 found that a total of 1,961 patients (20.5%) experienced a relapse involving various sites throughout the body (*i.e.*, bone marrow: 70.8%, CNS 21.9%, testicular 5.3%, and other extramedullary sites 3%) (Nguyen 2008). Malempati *et al.* (2007) recently summarized the data from the CCG-1952 study (NCI standard-risk ALL), which revealed an overall 6-year estimated EFS of 81.6%. Of the 2,174 patients enrolled in this study, 347 relapsed after achieving complete remission following induction therapy. Among the relapsed patients, 149 (42.9%) had isolated bone marrow relapse and 68 (19.6%) had a relapse involving both bone marrow and extramedullary tissues. Isolated extramedullary relapse was seen in 130 (37.5%) patients. A subsequent relapse event (*i.e.*, after the first relapse) was seen in 151 patients during the 2.4 year follow-up period. Among the patients with a second event, 21 (13.9%) died from either

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toxicity of the chemotherapeutic agents during reinduction therapy or reinduction failure leading to a major bone marrow or extramedullary relapse. A total of 41 patients (27.2%) died in second remission with 28 succumbing to complications from HSCT, 12 from chemotherapeutic agent toxicity and 1 from disease unrelated to leukemia. So far, the only factors predictive of survival after relapse are the site of relapse and the duration of the first complete remission (CR1). Risk-stratification strategies at initial diagnosis do not appear to be useful for predicting the outcome of this group of relapsed patients. There was no difference in EFS between the HSCT and chemotherapy treated patients for any relapse site or CR1 duration (**Figure 2.6**, Malempati 2007). Management of this group of patients has proved extremely challenging and the long-term survival rates (*i.e.*, 25-35%) for relapsed ALL patients continues to be very poor (Sadowitz 1993, Buchana 2000, Leahey 2000, Chessells 2003). Drug resistance in this heavily pretreated population may contribute to this poor response rate.

Despite substantial second remission rates (>90% in some studies) and wide availability of hematopoietic stem cell transplantation, most children with relapse die. The recent COG protocols, therefore, have maximized the therapeutic limits and increased dose intensity in primary therapy for newly diagnosed patients with ALL. Increasing the dose of chemotherapeutic agents does not decrease the relapse rate. Some patients pay a significant price with high rates of acute and chronic toxicities. More than two-thirds of "cured" patients report at least one late effect, and half of them experienced a condition that is severe to life threatening (Landier 2008). While the early data from

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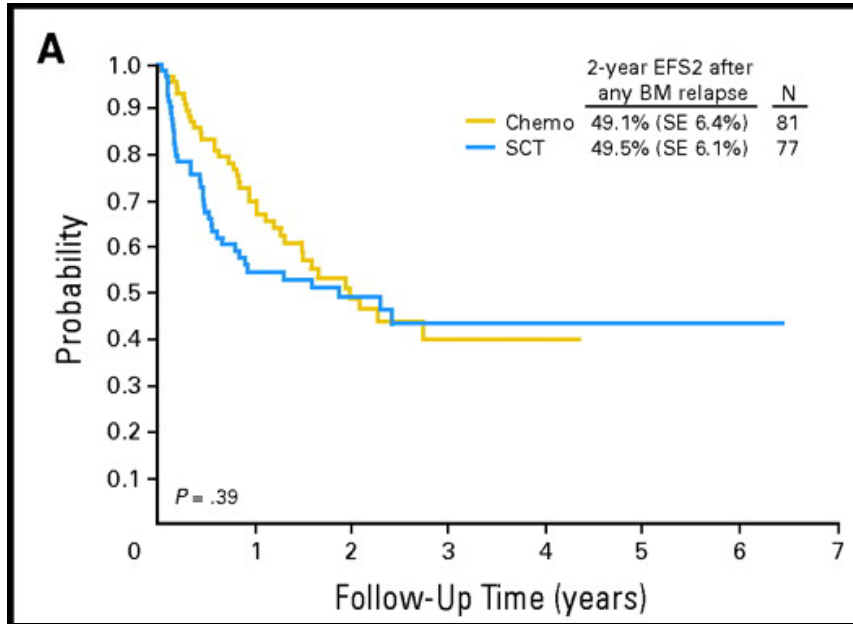
Figure 2.6 Comparison of second event-free survival (EFS2) after stem-cell transplantation and chemotherapy for bone marrow relapse. Kaplan-Meier estimate of with transplant *vs.* chemotherapy is shown. Adapted from (Malempati 2007).

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studies of these newer protocols are encouraging, the cure rate among relapsed patients has remained dismal (Friedrich 2009). This underscores the importance of optimal primary therapy.

More worrisome, however, is that the plateau in survival rates over the last 10 years suggests that the limits of current treatment strategies may have been reached. Moreover, the current risk-stratification model cannot identify approximately half of the non-responding patients, and it fails to predict the individual patient who is at greatest risk to experience severe toxicity at drug doses that would be well tolerated by most patients (Donadieu 1998). The low predictive value of the conventional prognostic indicators (*e.g.*, age, WBC, and karyotype) underscores the need to develop a better risk stratification strategy. Further improvement in treatment outcome and a reduction in drug toxicity will require the identification and application of new prognostic factors and treatment strategies. Ideally, these can used to optimize the treatment of patients as individuals. An in-depth understanding of the mechanisms of leukemia and host biology may make it possible to further refine risk stratification; thereby, resulting in the more efficient identification of prognostic subgroups of patients as well as appropriate therapeutic targets.

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Chapter 3

Pharmacogenetics of Acute Lymphoblastic Leukemia

Single nucleotide polymorphisms (SNPs) are the most frequent genetic variations. They constitute approximately 90% of all human genome variation; they occur every 100 to 300 bp (Lander 2001); and, by definition, all SNPs are present in at least 1% of population.

This genetic variation may play a very important role in the effectiveness and outcome of anticancer therapies. The chemotherapeutic drugs used to treat pediatric ALL have a narrow therapeutic range. Accordingly, they are associated with significant mortality in addition to relapse due to drug toxicity and morbidity. They are also associated with drug-induced second tumors. Drug-induced adverse events can be dose-limiting factors in many cases, and this affects treatment outcome. Studies have shown that adjusting dosage based on drug clearance improved ALL outcomes (Evans 1997 and 1998). Therefore, genetic variations in drug-metabolizing enzymes impact the balance between drug efficacy and drug toxicity – which, in turn, is likely to have an important impact on the treatment outcome in ALL.

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Most research to date has been focused on the contributions of acquired genetic abnormalities in leukemia blasts to the long-term outcome of the disease. Much less attention has been given to the role of host genetic variation in determining outcome of ALL therapy. Some insight into the molecular and cellular biology of chemotherapy responders *vs.* non-responders, therefore, may be informative. The ultimate question might be: Why do some patients fail to respond positively to therapy even though they have the same prognostic features as those that do respond well? Genetic polymorphisms in the genes that encode drug metabolizing enzymes and drug targets have been shown to influence patient response to drugs including chemotherapeutic agents. The case of Thiopurine s-methyltransferase (TPMT) exemplifies the value of this type of pharmacogenetic research. Studied for over 25 years and increasingly utilized at beside, TPMT is a drug-metabolizing enzyme that catalyses the S-methylation of cytotoxic and immunosupressant thiopurines such as 6-mercaptopurine (6-MP) to yield thioinosine monophosphate. This metabolite is then further metabolized by series of enzymes to yield mono, di, and triphosphates of 6-thioguanosine. These metabolites, termed 6-thioguanine nucleotides (TGNs), interfere with normal DNA and RNA synthesis which ultimately results in cell cycle arrest and apoptosis (Weinshilboum 1983, Lennard 1992, Relling 1999). Two major thiopurine metabolic pathways exist, oxidation catalysed by xanthine oxidase (XO) and methylation catalysed by TPMT (**Figure 3.1**) (Weinshilboum 2001 and 2003, Krynetski 2003). The TPMT pathway is the main mechanism of thiopurine

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metabolism in hematopoietic tissue because xanthine oxidase is not expressed in the hematopoietic cells (Lennard 1987).

Large inter-individual differences in TPMT enzyme activity were described over 30 years ago (Weinshilboum 1980). Subsequently, the genetic polymorphisms in the *TPMT* gene responsible for this phenotypic variation have been identified and characterized (Krynetski 1995, McLeod 2000). More than 21 different *TPMT* SNPs have been identified which may be associated with decreased levels of TPMT enzyme activity and/or thiopurine-induced toxicity (Salavaggione 2005). Of those, three SNPs termed **2, *3A* and **3C* account for more than 95% of the variability in TPMT enzyme activity (McLeod 2000, Wang 2003) (**Figure 3.2**). Approximately 90% of the population is homozygous for the wild-type alleles (*TPMT*1*) and are thus are characterized as having "normal" enzyme activity. Approximately, 5-10% of the population are heterozygous for TPMT and are characterized by an intermediate level of enzyme activity. Finally, 1 in 300 individuals carry two mutated alleles and are, therefore, severely TPMT-deficient (Weinshilboum 2000, Klemetsdal 1992, Yates 1997) (**Figure 3.3**). The most dramatic effect is seen in individuals with the *TPMT*3A* and **3B* mutations which are associated with a virtual absence of TPMT enzyme activity. TPMT-deficient patients accumulate excessive amounts of the active TGNs within their leukocytes following administration of 6-MP and azathioprine. This results in severe and potentially life-threatening hematopoietic toxicity (Evans 1991 and 2001, Relling 1999). These patients require up to a 90% reduction in the conventional drug dosage that is administered. Interestingly, this

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dosage reduction does not appear to compromise the treatment outcome (**Figure 3.4**) (Evans 1991, Relling 2006). Conversely, if TPMT-deficient patients are continually administered the conventional dosage, they develop severe hematopoietic toxicity which then necessitates the discontinuation of other chemotherapeutic drugs until their absolute neutrophil count recovers. Thus, this adverse drug reaction can compromises the overall therapeutic efficacy of the treatment protocol. TPMT deficiencies have also been linked to an increased risk of treatment-related acute myeloid leukemia and irradiation-induced brain tumors (Relling 1998 and1999b). To date, TPMT remains one of the few examples of pharmacogenetic research that has been successfully translated from bench to the bedside.

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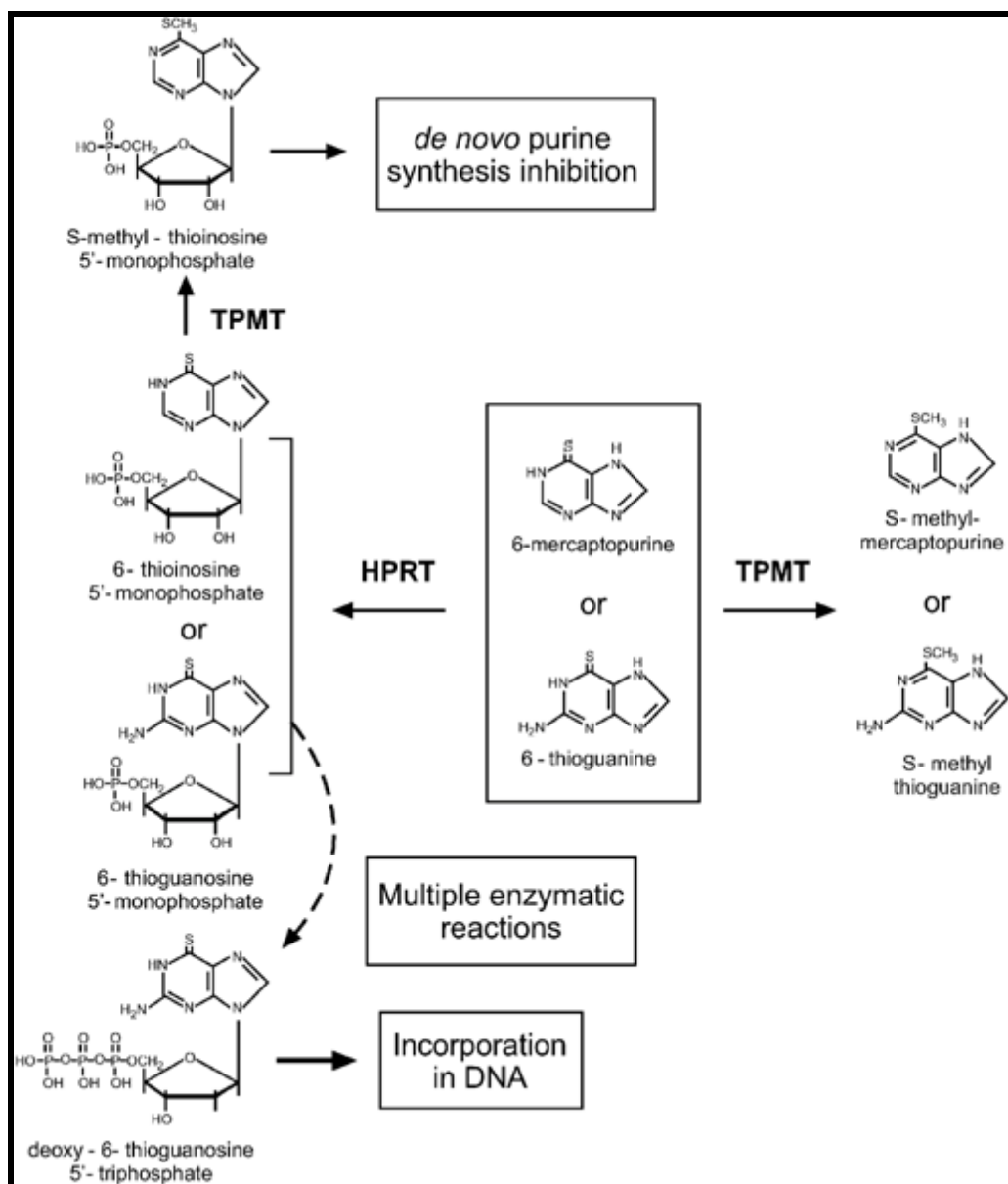
Figure 3.1 Thiopurine Metabolism. This figure shows a simplified schematic representation of the metabolic pathway for thiopurine drugs. The metabolic activation occurs as series reactions catalysed by hypoxanthine guanine phosphoriboxyltransferase (HPRT), inosine monophosphate dehydrogenase, and Guanine monphosphate synthetase. The figure was adapted from Krynetski (2003).
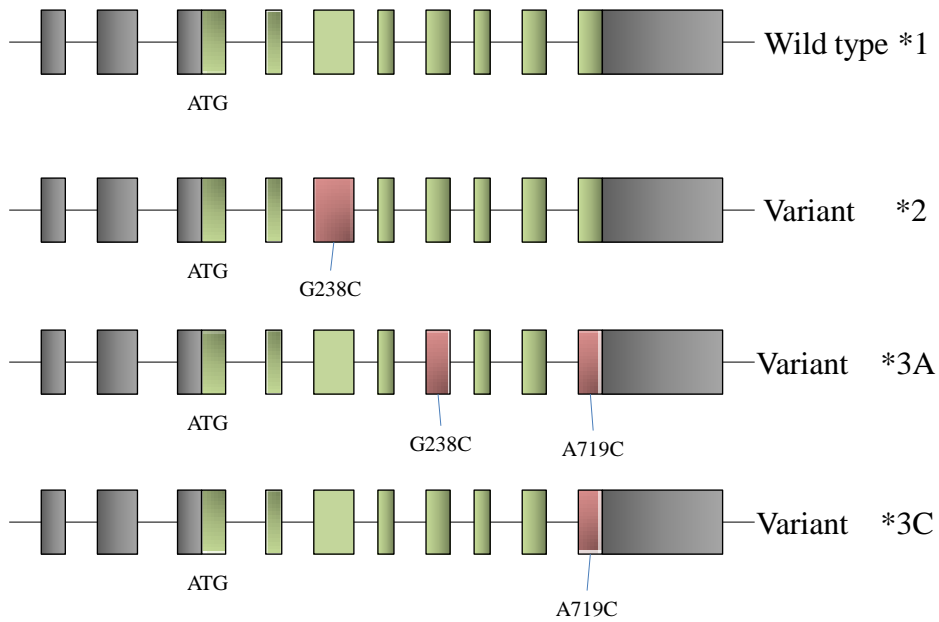
Figure 3.2 TPMT alleles. Boxes depict exons in the TPMT gene. Grey boxes are untranslated regions, green boxes represent open reading frames, and red boxes represent exons that contain mutations. This figure is adapted from Wang (2003).

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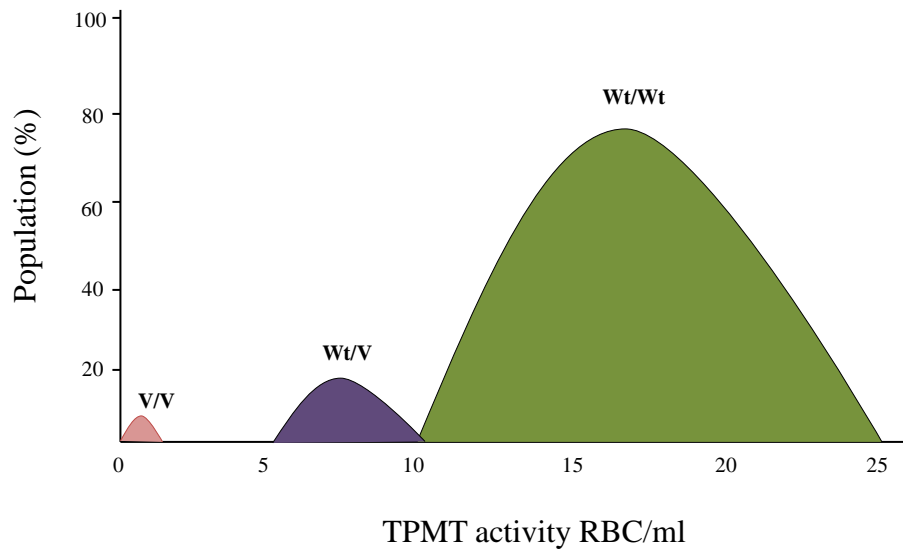
Figure 3.3 Frequency distribution of level of Red Blood Cell (RBC) TPMT activity in blood samples. The genotypes are indicated as v/v (homozygous mutant), Wt/v (heterozygous), and Wt/Wt (homozygous wild-type). This figure was modified from Wang (2003).
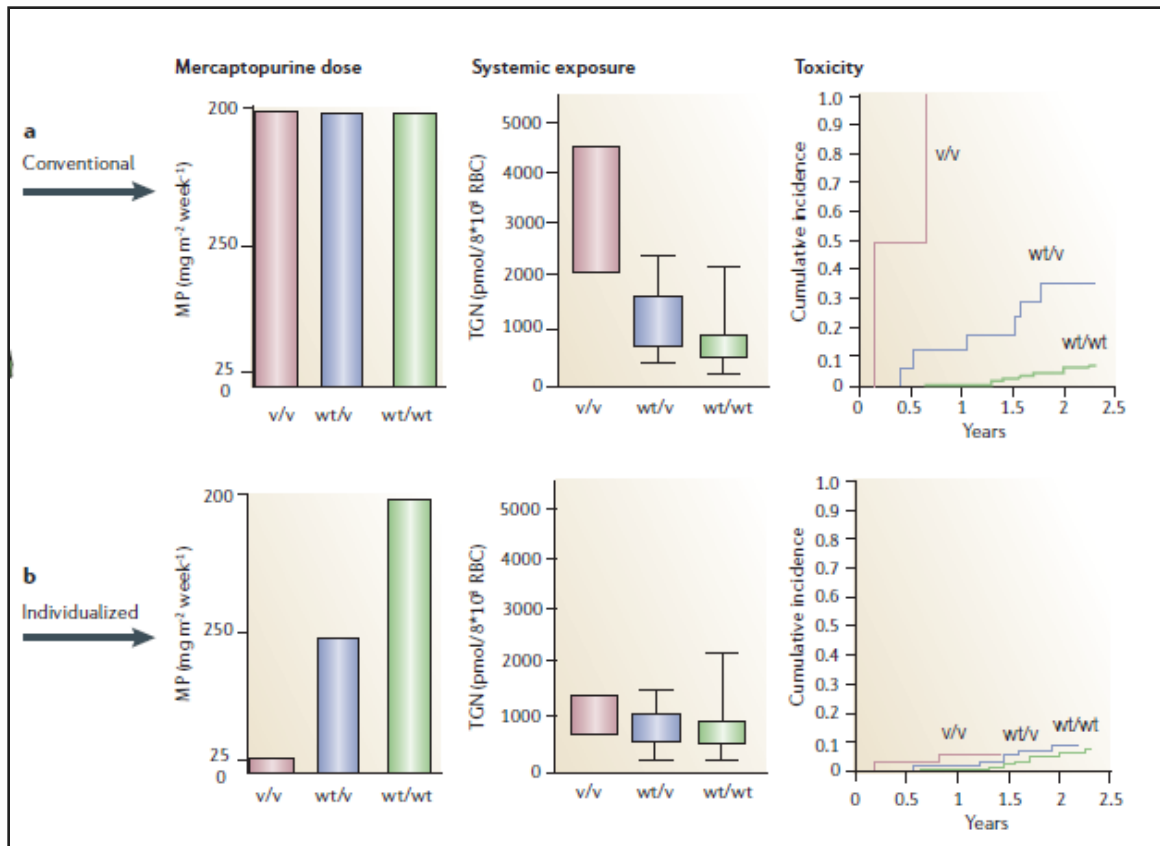
Figure 3.4 Effects of TPMT polymorphisms on the pharmacogenetics of mercaptopurine toxicity. a) MP given at conventional dosage. TPMT-deficient patients (v/v) show a tenfold higher systemic exposure to active thioguanine nucleotides (TGN) than do wild-type patients (wt/wt). Heterozygous patients (wt/v) show twofold higher TGN concentrations. The patients with higher concentrations of TGN exposure exhibit a significantly higher frequency of haematopoietic toxicity. b) When genotype-specific adjusted dosages are given (*i.e.*, individualized therapy), similar cellular TGN concentrations are achieved in all patients. All three TPMT drug metabolism phenotypes can be treated without acute toxicity. This figure was adapted from (Cheok 2006).

In order to assess the possible association of functional genetic variations with ALL treatment outcome, a candidate gene approach was employed for the current research. SNPs in genes that either mediate the disposition of chemotherapeutic agents or their effects may modify the risk of relapse and toxicity beyond what can be predicted from host and disease features alone (Relling 2001). The activity of drug-metabolizing enzymes determines the bioavailability and function of many chemotherapeutic agents. The conventional prognostic factors of age and WBC, however, have a low predictive value for treatment response. Unfortunately, even with large clinical trials, the ability to optimize treatment to the individual patient remains elusive. Major variables in predicting response to leukemia therapy include hereditary and acquired variability in drug disposition and metabolism. Although chemotherapy is the backbone of pediatric ALL treatment, there is a paucity of pharmacogenetic studies on the major therapeutic agents. In order to function effectively, most chemotherapeutic drugs undergo biotransformation to form reactive and cytotoxic metabolites. This biotransformation usually involves two stages. The first stage involves Phase I reactions which occur primarily through the cytochrome P450 family. Phase I reactions involve the introduction of small polar groups onto the parent drug by oxidation, reduction or hydrolysis. This step produces increased polarity in the metabolites which may then undergo further biotransformation. In the Phase II reactions, metabolites are conjugated with glutathione, acetate, sulfate or glycine to produce more water soluble compounds that can be excreted in the urine (Evans 1999).

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3.1. Drug-Metabolism Enzymes

The cytochrome P450 enzymes comprise a superfamily of 57 genes in humans – the majority of which are involved in the metabolism of numerous drugs and xenobiotic chemicals. Cytochrome P450 enzymes contain a heme group that can accommodate up to 6 ligands and has an absorption wavelength of 450 nm when bound to carbon-monoxide. The naming convention specifies family, subfamily, gene, and alleles. For example, *CYP*2D6*1 is allele 1 (wild type) within gene 6 of Subfamily D within Family 2 of the CYP450 superfamily (Danielson 2002). The majority of these enzymes belongs to the CYP1 and CYP3 families and is primarily associated with hydroxylation reactions. They incorporate one atom of molecular oxygen into the target substrate molecule and the remaining atom into a water molecule. This function facilitates the biotransformation of compounds that otherwise lack functional groups suitable for conjugation. Most chemotherapeutic drugs including cyclophosphamide, doxorubicin and vinca alkaloids are metabolized by cytochrome P450 enzymes. Therefore, the efficacy of many of these drugs is directly related to the levels of expression and activity of these enzymes.

3.1.1. CYP1A1

CYP1A1 is an enzyme responsible for the aryl-hydrocarbon hydroxylase activity. It is often associated with significant enzymatic inducibility and is highly expressed in lymphoid cell lines (Garte 1998). This tissue specificity might correlate to an increased risk of carcinogenesis in leukocytes. CYP1A1 is involved in the metabolic activation of

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several carcinogenic substances contained in tobacco products. Evidence exists that there is a significant influence of *CYP1A1*2* on the risk of lung cancer (Vineis 2003, Taioli 2003). Most chemotherapeutic agents involved in ALL treatment regimens are not directly metabolized by CYP1A1. However, synthetic glucocorticoids, which are important components of ALL treatment regimens, can induce the gene transcription which is reflected in greater catalytic activity of CYP1A1 (Bartsch 1995). Thus, if a patient is exposed to a carcinogenic substance (*e.g.*, tobacco smoke), the enhanced enzyme activity could activate, and thus increase, the toxicity of the carcinogens resulting in an increased risk of leukemia relapse. An association was reported between *CYP1A1*2A* and **2B* and an increased risk of developing leukemia (Sinnett 2000, Bowen 2003). However, this association was not observed in a case-controlled study with 550 leukemia patients (Roddam 2000). Still another study by Krajinovic *et al.* (2002a) reported that the *CYP1A1*2A* variant can increase the risk of leukemia and is associated with an unfavorable outcome in children with ALL. In their study of 320 patients treated with the Dana Farber Cancer Institute (DFCI) ALL protocol, sixty-four patients experienced an event, defined as relapse or death from disease during or after completion of therapy. The presence of at least one *CYP1A1*2A* allele was associated with an increased of relapse risk, Hazard Ratio (HR) = 2.3 (95% CI: 1.2-4.9). This study cohort included patients in the different risk groups whom were treated with various protocols. The current dissertation research sought to collect some data that could be evaluated for an association between CYP1A1*2B and the risk of disease relapse in standard-risk ALL.

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3.1.2. CYP3A4 and CYP3A5

The human *CYP3A* locus is located at 7q21.1 and consists of 4 function genes and 3 pseudogenes (Gellner 2001). The functional genes are *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*. Of the cytochrome P450 superfamily, the CYP3A subfamily is the most important for drug-metabolism, accounting for 60% of P450 enzymes in the liver and intestines (Danielson 2002). Thus far, polymorphisms in *CYP3A4* and *CYP3A5* are the most studied in hematologic malignancies. Most chemotherapeutic agents are substrates for CYP3A, including vincristine, cyclophosphamide, and epidophyllotoxins (Garte, 1998). The *CYP*3A4*1B polymorphism (-392A>G substitution), located in the regulatory region of the gene, alters the transcriptional efficiency of CYP3A4 and impacts its overall metabolic activity (Rebbeck 1998). However, its relevance for drug metabolism remains unclear (Hesselink 2004). On the other hand, the *CYP*3A5*3 and *CYP*3A5*6 polymorphisms induce splice site variants that substantially decrease functional levels of CYP3A5 compared with the wild type alleles (Kuehl 2001). Some studies have shown a significant association between *CYP3A4*1B/CYP3A5*3* and treatment-related leukemia, drug-induced toxicities, and higher etoposide clearance (Felix 1998, Kishi 2004 and 2007). Felix reported a protective effect, Odds Ratio (OR) = 0.09 (95% CI 0.01-0.87), of the *CYP3A4*1B* allele on secondary leukemia risk in a group of 99 *de novo* and 30 secondary leukemia samples. In contrast, Aplenc *et al.* (2003) found no statistically significant impact of these 3 SNPs (*CYP3A4*1B, CYP3A5*3, or CYP3A5*6*) on disease

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prognosis in 533 cases of childhood ALL. An association between *CYP3A4**1B and

CYP3A5*3C polymorphisms and a decreased risk of vincristine-associated peripheral

neuropathy, however was reported (Aplenc *et al.* 2003).

3.1.3. CYP2B6

The *CYP2B6* gene is located at 19q13.2 and is highly polymorphic. CYP2B6

accounts for approximately 3-5% of total microsomal cytochrome P450 protein in the

liver and is also expressed in intestine, kidney, and brain (Gervot 1999). Several

polymorphisms have been reported for *CYP2B6*. The *1459C >T* polymorphism

(Arg487Cys), termed **5* allele, corresponds to lower CYP2B6 protein levels when

compared with the wild-type allele (Lang 2001). Decreased enzymatic activity should

lead to decreased active metabolites, therefore inferior response to therapy and fewer

toxic side effects. Cyclophosphamide (CP) is a cytotoxic drug widely used in the

treatment of a variety of malignancies. CP is a prodrug requiring bioactivation to form

phosphamide mustard, which forms a strong bond with DNA, consequently inhibiting

both DNA replication and cell division, and ultimately leading to cell death (Sladek

1988). Various cytochrome P450 enzymes have been demonstrated to be involved 4-

hydroxylation of CP in humans (illustrated in **Figure 3.5**). Both CYP2B6 and the

previously discussed CYP3A4 are involved in the metabolic activation of the pro-drug

cyclophosphamide. CYP2B6 has higher affinity for the substrate and metabolizes the CP

by producing an active 4-hydroxy form, while CYP3A4 is responsible for the *N-*

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dechloroethylation of the drug to yield chloroacetaldehyde - a neurotoxin, which is responsible for some of the serious side effects of chemotherapy (Huang 2000). The primary metabolite, 4-hydroxycyclophosphamide, is in equilibrium with its open-ring tautomer (aldophosphamide) that undergoes chemical decomposition to form phosphamide mustard (a bifunctional DNA alkylator and the ultimate cytotoxic metabolite) and acrolein (Fleming 1997, Yu 1999) (**Figure 3.5**). Cyclophosphamide has a relatively narrow therapeutic index, and adverse effects include cardiotoxicity, nephrotoxicity, and myelosuppression. Studies have demonstrated wide inter-individual variation in the metabolism of cyclophosphamide in pediatric populations (Yule 1995). This makes a strong argument for studying the effect *CYP2B6* polymorphisms on drug response in childhood ALL.

3.1.4. GST

Glutathione-S-Transferases (GSTs) comprise a family of Phase II metabolic enzymes. GSTs function as dimers by catalyzing the conjugation of electrophilic substrates to glutathione. They are divided into eight classes, α through ɵ, based on their amino acid sequence and immunoreactivity (Hayes 1999). The *GSTM1*, *GSTP1* and *GSTT1* genes are members of the GST family and are located at chromosomal positions 1p13.3, 22q11.23 and 11q13 respectively. The most common polymorphisms include complete deletions of the *GSTM1* and/or *GSTT1* gene which are termed 'null' alleles as well as two SNPs in GSTP termed *GSTP1**B and *GSTP1**C (Cotton 2000,

49
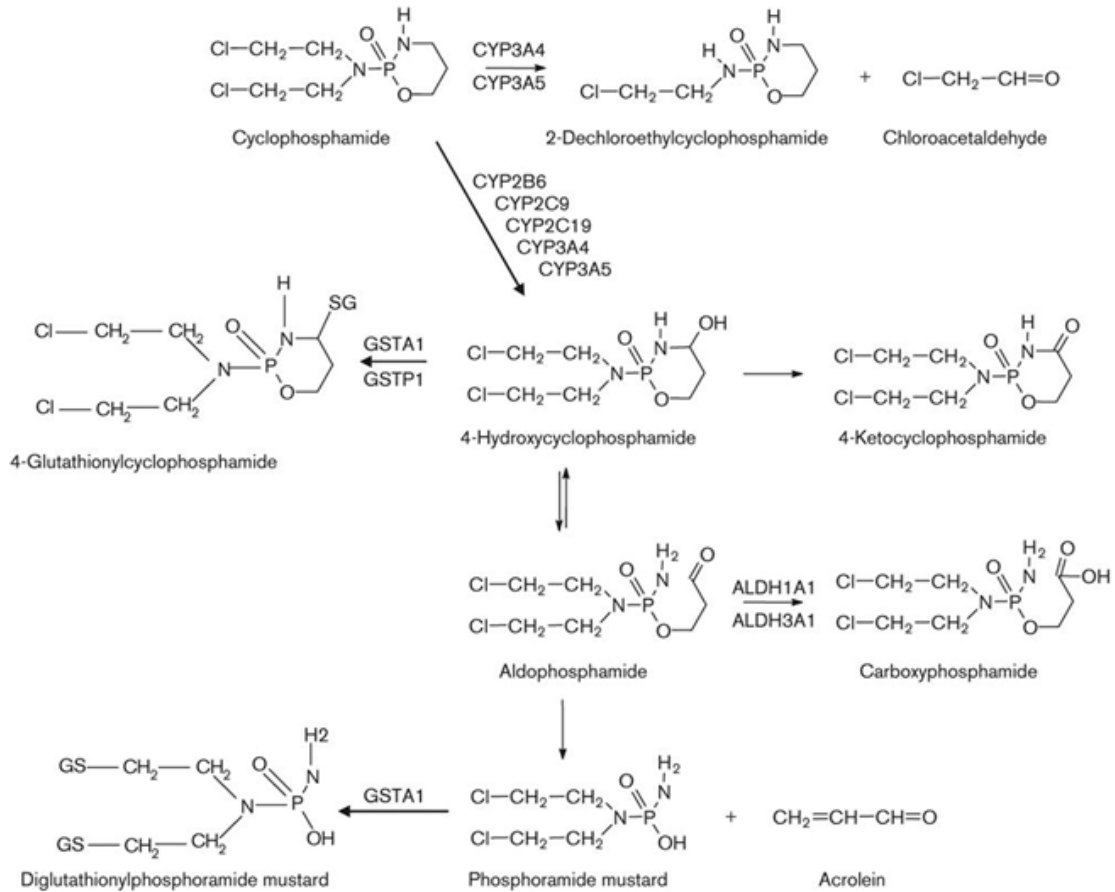
Figure 3.5 Metabolism of Cyclophosphamide. After cyclophosphamide administration, approximately 70-80% of the dose is converted into 4-hydroxycyclophosphamide (4OHCP) by various Cytochrome P450 enzymes including CYP2B6, 2C9, 2C19, 3A4, and 3A5, with CYP2B6 displaying highest 4-hydroxylase activity. 4OHCP is in equilibrium with its open-ring tautomer (aldophosphamide) which undergoes chemical decomposition to form phosphamide mustard (a DNA alkylator and the ultimate cytotoxic metabolite) and acrolein. 4OHCP and phosphamide mustard are detoxified by gluthione S-transferase (GST) to form 4-gluthionycyclophosphamise and digluthionycycyclophosphamide, respectively. This figure was adapted from Ekhart (2008).

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Hayes 2000, Agundez 2008). GST enzymes are involved in the detoxification of various exogenous substances, including chemotherapeutic agents (Tew 1994). Numerous studies have investigated the correlation between different GST genotypes (*GSTM1*, *GSTT1*, and *GSTP1*) and the susceptibility to various cancers (*e.g.*, lung, bladder and breast cancer) to treatment (Nazar-Stewart 1993, Nebert 1996). Some of these studies have shown an increased risk of cancer for individuals with specific GST genotypes – an outcome thought to be due to lowered enzyme activity. *GSTM1* and *GSTT1* null genotypes lead to an absence of enzyme activity. The *GSTP1*B* polymorphism is characterized by a single nucleotide 313A>G substitution in exon 5 which results in an amino acid change from isoleucine to valine. This mutation causes steric hindrance at the substrate-binding site of the enzyme thereby resulting in lower enzyme activity. Zielinska *et al.* (2005) recently assessed the role of *GST* variants in urinary excretion of unchanged ifosfamide and its side-chain metabolite. Specifically, they examined the levels of nephrotoxicity and neurotoxicity in 76 children who were treated with ifosfamide for different childhood neoplasms. They found that an individual's response to ifosfamide was associated with their *GSTP1* genotype. One study also demonstrated a protective effect of GST polymorphisms on relapse risk (Stanulla 2000). They reported a study of 64 pair of relapsed and non-relapsed patients from BFM-86 and BFM-90 ALL trials. The *GSTM1* and *GSTT1* null genotypes were associated with a protective effect on leukemia relapse, OR = 0.5 (95% CI 0.23-1.07) and OR = 0.36 (95% CI 0.13-1.23). The homozygous *GSTP1*B* (*i.e.*, Val105/Val105) genotype was noted to convey a similar protective effect,

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OR =0.33 (95% CI 0.09-1.23). However, a similar study from St. Jude Research Hospital (SJCRH) did not observe a statistically significant impact for *GST* genotypes (Chen 1997). They analyzed the *GSTM1* and *GSTT1* genotypes and disease-free survival in 161 of 525 patients enrolled in the SJCRH Total XI, Total XII and Total XIIIa ALL protocols. No association between *GST* genotypes and relapse risk was observed, although a subset analysis demonstrated that the GSTM1 null genotype was associated with a decreased risk of CNS relapse (98% *vs.* 94%, *p*= 0.054). As a cautionary note, however, these studies used ALL patients with different treatment regimens; including high-risk protocols.

3.2.   Drug response Pathways

Methotrexate (MTX) is a major component in the intensification and maintenance phases of childhood ALL therapy across cooperative group trials (Chabner 2001). MTX is a folate antagonist which competitively inhibits dihydrofolate reductase. This leads to impaired regeneration of tetrahydrofolate from dihydrofolate due to a lack of folate coenzymes and suppression of folate-dependent syntheses of DNA and RNA precursors as show in the **Figure 3.6.** This inhibition of nucleic acid synthesis results in cellular death. MTX cytotoxicity is also exerted through direct inhibition of enzymes associated with purine and pyrimidine synthesis (Calver 1999). While highly effective in eradicating leukemic cells, MTX is associated with significant gastrointenstinal, hepatic, neurological, or hematological toxicities as well as general immune suppression. For this

reason, dosing based on MTX pharmacogenetics plays an important role in high-dose MTX therapy. High-dose infusion of MTX remains an important aspect of ALL therapy. The advantage of this practice in high-risk ALL is still being investigated in the current COG trials. It was shown to be beneficial in ALL patients with testicular disease. Several studies from St. Jude Children Research Hospital have demonstrated good outcome by individualizing the MTX dosage according to patient's ability to clear the drug (Evans 1997 and 1998). Dosing adjustment is still part of St. Jude Total therapeutic protocols today. Unfortunately, individualizing MTX dose is not always a feasible option for patients treated elsewhere.

3.2.1. 5,10-methylenetetrahydrofolate reductase

The 5,10-methylenetetrahydrofolate reductase (MTHFR) is located at chromosomal position 1p36.3 with 11 exons that span 13.5 Kb. MTHFR is a key enzyme for intracellular folate homeostasis and metabolism. MTHFR catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folate metabolism pathway. The 5-methyltetrahydrofolate is a major circulating form of folate which provides a methyl group for the methylation of homocysteine to methionine which is required for nucleic acid methylation (Matherly 1996). The SNPs, *MTHFR* 667C>T and 1298A>G have been associated with decreased MTHFR activity and increased levels of homocysteine (Frosst 1995, Weisberg 1998, van der Put 1998). A change in reduced folate pool, derived from MTHFR activity, may significantly affect patient response to

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antifolate therapy. Several groups have reported a protective effect of these polymorphisms on leukemia susceptibility risk (Franco 2001, Alcasabas 2008). Conversely, reduced MTHFR activity has been linked to a higher risk of relapse and disease progression. Krajinovic *et al.* (2004) investigated the role of the *MTHFR* 667C>T and 1298A>G polymorphisms in 201 patients treated on Dana Farber Cancer Institute protocols for standard, high and very high risk ALL. The results showed that patients with the *MTHFR* 667T/1298A haplotype had an increased risk of relapse, HR = 2.2 (95% CI 1.0-4.7). Moreover, the effect of a thymidylate synthase (*TYMS*) triple repeat (associated with increased TYMS levels) combined with the *MTHFR* 667T/1298A haplotype demonstrated a highly significant reduction of EFS, HR = 9.0 (95% CI 1.0-42.8).   Aplenc *et al.* (2005) presented the findings from the Children's Cancer Group CCG-1891 trial, a national intermediate-risk ALL study involving 137 participating institutions. Intermediate-risk patients between 1 and 10 years of age at diagnosis with initial WBC counts of $<5 \times 10^4/\mu l$. The treatment had age-adjusted intrathecal therapy with weekly MTX (20 mg/m^2) during the maintenance phase. An adverse event was defined as leukemic relapse at any site. Controls were defined as patients that remained with continuous remission with <5% leukemia blasts in a bone marrow aspirate. The study set include 520 (43%) patients who had available samples that yielded DNA adequate for genotyping. This sample set consisted of 124 relapsed and 396 non-relapsed patients. It was reported that the *MTHFR* 677C>T variant allele showed a statistically significantly association with leukemia relapse, $\chi^2 = 4.38$, P = 0.036. The association

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remained significant (HR = 1.82 (95% CI 1.16-2.84)), when controlling for important covariates, and was found to be more predictive of relapse than other factors. While these are the two biggest studies on pediatric ALL to date, other groups have found no statistically significant effect (Rocha 2005, Chiusolo 2007). The wide range of results in the treatment response with respect to MTHFR polymorphisms may reflect differences in the treatment backbone of the protocol and dose adjustments made in some cases to limit toxicity (SJCRH protocols).

3.2.2.   Thymidylate synthase

The thymidylate synthase gene maps to chromosomal position 18p11.32 and contains 7 exons encoded across a 15.9 Kb genomic region (Kaneda 1990). TYMS is an essential enzyme for cellular proliferation. It catalyzes the transformation of deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymydine-5'-monophosphate (dTMP) which is an essential substrate for DNA replication. TYMS is an important target for methotrexate, which forms a stable complex with TYMS and folate cofactors to prevent DNA synthesis (Marsh 2001). The *TYMS* gene has a common length polymorphism characterized by the presence of 28-base pair tandem repeats (TR) in the 5' – untranslated region (5'-UTR). There are three predominant genotypes associated with the *TYMS* gene: 1) homozygous with two TR (2R/2R); 2) homozygous with three TR (3R/3R); and 3) heterozygous with both alleles (2R/3R). Increased *TYMS* mRNA expression and enzyme activity are associated with a greater number of tandem repeats

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(Horie 1995, Kawakami 1999, Pullarkat 2001). Individuals who were homozygous for the TR (3R/3R) were found to have a 4-fold greater relapse risk (OR = 4.1 (95% CI 1.9-9.0). This finding was confirmed in a subsequent investigation by the same research group at the same institution (Krajinovic 2002 and 2005). Two studies also examined the combined effects of multiple polymorphisms in several candidate genes in children with ALL who were treated using the DFCI or SJCRH Total XIIIb protocols. They demonstrated that patients who carried the 3R/3R genotype had a poorer outcome than patients with fewer TR genotypes (Costea 2003, Rocha 2005). In contrast to these results, however, other investigators have not been able to demonstrate similar associations (Lauten 2003, Pakakasama 2007). Lauten *et al.* (2003) assessed the 2R/3R genotype in a case-controlled study of 80 patients on the ALL BFM-86 and BFM-90 protocols. All patients were classified as standard or intermediate risk, and received identical cumulative MTX doses of $20g/m^2$ (5 g/m^2 X 4 doses). They showed an OR = 1.1 (95% CI 0.70-2.98) for 3R/3R genotype. These discordant findings are likely due to the heterogeneity of the study groups and their treatment protocols. Interestingly, there were significant differences in wild type and heterozygous frequencies between these two studies. One explanation for this could be due to preferential allele amplification in archived specimens (Miller 2002).

56
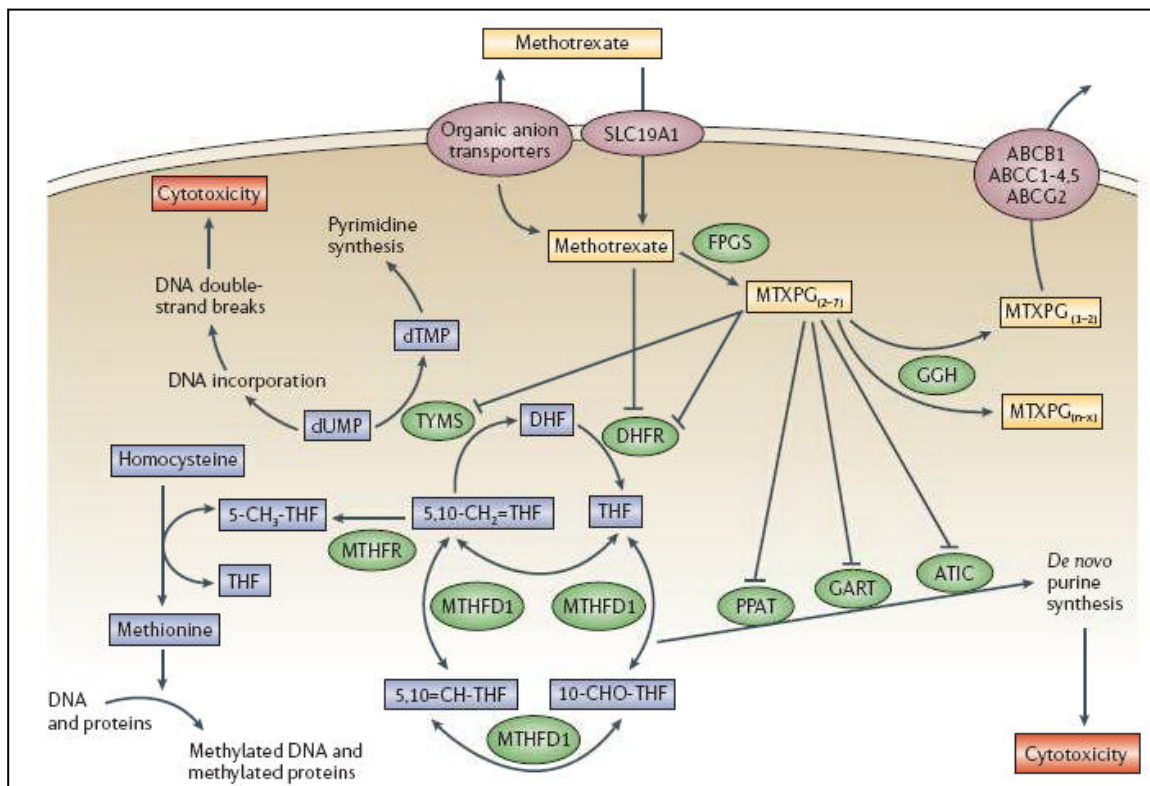
Figure 3.6 The folate pathway. This is a simplified schematic representation of the most important gene products involved in methotrexate (MTX) treatment response. MTX enters the cell through the reduced folate carrier. Its main intracellular target is dihydrofolate reductase (DHFR), inhibition of which results in accumulation of dihydrofolate (DHF) and depletion of cellular folates. MTX is a substrate for intracellular folypolyglutamate synthetase (FPGS), which catalyses the formation of $MTXPG_s$, which then inhibits multiple target enzymes. This figure was adapted from PharmGKB (2004).

3.3.   t(12;21)(p12;q22) in Acute Lymphoblastic Leukemia

The *ETV6/RUNX1* (formly *TEL/AML1*) gene rearrangement results from a cryptic, balanced, reciprocal chromosomal translocation, t(12;21)(p12;q22). This is the most frequently occurring genetic abnormality seen with childhood ALL (Shurtleff 1995, Romana 1995a) and it is associated with a favorable treatment outcome (McLean 1996, Rubnitz 1997 and 1999).

The *ETV6* gene (previously known as *TEL*) was first cloned in 1994 and is a member of the ETS family of transcription factors whose protein product is a nuclear phosphoprotein (Golub 1994 and 1997). It was subsequently found to be fused with *RUNX1* (previously known as *AML1*) in many cases of pediatric ALL (Golub 1995, Romana 1995b). This gene rearrangement disrupts the *ETV6* gene at the N terminus and fuses the helix-loop-helix (HLH) domain, inframe, to nearly all of *RUNX1*, including its DNA-binding Runt homology domain and its C-terminal transactivation region (see **Figure 3.7**).

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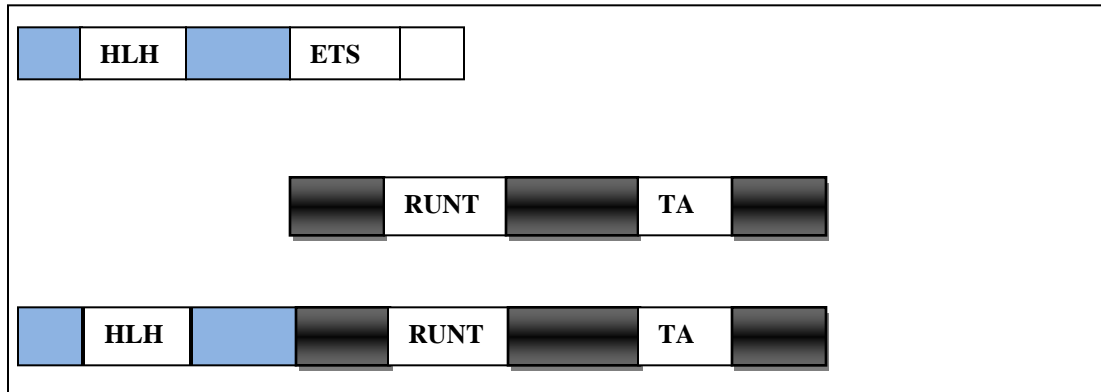
Figure 3.7 Schematic representation of the *TEL/AML1* gene rearrangement**.** *Source:* The figure was modified from Rubnitz *et al.* (1999).

Numerous studies have been published regarding the prognostic significance of the *ETV6/RUNX1* fusion gene with divergent results. Early studies showed excellent outcome. Both DFCI and St. Jude groups reported a favorable outcome for patients with *ETV6/RUNX1* (100% 8-year EFS and 92% 5-year EFS respectively). However, reports from the BFM groups showed that as many as 25% of relapsed patients carried the *ETV6/RUNX1* fusion gene (McLean 1996, Shurtleff 1995, Harbott 1997, Seeger 1998). Later, studies from the Pediatric Oncology Group (POG) and DFCI supported the excellent outcome for patients carrying *ETV6/RUNX1* fusion gene (Loh 1998, Pui 2000). Since all treatment protocols were based on the risk-stratification strategies, the heterogeneity of results may reflect the intensity of the treatment protocol and the assignment of patients to the risk group at diagnosis. For example, nearly half of the *ETV6/RUNX1*-positive patients in the first report from the DFCI study received intensive therapy (McLean 1996). This difference might potentially explain the improved outcome in this group of patients. Most recently, a DFCI prospective analysis of *ETV6/RUNX1-*

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positive patients treated on their 95-01 protocol found an *ETV6/RUNX1* prevalence of 26%, with a median follow-up of 5.2 years (Loh 2006). The 5-year EFS for *ETV6/RUNX1*-positive patients was 89% compared with 80% for *ETV6/RUNX1*-negative pre-B ALL patients ($P = 0.05$). The 5-year Overall Survival (OS) rate was 97% among *ETV6/RUNX1*-positive ALL patients compared with an OS of 89% among *ETV6/RUNX1*-negative ALL patients ($P = 0.03$). This is a result which confirms their previous findings.

Interestingly, among all the studies reviewed above, the unified pattern of relapse in *ETV6/RUNX1*-positive patients demonstrated that these patients tend to relapse long after therapy and remain sensitive to the same chemotherapy after relapse. This finding prompted the investigators to speculate about the origin of the relapsed clone. Using microsatellite markers, immunoglobulin and T-cell gene rearrangement analysis as well as fluorescent *in situ* hybridization techniques, Ford, *et al*. (2001) elegantly demonstrated that the clonal origin of two patients with late relapse was distinct from the initial leukemic clones despite the presence of the *ETV6/RUNX1* fusion transcript in the primary and relapsed leukemic cells. These investigators hypothesized that in some *ETV6/RUNX1*-positive relapsed patients most, but not all, the leukemic cells are eradicated by the initial therapy; then a second, independent transformation event occurs which gives rise to new *ETV6/RUNX1*-positive leukemic cells.

Most studies demonstrate that *ETV6/RUNX1*-positive patients have excellent treatment outcome compare to the *ETV6/RUNX1*-negative patients. Despite these

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findings, international collaborative groups have not been able to reach a consensus regarding the use of a patient's *ETV6/RUNX1* status as a prognostic indicator.

Based on the numerous supportive studies that have been reported to date, it would be unreasonable to completely ignore the potential for host genetic variation in *to* impact the chemo-sensitivity and treatment outcome. *In the current research, therefore, an attempt to further evaluate the possible association between treatment outcome and ETV6/RUNX1 fusion transcript status in a somewhat "homogenous" group of ALL patients.*

Summary: A wealth of research data support the view that genes involved in drug metabolism and genes that encode drug targets influence response to therapy and disease outcome in patients with ALL. It is important to recognize that these genetic variations may significantly associate with a specific drug response phenotype in the context of one treatment regimen but not in other regimens for the same disease. To evaluate the role of variants in drug-metabolism and drug-target genes in predicting therapeutic outcome, the current doctoral research study was conducted with the aim of assessing potential associations between disease remission, disease relapse rates and polymorphisms in the drug metabolism genes *CYP1A1*, *CYP2B6*, *CYP3A4*, *CYP3A5*, *GST*, *MTHFR*, and *TYMS*. A total of 125 patients with standard-risk ALL treated at The Children's Hospital, Aurora, Colorado served as the study population. This investigation has provided a unique opportunity to assess host genetic variations relative to treatment outcomes (specifically relapse) in a cohort of NCI-defined standard-risk ALL patients, all of whom

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presented with defined patient and tumor characteristics and who underwent similar

chemotherapeutic regimens.

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Chapter 4

Methods

4.1. Description of Study Population

Children with standard risk ALL, defined as age between 1 and 10 years at diagnosis and initial WBC count of $<5 \times 10^4$/µl, treated at The Children's Hospital between 1993 and 2005 were retrospectively included in this study.

The Center for Cancer and Blood Disorders at the Children's Hospital of Denver has a COMIRB-approved research protocol to collect diagnostic peripheral and bone marrow samples from newly diagnosed patients with leukemia. Patient cell bank samples had been previously collected in a de-identified manner and leftover clinical DNA samples were de-identified. This study was approved by COMIRB and the University of Denver IRB. There are no plans for further enrollment for the current study.

4.1.1. Inclusion criteria

Patients who met the age and WBC criteria as well and for whom bone marrow or peripheral blood samples at initial diagnosis were available along with a record of

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treatment outcome were included in this study. All samples were obtained from the COMIRB-approved cell bank in the Center for Cancer and Blood Disorders and the Molecular Diagnostic Laboratory at The Children's Hospital. Finally, to be accepted for inclusion in the current study, it was required that all samples had been collected per COMIRB 04-1041 approved protocols.

4.2. Nucleic Acid Extraction

Genomic DNA was extracted from peripheral blood or bone marrow aspirate using the Gentra PureGene Blood Kit (Gentra, Minneapolis, MN) following the manufacturer's instructions. Briefly, patient bone marrow (or peripheral blood) samples were collected in EDTA-tubes for routine clinical testing. An aliquot of 300 μl of bone marrow (or peripheral blood) was incubated for 10 minutes at room temperature in 900 μl of RBC lysis solution. White blood cells (WBCs) were then collected by centrifugation at 13,000 x g. for 20 seconds. The supernatant was carefully removed to avoid disturbing the WBC pellet and 600 μl of Cell Lysis Solution was added to the tube. The WBC pellet was resuspended by pipetting up and down to lyse the cells. When no visible cells remained, 200 μl of Protein Precipitation Solution was added to the cell lysate tube. The tube was then vigorously vortexed for 20 seconds and centrifuged for 15 minutes at 13,000 x g to pellet the protein. The supernatant containing the DNA was transferred to a clean 1.5 ml microfuge tube containing 600 μl of 100% isopropanol. The tube was inverted 20 – 30 times then centrifuged at 13,000 x g. for 5 minutes. The precipitated

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DNA was then visible as a small white pellet. The supernatant was discarded and the pellet was washed with approximately 300 µl of 70% ethanol by inverting the tube several times. The tube was centrifuged at 13,000 x g for 15 minutes. The ethanol was poured off and the tube was left open at room temperature for 5 – 10 minutes (taking care not to over dry the DNA pellet). The DNA pellet was then resuspended in approximately 50 µl of DNA Hydration Solution (10 mM Tris (pH 7.6) and 1 mM EDTA), vortexed for 5 seconds at medium speed and then incubated at 65 ^{0}C for 15 – 30 minutes to accelerate rehydration. The rehydrated DNA was allowed to sit at room temperature overnight to ensure complete the rehydration. The DNA concentration was measured using the NanoDrop spectrophotometer and all DNA samples were then diluted with DNA Hydration Solution to the final working concentration as 50 ng/µl. The working DNA solution was stored at – 70 ^{0}C until analyzed. Detailed information on the DNA extraction technique may be found in **Appendix 1.**

4.3. Genetic Polymorphism Detection

Nine SNPs and two genetic deletion/duplication mutations in the Phase I and II metabolism enzymes (CYP and GSP respectively) and drug-target (MTHFR and TS) genes were chosen for genotyping based on the important roles they played in the pharmacokenetics and pharmacodynamics of chemotherapeutic drugs. *Taq*Man® (Applied Biosystems (ABI), Foster City, CA) genotyping techniques were utilized for SNP genotyping. Polymerase chain reaction (PCR) and argarose gel electrophoresis were

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employed for gene deletion or duplication detection. The genetic polymorphisms content

sequences are presented in **Table 4.1**. The details of the detection procedures are

described below.

Table 4.1 Content sequences for the genetic polymorphisms

Gene	SNP_ID	ABI_ID	Content Sequence
GSTP1	rs1138272	1049615_20	GATACATGGTGGTGTCTGGCAGGAGG[C/T]GGGCAAGGATGACTATGTGAAGGCA
GSTP1	rs1695	3237198_20	GCGTGGAGGACCTCCGCTGCAAATAC[A/G]TCTCCCTCATCTACACCAACTATGT
CYP3A5	rs10264272	30203950_10	TCTAAGAAACCAAATTTAGGAACTT[C/T]TTAGTGCTCTCCACAAAGGGGTCTT
CYP3A5	rs776746	26201809_30	TCTTTAAAGAGCTCTTTGTCTTTCA[A/G]TATCTCTTCCCTGTTTGGACCACAT
CYP3A4	rs2740574	N/A	TGAGGACAGCCATAGA GACAAGGGCA[A/G] GAGAGAGGCG ATTTAATAGAT
CYP2B6	rs3211371	30634242_40	GCAAAATACCCCCAACATACCAGAT[C/T]GCTTCCTGCCCCGCTGAAGGGGCTG
CYP1A1	rs1048943	25624888_50	GCAAGCGGAAGTGTATCGGTGAGACC[C/T]TTGCCCGCTGGGAGGTCTTTCTCTT
MTHFR 677	rs1801133	N/A	CTTGAAGGAGAAGGTGTCTGCGGGAG[C/T]CGATTTCATCATCACGCAGCTTTTC
MTHFR 1298	Rs1801131	N/A	GTGGGGGGAGGAGCTGACCAGTGAAG[A/C]AAGTGTCTTTGAAGTCTTCGTTCTT

rs, dbSNP identifier; ABI, Applied Biosystems

4.3.1. Genotyping by *Taq*Man® Drug Metabolism Genotyping Assays

*Taq*Man® Drug Metabolism Genotyping Assays were used to detect *GSPT1*, *CYP3A5*, *CYP2B6*, and *CYP1A1* polymorphisms. The assay are based on the 5' nuclease chemistry for amplifying and detecting specific genetic polymorphisms in DNA samples. These assays were developed using ABI's bioinformatic assay design process, and genomic information from public SNP databases and public genome assemblies. These assays are designed and optimized to work with *Taq*Man® Universal PCR Master Mix (ABI, Foster City CA) and only 3 to 20 ng of DNA is required for each reaction. Each assay contains sequence-specific forward and reverse primers that amplify the polymorphic sequence of interest; one probe labeled with a VIC® reporter dye and a non-fluorescent quencher (NFQ) dye to detect the "Allele X" sequence and; a second probe labeled with a 6-FAM™ reporter dye and a NFQ dye to detect the "Allele Y" sequence. During PCR, each *Taq*Man® probe anneals specifically to its complementary sequence between the forward and reverse primer sites. Ampli*Taq* Gold® DNA polymerase extends the primers bound to the DNA template and cleaves only the probe that are bound to the target. Cleavage separates the reporter dye from the quencher dye, resulting in increased fluorescence by the reporter molecule. By detecting the increase in fluorescence signal, it is possible to determine which alleles are present in the sample. Even single nucleotide mismatches between a probe and the target sequence alter the efficiency of probe binding to the target, which in turn reduces the amount of reporter dye cleaved from a quenched probe. **Figure 4.1** shows the principle of this assay. A substantial increase of only VIC®

67
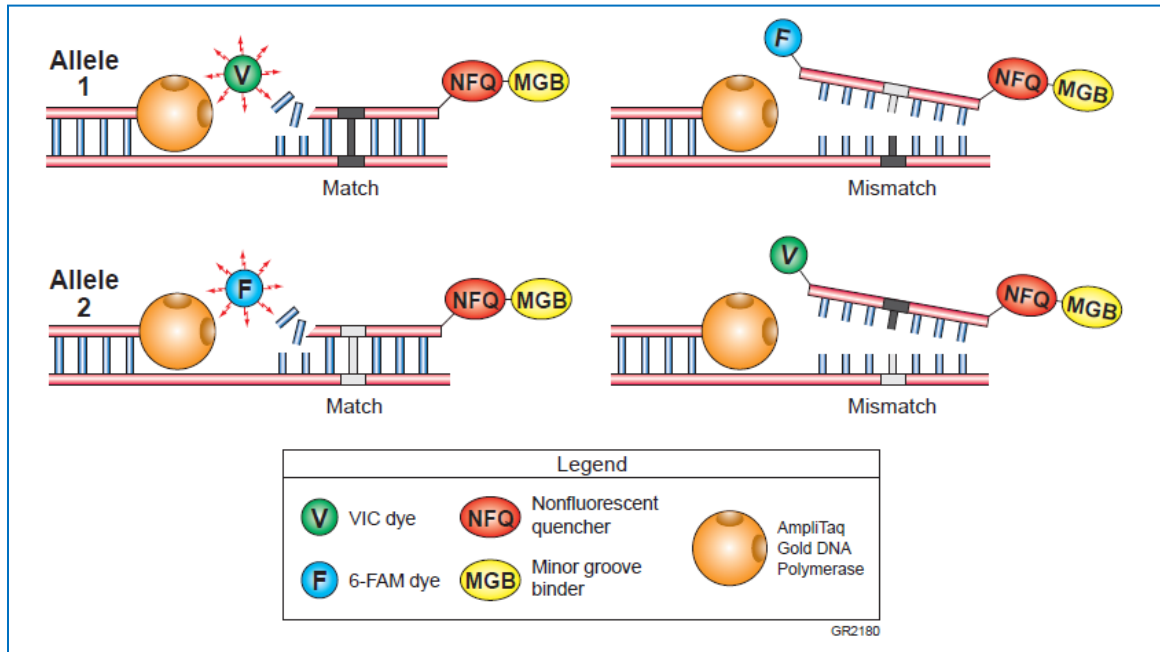
Figure 4.1 Principle of *Taq*Man® Chemistry. Allelic discrimination probes are labled with VIC® dye at the 5' end of the allele 1 probe and 6-FAM™ dye at the 5' end of allele 2 probe. A NFQ and a MGB are linked to the 3' end of each probes. During PCR, Taq DNA polymerase is more likely to displace a mismatched probe without cleaving it. Conversly, Taq DNA polymerase cleaves a probe that is hybridized (matched) to the target temple, reulting in an increased flurescence by the reporter dye. The uncleaved probe (mismatched) will not generate an increased fluorescence due to the presence of quencher dye in a close proximity. Thus, an increased fluorescence signal (6-FAM™ or VIC®) during PCR amplification indicates which allele is present in the sample.
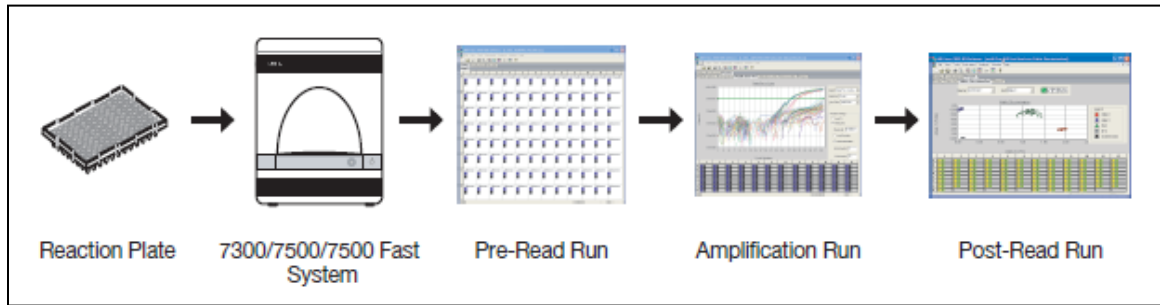
Figure 4.2 Workflow of *Taq*Man® genotyping. The above is a schematic representation of the workflow involved in the *Taq*Man® genotyping. From Left to right: reaction plate was prepared with reaction mix and template DNA, followed by PCR amplification set up with an new plate read (Pre-Read) was performed, then a real-time PCR was performed, followed by the post-read run whcih the SDS software automatically subtrates the baseline fluoresence determined at the pre-read and allele calls were assigned by the SDS software.

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dye signal indicates homozygosity for Allele 1 and *vise versa* a substantial increase of only 6-FAM™ signal will indicate homozygosity for Allele 2. Heterozygosity for Allele 1 and 2 will be represented by the increase in both VIC® and 6-FAM™ signals. An "allelic discrimination experiment" is constructed according to the Applied Biosystems 7500 "Allelic Discrimination: Getting Started Guide". **Figure 4.2** illustrates the complete process for an allelic discrimination experiment.

Each genotyping reaction contains 12.5 μl of 2X *Taq*Man® Universal PCR Master Mix (UMM), 1.25 μl of Assay Mix (primers and probes), 10.25 μl of DNase-free water, and 1.0 μl of sample DNA (50 ng/μl). The UMM, Assay Mix and DNase-free water were combined in a microcentrifuge tube to create a master mix (MM). The MM was flicked and inverted several times to mix the contents. Aliquots of 24.0 μl of MM were then pipetted into the required number of wells on an optical 96-well plates (ABI, Foster City, CA). Template DNA (1 μl of 50 ng/μl stock) was then added to each well and the reaction plate was sealed with optical caps. Two, "no template" negative controls (Dnase-free water) were included in each assay. The ABI 7500 Real-Time PCR System was slected to perform all allele discrimination experiments. This system allows real-time analysis of PCR, which provides for greater assay accuracyand is helpful for troubleshooting. The thermal cycling conditions employed are shown below.

Taq Gold® polymerase activation	PCR (50 cycles)	
HOLD	Denature	Anneal/Extend
10 min at 95 ^{o}C	15 sec at 92 ^{o}C	90 sec at 60 ^{o}C

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An Allelic Discrimination (AD) Plate Document was set up on the ABI 7500 Real-Time PCR System in which 6-FAM™ and VIC® were selected as detectors for the wildtype and mutant alleles, respectively. The amplification run was then performed according to manufacturer's instruction (refer to **manufacturer's instructions** for details). All data generated during the run were saved to the AD plate document specified previously. The AD data collected during the run were then analyzed by the 7500 SDS software. Briefly, the raw data were converted the raw were organized in terms of fluorescence signal *vs.* filters. The SDS software plots the results from the AD run on a scatter plot of Allele X *vs.* Allele Y. Each well of the reaction plate is represented an "X" (Undetermined) on the plot as shown in **Figure 4.3**. The clustering of points can vary on the plot due to differences in the content of reporter dye fluorescent intensity after PCR amplification. Allele calls are then assigned by selecting "Automatic Allele Calling" under the "Analysis Setting" and then selecting the "Reanalyze" tab. Samples are then grouped on the plot as "Allele X", "Allele Y", "Both Alleles X & Y", "Undetermined" and "NTC" (illustrated in **Figure 4.4**). The allele calls for each sample well are then listed in the Call column (**Figure 4.5**) (refer to **manufacturer's instructions** for details).

71
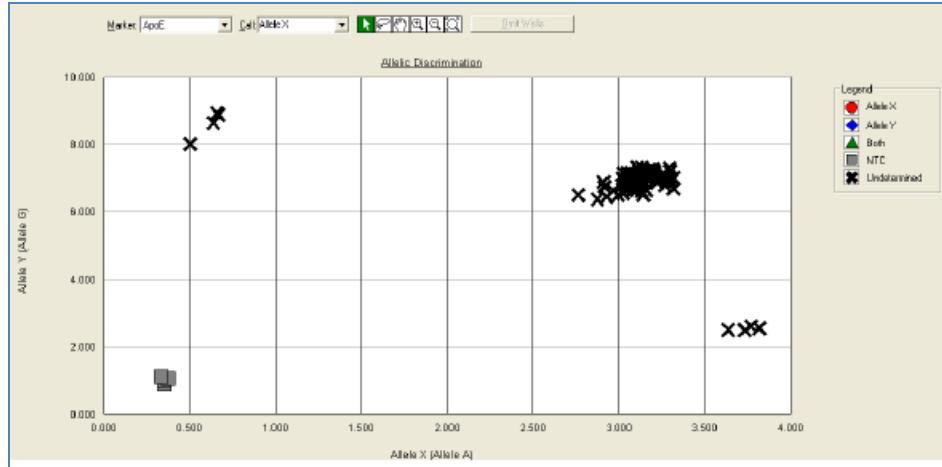
Figure 4.3 Allelic discrimination cluster plot. After a post-read, the 7500 SDS software display a scatter plot of Allele X *vs*. Allele Y with each wells represented by an X. The X represents the ratio of dye fluorescence intensity from Allele X dye and/or Allele Y dye after PCR amplification. The horizontal axis (Allele X) and vertical axis (Allele Y) indicate dye fluorescence intensity.
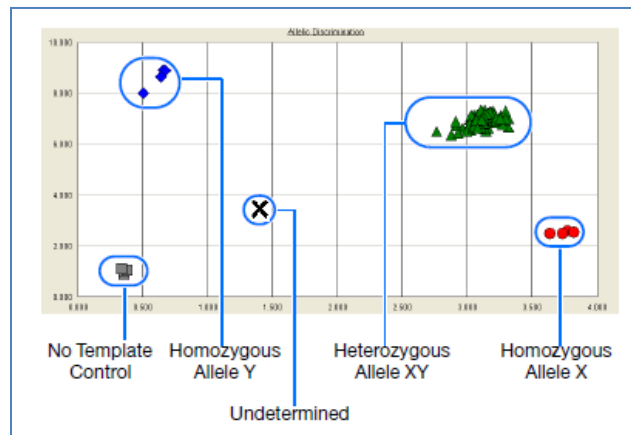
Figure 4.4 Allelic discrimination post-read plot. After 7500 SDS software analysed the ratio of raw fluorescence data, a post-read plot was displayed. A genotype was assigned for each sample in each well according to the relative dye fluorescence intensity.

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Figure 4.5 Allelic discrimination genotyping reports. The post-read data can be also displayed as a report form. (a) displays the genotyping results in a table format, (b) represents the allele calls listed as the name of the dye-related probes, (c) the user-defined column can be used to add comments.

4.3.2. Genotyping by *Taq*Man® Genotyping Assays

CYP3A4, MTHFR 667, and MTHFR 1298 polymorphisms were genotyped using laboratory developed assays with *Taq*Man® chemistry, samples handing steps and data analysis methods as described in the preceeding section. The forward primer, reverse primer, wildtype allele probe (VIC® labeled), and mutant allele probe (6-FAM™ labeled) were selected from the National Cancer Institute's SNP500 database (http://snp500cancer.nci.nih.gov). Primer and probe sequences are listed in **Table 4.2.**

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Table 4.2 Primers and Probes for *CYP3A4* and *MTHFR* Genotyping

Gene		Concentration	5' dye	Sequence	3' dye	Allele
CYP 3A4 (rs2740574)	Probe 1	200 nM	FAM	AAGGGCAGGAGAGAG	MGB	G
	Probe 2	200 nM	VIC	AAGGGCAAGAGAGAG	MGB	A
	Primer F	900 nM		TGGAATGAGGACAGCCATAGAGA		
	Primer R	900 nM		AGTGGAGCCATTGGCATAAAATCT		
MTHFR 667 (rs1801133)	Probe 1	200 nM	FAM	ATGAAATCGACTCCCGC	MGB	T
	Probe 2	200 nM	VIC	ATGAAATCGGCTCCCGC	MGB	C
	Primer F	900 nM		GCACTTGAAGGAGAAGGTGTCT		
	Primer R	900 nM		TGTGTCAGCCTCAAAGAAAAGCT		
MTHFR 1298 (rs1801131)	Probe 1	200 nM	FAM	AGACACTTGCTTCACT	MGB	C
	Probe 2	200 nM	VIC	CAAAGACACTTCTTC	MGB	A
	Primer F	900 nM		GGAGGAGCTGCTGAAGATGTG		
	Primer R	900 nM		CCCGAGAGGTAAAGAACAAAGACTT		

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4.3.3. Genotyping for *TYMS, GSTM1, and GSTT1*

Genotyping for the *TYMS* tandem repeats was performed by polymerase chain reaction assay as previously described with modification (Hishida *et al.*, 2003). Briefly, the reaction consisted of 2 μl of (50 ng/μl) genomic DNA, 2.5 μl of GeneAmp$^®$ 10X PCR Gold Buffer with 15 mM MgCl$_2$ (ABI, Foster City, CA), 0.5 μl of DMSO (Sigma, MO), 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 2 μl of 2.5 mM dNTPs, 0.2 μl of 5U/μl Ampli*Taq* Gold$^®$ and 16.8 μl DNase-free water. The sequence of the forward primer was (5'-CGT GGC TCC TGC GTT TCC-3') and the sequence of the reverse primer was (5'-GAG CCG GCC ACA GGC AT-3). Amplification was performed in a Veriti 96 Well Thermal Cycler (ABI, Foster City, CA) with initial denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 20 seconds. There was also a final elongation step at 72°C for 10 minutes. Amplified PCR products were electrophoresed through a 3% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer (Qiagen, Valencia, CA) and visualized with ethidium bromide staining to detect the 2R (210 base pair; bp) and 3R (238 bp) alleles as illustrated in **Figure 4.6.**

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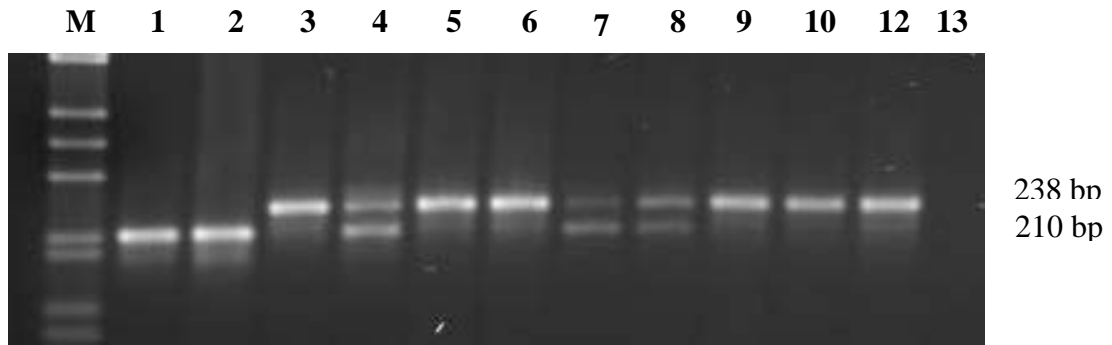
Figure 4.6 Genotyping for *TYMS* tandem repeats. PCR products were separated by electrophoresis to determine genotypes. The 2R and 3R alleles were identified by a band of 210 and 238 bp, respectively, as indicated. Lane M indicates a DNA ladder; Lan1 and 2 represent 2R/2R genotype; Lane 4, 7, and 8 represent 2R/3R genotype; Lane 3, 5-6, 9-12 represent 3R/3R genotype; Lane 13 represents no template control.

GSTT1 and *GSTM1* gene deletions, designated as null allels, were identified by PCR amplification as previously described (Spurdle *et al.*, 2001), using primers located within the *GSTT1* and *GSTM1* genes to detect the presence of these gene sequences. A 5'-untranslated region of the estrogen receptor (ER) gene was amplified as a positive amplification control to ensure a succesful amplification reaction. Primer sequences and product sizes are listed in **Table 4.3**. Each PCR reaction took place in a 25 µl reaction volume which contained 100 ng of genomic DNA, forward/reverse primers (400 nM), dNTPs (200 nM), 1X Gene- AMP® Gold PCR Buffer with 1.5 mM $MgCl_2$, and 1 U Ampli*Taq* Gold® (Applied Biosystems, CA). Amplification was performed in a Veriti 96 Well Thermal Cycler (ABI, Foster City, CA) with initial denaturation at $95^{o}C$ for 5 minutes followed by 45 cycles of $95^{o}C$ for 15 seconds, $61^{o}C$ for 20 seconds and $72^{o}C$ for 20 seconds. There was also a final elongation step at $72^{o}C$ for 10 minutes. Amplified PCR products were electrophoresed through a 3% agarose gel in 1X TAE buffer (Qiagen, Valencia, CA) and visualized with ethidium bromide staining to detect the *GSTT1* and *GSTM1* null alleles as illustrated in **Figure 4.7**. All reactions were expected to show amplification of the ER positive amplification control (181 bp).The absence of a 114 bp or 131 bp fragment indicated *GSTM1 or GSTT1* null genotypes, respectively. Patient samples showing amplification of the target PCR products were defined as having a non-null genotype.

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Table 4.3. Primer sequences used for PCR detection of *GSTT1* and *GSTM1* genotypes

Gene	Amplicon size	Genotype	Forward primer	Reverse primer
GSTT1	131 bp	Null allele	5'GGTCATTCTGAAGGCCAAGG 3'	5' TTTGTGGACTGCTGAGGACG 3'
GSTM1	114 bp	Null allele	5'' TGCTTCACGTGTTATGGAGGTTC 3'	5'' GTTGGGCTCAAATATACGGTGG 3''
ER 5'	181 bp	DNA control	5'' CAAGTCTCCCCTCACTCCCC	5'' GTGCGAGTGGCTCAGTGTGT 3''

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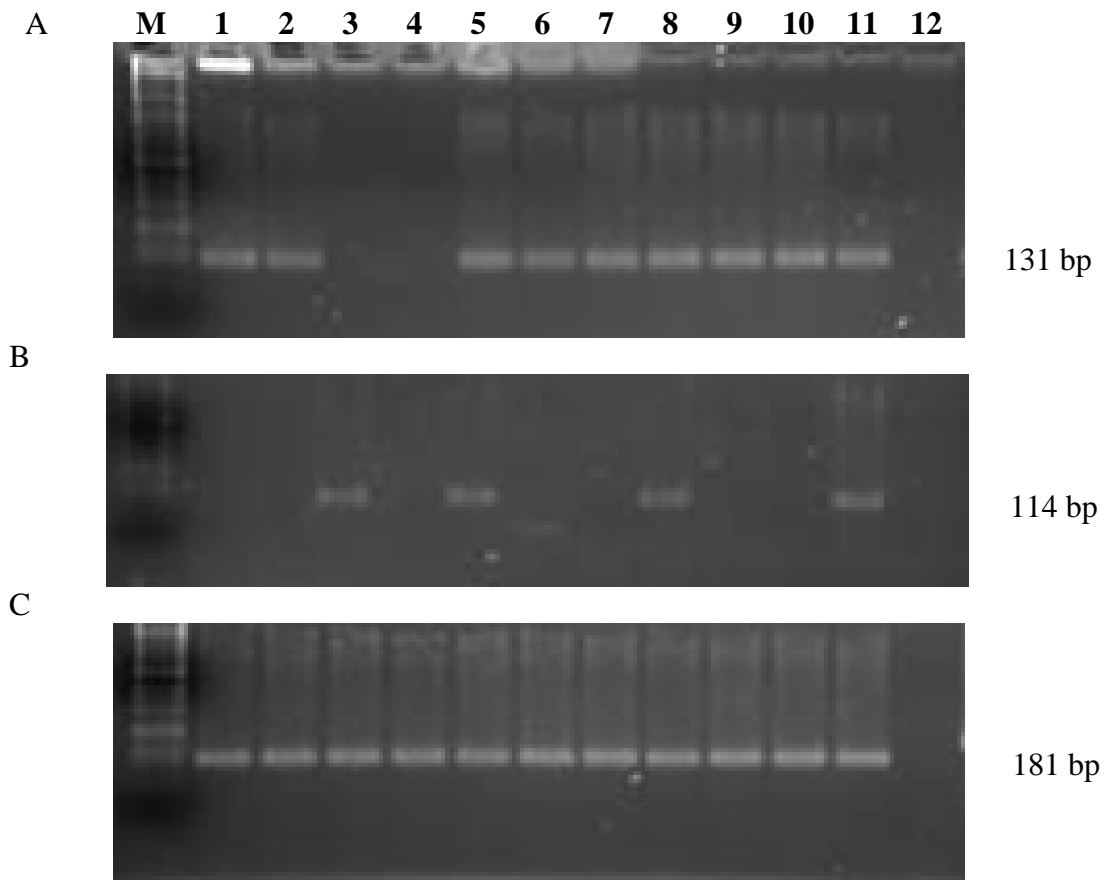
Figure 4.7 Representative cases of *GSTT1* and *GSTM1* genotyping. Lane **M** indicates DNA ladder. (A) GSTT1 genotyping examples. Lane 3 and 4 represent null genotypes while the remainder of the lanes represent non-null genotypes. (B) GSTM1 genotyping examples. Lanes 3, 5, 8, and 11 indicated non-null genotypes. (C) ER PCR products. Lane 12 was a no-template control (water) and is used to indicate the absence of contamination in the stock PCR reagents. Lane 4 indicates a double null genotype for *GSTM1/GSTT1*.

4.4.   Statistical Analysis

The primary outcome of interest in the current study was disease relapse. For analysis of relapse risk, cases of relapse were defined as patients who experienced leukemia relapse at any site. The event-free group was defined as those patients who remained in continous remission (<5% lymphoblasts in bone marrow aspirate).

The majority of the 125 patients with standard-risk ALL yielded adequate DNA for complete genotyping. The study set included 16 patients who experienced disease relapse and 109 patients in continous remission. Statistical analyses were performed with SAS/STAT software (SAS Institute Inc., NC) at the Colorado Biostatistics Consortium. For statistical analyses, A p value less than 0.05 was considered significant.

Kaplan-Meier survival curve is the most common method to describe survival characteristics. It tracks how many individuals have not experienced the event at a given time or during a given time interval. The data is then plotted over the entire time of the study. The survival curve represents an estimated probability (Y-axis) of surviving in a given length of time (X-axis). This analysis allows estimation of survival over time. Hazard, defined as the probability of the endpoint (*e.g*., leukemia relapse), is a mesure of how rapidly the event occurs. The hazard ratio (HR) compares the hazards in two groups (*e.g*., relapse and EFS) and is calculated with its 95% confidence interval. HR is a estimate of relative risk of event.

Univariate analysis of 12 genotypes and disease relapse was performed with Chi-square or Fisher's exact tests to compare the genotypes frequencies in the relapse and

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event-free groups. In order to estimate the relative risk of disease relapse, hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using a logistic regression model to evaluate the significance of differences in survival between the relapsed and event-free survival groups. Life table estimates were calculated by the Kaplan-Meier method and survival curves were generated for a graphical presentation of event-free survival probabilities. Survival time was defined as the time between diagnosis and the date of an event or last follow-up. A log-rank test was ultilized to compare time-to-relapse values (Kaplan & Meier, 1958).

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Chapter 5

Results

A total of 125 patients with a diagnosis of standard-risk acute lymphoblastic leukemia treated from 1998 through 2005 at the Center for Cancer and Blood Disorder at The Children's Hospital in Denver were included in this study. Standard-risk ALL was defined according to NCI criteria based on age (1 – 9.99 years) and initial WBC count (less than 50,000/µl) at diagnosis. Genotype frequencies are shown in **Table 5.2**. Among this cohort, there were 72 (58%) males and 53 (42%) females which is comparable to the national frequency for standard-risk ALL. There were 16 patients who had experienced disease relapse and 109 patients who had remained event-free. This gives a 5-year EFS of 87.2% which is slightly better than the national average of 81.6% (p = 0.14) (Matloub, 2006). Patient ethnicity data were not collected because 91% of ALL patients treated at The Children's Hospital in Denver are of Caucasian ethnicity. **Table 5.1** shows the ethnic distribution among the ALL patients treated at The Children's Hospital in Denver.

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Table 5.1 ALL patient distribution

Ethnic Categories	Gender		
	Females	Males	Total
Hispanic or Latino	9	12	**21**
Not Hispanic or Latino	55	74	**129**
Ethnic Categories: Total of All Subjects	**64**	**86**	**150**
Racial Categories			
American Indian/Alaska Native	2	2	**4**
Asian	2	3	**5**
Native Hawaiian or Other Pacific Islander	0	0	**0**
Black or African-American	2	3	**5**
White	58	78	**136**
Racial Categories: Total of All Subjects	**64**	**86**	**150**

Note: These classifications are constructed according to "Revisions to the Standards for the Classification of Federal Data on Race and Ethnicity" by the Office of Management and Budget (October 30, 1997).

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Table 5.2 Genotype frequencies distribution

Genotype	Relapsed n (%)	EFS n (%)	P^1
CYP 1A1			0.473
AA	13 (86.7)	85 (78.7)	
AG	2 (13.3)	23 (21.3)	
GG	0 (0)	0 (0)	
CYP 2B6			0.923
CC	13 (81.3)	85 (78.7)	
CT	3 (18.7)	21 (19.5)	
TT	0 (0)	1 (0.9)	
CYP 3A4			0.888
AA	15 (93.8)	98 (90.7)	
AG	1 (6.2)	9 (8.4)	
GG	0 (0)	1 (0.9)	
CYP 3A5_1			**0.009**
CC	15 (93.8)	108 (100)	
CT	1 (6.2)	0 (0)	
TT	0 (0)	0 (0)	
CYP 3A5_2			0.460
AA	14 (87.5)	80 (74.1)	
AG	2 (12.5)	24 (22.2)	
GG	0 (0)	4 (3.7)	
*GST*P1_1			0.502
CC	15 (93.8)	90 (82.6)	
CT	1 (6.2)	16 (14.7)	
TT	0 (0)	3 (2.7)	
*GST*P1_2			0.680
AA	6 (37.5)	36 (33)	
AG	6 (37.5)	53 (48.6)	
GG	4 (25)	20 (18.4)	

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Table 5.2 (continued)

Genotype	Relapsed n (%)	EFS n (%)	P^1
MTHFR 677			0.356
CC	7 (43.8)	40 (37.4)	
CT	8 (50)	44 (41.1)	
TT	1 (6.2)	23 (21.5)	
MTHFR 1298			0.676
AA	7 (43.8)	60 (55.5)	
AC	7 (43.8)	37 (34.3)	
CC	2 (12.5)	11 (10.2)	
*GST*M1			0.144
present	12 (75)	59 (55.7)	
null	4 (25)	47 (44.3)	
*GST*T1			0.662
present	14 (87.5)	89 (83.2)	
null	2 (12.5)	18 (16.8)	
TS tandem repeat			0.348
2R/2R	5 (31.3)	27 (25.2)	
2R/3R	9 (56.2)	48 (44.9)	
3R/3R	2 (12.5)	32 (29.9)	
TEL/AML1			0.096
Present	0 (0)	16 (15.1)	
Not present	16 100)	90 (84.9)	

1. Chi-square test

5.1. Phase I Drug-Metabolism Enzyme Polymorphisms

A total of 123 patient samples were successfully genotyped for the *CYP*1A1 (rs1048943) and *CYP*2B6 (rs3211371). Genotypic frequencies are shown in **Table 5.3**. These are comparable to the published frequencies in the NCI SNP500. Among the 123 patient samples, 25 patients were found to be heterozygous for *CYP*1A1 and 2 of them were in the relapsed group. No individuals who were homozygous for the mutant allele were observed in this study cohort. There was no statistically significant difference between the EFS group and the disease relapsed group with regard to genotypic frequencies ($P = 0.473$, χ^2). The *CYP*2B6 heterozygous genotype (CT) was found in 24 patients while the homozygous genotype (TT) was found in just 1 patient. A statistically significant difference was not observed in the genotype distribution between the relapsed group (CC, 81.3% and CT, 18.7%) and the EFS group (CC, 79.4%; CT, 19.5%; TT, 0.9%). The TT genotype was not observed in the relapsed group. This is likely due to its low allele frequency.

A total of 124 patients were genotyped for *CYP* 3A4 (rs 2740574), *CYP3A5* (rs 10264272), and *CYP3A5* (rs776746). Genotypic frequencies are comparable to published reports including the NCI SNP 500. There is no statistically significant difference between the relapsed and EFS groups for the *CYP*3A4 (rs2740574) and *CYP*3A5 (rs776746) genotypes frequencies ($p = 0.888$ and 0.460, respectively). There is also no statistically significant difference of risk of relapse (HR = 0.67, 95% CI = 0.09 – 2.80, *P* = 0.70 and HR = 0.43, 95%CI = 0.10 – 1.90, *P* = 0.27 respectively). No *CYP3A4* GG

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genotype was observed and the AG genotype was observed in only 1 patient and that individual was in the relapsed group. In the EFS group, the AG genotype was found in 9 patients and the GG genotype was found in 1 patient.

5.2.   Phase II Drug-Metabolism Enzyme Polymorphisms

With regard to the glutathione *S*-transferase (*GST*) genotypes, four *GST* polymorphisms were analyzed. They include 1) the coding region SNPs at codon 105 (rs1138272) defined as *GSTP1* (C > T) and codon 114 (rs1695) defined as *GSTP1* (A > G), 2) as well as the *GSTM1* and *GSTT1* deletions. The homozygous deletions defined as *GSTM1* null and *GSTT1* null lead to the absence of phenotypic enzyme activity.

 The genotypic frequencies for the current study cohort were found to be consistent with the published reports included in NCI SNP500. The genotype prevalence in the entire study population was as follow: *GSTP1* (rs 1138272) CC: 84.0 %; CT: 13.6%; TT: 2.4%. No TT genotype was observed in the relapsed group. *GSTP1* (rs1695) AA: 33.6%; AG: 47.2%; GG: 19.2%; *GSTM1* present: 58.2%; *GSTM1* null: 41.8%; *GSTT1* present: 83.7%; *GSTT1* null: 16.3%. **Table 5.4** shows the distribution of *GSTM1*, *GSTT1* and *GSTP1* genotypes in the EFS and relapsed groups and the association of these genotypes with the

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Table 5.3 Analysis of *CYP1A1, CYP2B6, CYP3A4, and CYP3A5* polymorphisms and risk of leukemia relapse

Genotype	Relapsed Group n (%)	EFS Group n (%)	P*	HR (95% CI)	P-value
CYP1A1			0.473		
CC	13 (86.7)	85 (78.7)			
CT	2 (13.3)	23 (21.3)		0.557 (0.126, 2.472)	0.410
TT	0 (0)	0 (0)			
CYP2B6			0.923		
CC	13 (81.3)	85 (79.4)			
CT	3 (18.7)	21 (19.5)			
TT	0 (0)	1 (0.9)		0.795 (0.226, 2.796)	0.721
CYP3A4			0.888		
AA	15 (93.8)	98 (90.7)			
AG	1 (6.2)	9 (8.4)		0.671 (0.089, 5.084)	0.699
GG	0 (0)	1 (0.9)			
CYP3A5 (rs10264247)			0.009		
CC	15 (93.8)	108 (100)			
CT	1 (6.2)	0 (0)		5.697 (0.743, 43.694)	0.094
TT	0 (0)	0 (0)			
CYP3A5 (rs776746)			0.460		
AA	0 (0)	4 (3.7)			
AG	2 (12.5)	24 (22.2)		0.433 (0.098, 1.904)	0.268
GG	14 (87.5)	80 (74.1)			

HRs were calculated for *CYP1A1* CT *vs.* CC, CYP2B6 CT/TT *vs.* CC, CYP3A4 AG/GG *vs.* AA, CYP3A5 (rs10264247) CT *vs.* CC, and CYP3A5 (rs776746) AA/AG *vs.* GG, respectively. *P**, P value was estimated by chi-square test. HR: Hazard Ratio; CI: Confidence Interval; EFS: Event-Free Survival

occurrence of disease relapse. In terms of the risk of disease relapse for the current study cohort, the *GSTM1* null genotype appeared to confer a 2.5-fold protective effect compared to patients that were homozygous for the non-null condition. This apparent protective effect, however, was not statistically significant (HR = 0.394; 95% CI = 0.127 – 1.2254, P = 0.107). Relative to the presence of the *GSTT1* gene, the *GSTT1* null genotype was also associated with an apparent reduction in the risk of leukemia relapse. This apparent effect, however, was not statistically significant (HR = 0.662; 95% CI = 0.150 – 2.915, P = 0.585). A 3-fold statistically non-significant difference in risk of relapse (HR = 0.341; 95% CI = 0.045 – 2.581, P = 0.298) was associated the *GSTP1* (rs1138272) CT & TT genotypes in comparison to the CC genotype. No association with leukemia relapse was found for the GSTP1 (rs1965) polymorphisms. The HR for the GG & AG genotypes compared to AA genotype was 0.828 (95% CI = 0.301 – 2.279, P = 0.715).

GST enzymes exhibited broad substrate specificity toward a variety of substances (Tew 1994). Given the profound effect of GSTM1 and GSTT1 null genotypes (absence of enzyme activity), the association of *GSTM1* and *T1* genotype combination with the risk of leukemia relapse was investigated in this study cohort. Patients were grouped according to their genotype as either: 1) *GSTM1* or T1 showed a null genotype or 2) both *GSTM1* and T1 were present. The risk of relapse increased 2.7-fold when both *GSTM1* and *GSTT1* were present compared to when either *GSTM1* or *GSTT1* was a null genotype

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(HR = 2.7, 95% CI = 0.9 – 7.9, *P* = 0.063). This result approached but did not reach statistical significance.

5.3.   **Drug-Target Polymorphisms**

Genotype distributions for the MTHFR and TYMS enzymes, which are the critical components of the folate metabolic pathway, are shown in **Table 5.5**. Overall genotype frequencies were as follow: The frequencies of *MTHFR* genotypes involving the C/T polymorphism at nucleotide position 677 in the current study cohort were 38.2%, 42.3%, and 19.5% for the CC, CT, and TT genotypes, respectively. There was no statistically significant association between the relapsed and EFS groups in regard to genotype distribution. A slightly higher TT genotype was noted for the current study cohort but the C and T allele frequencies were within the observed range of published reports. The MTHFR 677 TT genotype was observed in 6.2% and 21.5% of patients among the relapsed and EFS groups, respectively. This difference did not reach statistical significance (*P* = 0.356, chi-square test).

The frequencies of MTHFR genotypes involving the A/C polymorphism at nucleotide position 1298 in the current study cohort were 54.0%, 35.5%, and 10.5% for the AA, AC, and CC genotypes, respectively. There was no statistically significant difference regarding to the CC genotype distribution in the current study cohort which showed a frequency of 12.5% and 10.2% in the relapsed and EFS groups respectively

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Table 5.4 Analysis of *GSTP1*, *GSTM1* and *GSTT1* polymorphisms and risk of leukemia relapse

Genotype	Relapsed Group n (%)	EFS Group n (%)	P*	HR (95% CI)	P-value
GSTP1 (rs1138272)			0.502		
CC	15 (93.8)	90 (82.6)		0.341 (0.045, 2.581)	0.298
CT	1 (6.2)	16 (14.7)			
TT	0 (0)	3 (2.7)			
GSTP1 (rs1695)			0.680		
AA	6 (37.5)	36 (33.0)			
AG	6 (37.5)	53 (48.6)		0.828 (0.301, 2.279)	0.715
GG	4 (25.0)	20 (18.4)			
GSTM1			0.144		
present	12 (75.0)	59 (55.7)			
null	4 (25.0)	47 (44.3)		0.394 (0.127, 1.224)	0.107
GSTT1			0.662		
present	14 (87.5)	89 (83.2)			
null	2 (12.5)	18 (16.8)		0.662 (0.150, 2.915)	0.585

HRs were calculated with *GSTP1* (rs1138272) CC/CT *vs.* TT, *GSTP1* (rs1695) AA *vs.* AG/GG, *GSTM1* null *vs.* present, *GSTT1* null *vs.* present, respectively. *P**, P value was estimated by chi-square test. HR: Hazard Ratio; CI: Confidence Interval; EFS: Event-Free Survival

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(P = 0.676, chi-squred test). The MTHFR 1298 CC genotype showed a slightly increased HR relative to the AA and AC genotypes (HR = 1.35, 05% CI = 0.307 – 5.962, P = 0.689) but this did not reach statistical significance.

The *TYMS* gene has a promoter enhancer region polymorphism in the 5'-untranslated region, characterized by a double (2R) or triple (3R) 28-bp tandem repeat sequence. As shown in **Table 5.5**, the genotype frequencies of genotypes involving this *TYMS* polymorphism in the study cohort were 26.0%, 46.3%, and 27.6% for the 2R/2R, 2R/3R, and 3R/3R genotypes, respectively. This distribution is comparable to published reports. Among the relapsed and EFS groups, the polymorphism distribution was 31.3% and 25.2% for 2R/2R, 56.2% and 44.9% for 2R/3R, and 12.4% and 29.9% for 3R/3R respectively. This difference was not statistically significant (P = 0.356, chi-squred test). The 3R/3R patients had an apparent decreased risk of leukemia relapse when compared to 2R/2R and 2R/3R genotypes in the study cohort (HR = 0.401, 95% CI = 0.091 – 1.767, P = 0.227). This apparent difference, however, did not reach statistical significance.

5.4.   Combinational Analysis of CYP and GST Genotypes

CYP and GST genotypes may confer varying degrees of metabolic activities for chemo-theraputic agents. For this reason, the potential association between CYP-GSTM1/T1 combinations and the risk of leukemia relapse was investigated for the current study cohort. Hence, the study cohort was grouped into three clinically pre-

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defined "drug exposure groups" based on their known genotypes, as: 1) High-drug exposure group, 2) Low drug-exposure group, and 3) Intermediate-drug exposure group. **Table 5.6** lists the features of these groups.

Table 5.7 lists the "drug exposure group" with regard to the risk of leukemia relapse in the study cohort. Patients who were in the low-drug exposure group had a 2.2-fold increased risk of relapse compared to the high-drug exposure group. When the high-drug exposure group was compared to the low and other-drug exposure groups, this phenomenon remainded with a 2.4-fold increased risk of relapse for the low and other-drug exposure groups compared to the high-drug exposure group. The Kaplan-Meier survival curves reflected these differences in the EFS.

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Table 5.5 Analysis of *MTHFR* 677 C /T, 1298 A/C and *TYMS* tandem repeat polymorphisms and risk of leukemia relapse

Genotype	Relapsed Group n (%)	EFS Group n (%)	P*	HR (95% CI)	P-value
MTHFR 677 C/T			0.356		
CC	7 (43.8)	40 (37.4)			
CT	8 (50.0)	44 (41.1)			
TT	1 (6.2)	23 (21.5)		0.273 (0.034, 2.184)	0.221
MTHFR 1298 A/C			0.676		
AA	7 (43.8)	60 (55.5)			
AC	7 (43.8)	37 (34.3)			
CC	2 (12.5)	11 (10.2)		1.353 (0.307, 5.962)	0.689
TYMS 28bp repeats			0.348		
2R/2R	3 (31.1)	27 (25.2)			
2R/3R	9 (56.2)	48 (44.9)			
3R/3R	2 (12.5)	32 (29.9)		0.401 (0.091, 1.767)	0.227

HRs were calculated with *MTHFR* 677 TT *vs.* CT/CC, *MTHFR* 1298 CC *vs.* AC/AA, and *TS* 3R/3R *vs.* 2R/3R & 2R/2R respectively. *P**, P value was estimated by chi-square test. HR: Hazard Ratio; CI: Confidence Interval; EFS: Event-Free Survival

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Table 5.6 'Drug Exposure Group'' Features

Genotype Group Designation	Criteria	Hypothesized Effects	Hypothesized Outcome
High-drug exposure group	No variants in either *CYP3.A4* or *CYP3.A5*; *GSTs* both null	Rapid activation, slow detoxification	Best
Low-drug exposure group	Variants in either *CYP3.A4* or *CYP3.A5*; GSTs both present	Slow activation, rapid detoxification	Worst
Intermediate-drug exposure group	All other *CYP3.A4/3.A5/GST* combinations	Intermediate activation, intermediate detoxification	Intermediate

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Table 5.7 "Drug exposure group" and risk of leukemia relapse

Features	Relapsed Group n (%)	EFS Group n (%)	P*	HR (95% CI)	P-value
Low Drug Exposure	1 (6.25)	6 (5.61)	0.602	2.2 (0.3, 19.2)	0.47
High Drug Exposure	5 (31.25)	55 (51.40)			
Low + Intermediate Group	11 (68.75)	52 (48.60)	0.133	2.4 (0.8, 6.9)	0.11
High Drug Exposure	5 (31.25)	55 (51.40)			
GST either null	5 (31.25)	58 (54.21)	0.087		
GST both present	11 (68.75)	49 (45.79)		2.7 (0.9, 7.9)	0.063

P, *P* value was estimated by chi-square test. HR: Hazard Ratio; CI: Confidence Interval; EFS: Event-Free Survival

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Chapter 6

Discussion

Acute lymphoblastic leukemia (ALL) is the most common childhood malignacy. Tremendous progress has been made in the therapeutic outcome of ALL over the last four decades.The current cure rate routinely exceeding 80% in most collaborative groups (Pui, 2008 &2009). Standard-risk ALL has seen 6-year event free survival reach 82% with an overall survival rate of 92% (Malempati 2007, Matloub 2006). Despite these successes, the outcome of relapsed ALL remains poor and it is the most common cause of cancer-related death in children. The 5-year overall survival estimate after bone marrow relapse is approximately 24% (Gaynon 1998, Rivera 2005). Currently there is no consensus on the optimal therapy for relapsed ALL patients who attain a second disease remission. As a result the management of relapsed ALL patients remains a major challenge for pediatric oncologists.

In an attempt to discover potential markers to identify patients with ALL who may be at risk of leukemia relapse, a small-scale retrospective study was conducted on 125 patients with standard-risk acute lymphoblastic leukemia who had been treated at

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The Children's Hospital in Denver. The rationale for the current study cohort was: 1) standard-risk ALL is the largest cohort of childhood acute lymphoblastic leukemia; 2) the cohort comprised a single class of patients who had been uniformly treated in a single treatment center. This approach made it possible to determine whether interindividual variations in treatment response can be explained by genetic polymorphisms that influence drug disposition. In this study cohort, there were 58% males and 42% females. Among male participants, 56% and 58% of males were in the relapsed and EFS groups, respectively. Gender did not present any significant difference with regard to the risk of leukemia relapse ($P = 0.91$). This finding is in agreement with all previous studies.

6.1. Phase I Drug Metabolism Enzymes

As described in the previous chapters, cytochrome P450 enzymes play a critical role in the metabolism of several chemotheraputic agents including cyclosphamide, doxorubicin and glucocorticoids that are commonly used in both intial induction and maintenance phase of pediatric ALL therapy. It had been suggested that polymorphisms in the genes that encode these enzymes might contribute to treatment outcome (Relling 2001, Fleury 2004, Aplenc 2003). In the current study, no statistically significant association was found between an altered risk of leukemia relapse and polymorphsims in the *CYP1A1* (rs1048943), *CYP2B6* (rs3211371), *CYP3A4* (rs2740574) or *CYP3A5* (rs10264272) genes. The one exception was *CYP3A5* (rs776746) which was found to be associated with an increased risk of relapse in patients with the variant genotype (HR =

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5.70, 95% CI = 0.743 – 43.694, *P* = 0.09). No patients in the EFS group carried the variant alleles and only one patient in the relapsed group carried heterzygous allele (*P* = 0.01). Although this allele frequency difference reached statistical significance, it should be noted that due to the small population of the relapsed group, this result was based on a single patient who carried the variant genotype. This finding should not be considered significant. The Kaplan-Meier survival curves shown in **Figures 6.1, 6.2, 6.3, 6.4, and 6.5** illustrate these findings.

Aplenc *et al*. (2003) reported a study on the CCG-1891 standard-risk ALL trial. Genotypes were determined for 533 of the 1203 patients enrolled in the trial which included 121 relapsed patients and 412 control patients who were in remission status. In that study, no association was found between a risk of leukemia relapse and CYP3A4 (rs2740574) and CYP3A5 variants (rs10264272; rs776746) (OR = 1.19, 1.02, and 0.79 respectively). Similarly, no association between gene-gene interactions and relapse risk was observed either. The study did, however, detect an association between the *CYP3A4*/*CYP3A5* variants and decreased toxicity. Fleury *et al*. (2004) subsequently reported their finding of no association between *CYP3A4* variants and ALL treatment outcome. The study included 222 patients treated on DFCI protocols 87-01, 91-01, or 95-01. There were 33 patients who experienced a relapse and 20 who died. Rocha *et al.* (2005) reported a St. Jude study with 247 patients with newly diagnosed ALL who were treated on the using the Total XIIB protocol. There were 47 adverse events including

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relapse, second malignancies, and deaths in CR. No association was observed, however, between the risk of relapse and *CYP3A4* or *CYP3A5* genotypes.

The only positive association between CYP variants and risk of leukemia relapse was found in a Canadian study. Krajinovic *et al.* (2002a) conducted a study on 320 pediatric ALL patients treated on DFCI protocols 87-01, 91-01, and 95-01. Sixty-four patients experienced an event which was defined as disease relapse or a fatal outcome during or after the first CR. They studied risk of relapse and functional polymorphisms in genes encoding drug metabolizing enzymes including CYP1A1, CYP2D6, CYP2E1. In this study, the presence of *CYP1A1*2A* variants was associated with an increased risk of relapse (HR = 2.3, 95% CI = 1.1-4.9, *P* = 0.03). Although they noted that CYP1A1 is not thought to metabolize any of the chemotherapeutic agents used with the DFCI protocols.

In the current study, CYP2B6 variant allele frequency difference was not observed between the EFS group and relapsed group (19.5% vs. 18.7% respectively, *P* = 0.923). There was no effect of CYP2B6 variant on the risk of relapse (HR = 0.80, 95% CI = 0.23-2.80, *P* = 0.721). To the best of the current author's knowledge, there are no reports regarding *CYP2B6* polymorphisms and the risk of leukemia relapse in pediatric ALL. Cyclophosphamide is given as a prodrug which must undergo activiation during phase I metabolism by cytochrome P450 enzymes including CYP2B6. The active metabolite, 4-hydroxy-cyclophosphamide, is responsible for cyclophosphamide's alkylating effect (Huitema 2000). CYP2B6 should play a major role in this process because it has a higher affinity for the substrate than CYP2C9, CYP2C19, CYP3A4, or

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CYP3A5 (Patterson 2002, Wall 2003). Ekhart et al. (2008) conducted a study to evaluate effects of genetic polymorphisms on the pharmacokinetics of cyclophosphamide in 124 cancer patients treated with a high-dose chemotherapy (4-6 g/m^2 of cyclophosphamide). They found no effect of polymorhphisms in the *CYP2B6*, *CYP2C9*, *CYP3A4*, *CYP3A5 GSTA1*, *GATP1*, *ALDH1A1*, and *ALDH3A1* genes on the clearance of cyclophosphamide. These results may indicate the *CYP2B6* variants alone are not crucial factors affecting the pharpharcokinetics of cyclophosphamide. The findings from the current study certainly agree with Ekhart's results.

From the CCG-1891 study, Aplenc *et al.* (2003) observed that CYP3A4 and CYP3A5 variants were associated with decreased chemotherapeutic toxicity; specifically peripheral neurotoxicity. All patients were followed for more than 5 years after completion of therapy. Toxicity data were collected prospectively during the study. As there were no toxicity data available from the study cohort employed for the current research, an association analysis could not be performed.

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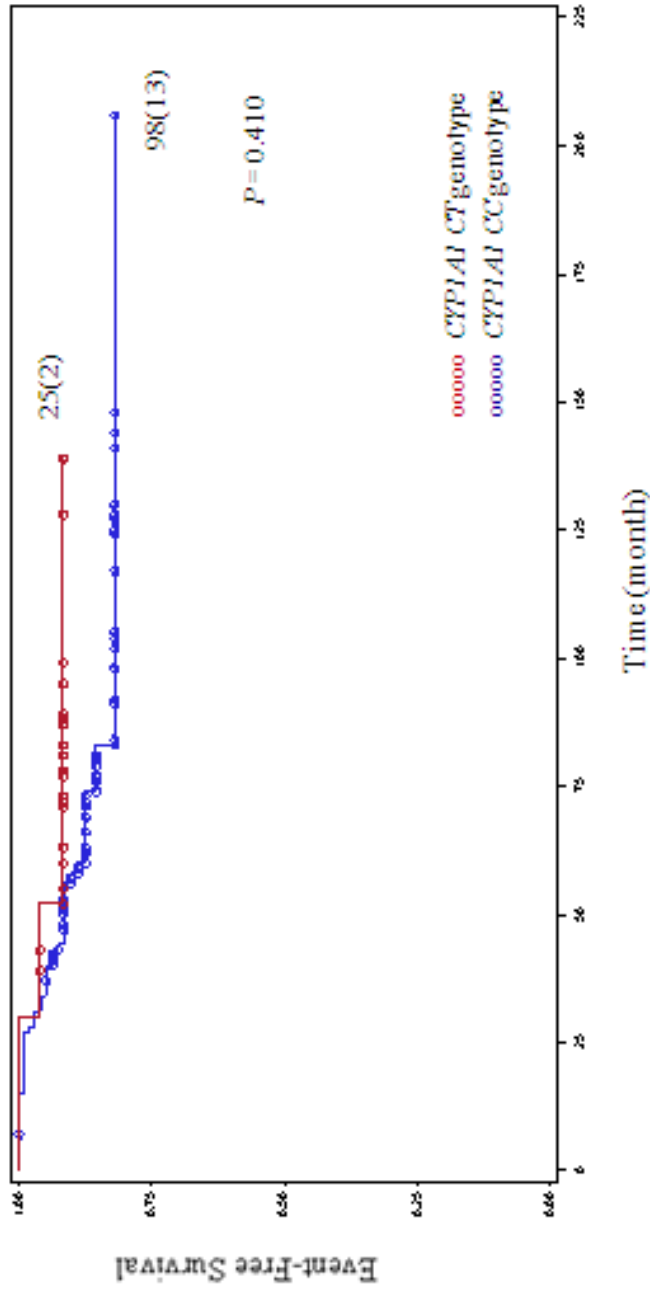
Fig. 6.1 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *CYP1A1* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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Fig. 6.2 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *CYP2B6* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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Fig. 6.3 Kaplan–Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *CYP3A4* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated

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Fig. 6.4 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *CYP3A5* (rs10264272) genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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Fig. 6.5 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *CYP3A5* (*rs776746*) genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

Summary: It should be noted that all of the above studies have limitations. These studies included patients from different treatment protocols who, therefore, received different types of corticosteroids and dosages (even within DFCI protocols). The small samples sizes (drawn from patient subsets) diminish the statistical power of the analyses that were performed. Another issue of concern is how representative the genotypes are since the DNA was extracted from the leukemic blasts rather than from from patient germline cells. Caution, therefore, should be taken with respect to drawing broad conclusions pertaining to phase I drug metabolizing enzyme polymorphisms and treatment responses. The findings from the current study were in agreement with some previous studies but not others.

6.2. Phase II drug metabolism enzymes

Phase II drug metabolism involves conjugation of molecules to the products of phase I reactions to facilitate excretion. Glutathione S-transferases (GSTs) function as dimers by catalyzing the conjugation of mutagenic electrophilic substrates to glutathione. Some of the most studied GST polymorphisms including *GSTP1*B* (rs1695) which alters substrate affinity, *GSTP1*C* (rs1138272) which alters the catalytic activity and the *GSTM1* and *GSTT1* null genotypes - both of which lead to loss of enzyme activity. GST enzymes metabolize numerous chemotherapeutic agents such as glucocorticosteroids, vincristine, methotrexate, and cyclophosphamide. Consequently, polymorphisms in any

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of these *GST* genes have significant potential to influence the treatment response of patients with pediatric ALL.

In the current study, *GSTP1*C* was observed in 94% of relapsed patients and 83% of EFS patients. This finding represents an apparent 3-fold difference in risk of leukemia relapse although it did not reach statistical significance (HR = 0.341, 95% CI = 0.05-2.58, *P* = 0.298). *GSTP1*B* did not show a significant difference between the relapse group and the EFS group as can be seen in **Figures 6.7 and 6.8**. A protective effect was observed, however, with the *GSTM1* and *GSTT1* null alleles (HR = 0.394 and 0.662, respectively). Seventy-five percent of relapsed patients had the GSTM1 gene present *vs.* 56% of EFS group. Thus, these genotypes appeared to be associated with a more than 2-fold decreased in the risk of relapse. This association, however, did not reach statistical significance in the current study as can be seen in **Figures 6.9 and 6.10**. The *GSTM1* and *GSTT1* genotypes were also grouped for the purposes of statistical analysis. In the current study, the patients having both the *GSTM1* and *GSTT1* non-null genotype had a 2.7-fold (HR = 2.7, 95% CI=0.9-7.9, *P* = 0.063) increased risk of relapse relative to those patients who carried either a *GSTM1* null or *GSTT1* null genotype. **This finding (Figure 6.6) was the strongest association in the current study.** These findings are supported by the BFM case-control study with 128 patients treated on ALL-BFM trails 86 and 90 (Stanulla, 2000). In that study, the *GSTM1* and *GSTT1* null genotypes conferred a 2-fold and 2.8-fold difference in risk of relapse when compared to the presence of *GSTM1* or *GSTT1*. A significant reduction in CNS relapse risk in patients who carried the *GSTP1*B*

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genotype was also found although the effect was only observed in patients with intermediate or high risk of treatment failure. Furthermore, analyses that examined the risk of relapse with the normal genotype (non protective) *vs.* any one, two or three protective genotypes was performed. These analyses demonstrated an effect with the addition of each protective genotype (OR = 0.53, 95% CI = 0.24-1.19, *P* = 0.123 and OR = 0.29, 95% CI = 0.06-1.37, *P* = 0.118 respectively) compared to the reference group with no low-risk genotype (GSTM1 null, GSTT1 null, and GSTP1 Val_{105}/Val_{105}).

Rocha (2005) reported on a St. Jude Total Therapy XIIIB trial with 246 patients. After correcting for other risk factors such as WBC, certain translocations, day 19 marrow, treatment arms and risk group, it was demonstrated that the *GSTM1* non-null genotype was associated with a significantly increased risk of hematologic relapse (HR = 18.1, 95% CI=3.9-84.3, *P* = 0.002). Other studies, however, did not find a statistically significant association. Chen *et al.* (1997) reported on a St. Jude study of Total Therapy XI, XII, and XIIIA trials with 161 of 525 patients enrolled and found no significant inpact impact of *GSTM1* and *GSTT1* null genotypes on risk of leukemia relapse. Interestingly, in a subset analysis an apparent association was noted between the *GSTM1* null genotype and a decreased risk of CNS relapse although this did not reach statistical significance (HR = 0.90, 95% CI = 0.83 – 0.97, *P* = 0.054).

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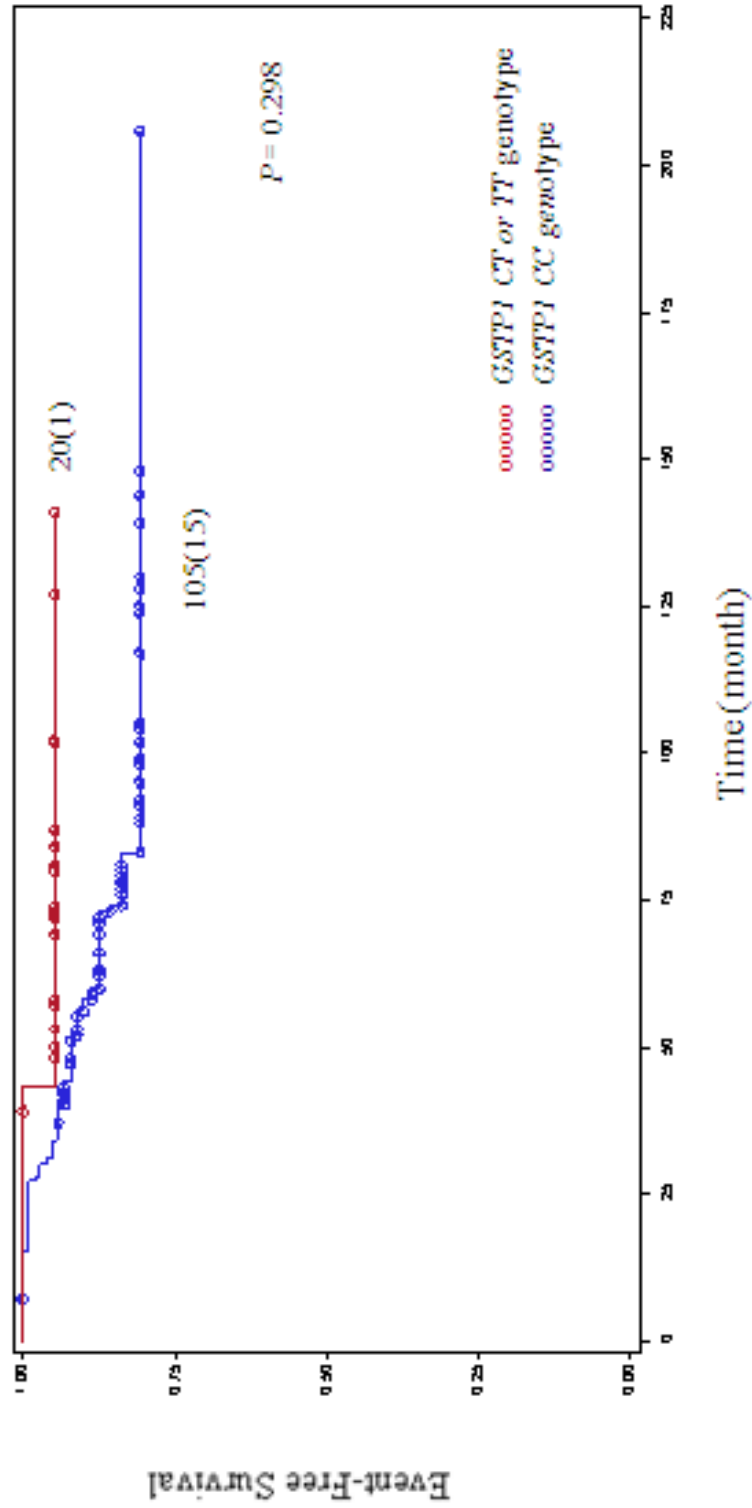
Fig. 6.7 Kaplan–Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *GSTP1* (rs1138272) genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.
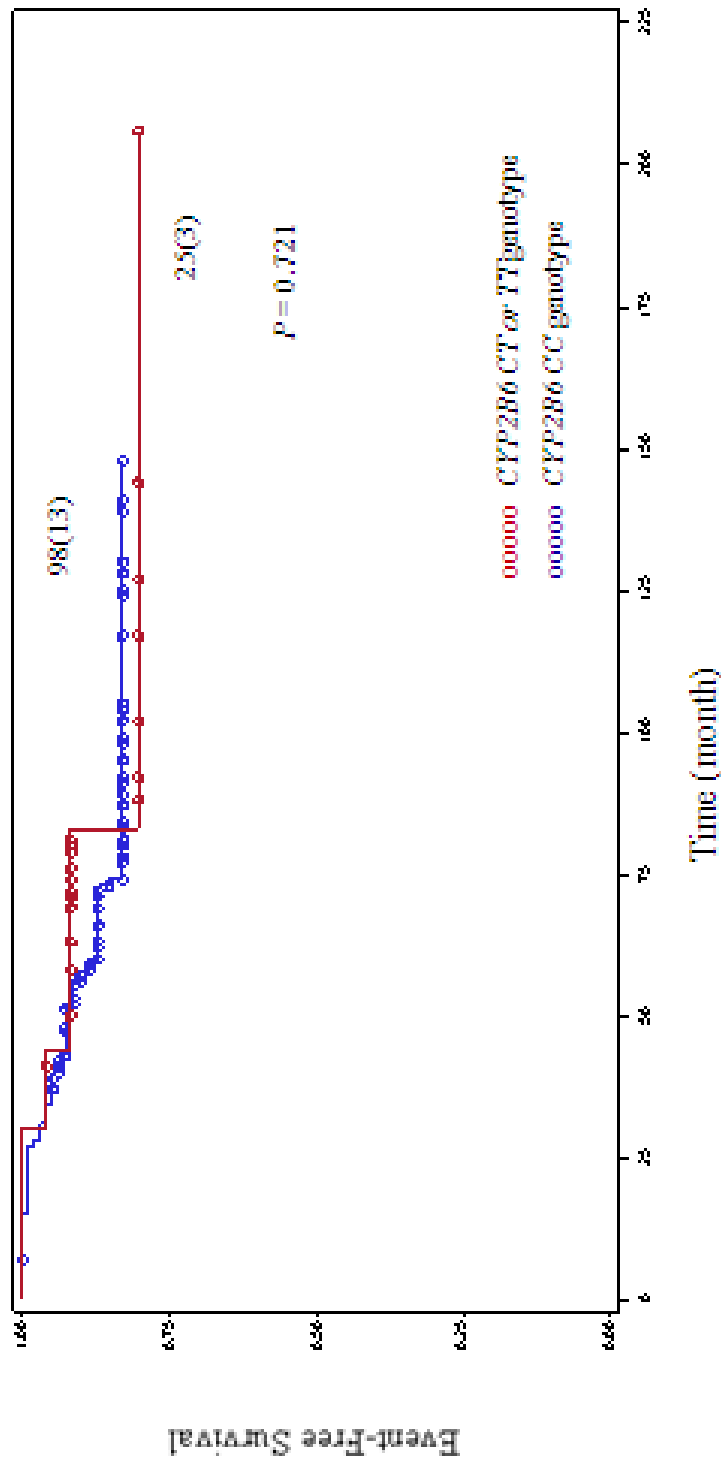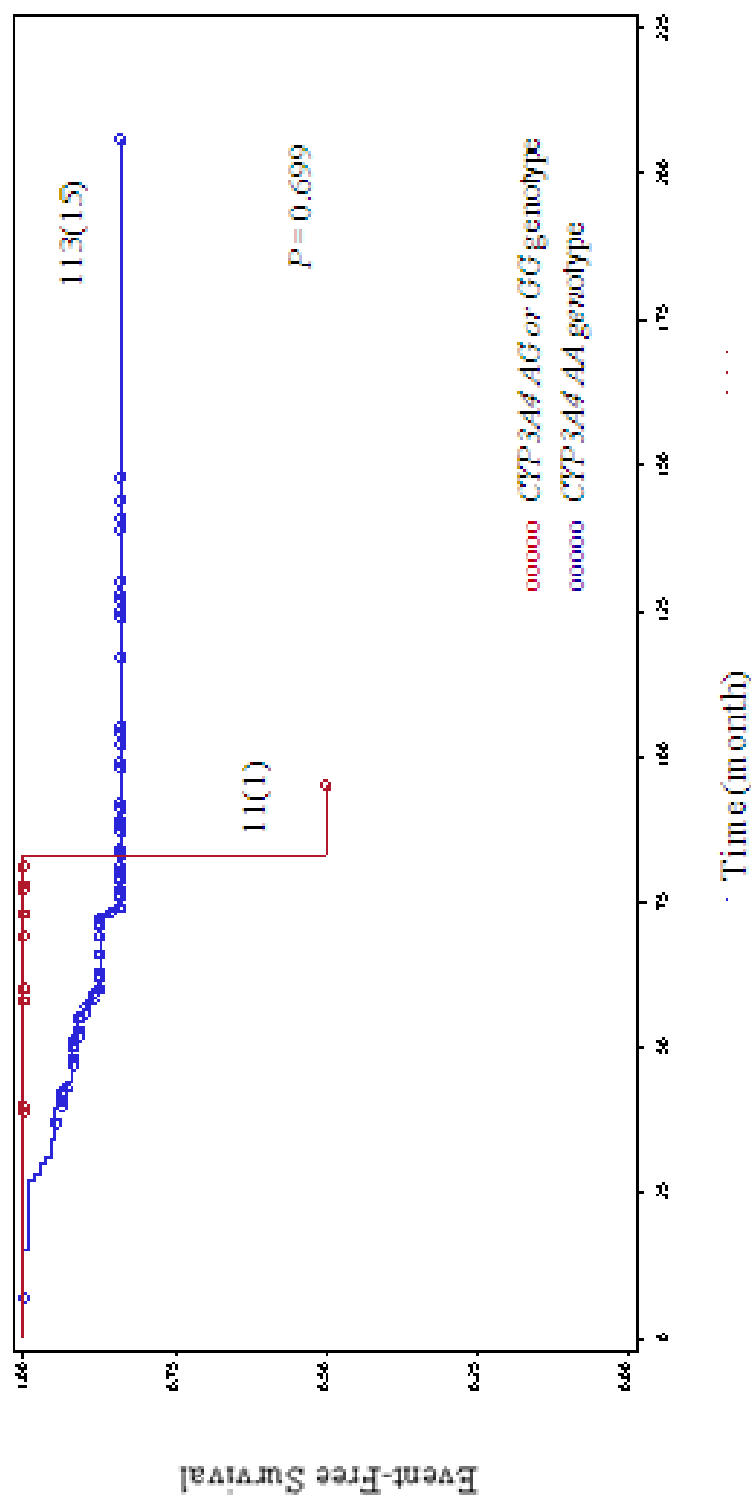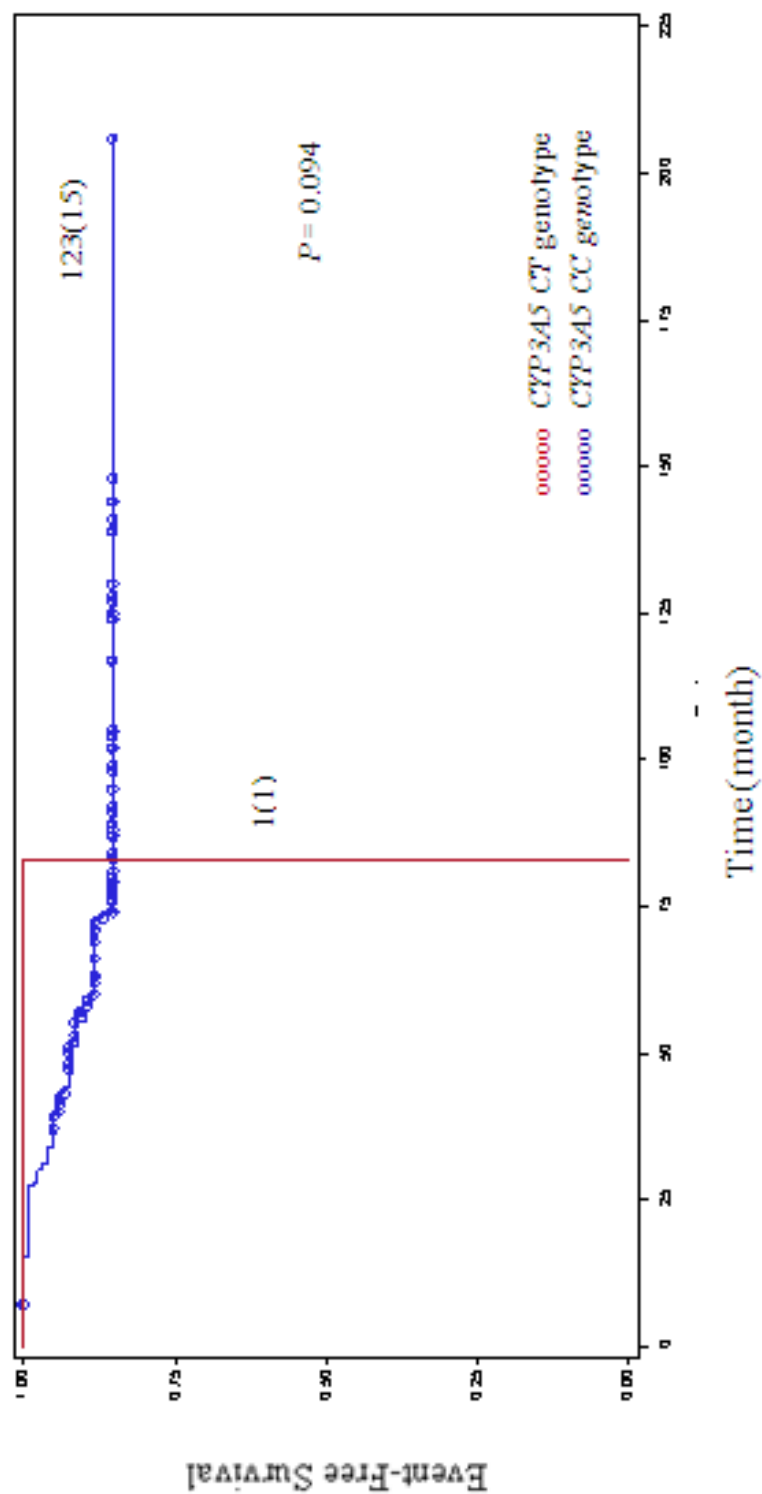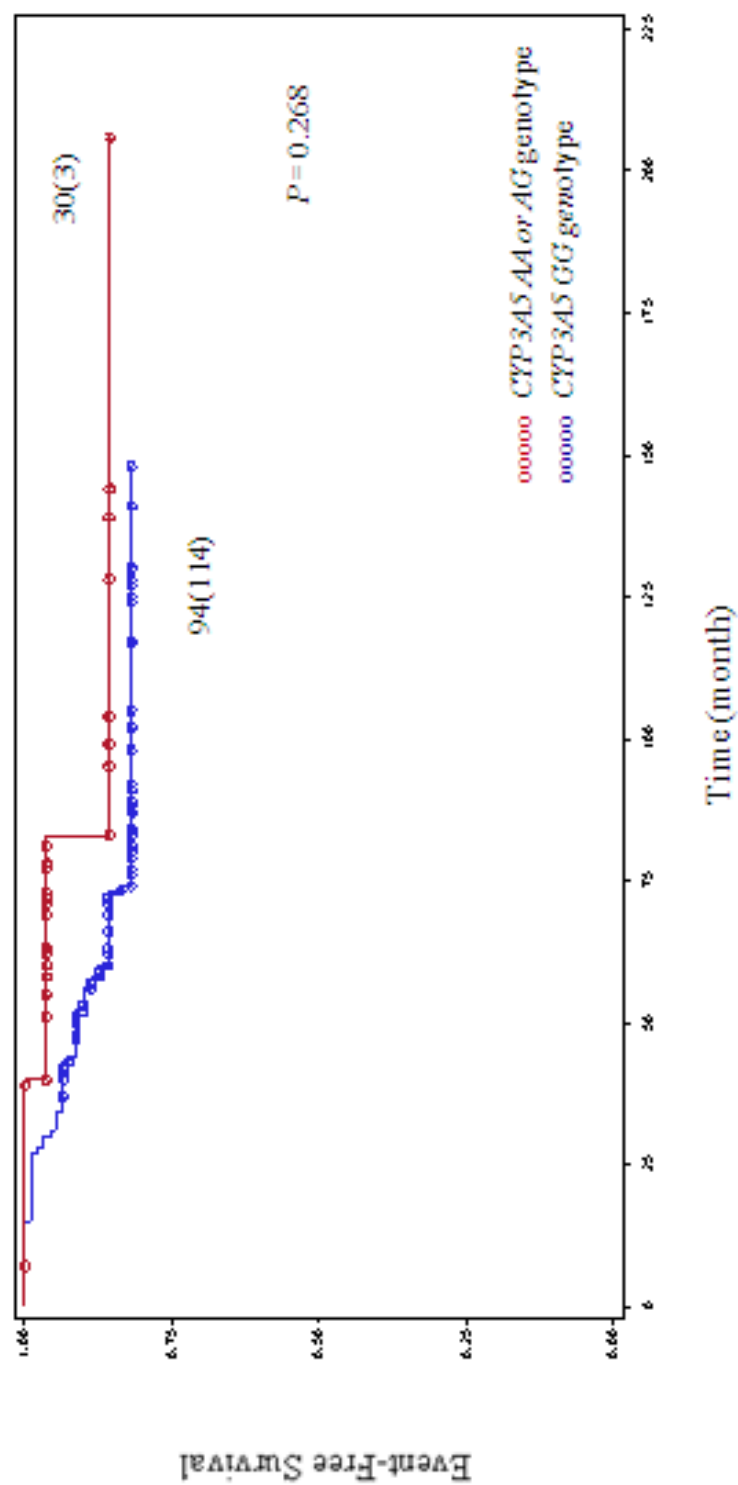
110
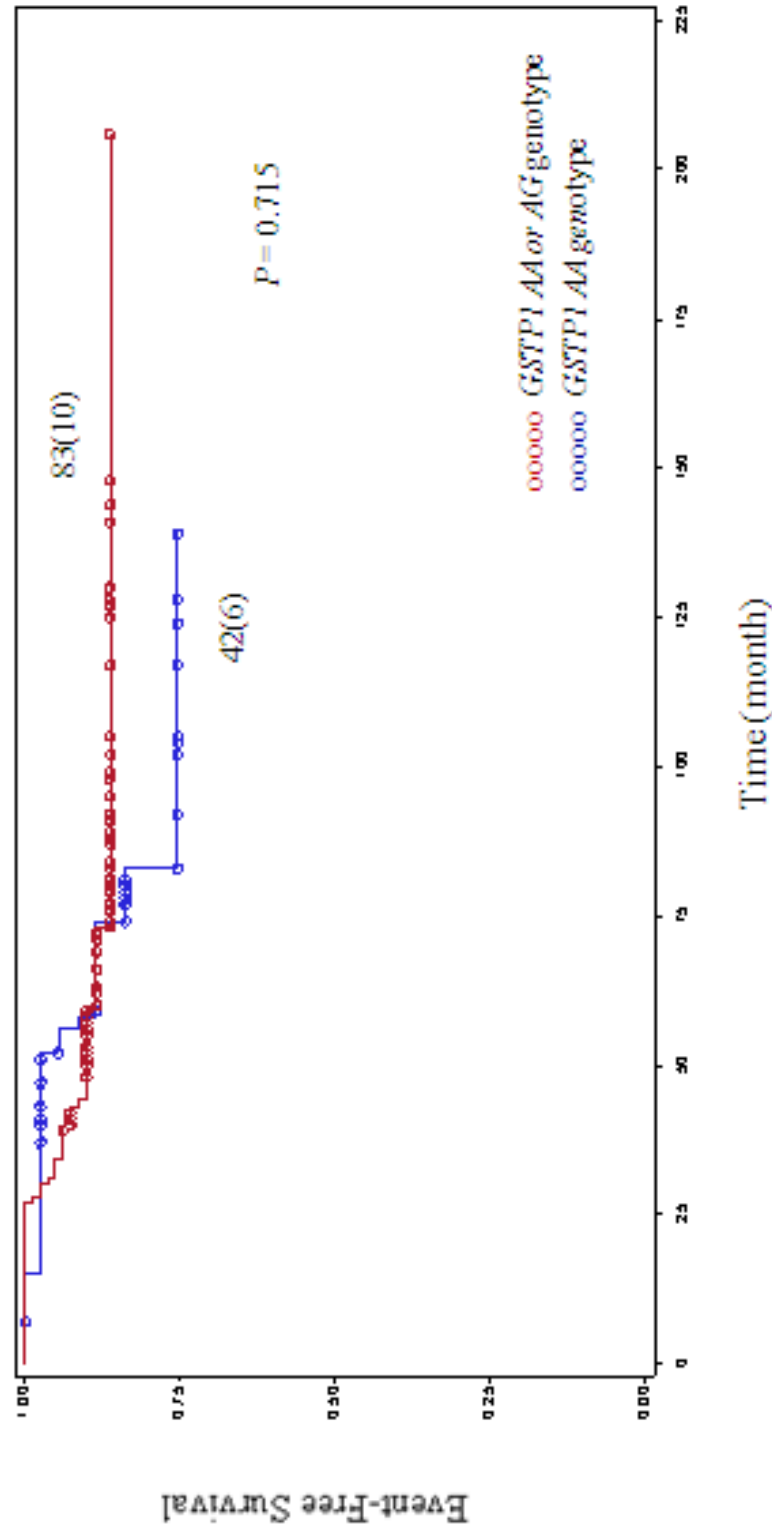
Fig. 6.8 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *GSTP1* (rs1695) genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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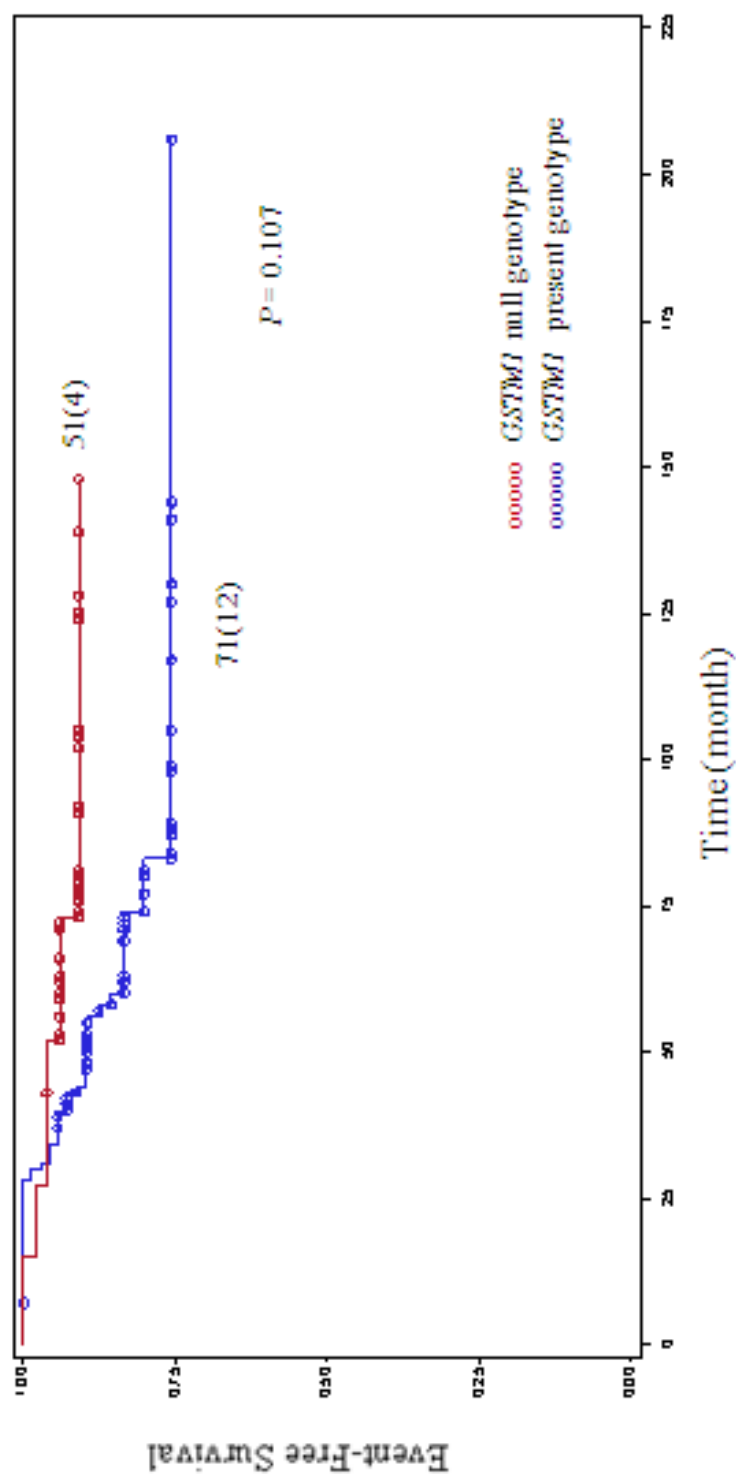
Fig. 6.9 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *GSTM1* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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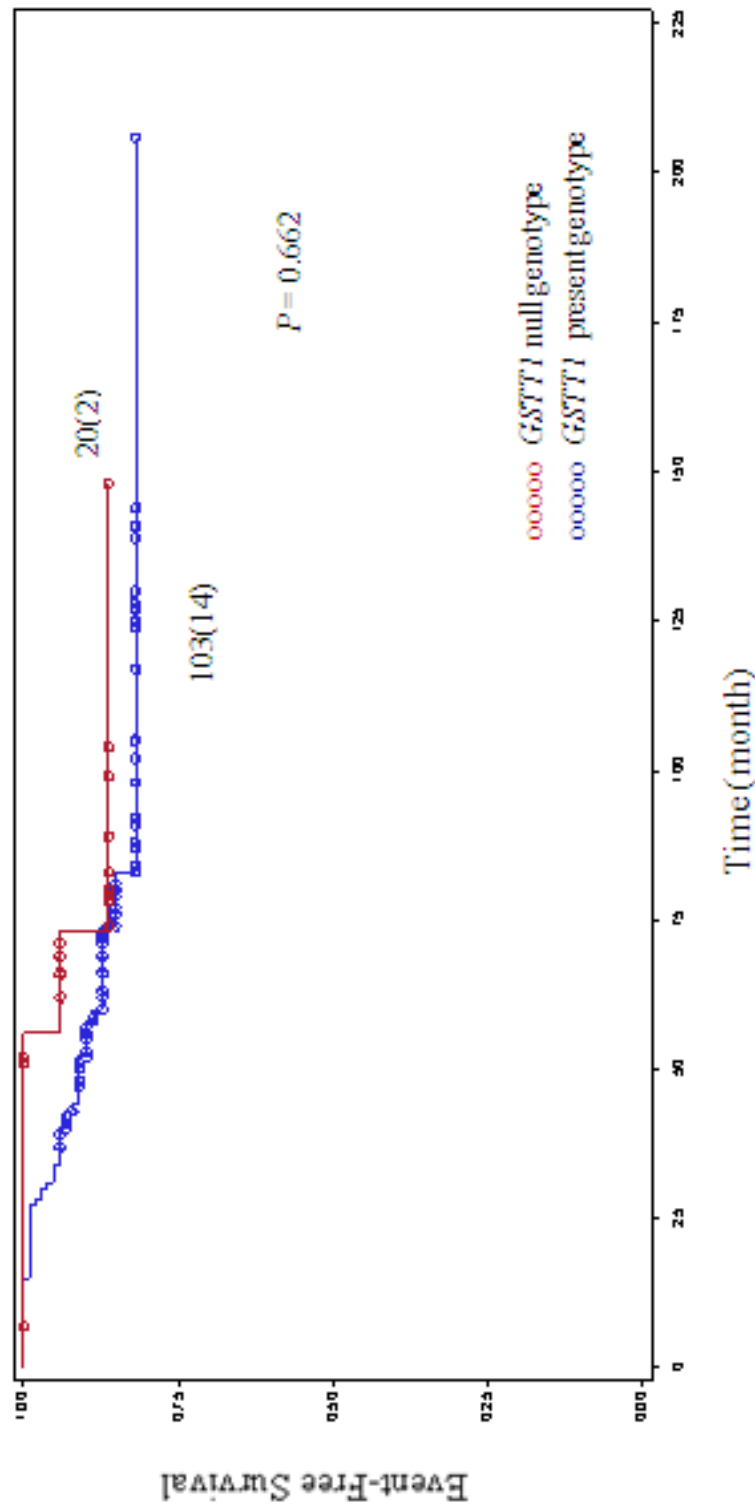
Fig. 6.10 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *GSTT1* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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Thus far, the largest studies indicated no association between GST genotypes and risk of disease relapse. Davies (2002) studied 710 patients including 107 who had experienced bone marrow relapse and 25 who had experienced CNS relapse. The patients were from the CCG protocols 1881, 1882, 1883 and 1901 representing a total of 4087 enrolled patients. In the analysis, an association between *GSTM1* and *GSTT1* null genotype and the risk of disease relapse was not observed. A Kaplan-Meier survival curve also demonstrated the lack of a difference in the overall survival with either genotype. One caveat, that should be noted, however, is that despite the large sample size, the genotyped patients had a lower relapse rate than the aggregate of all enrolled patients. Krajinovic (2002a), based on a similiar study to that described in the prior section on patients treated with DFCI protocols, found no association between the *GSTM1*, *GSTT1* or *GSTP1* genotypes and the risk of leukemia relapse. Again, however, it is important to emphasize that the patient population consisted of different risk groups.

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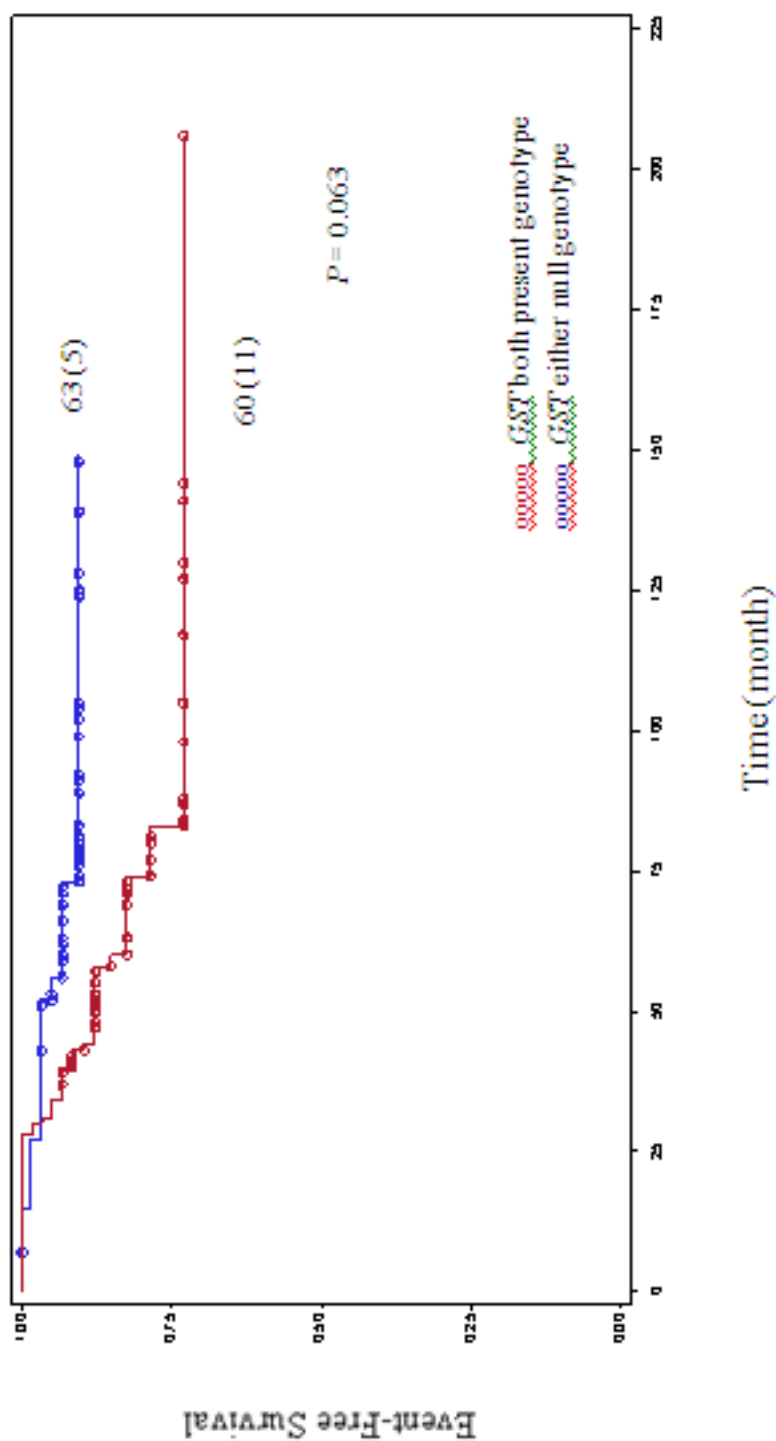
Fig. 6.6 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *GSTM1* and *GSTT1* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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Summary: The current study demonstrated an association between *GSTM1* deletion and a decreased risk of disease relapse (HR = 0.39, 95% CI = 0.13-1.22, *P* = 0.107) in the pediatric standard-risk ALL patients treated at The Children's Hospital in Denver, CO. When the *GSTM1* and *GSTT1* genotypes were combined in the analysis, there was an even stronger apparent association with the risk of relapse (HR = 2.73, 95% CI = 0.90-7.90, *P* = 0.063). Although neither of these reached a statistical significance (*P* = 0.05) but the clinical effect was evident with 75% of patients in the relapse group having the *GSTM1* non-null genotype *vs.* 56% of patients in the EFS group. The Kaplan-Meier survival analysis shown in **Figure 6.6** also illustrates these effects. Sixty-nine percent of relapsed patients have both *GSTM1* and *GSTT1* non-null genotypes *vs.* 46% in the EFS group. With the excellent EFS rate in the standard-risk ALL, the significance of this finding should not be underestimated. Of course, the aforementioned studies all have some limitations. Most were drawn from different risk groups of patients and as a result the treatment regimens differed greatly. **This illustrates the importance of larger prospective clinical trials with well defined inclusion criteria.**

6.3. Drug Target Enzymes

Methotrexate (MTX) is a common therapeutic agent for both malignant and nonmalignant diseases. It plays a central role in childhood ALL across all treatment protocols. MTX interferes with the natural folate cycle by DHFR, TYMS, and MTHFR and thereby leads to a reduced folate pool, inhibition of nucleic acid synthesis, and

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eventually cell death. Polyglutamated MTX also directly inhibits the enzymes synthesizing thymidylate and purine. Polymorphisms in the genes encoding those enzymes, therfore have the potential to influence the treatment outcome in ALL patients. The *MTHFR* gene has multiple polymorphic sites with two common non-synonymous variants, 677C>T and 1298A>C which are associated with reduced enzyme activity. The *MTHFR* 677 CT heterozygous genotype is associated with a 40% decrease in enzymatic activity while the TT homozygous genotype is associated with a 70% reduction in enzymatic activity (Schwahn 2001). The *TYMS* gene has a 28-bp insertion in the promoter region. Triple repeats (3R) of this insertion have been associated with a higher level of *TYMS* expression. Since TYMS is another important target for MTX genetic polymorphisms in the *TYMS*, may influence the outcome of ALL treatment.

In the current research, a slightly higher percentage of MTHFR 677 variant genotypes were observed in the study cohort, *i.e.*, 42% CT heterozygous genotype *vs.* 20% homozygous TT genotype. It is unclear why the study population employed for the current research had higher than anticipated frequencies of variant alleles. However the genotype frequencies were found to be in Hardy-Weinberg equilibrium. Some researchers have expressed concerns that preferential allele amplification might result in an analytical genotyping error (Miller 2002). Genotyping results from the current study were independently confirmed by two individuals. Furthermore, the genotyping assays were validated throught the use of mutiple assay proficiency panels from the College of American Pathologists (CAP) and clinical samples (**Appendix. 2**). The results from the

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current study seem to suggest a protective effect in patients with the *MTHFR* 677 TT genotype for the risk of disease relapse. This apparent effect, however, did not reach the statistical significance ($P = 0.221$). As illustrated in **Figure 6.11,** the Kaplan-Meier survival curve clearly shows that there is no difference between risk of relapse with a *MTHFR* 677 TT genotype compared to either the CT or CC genotypes. Several reports have, however, demonstrated a protective effect of *MTHFR 667* and *MTHFR 1298* polymorphisms on leukemia susceptibility risk (Franco 2001). Taub (2002) reported that leukemic cells with the *MTHFR* 677 variant alleles had an increased sensitivity (*in vitro*) to MTX. This finding suggested that patients who carried the variant genotypes should have a decreased risk of leukemia relapse. Most of clinical trials have appeared to indicate either an increased risk of relapse or decreased EFS.

With regard to *TYMS* polymorphisms in the current study, a higher frequency of the 3R/3R genotype was found in the EFS group. Thirty-percent of patients in the EFS group were observed to have a 3R/3R genotype while only 13% of those in the relapsed group showed this genotype. The 3R/3R genotype, therfore appeared to be associated with a decreased risk of relapse in the current study cohort (HR = 0.40, 95% CI = 0.09-1.77, $P = 0.227$), although this did not reach statistical significance. Most of recent studies have indicated that patients with a 3R/3R genotype were at higher risk of disease

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Fig. 6.11 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *MTHFR 677* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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relapse. Krajinovic *et al.* (2002b) evaluated 205 patients treated with DFCI protocols as described in the MTHFR section. Patients in that study with the 3R/3R genotype were found to have a higher risk of adverse event (OR = 4.8, 95% CI=1.1-20.1, *P* = 0.04). This effect remained when the control group contained only patients with a CR > 5 years. The survival analysis demonstrated similar statistical significance.

Lauten (2003) reported their findings with patients treated on BFM protocols 86 or 90. The increased risk of relapse that had been reported by others for the 3R/3R genotype was not evident in Lauten's case-control study of 80 pediatric ALL patients who received the same amount of MTX (4 g/m^2). Likewise, the St. Jude Total Therapy XIIIB study did not observe an impact related to the 3R/3R genotype (Rocha 2005). Interestingly, the same group of investigators (Relling 2004) reported that patients with the 2R/2R genotype had a significantly increased risk for a specific adverse event; that being osteonecrosis of the hip (OR = 7.2, 95% CI=1.1-48.9, *P* = 0.044).

During the current study, a gene expression profiling and MTX response study of ALL was published by St. Jude investigaters (Sorich 2008). In that study, genome-wide expression profiling was conducted to identify genes whose expression appeared to be linked to *in vivo* MTX response based on a drop in the WBC on day 3 following high-dose MTX admnistration. A total of 293 patients with newly diagnosed ALL, who were enrolled in the Total Therapy XIII and XV protocols, were included in the study. The study found 48 genes and 2 cDNA clones that were stronly linked to the *in vivo* MTX response. Among those were genes involved in nucleotide metabolism (*TYMS* and

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CTPS). The study showed that low expression of TYMS was significantly associated with poor *in vivo* MTX response. Univariate hazard analysis of the risk of relapse produced HR of 0.60 ($P = 0.008$). Interestingly, other known genes involved in the folate metabolism pathway were not among the top 50 putatively associated genes. This finding suggested that *de novo* MTX resistance may be acquired during treatment rather than predisposed as was previously thought. Unfortunately RNA is not available for a similar analysis of the patients enrolled in the current study.

Summary: The current study did not find that either *MTHFR* or *TYMS* variants were associated with an increased risk of leukemia relapse (**Figure 6.12 and 6.13**). On the contrary, the results suggested that they might have a slight protective effect against the risk of relapse. These discordant findings may be due to difference in study design, population size, and/or therapeutic regime (*e.g.*, schedule of treatment, dosage, and coadministration of other therapeutic drugs). As a consequence of the heterogeneity of the available studies, comparisons among these studies are very difficult if not impossible. None of the studies mentioned the quality of the genomic DNA that was used in the analysis. As mentioned above, this illustrated again the importance of larger, prospective trials that consistently and carefully assessed study end points. Moreover, these trials need to collect comprehensive data on variables such as chemotherapy dosage, treatment compliance data, malignacy phenotype and genotype, etc..
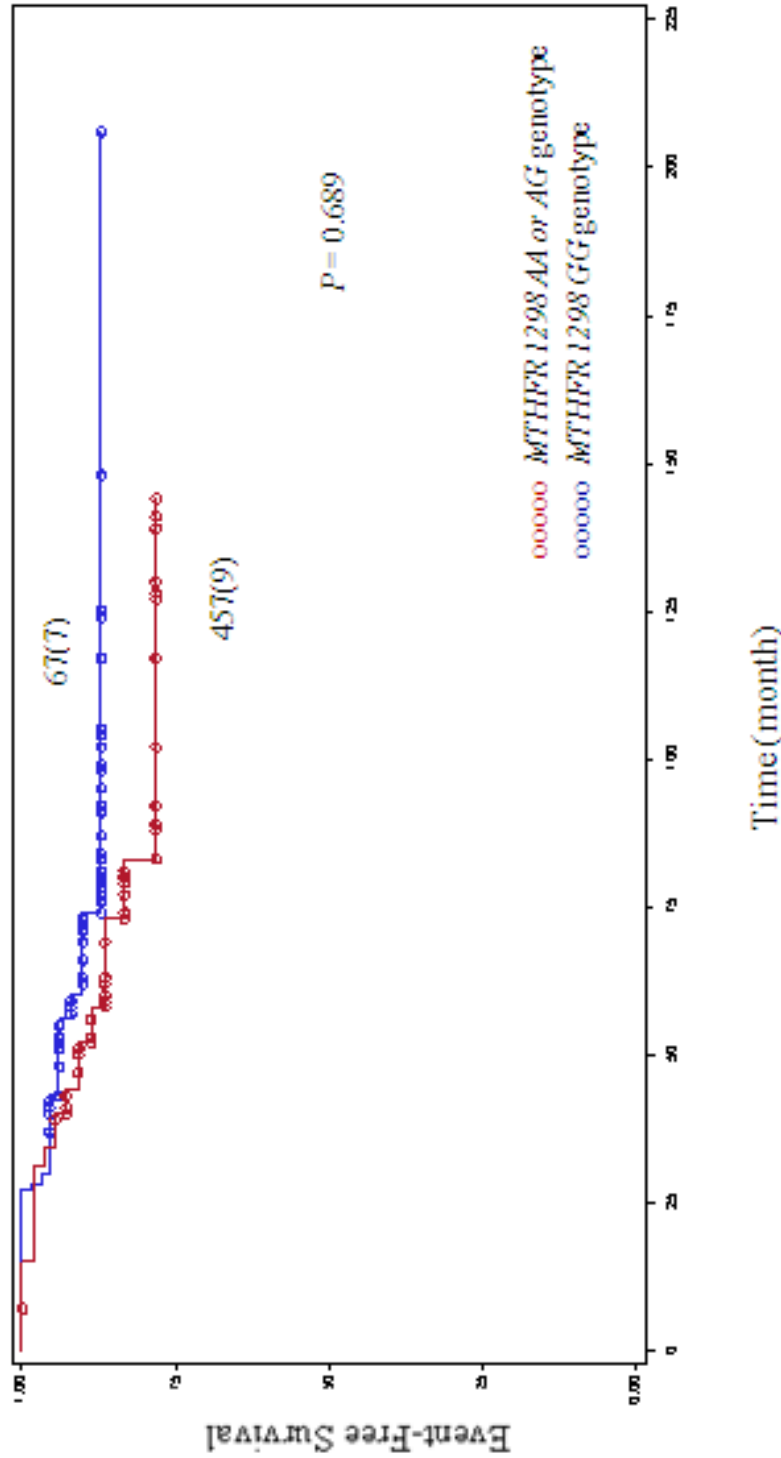
Fig. 6.12 Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *MTHFR 1298* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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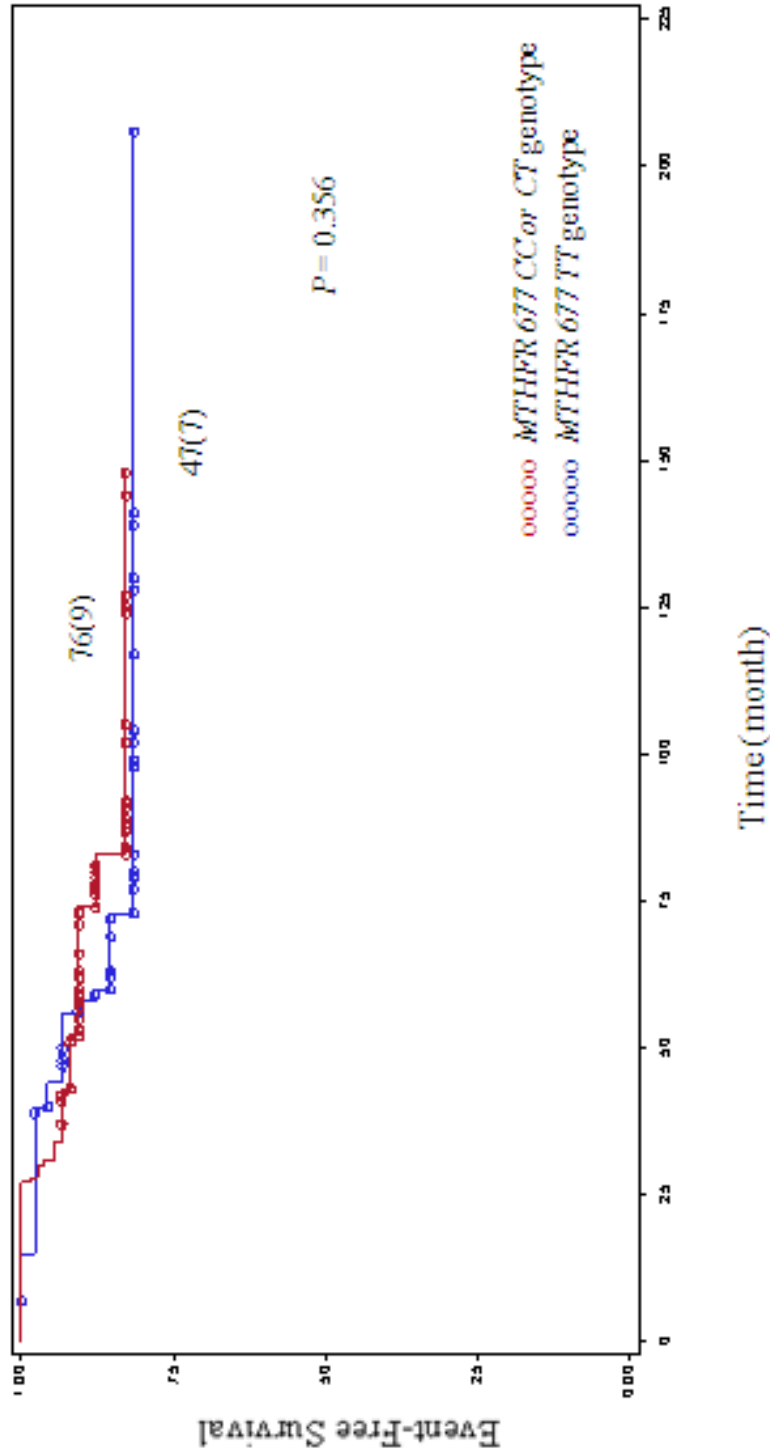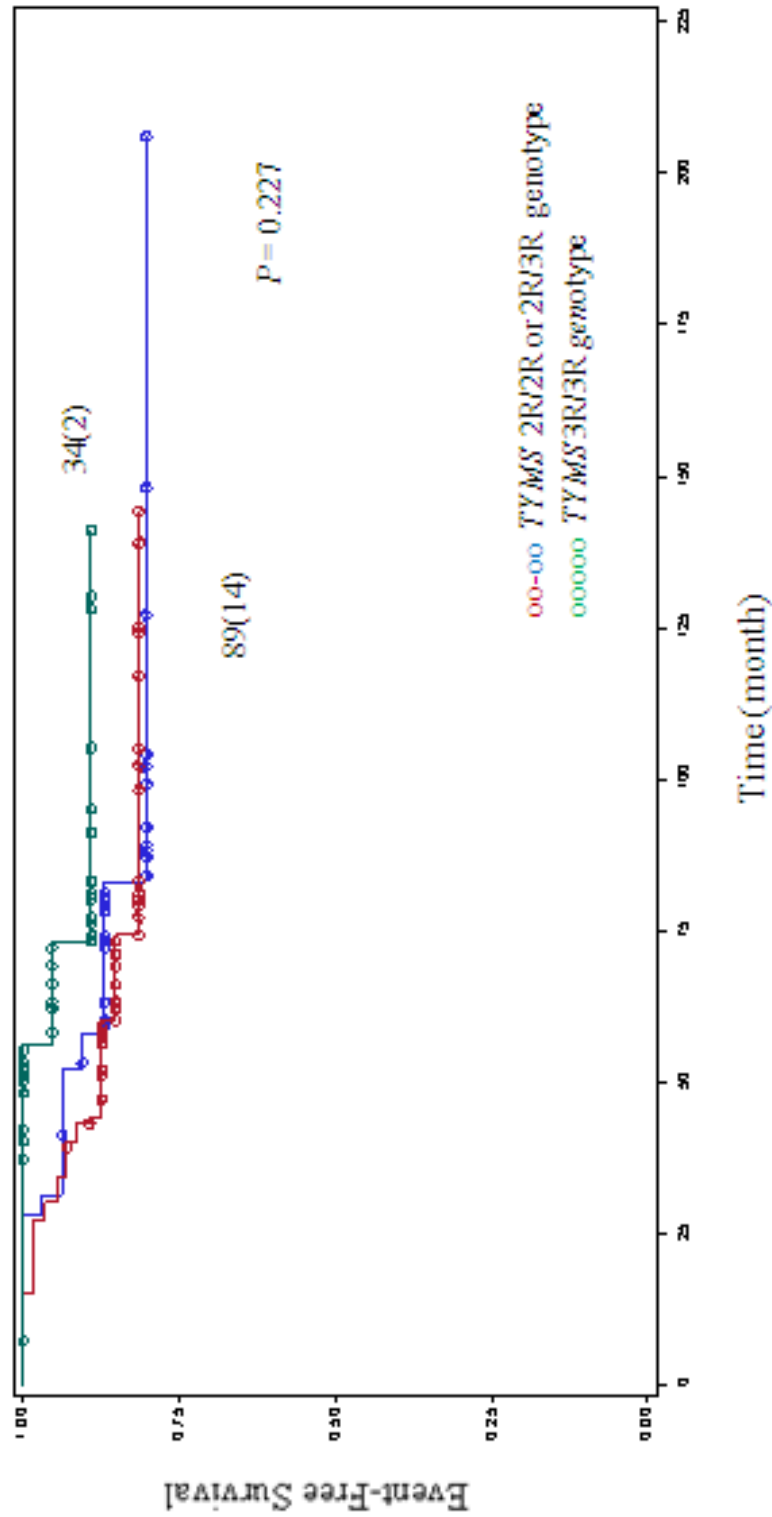**Fig. 6.13** Kaplan-Meier estimates of survival probabilities for patients with acute lymphoblastic leukemia (ALL), according to the *TYMS* genotype. The numbers of patients in each curve, numbers of patients with an event (numbers in parentheses), as well as the *P* value (estimated by log-rank test for the survival difference between the patient groups) are indicated.

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6.4. Conclusion and Future Perspectives

The current study provides important new information on the potential role of drug metabolizing enzyme polymorphisms and the risk of leukemia relapse in pediatric patients treated with standard-risk acute lymphoblastic leukemia at The Children's Hospital in Denver, CO. This study found an association between the *GSTM1* null genotype and a decreased risk of relapse (HR = 0.394, 95% CI=0.124-1.224, *P* = 0.144). An even greater effect was observed in terms of an increased risk of disease relapse for the combination of both *GSTM1* and *GSTT1* genes being non-null versus the presence of either the *GSTM1* or *GSTT1* null genotype (HR = 2.73, 95% CI=0.0-7.9, *P* = 0.063). Both conditions approached (but did not reach) statistical significance, however, the clinical impact could be more important as this appears to be the first study to demonstrate the effect of *GSTM1* and *GSTT1* null genotype with risk of leukemia relapse with standard-risk pediatric ALL. It is important to recognize that these findings should only be applied in the conext of this specific treatment protocol but not other protocols.

None of the other genetic polymorphisms in the current study showed a significant association with the risk of disease relapse after successful induction therapy. Moreover, the current study did not show a significant association between either the *MTHFR* 677 variants or the *TYMS* 3R/3R genotype and risk of relapse as described in previously reported CCG studies. This discrepancy might be explained by: 1) the limited scale of the current study which may not have a sufficiently large patient population, (especially in the relapsed group), to detect a modest host genetic effect on the risk of

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relapse; 2) the presence of treatment heterogeneity may obscure modest genetic effects, and/or 3) the complexity of the treatment protocol which not only involves multiple interacting chemotherapeutic drugs but also over-the-counter medications and herbal supplements. Patient compliance is also an important factor. Unfortunately, the ability to capture and quantify these variables in most association studies is extremely limited.

Clearly, the relatively small scale of the current study necessitates that the findings should be interpreted with caution. The findings from the current study may be protocol specific and a genetic polymorphism that is a significant indicator of relapse in the current study may emerge as irrelevant in another. The improvements that have been achieved in pediatric ALL outcome are largely attributed to refinements in multi-drug chemotherapy protocols. They are administered at maximum tolerant doses for the pediatric population. It is therefore unlikely that we can further improve the outcome of ALL patients by dose-escalation of existing therapies due to the enormous toxicity that these drugs have on the active developmental pathways of pediatric patients. In the last 5 years, pharmacogenomic studies have shifted to high-resolution genome-wide association studies in an effort to better understand the fundamental molecular biology of acute ALL and to identify potential markers related to treatment outcome as well as to identify novel therapeutic targets. Despite these advances, the pharmacogenetic of ALL remains as one of the most promising areas for continued investigation. The findings from the current study underscore the need for and the value of prospective, large multi-institutional studies as a major thrust in the ongoing effort to improve the survival of ALL patients.

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ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AZA	azathioprine
BFM `	Berlin-Frankfurt-Munster Study Group
BCB	comple blood count
CCG	children's cancer group
CI	confidence interval
CP	cyclophosphamide
CR	complete remission
Ct	cycle threshold
CYP	cytochrome P450
DFCI	Dana Farber Cancer Institute
DHFR	dihydrofolate reductase
EFS	event-free survival
FAB	French-American-British
6-FAM™	6-carboxyfluorescein
GST	glutathione s-transferase
HSC	hematopoietic stem cell
HPRT	hypoxanthine phosphoribosyl transferase
HR	hazard ratio
MGB	minor groove binding
6MP	6-mercaptopurine
MTHFR	5, 10-methylenetetradyfrofolate reductase
MTX	methotrexate
NFQ	nonfluorescent quencher
OR	odds ratio
POG	pediatric oncology group
RBC	red blood cell
SJCRH	St Jude Children's Research Hospital
TPMT	thiopurine s-methyltransferase
TYMS	thymidylate synthetase
WBC	white blood cell count
WHO	world health organization

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Appendices

Appendix 1. DNA Isolation from Blood or Bone Marrow (BM) – Manual Extraction

I. **PRINCIPLE**

Red blood cells (RBCs) are first removed by preferential lysis. WBCs are then lysed to release lipids, nucleic acids, and proteins. The proteins and lipids are removed by high-salt precipitation while DNA is left in solution. DNA is finally precipitated from the solution with isopropanol, washed with 70% ethanol and allowed to dry before re-hydrating in a Tris-EDTA buffer.

II. **SPECIMEN REQUIREMENTS**

   A.   Acceptable specimens:
1. Blood or bone marrow (BM) aspirate collected in EDTA anti-coagulant (preferred). Store at 2-8°C upon receipt. For best results, samples should be left at room temperature for no more than 24 hours prior to processing.
2. Blood or BM collected in ACD (yellow top) or citrate (blue top) are also acceptable
3. Slides from EDTA- preserved blood or BM, dried, room temp.

   B.   Unacceptable specimens:
1. Heparin is a known inhibitor of the PCR; therefore, specimens collected in heparin are generally not acceptable for PCR-based assays.
2. Clotted specimens will be noted but may be accepted at the discretion of the technologist.
   - a. If a BM sample (irreplaceable) is clotted and WBCs cannot be obtained from the sample, it may be processed according to the DNA Isolation from Fresh or Frozen Tissue Protocol.
   - b. If a blood sample is clotted and WBCs cannot be obtained from the sample, another sample must be obtained.

III. **REAGENTS/EQUIPMENT/SUPPLIES**

   A.   Reagents
1. RBC Lysis Solution, 1000 mL, Cat# 158904, QIAGEN. Store at room temperature.
2. Cell Lysis Solution, 1000 mL, Cat# 158908, QIAGEN. Store at room temperature.
3. Protein Precipitation Solution, 350 mL, Cat. # 158912, QIAGEN Store at room temperature.
4. 2-propanol (isopropanol), molecular biology grade, 500 mL, Cat# I9516-500ML, SIGMA. Store at room temperature.
5. Glycogen (20 mg/ml), 100 µL, Cat# 10814-010, INVITROGEN. Store at -20°C.

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6. 70% Ethanol (v/v), prepared in molecular-grade water from 100% ACS/USP grade ethyl alcohol obtained from TCH pharmacy. Store at room temperature.
7. DNA Hydration Solution, 100 mL, Cat# 1045698, QIAGEN. Store at room temperature.

B.   Equipment
  1. Centrifuges
     a.   Eppendorf 5415R or equivalent benchtop microcentrifuge
     b.   Beckman CPKR or equivalent floor-model centrifuge (for large-scale preps)
  2. Pipettors – Use only those designated for Specimen Processing, labeled as SP##. Use only aerosol-resistant tips (ARTs) for specimen processing.
  3. NanoDrop ND-1000 spectrophotometer

C.   Supplies

  1. 1.7 mL clear snapcap microtubes, Cat# 22-281, Genesee Scientific
  2. 15 mL BD Falcon conical polypropylene centrifuge tube, Cat# C3977-4, Cardinal Health

IV.   **SPECIAL SAFETY PRECAUTIONS**
The reagents used in this procedure may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS and follow the handling instructions. Wear appropriate personal protective equipment. Place all hazardous waste in the appropriate container.

Stock volumes of alcohols used in this procedure are kept in the flammable liquid storage cabinet in the reagent prep room. Smaller volumes sufficient for daily use (less than 500 mL) may be aliquoted and stored in the specimen processing room on the benchtop.

Protein Precipitation Solution is known to produce a toxic gas when mixed with bleach. **Empty reagent bottles should not be re-used for hazardous waste**. In the event of a spill, blot up excess with paper towels, then use water to thoroughly clean the area. Spray-bottle bleach may then be used as necessary for decontamination.

V.   **QUALITY CONTROL** – Individual reagents are QC'ed and results are documented.

VI.   **PROCEDURE** -

NOTE: All work must be performed in the biological safety cabinet (hood) with the blower ON until the final DNA product is ready for hydration, at which point it may safely be manipulated on an open bench.

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A.   RBC lysis

    1.   To a clear 1.7 mL microtube labeled with MDL number, add 900 µL of RBC Lysis Solution. Add 300 µL of well-mixed patient specimen. Cap and invert several times to mix. Incubate a minimum of 5 minutes. Spin tubes for 20 seconds at 8000 rpm in the benchtop microfuge.

    2.   Observe the size of the WBC pellet; it should be approximately equal to the "ideal" pellet size illustrated in Fig. 1 below. If larger than the illustration, vortex briefly to re-suspend the cells, then divide into 2 or more tubes and re-spin.

    3.   Pipet off the supernatant into the blood/BM waste bottle, leaving behind 10-20 µL of residual fluid. (For Recap and vortex vigorously to resuspend the WBCs.
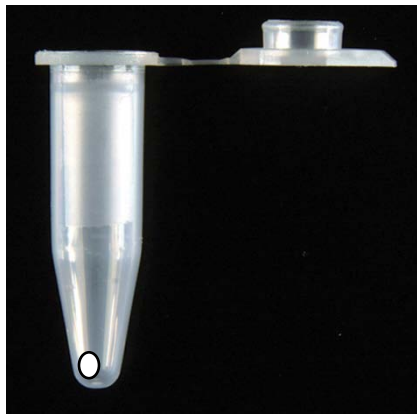
Figure 1. "Ideal" WBC   pellet size

B.   Cell lysis
    1.   Add 600 µL of Cell Lysis Solution and pipet up and down to mix
       a.   Blood or BM is processed in a 1.7 mL microtube at a 1:3 ratio with RBC Lysis Solution (300 µL sample and 900 µL RBC Lysis Solution). Scale up by using multiple microtubes, or scale down by using less reagent according to the specimen volume available. For most procedures two microtubes are preferred.
          1.   Add 300 µL whole blood (or BM) to a 1.7 mL clear microtube labeled with the MDL number and containing 900 µL RBC Lysis Solution. Process 2 microtubes per sample if possible. Invert 10 times. Incubate 5 minutes at room temperature.
          2.   For slides begin at step IX.A.1.f.
       b.   Centrifuge microtubes for 20 seconds at 8,000 rpm in the benchtop microfuge.
          1.   Observe the WBC pellet, if the pellet is obscured by RBCs still, then remove as much of the RBC lysis supernatant as possible with a pipette and add another 900 µL RBC Lysis Solution. Vortex briefly and incubate 5 minutes at room temperature and

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re-centrifuge as above. (Some nucleated RBCs may be present, particularly if the sample is a BM, and these cells may not lyse.)
2. If the WBC pellet is clearly visible remove as much supernatant as possible with a pipette leaving behind the WBC pellet and about 10µL of the residual liquid. **BM NOTE:** Be sure to draw off all fat floating on the surface of the supernatant, otherwise it will contaminate the DNA in subsequent steps.
3. Note the size of the WBC pellet to determine whether the pellet should be divided into more tubes due to its large size or if a smaller volume of cell lysis solution should be used in the subsequent steps due to its small size. See Illustration I for an example of "good" WBC pellet size.
4. Additionally, if more sample is available and the WBC pellet is small, process more blood or BM to attain enough WBCs to get plenty of DNA for the tests ordered.
c. To facilitate cell lysis, vortex the microtube vigorously to resuspend the white cells in the residual liquid.
d. Add 300 µL (small WBC pellet) or 600 µL ("good" WBC pellet size) of Cell Lysis Solution to the resuspended cells and pipette up and down 5-10 times to lyse the WBCs. Samples are stable in Cell Lysis Solution for at least 18 months at room temperature.
e. For slides, begin by washing each with ~100 µL of Cell Lysis Solution and use the side of a pipettor tip to scrape the material from the slide into a 1.7 mL clear microtube labeled with the MDL number.

2. Protein Precipitation
a. Add 100 µL (to the 300 µL volume) or 200 µL (to the 600 µL volume) of Protein Precipitation Solution to the cell lysate. (33 µL protein precipitation solution per 100 µL cell lysate).
b. Vortex vigorously at high speed for at least 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate. Watch for precipitation of the proteins into many red particles in the solution. The solution is well mixed when this appears. If this does not occur after 20 seconds, vortex vigorously until precipitation is noted or until another 30 seconds have elapsed, whichever comes first. Not all samples will appear precipitated; however the proteins will spin-down upon centrifugation.
c. Centrifuge at 13,000-16,000 rpm for 3 minutes. The precipitated proteins will form a tight, dark brown pellet. If the pellet is not tight at the bottom of the microtube, centrifuge for an additional 10 minutes at 13,000-16,000 rpm.

3. DNA Precipitation
a. Carefully pour the supernatant containing the DNA into a 1.7 mL clear microtube containing 300 µL 100% Isopropanol (for samples using only 300 µL cell lysis solution) or 600 µL 100% Isopropanol (for samples using 600 µL cell lysis solution). (1 to 1 ratio isopropanol and original cell lysis volume).
b. Mix the sample by inverting at least 15 times. DNA may or may not be observed.

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                1. If DNA is observed continue inverting until the mass of DNA is a tighter clump rather than a loose, foamy appearing substance.
                2. If DNA is not observed add 1 µL of Glycogen and continue inverting another 15 times.
        c. Centrifuge at 13,000-16,000 rpm for 3 minutes if DNA was observed while inverting. Centrifuge for 10 minutes if DNA was not observed while inverting and Glycogen was added. The DNA pellet will be visible as a small white pellet.
        d. Pour off the supernatant, discarding it in the designated hazardous waste container (Alcohol), and drain the microtube briefly on clean absorbent paper, making sure the DNA pellet remains in the microtube.
        e. Add 300 µL 70% Ethanol and gently swirl the liquid over the DNA pellet by partial inversion 10 times to wash the DNA pellet.
        f. Centrifuge at 13,000-16,000 rpm for 3 minutes.
        g. Remove the supernatant with a pipette tip and discard in the designated hazardous waste container (Alcohol), leaving behind only the DNA.
        h. Allow DNA to dry at room temperature for 10 minutes or until there is no moisture in the tube. The white DNA pellet will turn translucent.

    4. DNA Hydration
        a. Add DNA hydration solution according to the size of the DNA pellet.
                1. If DNA was visible when precipitating (at step IX.A.3.b) add a minimum of 25 µL DNA hydration solution.
                2. If Glycogen was used to pellet the DNA, hydrate with a maximum of 12 µL DNA hydration solution.
                3. Do not over-hydrate. A sample is easy to dilute but difficult to re-concentrate.
                4. Generally, 50 µL DNA hydration solution will achieve the desired 0.500 µg/µL concentration if the original WBC pellet appeared as shown in Illustration I.
        b. If the specimen is required the same day, vortex 5 seconds at medium speed to mix and briefly centrifuge to collect sample at the bottom of the microtube before incubating at 56°C.
        c. If the specimen is to be tested on subsequent days, allow the hydrated sample to sit at room temperature overnight.
        d. Combine all microtubes that originated from the same sample and label this tube with the MDL number on a white LabPal label (i.e. 093455 for 09-MD-3455).

B. MEASUREMENT OF DNA CONCENTRATION
    1. Determine DNA concentration and purity using the NanoDrop ND1000. Pipette 2.0 µL of hydrated DNA onto the NanoDrop platform (as little as 1 µL may be used). Good quality DNA should have a 260/280 ratio between 1.7 and 2.0.
    2. Optimal DNA concentration is between 0.1 and 0.5 µg/µl. If the concentration is higher than this, add additional DNA hydration solution to reach this target concentration.
    3. Record the DNA concentration (µg/µl) and purity on the side of the sample microtube.

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4. Record DNA concentration and purity on the extraction sheet to be entered in the MDL database.
5. Refer to the NanoDrop ND1000 Spectrophotometer Procedure for additional information.

C. DNA STORAGE
1. Store DNA at room temperature overnight to allow for complete hydration.
2. DNA long term storage is in the -70°C freezer in a designated storage box to be held for testing, potential further testing and/or to be used in quality control testing.
3. The adequacy of storage at –70°C is monitored by analysis of current testing performance using controls stored under identical conditions.
4. Specimens are maintained in a limited access, secured area at all times. The Molecular Diagnostic Laboratory specimens are stored in a coded manner. Laboratory personnel are present when the lab is open. The laboratory has no public access.

VII. Reference Range

VIII. Method Limitations

IX. **REFERENCES**
A. Gentra Puregene Handbook, 4/2010, Qiagen

X. Related Documents
A. NanoDrop ND1000 Spectrophotometer Procedure

XI. Attachments
A. Document Historical Record
B. DNA Isolation Blood BM Summary Flowchart

Illustration I
Ideal WBC pellet size. If much larger than this (for example 2x as large), please aliquot into additional clear microtubes. If much smaller than this (for example ½ as large), please use the smaller amount (300 µL) of Cell Lysis Solution and process more of the sample to attain more WBCs.

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Appendix 2. Clinical validation: *MTHFR* C677T and *MTHFR* A1298G

TCH MDx Lab Results

		MTHFR 677 Wild type	MTHFR 677 Variants
ARUP Laboratories Results	MTHFR 677 Wild type	7	0
	MTHFR 677 Variants	0	26

TCH MDx Lab Results

		MTHFR 677 Wild type	MTHFR 677 Variants
CAP proficiency Survey	MTHFR 677 Wild type	2	0
	MTHFR 677 Variants	0	6

TCH MDx Lab Results

		MTHFR 1298 Wild type	MTHFR 1298 Variants
ARUP Laboratories Results	MTHFR 1298 Wild type	4	0
	MTHFR 1298 Variants	0	4

Appendix 3. Patient genotyping results

Research ID	Month	Gender	1A1	2B6	3A4	3A5-1	3A5-2	GSTP1-1	GSTP1-2	TYMS	MTHFR 667	MTHFR 1298	GSTM1	GSTT1
47	15	F	1	1	1	1	3	1	1	2	1	3	2	1
48	44	M	1	2	1	1	3	1	2	2	1	3	1	1
49	43	M	1	1	1	1	3	2	3	2	2	1	1	1
39	60	M	1	1	1	1	3	1	1	2	1	2	1	1
50	83	M	1	2	2	2	2	1	2	1	2	1	1	1
59	28	F	1	1	1	1	3	1	1	1	2	1	1	1
29	74	F	1	1	1	1	3	1	3	2	3	1	2	1
31	73	M	0	1	2	1	3	1	1	3	1	2	2	2
16	27	M	1	1	1	1	3	1	2	2	2	1	2	1
44	58	M	1	1	1	1	3	1	1	1	1	2	1	1
6	52	F	2	1	1	1	3	1	2	2	2	2	2	1
22	30	F	2	2	1	1	3	1	3	1	2	1	1	1
90	31	M	1	1	1	1	3	1	3	3	2	2	1	1
120	56	M	1	1	1	1	3	1	1	2	1	1	1	2
116	34	F	1	1	1	1	3	1	2	3	2	2	1	1
118	40	F	1	1	1	1	2	1	3	2	1	2	1	1

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2	3	7	8	10	11	12	13	14	15	17	18	19	20	21	23	24	25	26
1	1	1	1	2	1	1	2	1	1	1	0	1	1	1	1	2	1	1
1	1	2	1	2	2	2	1	2	1	1	0	2	2	2	2	1	1	1
2	1	1	1		2	2	1	2	1	3	1	2		1		2	2	2
2	1	3	2	1	1	2	1	1	1		2	2	3	1	2	2	3	3
2	2	3	2	1	2	2	2	2	3	1	3	1	1	1	1			
2	2	1	2		3	1	3	1	2	1	2	3	1	2	3	2	2	3
1	1	1	1	3	1		1	1	1	1	1		2	1		2	
2	3	3	3	1	3	3	2	1	3	3	3	3	3	3	3	3	3	3
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
2	1	1	1		2	1	1		2		1	2	1	1	1	3	1
1	1	1	1	1	1	2	2	1	1	2	1	1	1	1	2	2	2
F	F	M	M	F	M	F	M	M	M	F	F	F	M	F	M	M	F	F
2	3	7	8	10	11	12	13	14	15	17	18	19	20	21	23	24	25	26

ID	27	28	30	32	33	34	40	41	42	43	45	46	51	52	53	54	55	56	57
	F	M	M	M	F	M	M	F	M	M	F	M	F	F	M	F	M	M	F
	1	1	1	2	1	2	1	1	1	2	1	1	2	1	1	1	1	1	1
	1	2	2	1	2	1	2	1	2	1	1	2	1	2	1	1	2	1	1
	1	2	2	1	1	2	1	1	2	1	1	2	1	1	1	2	1	1	1
	1	1	1	2	1	2	3	1		1			1		1		1		
	3	3	3	1	3	3	2	2	3	3	2	2	3	2	3	3	3	2	3
	2	2	1		2		1	1		1			1	1		2		1	
	2	3	2	2	1	3	2	2	3	2	2	1	2	1	3	3	2	2	
	1	2	2	2	1	2	2	1	3	1	3	3	1	3	1	3	2		
	3	2	2	1	3	1	1	2	3	2	1	1	2	1	2	1	3		
	1	2	3	1	1	2	2	1		2	2	1	3	2	2	1			
	1	2	2	2	2	2	1	1	1	2	1	1	2	2	2	1	2		
	1	1	1	1	2	1	1	2	1	1	1	1	2	1	1	2	1		

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ID	Sex												
61	M	1	1	1	1	3	1	1	2	2	1	2	1
62	F	1	1	1	1	3	2	2	3	1	2	1	1
64	M	2	1	2	1	3	2	1	3	2	1	1	2
65	F	2	1	1	1	2	1	1	2	1	2	1	2
66	F	1	2	1	1	3	2	2	2	2	2	1	1
68	F	1	1	1	1	2	2	1	1	2	1	1	1
69	M	2	2	1	1	3	2	1	2	2	1	2	2
70	M	1	1	1	1	3	2	1	3	2	2	1	1
71	M	1	1	1	2	3	3	2	1	2	2	2	1
72	F	1	2	1	1	3	2	1	2	2	2	1	2
73	M	1	1	1	1	3	1	1	2	1	2	2	1
74	F	1	2	1	2	3	1	2	1	1	3	1	2
75	F	1	1	1	1	2	1	1	1	1	1	2	1
76	M	1	2	1	1	3	1	2	1	2	2	1	2
77	F	2	1	1	2	3	3	1	3	1	1	2	2
78	M	2	1	1	1	2	2	1	2	1	1	2	1
79	F	1	1	1	1	3	2	1	1	1	1	1	1
80	F	1	1	1	1	3	2	1	2	1	1	2	1
81	F	2	1	1	2	3	2	2	2	3	1	2	1

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ID	Gender	1	2	3	4	5	6	7	8	9	10	11	12
82	M	1	2	1	1	3	1	2	2	2	2	1	1
84	F	1	1	1	1	3	2	3	3	3	1	2	1
85	M	1	1	1	1	3	2	1	2	2	1	1	1
87	F	2	1	2	1	3	1	3	1	1	3	1	1
88	M	1	1	1	1	1	1	2	3	2	2	2	1
89	F	2	1	1	1	2	1	1	2	3	1	1	1
91	F	2	1	1	1	3	1	2	2	2	1	2	1
92	F	1	2	1	1	2	2	3	3	1	1	1	1
93	M	2	2	1	1	3	1	1	1	3	1	2	2
94	M	1	1	1	1	3	1	2	2	3	1	1	0
96	M	1	1	1	1	3	1	3	3	1	3	0	1
98	M	2	1	1	1	3	1	3	3	2	1	2	2
99	M	1	2	1	1	2	1	2	2	2	1	2	1
100	M	1	1	1	1	2	1	2	2	3	1	2	1
102	M	1	1	1	1	3	1	2	2	3	2	1	1
103	M	1	1	1	1	3	1	1	2	1	1	1	1
104	M	1	1	1	1	3	1	2	3	3	2	2	1
105	F	1	1	1	1	3	1	2	2	2	1	1	2
107	F	1	1	1	1	3	1	2	1	1	3	2	1

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ID													Gender
108	1	2	2	2	2	1	1	2	1	1	1	2	M
109	1	1	3	2	2	2	1	2	1	2	1	1	M
110	1	2	2	1	1	3	1	3	1	1	1	1	M
111	1	2	1	2	2	3	1	3	1	1	1	1	M
112	1	1	2	2	3	1	1	3	1	2	2	1	M
113	1	1	1	3	2	2	2	2	1	1	1	1	F
114	1	1	1	2	2	2	2	3	1	1	1	1	M
115	1	2	2	1	2	2	1	3	1	1	1	1	M
117	1	2	3	1	2	2	1	3	1	1	1	2	F
119	1	1	2	3	3	3	1	2	1	1	1	1	M
121	1	2	2	3	1	3	3	3	1	1	1	1	M
122	1	1	1	2	1	1	3	3	1	1	1	1	M
123	2	2	1	3	2	2	1	3	1	1	1	2	M
124	1	0	2	2	3	2	1	3	1	1	1	1	F
125	1	1	1	2	3	3	1	3	1	1	1	1	M
126	1	2	1	3	3	1	1	3	1	1	1	1	M
127	1	1	2	2	2	2	1	3	1	1	1	1	M
128	1	1	2	3	2	3	1	3	1	1	1	1	F
129	2	1	2	2	3	3	1	2	1	1	1	1	M

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130		M	1	2	1	1	3	2	2	3	1	3	1	1
131		F	1	1	1	1	3	1	2	3	1	1	1	1
132		M	0	0	0	0	0	2	2	0	0	0	1	1
133		M	1	1	2	1	3	1	1	2	1	3	1	1
134		M	2	1	1	1	3	1	1	2	3	1	2	1
135		M	1	1	1	1	3	1	1	2	2	2	1	1
136		M	1	1	1	1	3	2	2	3	3	1	1	1
137		M	1	2	1	1	3	1	1	0	2	1	1	1
139		F	1	1	1	1	2	2	2	1	2	2	1	1
140		F	1	1	2	1	3	1	2	3	2	2	1	1
141		F	2	1	2	1	2	2	2	3	1	1	1	1
142		F	1	1	1	1	3	1	1	2	1	2	1	1
143		F	1	1	1	1	3	1	1	3	3	1	1	1
147		M	1	1	1	1	3	1	1	2	2	1	1	1

Note 1: Samples heighted in blue are from relapsed patients.
Note 2: 1 = wild type; 2 = heterozygous; 3 = homozygous mutant
Note 3: 1 = gene present, 2 = null genotype for *GSTM1* and *GSTT1*
Note 4: 1 = 2R/2R, 2 = 2R/3R, and 3 = 3R/3R genotype for *TYMS*
Note 5: month = duration of complete remission or the last follow-up
Note 6: 0 = no DNA is available for genotyping

Appendix 4. Data Coding:[i]

```
   if CYP1A1=2 then CYP1A1g=2;
   if CYP1A1=1 then CYP1A1g=1;***reference group;

   if CYP2B6=3 then CYP2B6g=2;
   if CYP2B6=2 then CYP2B6g=2;
   if CYP2B6=1 then CYP2B6g=1;***reference group;
   if CYP3A4=3 then CYP3A4g=2;
   if CYP3A4=2 then CYP3A4g=2;
   if CYP3A4=1 then CYP3A4g=1;***reference group;

   if CYP3A5_1=2 then CYP3A5_1g=2;
   if CYP3A5_1=1 then CYP3A5_1g=1;***reference group;
   if CYP3A5_2=1 then CYP3A5_2g=2;
   if CYP3A5_2=2 then CYP3A5_2g=2;
   if CYP3A5_2=3 then CYP3A5_2g=1;***reference group;
   if GSTP1_1=3 then GSTP1_1g=2;
   if GSTP1_1=2 then GSTP1_1g=2;
   if GSTP1_1=1 then GSTP1_1g=1;***reference group;


   if GSTP1_2=3 then GSTP1_2g=2;
   if GSTP1_2=2 then GSTP1_2g=2;
   if GSTP1_2=1 then GSTP1_2g=1;***reference group;

   TSg=TS;

   if MTHFR667=3 then MTHFR667g=2;
   if MTHFR667=2 then MTHFR667g=2;
   if MTHFR667=1 then MTHFR667g=1; ;***reference group;


   if MTHFR1298=3 then MTHFR1298g=2;
   if MTHFR1298=2 then MTHFR1298g=2;
   if MTHFR1298=1 then MTHFR1298g=1;***reference group;


   if GSTM1=2 then GSTM1g=2;
   if GSTM1=1 then GSTM1g=1;***reference group;

   if GSTT1=2 then GSTT1g=2;
   if GSTT1=1 then GSTT1g=1;***reference group;




if CYP3A4=1 and CYP3A5_1=1 and GSTM1=1 and GSTT1=1 then DrugExpo=3;
 if (CYP3A4=1 or CYP3A5_1=1) and (GSTM1=2 or GSTT1=2 ) then DrugExpo=3;
 if CYP3A4=2 and CYP3A5_1=1 and GSTM1=. and GSTT1=. then
DrugExpo=3;***All others;
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  if (CYP3A4=1 and CYP3A5_1=1) and (GSTM1=2 or GSTT1=2 ) then
DrugExpo=2;***High drug exposure;

  if (CYP3A4 >1 or CYP3A5_1=2) and (GSTM1=1 and GSTT1=1 ) then
DrugExpo=1;***low drug exposure;

 *** 1 vs 2;

   if DrugExpo =1 then DrugExpoA=1;***low drug exposure;
   if DrugExpo = 2 then DrugExpoA=0;***High drug exposure;

 *** 1+3 vs 2;
 if DrugExpo in (1, 3) then DrugExpoB=1;***low drug exposure + all
others;
 if DrugExpo = 2 then DrugExpoB=0;***High drug exposure;


*** Drug shorter stay vs longer stay;
 if GSTM1=1 and GSTT1=1 then DrugExpo2=1;***Drug stay shorter;
 if GSTM1=2 or GSTT1=2 then DrugExpo2=0;***Drug stay longer;
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