Thiopurine Methyltransferase Genotyping in Childhood Acute Lymphoblastic Leukemia Patients in Gaza Strip

Prepared By
Wael M. Harb

Supervised by
Dr. Basim Ayesh       Dr. AbdAllah Abed

A thesis Submitted to the Faculty of Science in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biological Sciences – Medical Technology

1431 – 2010 م
DECLARATION

I hereby declare that this submission is my own original work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor materials which to a substantial extent has been accepted for award of any other degree of the university or other institute, except where due acknowledgement has been mad in the text.

Signature       Name       Date
Wael            Wael M. Harb       June-2010

Copyright.
All Rights Reserved: No part of this work can be copied, translated or stored in a retrieval system, without prior permission of the authors.
ABSTRACT

Thiopurine methyltransferase genotyping in childhood acute lymphoblastic leukemia patients in Gaza Strip

Background
The genetic polymorphism of thiopurine methyltransferase (TPMT) is well characterized in most populations. Three common polymorphic alleles are associated with impaired activity of the enzyme. These are TPMT*2 (238G>C), TPMT*3A (460G>A, 719A>G) and TPMT*3C (719A>G).

Objective
The aim of the present study was to determine the frequency of the functional TPMT polymorphisms and their association with the occurrence of adverse events, during 6-mercaptopurine therapy in pediatric acute lymphoblastic leukemic (ALL) patients in Gaza Strip.

Methods
A total of 56 DNA samples from all pediatric ALL patients admitted to the pediatric hematology departments of Gaza strip hospitals were analyzed. Genomic DNA from peripheral blood leukocytes was isolated and the TPMT*2, TPMT*3A and TPMT*3C allelic polymorphism was determined by PCR- RFLP and allele specific PCR technique.

Results
No TPMT*2, *3C mutant alleles were detected. Only the TPMT*3A allele was detected in one patient out of 56 DNA samples of pediatric ALL patients in Gaza strip. Thus, frequency of TPMT*3A allele was 0.89%. Fourteen patients of ALL were suffering from myelotoxicity during 6MP therapy. From our results, no significant association could be established between clinical and laboratory data and/or the presence of the mutation in TPMT gene. The calculated incidence of ALL in children in Gaza Strip is 2 cases per 100000 children.
The incidence of ALL is higher in males than in females. The mean age of ALL incidences in Gaza Strip is 4.4 ± 2.6 years.

**Conclusion**
This is the first analysis of the polymorphisms of the TPMT gene in childhood ALL patients in Gaza Strip. Other types of mutation in TPMT gene may be responsible for the observed myelotoxicity among the investigated patients. Factors other than TPMT polymorphisms may also be responsible for the development of toxicity. Therefore, more studies are recommended to be performed in order to investigate such factors.

Keywords: Polymorphisms, Thiopurine S-methyltransferase, Gaza Strip, Acute lymphoblastic leukemia
ملخص الدراسة:

التنوع الجيني في جين TPMT

لدى الأطفال المرضى بإيبياض الدم الليمفاوي الحاد في قطاع غزة

التنوع الجيني في جين TPMT موجود في معظم الشعوب، وهم ثلاث أنواع من الطفرات الشائعة والتي تمثل TPMT*3A (460G>A, 719A>G), وTPMT*2 (238G>C), TPMT*3C (719A>G).

أهداف الدراسة هو دراسة توزيع الانواع الجينية ونسبة التنوع في جين TPMT وعلاقته بالنقص الحاد في عديد كرات الدم البيضاء ونقص الصفائح الدموية أو أي عوامل سمية أخرى عند إعطاء المرضى الجرعة الثابتة من 6-MP.

تم في هذه الدراسة فحص عينة مكونة من 60 طفل يعانون من إيبياض الدم الليمفاوي الحاد في قطاع غزة تتراوح أعمارهم بين 6 شهور و12 سنة. تم فصل عينات الدم أخذت من المرضى بالطرق المعروفة في تحديد PCR- RFLP and allele specific PCR technique واستخدام نهجين لفحص ومحاكاة هذه الطفرات الشائعة في هذا الجين.

أظهرت نتائج هذه الدراسة وجود نوع واحد من الطفرات وهو البديل TPMT*3A والذي كان معدل انتشاره يشكل 89.0%. ولم توجد طفرات في أي من البديل 2, TPMT*3C, TPMT*2. كما أنه تم توجد علاقة محددة بين الناحية الإكلينيكية للمريض والنتائج لفحص المختبر ووجود هذا النوع من الطفرات في جين TPMT. وجد أيضا معدل إصابة الأطفال بإيبياض الدم الحاد سنويا 2 من بين كل 1000 طفل. وكان متوسط أعمارهم عند الإصابة 4.43 سنة.

2.57% كان مرض سرطان الدم الحاد الليمفاوي شائع في الذكور أكثر من الإناث.

تنتجه من هذه الدراسة وهي الأولى من نوعها التي تجري في قطاع غزة لفحص التنوع الجيني في جين TPMT، أنه ربما يكون هناك نوع آخر من الطفرات موجود أو عوامل أخرى مرتبطة بالنقص الحاد في كريات الدم البيضاء والصفائح الدموية الموجودة لدى المرضى. لذلك يستحسن إجراء أبحاث أخرى تتناول هذه الأساليب الممكنة.
DEDICATION

This work is dedicated to:

My parents, who encouraged me accomplish this work successfully.

To my wife who supports me.

To our Palestinian people who are suffering from the siege.
I would like to thank all those people who have helped in the completion of this thesis. First I'm grateful to Dr. Basim Ayesh and Dr. AbdAllah Abed for their grate efforts and supervision on this thesis.

I would like to thank the physician Mohamed Abu Shaban who provided me with information about childhood ALL patients in Gaza strip.

My special thanks are to the master degree program coordinators at the Islamic University, especially Dr. Aboud Al Kishawi and Mr. Mohammed Abu Oda who are still struggling for the success of this program.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Item</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>I</td>
</tr>
<tr>
<td>Abstract</td>
<td>II</td>
</tr>
<tr>
<td>Arabic Abstract</td>
<td>IV</td>
</tr>
<tr>
<td>Dedication</td>
<td>V</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>VI</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>VII</td>
</tr>
<tr>
<td>List of Tables</td>
<td>IX</td>
</tr>
<tr>
<td>List of Figures</td>
<td>IX</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>X</td>
</tr>
</tbody>
</table>

## Chapter One: Introduction

1.1. Overview ........................................... 1
1.2. Aim of the study .................................. 3
1.3. Significance ...................................... 4

## Chapter Two: Literature review

2.1. Background of Acute lymphoblastic leukemia .......... 5
2.2. Genetics of leukemia ................................. 7
2.2.1. Primary abnormalities ............................. 7
2.2.1.1. B lineage ALL ................................ 7
2.2.1.2. T-lineage ALL ................................ 8
2.3. Risk Classification Systems and Risk-Adapted Therapy .... 8
2.4 Clinical manifestation ............................... 9
2.5. Diagnosis of ALL .................................... 10
2.5.1. Laboratory studies ................................ 10
2.5.1.1. Blood count ................................ 10
2.5.1.2. Bone marrow investigation .................... 11
2.5.1.3. Immunophenotyping ............................. 11
2.5.1.4. Morphological classification ................... 12
2.5.1.5. Cytochemistry ................................ 12
2.5.1.6. Cytogenetics ................................ 12
2.6. Management of ALL ................................... 12
2.7. 6-mercaptopurine ................................. 14
2.7.1 Drug discovery ................................... 14
2.8. Metabolism of 6-mercaptopurine (6MP) .................. 15
2.9. Pharmacogenetics plays a role in drug toxicity and efficacy .... 16
2.10. Thiopurine S-methyltransferase ...................... 17
2.11. Molecular basis for altered TPMT activity ........... 18
2.12. Genetic polymorphism and alleles of TPMT ........... 19
2.13. Relationship between TPMT and toxicity ............. 20
2.14. Ethnic variations in TPMT mutations ................. 22
2.15. Difference between phenotype and genotype .......... 22
2.16. frequency of TPMT allele distribution in different population ... 22
2.17. Prevalence of ALL in Gaza strip .................... 24
### Chapter Three: Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Study Population</td>
<td>25</td>
</tr>
<tr>
<td>3.2. Ethical Considerations</td>
<td>25</td>
</tr>
<tr>
<td>3.3. Materials</td>
<td>25</td>
</tr>
<tr>
<td>3.3.1. Chemicals and Reagents</td>
<td>25</td>
</tr>
<tr>
<td>3.3.2. Disposables</td>
<td>26</td>
</tr>
<tr>
<td>3.3.3. Major equipment used in the present study</td>
<td>26</td>
</tr>
<tr>
<td>3.4. Methods</td>
<td>26</td>
</tr>
<tr>
<td>3.4.1. Blood Collection</td>
<td>26</td>
</tr>
<tr>
<td>3.4.2. DNA Extraction</td>
<td>27</td>
</tr>
<tr>
<td>3.4.3. TPMT genotyping</td>
<td>27</td>
</tr>
<tr>
<td>3.4.4. Data collection and analysis</td>
<td>29</td>
</tr>
</tbody>
</table>

### Chapter Four: Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1. Description of the study population</td>
<td>30</td>
</tr>
<tr>
<td>4.2. Management of patients</td>
<td>32</td>
</tr>
<tr>
<td>4.3. Genotyping of DNA samples</td>
<td>33</td>
</tr>
</tbody>
</table>

### Chapter Five: Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1. Conclusions</td>
<td>39</td>
</tr>
<tr>
<td>6.2. Recommendations</td>
<td>39</td>
</tr>
<tr>
<td>References</td>
<td>40</td>
</tr>
</tbody>
</table>

### Appendix

<table>
<thead>
<tr>
<th>Annex</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annex 1</td>
<td>51</td>
</tr>
<tr>
<td>Annex 2</td>
<td>52</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table | Page  
---|---
Table 2.1: Clinical finding in ALL patients | 10
Table 2.2: Immunological classification of childhood ALL | 11
Table 2.3: Description of the phases of chemotherapy in patients with ALL | 13
Table 2.4: TPMT genotype classification and implications to therapy | 21
Table 2.5: Summary of different studies of TPMT allele variation in different population | 23
Table 3.1: A list chemicals and reagents | 25
Table 3.2: List of disposable | 26
Table 3.3: A list of major equipment used in this study | 26
Table 3.4: Description of primers and restriction enzyme and there | 28
Table 4.1: Complications associated with 6MP | 32
Table 4.2: Frequencies of TPMT in 56 samples of Gaza pediatric patient with ALL | 35

LIST OF FIGURES

Figure | Page  
---|---
Figure 2.1: Blood cell development | 6
Figure 2.2: Chemical structure of 6MP | 14
Figure 2.3: Scheme of thiopurine drug metabolism | 16
Figure 2.4: Five stages of pharmacogenetics and pharmacogenomics in cancer therapy | 17
Figure 2.5: Position of TPMT gene on chromosome 6 | 18
Figure 2.6: Allelic variants of the TPMT gene | 20
Figure 2.7: Distribution of pediatric ALL patient in Gaza strip from year 2006 to 2009 years | 34
Figure 4.1: Distribution of cases by gender | 30
Figure 4.2: Distribution of cases by age | 31
Figure 4.3: Electrophoresis patterns for TPMT*2 (238G>C) | 33
Figure 4.4: Electrophoresis patterns for Electrophoreses pattern for detection 460G>A | 34
Figure 4.5: PCR product digest with restriction enzymes Accl to detect the 719A>G mutation | 34
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MP</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>6-MTIMP</td>
<td>6-methyl thioinosine 5'-monophosphate</td>
</tr>
<tr>
<td>6MMPRS</td>
<td>6-Methylmercaptopurine ribnucleotides</td>
</tr>
<tr>
<td>6-TITP</td>
<td>6-thioinosine 5'-triphosphate</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>BFM</td>
<td>Berlin-Frankfurt-Münster protocols</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood cell count</td>
</tr>
<tr>
<td>CCG</td>
<td>Children’s Cancer Group</td>
</tr>
<tr>
<td>CNS</td>
<td>Center Nerves System</td>
</tr>
<tr>
<td>COG</td>
<td>Children’s Oncology Group</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetic acid</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>HGPRT</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>meMP</td>
<td>methylmercaptopurine</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal Residual Disease</td>
</tr>
<tr>
<td>MTX</td>
<td>Methatrxate</td>
</tr>
<tr>
<td>NCI</td>
<td>Pediatric National Cancer Institute</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>POG</td>
<td>Pediatric Oncology Group</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia Chromosome</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide polymorphism</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
</tbody>
</table>
T  Thymine
TGDP  Thioguanosine diphosphate
TGNs  Thioguanine Nucleotides
TGMP  thioguanosine monophosphate
TGTP  Thioguanosine triphosphate
Thr  Threonine
TIMP  Thioinosine monophosphate
TPMT  Thiopurine S-methyltransferase
Tyr  Tyrosine
U  Unit
WBC  White Blood Cell
Xo  Xanthine Oxidase
μl  Microliter
Chapter One

Introduction

1.1. Overview

Acute leukemia is a malignant disorder in which hemopoietic blast cells constitute more than 30% of bone marrow cells. The primitive cells usually accumulate in the blood, they infiltrate other tissues and cause bone marrow failure (Mehta and Hoffbrand, 2000). Acute leukemia constitutes about 97% of all childhood leukemias and consists of 75% acute lymphoblastic leukemia (ALL). Acute myeloblastic leukemia (AML) also known as acute nonlymphocytic leukemia (ANLL) 20%; acute undifferentiated leukemia (AUL) <0.5% and rarely acute mixed-lineage leukemia (AMLL). On the other hand chronic myeloid leukemias constitute about 3% of all childhood leukemias and consist of philadelphia chromosome–positive (Ph1-positive) myeloid leukemia and juvenile myelomonocytic leukemia (JMML) (Lanzkowsky, 2005).

Acute lymphoblastic leukemia like other malignancies of hematologic origin is believed to involve a transformation event that occurs in a single progenitor cell that gains the capability for indefinite clonal expansion. The leukemogenic event may occur in committed lymphoid cells of B or T lineages or in early precursors, which gives rise to the different subtypes of ALL based on the stage of lymphoid differentiation of the cell (Reaman, 2002).

The incidence of ALL is 3 - 4 cases per 100,000 white children and 2500–3000 children are diagnosed in the developed countries per year (Mehta and Hoffbrand, 2000). The peak incidence in children is at age 2-5 years (eMedicine, 2010).

According to Palestinian Ministry of health hospital records, in Gaza Strip, the total number of recorded childhood ALL patients in 2006 was 15 patients, 14 patients in 2007, 15 patients in 2008 and 10 patients in 2009.

The treatment protocol for ALL involves 6-mercaptopurine as a chemotherapeutic drug. The first phase of therapy (remission – induction) is with high dose of intensive combination chemotherapy to reduce or eradicate leukaemic cells from the bone marrow and re–establish normal haemopoiesis.
Remission induction regimes comprise vincristine, prednisolone and L-asparaginase often with daunorubicin and Cyclophosphamide. Post-remission therapy is with two or three intensification blocks with additional drugs. Patients then receive maintenance chemotherapy for a further 2 to 3 years with daily 6-mercaptopurine, weekly methotrexate and monthly vincristine and prednisolone (Mehta and Hoffbrand, 2000). Duration of therapy differs among centers and protocols, but on average, therapy lasts 2.5 years.

6-Mercaptopurine, 6-thioguanine and azathioprine are thiopurine drugs that are usually used to treat acute lymphoblastic leukemia, autoimmune disorders, inflammatory bowel disease and organ transplant recipients (Paterson and Tidd, 1975; Lennard, 1992). Both 6-Mercaptopurine and 6-thioguanine are prodrugs that require activation by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) to exert a cytotoxic effect. Alternatively, these agents can undergo S-methylation catalyzed by thiopurine methyltransferase (TPMT) to methylmercaptopurine (meMP) or oxidation to thioric acid via xanthine oxidase. Metabolism via either TPMT or xanthine oxidase reduces formation of the active thioguanine nucleotides (Krynetski et al., 1996; Krynetski and Evans, 1999).

Variation in TPMT activity regulates thiopurine toxicity and therapeutic efficacy of thiopurine drugs. Approximately 1 in 300 of population have low activity, 6-11% have intermediate activity and 89-94% have high activity (McLeod et al., 1994; Krynetski et al., 1996). Patients with low or undetectable levels of TPMT activity develop severe myelosuppression when treated with standard doses of thiopurines, although heterozygous patients with intermediate TPMT activity may have increased mercaptopurine toxicity. This is variable, and the majority of patients tolerate normal doses of Purinethol while patients with very high TPMT are more likely to have a reduced clinical response to these agents (Lennard et al., 1990; Lilleyman and Lennard, 1994).

TPMT is encoded by a 34 kb gene consisting of 10 exons and nine introns and has been localized to chromosome 6p22.3 (Krynetski et al., 1996; Szumlanski et al., 1996). TPMT enzyme activity is largely influenced by variants (polymorphisms) in the TPMT gene. People heterozygous for TPMT mutations have intermediate activity while those homozygous for the mutation have low activity (Evans et al., 2001).
Over 23 variants of the TPMT gene are associated with decreased TPMT activity (Schaeffeler et al., 2006). Three variant alleles, TPMT*2, TPMT*3A and TPMT*3C, account for 80–95% of intermediate or low activity cases (Yates et al., 1997). Although the overall prevalence of TPMT deficiency is similar between different ethnic groups, the frequency of variant alleles differs between different populations. For example, there is a higher representation of the TMPT*3A allele in South Asians compared with the high carriage of TPMT*3C in East and West Africans (McLeod and Siva, 2002).

Genotyping or phenotype techniques can be used to identify TPMT allelic variation and the possible risk of developing profound neutropenia (Sanderson et al., 2004). Polymerase chain reaction PCR -RFLP and allele specific PCR 'technique are used for genotyping the TPMT polymorphism. It produces a relatively rapid result, is not expensive and is not subjected to interference by exogenous factors. Furthermore, PCR-RFLP genotyping has been found to be well correlated with blood TPMT enzymatic activity with over a 95% concordance rate, and to have a high sensitivity and specificity (Otterness et al., 1997; Yates et al., 1997).

1.2. Aim of the study

The aim of the present study is to determine the frequency of the functional TPMT polymorphisms and their association with the occurrence of adverse events toxicity, during mercaptopurine therapy in childhood acute lymphoblastic leukemic patients in Gaza Strip.

The specific objectives were

- To detect the prevalence of homozygous and heterozygous mutations of (TPMT) gene in childhood ALL in Gaza strip. These are 238G>C, 460G>A and 719A>G.
- To determine the most common alleles resulting from these mutations (TPMT*2, TPMT*3A and TPMT*3C).
- To investigate the effect of TPMT genotype in children receiving 6-mercaptopurine during the early treatment of childhood ALL (If any).
To highlight the importance of TPMT polymorphism detection in Gaza Strip in prevention of myelotoxicity and in guiding initial mercaptopurin dosage.

1.3. Significance

Thiopurines are very useful drugs, but they have a relatively narrow therapeutic index, with life-threatening myelosuppression as a major toxicity. Substantial dose reductions are generally required for homozygous TPMT deficiency patients to avoid the development of life threatening bone marrow suppression as a result of reduced TPMT enzyme activity. If a patient has clinical or laboratory evidence of severe toxicity, particularly myelosuppression, TPMT testing should be considered. Therefore, TPMT genotyping allows for dose prediction and adjustment to prevent dangerous side-effects in patients found to have lower activity than normal. According to my knowledge there is no previous study of TPMT genotype in Gaza Strip.
Chapter two

Literature review

2.1. Background of acute lymphoblastic leukemia

In ALL, the lymphocytes multiply too quickly and live for too long, so there are too many of them circulating in the blood. These abnormal lymphocytes are not fully developed and do not work properly. Over a period of time, they replace the normal white cells and red blood cells and platelets in the bone marrow (European Medicines Agency, 2009). The bone marrow stem cells are defined by a combination of the traits of self-maintenance and the ability to produce multiple, varied offsprings. Putting this in more biological terms, stem cells have the unique and defining characteristics of self-renewal and of differentiation into multiple cell types (Figure 2.1). Thus, with each cell division there is an inherent asymmetry in stem cells that is generally not found with other cell types (Provan and Gribben, 2005).

ALL is the most common type of leukaemia in young children, but the disease also affects adults, especially those aged 65 years and older. Many people with ALL can be cured. However, despite the available treatments, ALL remains a serious and life-threatening disease in some patients (European Medicines Agency, 2009). In patients under 15 years old, they account for over 30% of all malignant diseases (Pui et al., 2008).

Acute leukemias represent a clonal expansion and arrest at a specific stage of normal myeloid or lymphoid hematopoiesis (Mehta and Hoffbrand, 2000). The leukemogenic event may occur in committed lymphoid cells of B- or T-cell lineages or in early precursors, which gives rise to the different subtypes of ALL based on the stage of lymphoid differentiation of the cell in which the event occurred (Reaman, 2002). About 80% of all cases of ALL express cell-surface markers indicative of a precursor B-cell lineage. Only 1% to 2% of cases express a phenotype typical of a mature B cell. T-cell ALL accounts for about 15% to 20% of cases and is commonly associated with features at diagnosis, such as older age male predominance, high white blood cell count (WBC) and-
extramedullary disease, all of which indicate the need for increased intensity of chemotherapy (Pizzo and Poplack, 2002).

Figure 2.1. Blood cell development.
A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell (National Cancer Institute USA., 2010).

Cure rates of B-cell–progenitor ALL among children are very high, but they are not as high among adults. Approximately one fifth of children with B-cell–progenitor ALL have recurring disease, which is difficult to treat. Thus, this form of ALL varies with respect to the potential for relapse and resistance to chemotherapy. It is generally accepted that B-cell–progenitor ALL originates in B-cell–restricted progenitors that have accumulated critical genetic lesions. Cytogenetic studies of B-cell–progenitor ALL have revealed aberrations in chromosomal sites of regulatory molecules that are implicated in signaling and transcriptional regulation (Greaves and Wiemels, 2003). Approximately 10% of children diagnosed with ALL will die as a result of their disease (Canadian Cancer Society, 2007). compared to 91% in the 1960’s (Rivera et al., 1993).
2.2. Genetics of leukemia

2.2.1. Primary abnormalities

Acquired genetic changes are considered to be central to the development of leukemia. These changes affect the number (ploidy) and/or the structure of chromosomes, such as translocations, inversions, deletions, point mutations and amplifications (Hoffbrand et al., 2005). These genetic alterations contribute to the leukemic transformation of hematopoietic stem cells or their committed progenitors by changing cellular functions. They alter key regulatory processes by maintaining or enhancing an unlimited capacity for self-renewal, subverting the controls of normal proliferation, blocking differentiation, and promoting resistance to death signals (apoptosis) (Pui et al., 2003). The primary mechanisms of leukemia induction include aberrant activation of proto-oncogenes (e.g. MYC, TAL1, LYL1, LMO2 and HOX11) and generation of fusion genes encoding active kinases (e.g. BCR–ABL) or transcription factors (e.g. TEL–AML1, E2A–PBX1 and MLL linked to one of many fusion partners) (Hoffbrand et al., 2005).

2.2.1.1. B lineage ALL

The t (8; 14) is pathognomonic for the surface immunoglobulin (Ig)-positive B-ALL with L3 morphology (this is considered to be the leukemic form of Burkitt’s lymphoma). In B-ALL, the c-myc proto-oncogene is juxtaposed with Ig loci (IgH, IgK, or IgL), thereby expressing a dysregulated c-myc protein. In B-precursor ALL the t (1; 19) fuses the E2A and PBX-1 genes and produces a fusion protein that activates transcription. About 2–5% of children and up to 20% of adult cases with ALL have the Philadelphia chromosome (Phl, t9; 22) (Munker et al., 2007). TEL-AML1 and E2A-PBX1, is the HOX gene–mediated transcriptional cascade. The11 (11q23) MLL produce protein is required to maintain this transcription, whereas members of the polycom b group (PcG) family of proteins repress HOX gene transcription. Whose products influence self-renewal, proliferation, and differentiation of hematopoietic stem cells and their committed progenitors (Buske et al., 2000).
2.2.1.2. T-lineage ALL

In T lineage ALL a number of chromosomal aberrations are also found including t(1;14), t(11;14), and t(7;9). In t(1;14) the SCL (or TAL-1) gene is dysregulated. In such cases, the T-cell antigen receptor alpha/delta is juxtaposed with either the TTG1 or TTG2 loci. Transgenic mice that overexpress the TTG1 or TTG2 genes develop T-cell lymphomas. In leukemic cells with t(7;9), a truncated form of the TAN-1 protein is expressed (Munker et al., 2007). The TEL-AML1 transcription factor gene results from the translocation t(12;21). A prominent effect of the TEL-AML1 fusion protein is inhibition of the transcriptional activity (Hiebert et al., 1995). Indeed, LMO2 is one of genes activated in T-ALL (Munker et al., 2007).

2.3. Risk classification systems and risk-adapted therapy

Since intensification of treatment has contributed to the improvement in event-free survival (EFS) in children with ALL, children who have historically had a very good outcome are treated with modest therapy and spared toxicity, while allowing children with a historically lower probability of long-term survival to receive more-intensive therapy to maximize cure (Smith et al., 1996). The Pediatric National Cancer Institute (NCI) criteria were based on factors that had international acceptance and reproducibility, including age, initial white blood count (WBC) and the presence of extramedullary disease at diagnosis. Pediatric Oncology Group (POG) and Children’s Cancer Group (CCG) refined therapy based on additional risk factors such as ploidy, blast karyotype, and early morphologic response. As a result of the merger between CCG and POG, the Children’s Oncology Group (COG) developed a consensus classification strategy for treatment assignment based on the retrospective analysis of over 6000 children and adolescents with ALL from CCG and POG data. Based on this analysis, patients with precursor B-cell ALL are initially assigned to a standard-risk or high-risk group based on age and initial WBC (ages 1 to 9 years, and WBC < 50,000 cells/µL is considered standard risk) or age ≥10 years and/or WBC ≥50,000/µL are in higher risk (Smith et al., 1996; Schultz et al., 2007).
All children with T-cell disease phenotype are considered high risk regardless of age and initial WBC. Early treatment response and cytogenetics are subsequently used to modify initial risk-group classification. Patients are classified as very high risk if they have any of the following features (regardless of initial risk group): t(9;22) and/or BCR/ABL fusion gene, hypodiploidy with less than 44 chromosomes, and induction failure (Schultz et al., 2007). In a recent analysis of 706 children with B-lineage ALL enrolled in four consecutive treatment protocols at St. Jude Children’s Research Hospital from 1991 to 2006, those with hyperdiploidy (> 50 chromosomes), TEL-AML1 fusion gene and t(1;19) E2A-PBX1 fusion had the most favorable outcome, whereas those with the t(9;22) BCR-ABL or t(4;11) MLL-AF4 fusion had a very poor outcome (Pui et al., 2008). Different approaches to risk classification are being used by other cooperative groups.

The Berlin-Frankfurt-Munster Group (BFM) categorizes risk almost solely on treatment response criteria. Minimal residual disease (MRD) measurements at two time points are used in addition to prednisone prophase response. All patients with either t (9; 22) or t (4; 11) are considered high risk, regardless of marrow response (Schrappe et al., 2000). The Dana-Farber Cancer Institute (DFCI) ALL Consortium is testing a new risk classification system for patients with precursor B-cell ALL. Patients are initially classified according to age and WBC and presence of central nervous system (CNS) disease as standard or high risk children with ALL. CNS disease at diagnosis are a higher risk for treatment failure compared with patients not meeting the criteria for CNS disease at diagnosis (Dana-Farber Cancer Institute, 2007).

2.4. Clinical manifestation

ALL is involved with a wide range of symptoms as illustrated in Table 2.1.
Table 2.1. Clinical finding in acute lymphoblastic leukemia (Ciesla, 2007).

<table>
<thead>
<tr>
<th>Pathogenesis</th>
<th>Signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone Marrow Infiltration</strong></td>
<td>Neutropenia, Anemia, Thrombocytopenia</td>
</tr>
<tr>
<td></td>
<td>Fever, infection, Pallor, dyspnea, lethargy, Bleeding, petechiae, ecchymosis,</td>
</tr>
<tr>
<td></td>
<td>intracranial hematoma and gastrointestinal or conjunctival hemorrhage (rare)</td>
</tr>
<tr>
<td><strong>Medullary Infiltration</strong></td>
<td>Marrow</td>
</tr>
<tr>
<td></td>
<td>Bone pain and tenderness, limp, arthralgia</td>
</tr>
<tr>
<td><strong>Extramedullary Infiltration</strong></td>
<td>Liver, spleen, lymph nodes, thymus</td>
</tr>
<tr>
<td></td>
<td>Central nervous system</td>
</tr>
<tr>
<td></td>
<td>Organomegaly, Neurological complications including dizziness, headache, vomiting,</td>
</tr>
<tr>
<td></td>
<td>alteration of mental function</td>
</tr>
<tr>
<td>Gums, mouth</td>
<td>Gingival bleeding and hypertrophy, Lesions or granulocytic sarcoma</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
</tr>
</tbody>
</table>

2.5. Diagnosis of ALL

2.5.1. Laboratory studies

2.5.1.1. Blood count

First, a moderate to marked reduction of hemoglobin with normocytic and normochromic red cell morphology. Low hemoglobin indicates longer duration of leukemia, while higher hemoglobin indicates a more rapidly proliferating leukemia. Second, the white blood cell count may give a Low, normal or increased number. Third, blood smear usually shows very few to few blasts (in patient with leucopenia). When the WBC count is greater than 10000/mm, blasts are usually abundant. Eosinophilia is occasionally seen in children with ALL, while 20% of patients with AML have an increased number of basophils. Fourth, 92% of patients have platelets count below normal (thrombocytopenia) (Mehta and Hoffbrand, 2000).
2.5.1.2. Bone marrow investigation

The hallmark of the diagnosis of ALL is the blast cells, relatively undifferentiated cells with diffusely distributed nuclear chromatin, one or more nucleoli and basophilic cytoplasm (Mehta and Hoffbrand, 2000). Bone marrow is usually replaced by 80-100% blasts. Megakaryocytes are usually absent. Leukemia must be suspected when the bone marrow contains more than 5% blasts.

2.5.1.3. Immunophenotyping

A panel of monoclonal antibodies is used to differentiate ALL from AML. A further panel of B-and T-lineage markers and lymphocyte maturation markers are used to sub classify ALL (Table 2.2).

Table 2.2. Immunological classification of childhood acute lymphoblastic leukaemia (Provan, et al., 2004).

<table>
<thead>
<tr>
<th>Immunological subgroup</th>
<th>Immunophenotypic profile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B lineage</strong></td>
<td></td>
</tr>
<tr>
<td>Pro B-ALL</td>
<td>HLA-DR+, TdT+, CD19+ (5% children; 11% adults).</td>
</tr>
<tr>
<td>Common ALL</td>
<td>HLA-DR+, TdT+, CD19+, CD10+ (65% children; 51% adults).</td>
</tr>
<tr>
<td>Pre B-ALL</td>
<td>HLA-DR+, TdT+, CD19+, CD10+, cytoplasmic IgM+</td>
</tr>
<tr>
<td><strong>B-cell ALL</strong></td>
<td>HLA-DR+, CD19+, CD10+, surface IgM+ (3% children; 4% adults).</td>
</tr>
<tr>
<td><strong>T lineage</strong></td>
<td></td>
</tr>
<tr>
<td>Pre-T ALL</td>
<td>TdT+, cytoplasmic CD3+, CD7+ (1% children; 7% adults).</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>TdT+, cytoplasmic CD3+, CD1a/2/3+, CD5+ (11% children; 17% adults).</td>
</tr>
</tbody>
</table>
2.5.1.4 Morphological classification
According to the French–American–British (FAB), classification of ALL, L1 indicates a small monomorphic type with small homogeneous blasts, single inconspicuous nucleolus, regular nuclear outline; commonest subtype; L2 indicates a Large heterogeneous type, larger blasts, more pleomorphic and multinucleolate, irregular frequently clefted nuclei with conspicuous nucleoli and L3 Burkitt cell type indicates a large homogeneous blasts, abundant strongly basophilic cytoplasm with vacuoles; associated with B-cell phenotype (Provan et al., 2004).

2.5.1.5. Cytochemistry
ALL is frequently diagnosed by staining with Sudan black: ALL negative, AML positive, Enzymatic peroxides: ALL negative, AML positive and PAS: in ALL positive, AML negative (Mehta and Hoffbrand, 2000).

2.5.1.6. Cytogenetics
The demonstration that specific chromosomal abnormalities are associated with distinct subtypes of acute leukaemia has had enormous implications for the diagnosis and management of acute leukaemia and it is now clear that distinct cytogenetic abnormalities present in newly diagnosed patients with acute leukaemia provide vitally important prognostic information (Provan, 2003).

2.6. Management of ALL
Leukemia is mostly managed by 3 phases of chemotherapy (Mehta and Hoffbrand, 2000):
In the first phase, Remission – induction, main dose intensive combination chemotherapy is administrated to reduce or eradicate leukaemic cells from the bone marrow and re-establish normal haemopoiesis. Remission induction include comprise vincristine, prednisolone and L-asparaginase often with dounorubicin, cyclophosphamide. In children with ALL, rates of complete remission are ranging from 90 to 95 percent and rates of long-term disease-free survival, approaching 70 % can be achieved (Rivera et al., 1995). A second
consolidation therapy is applied with two or three intensification blocks with additional drugs.

Table 2.3. Description of the phases of chemotherapy in patients with acute lymphoblastic leukemia (Day and Henry, 2002).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Length of Treatment</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1 – Remission Induction</td>
<td>4-8 weeks</td>
<td>This phase is intended to put the patient into clinical remission. Ninety-five percent of children reach this endpoint.</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>Throughout entire</td>
<td>This starts during the induction phase and continues throughout therapy. This is designed to prevent relapse with leukemic meningitis.</td>
</tr>
<tr>
<td>Prevention Therapy</td>
<td>therapy</td>
<td></td>
</tr>
<tr>
<td>Phase 2 – Consolidation</td>
<td>3-9 months</td>
<td>This is designed to take care of the small number of leukemic lymphoblasts that remain after clinical remission is achieved.</td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 3 – Maintenance Therapy</td>
<td>Continues until</td>
<td>This is a less intensive continuation of the chemotherapy regimen, designed to keep patients in remission.</td>
</tr>
<tr>
<td></td>
<td>months 30-36</td>
<td></td>
</tr>
</tbody>
</table>

Finally patients receive maintenance chemotherapy for a further 2 to 3 years (Table 2.3) with daily mercaptopurine, weekly methotrexate and monthly vincristine and prednisolone. Thiopurines are very useful drugs, but they have a relatively narrow therapeutic index, with life-threatening myelosuppression as a major toxicity (Paterson and Tidd, 1975; Lennard, 1992). They are primarily used as antineoplastic and immunosuppressive agents. 6-Mercaptopurine, (IUPAC name: 3, 7-dihydropurine-6-thioneis) are components of essentially all modern treatment protocols for ALL (Krynetski et al., 1995).

In Gaza strip Maintenance chemotherapy for ALL according to Berlin-Frankfurt-Mu¨nster (BFM) 2002 protocol 6MP 50 mg/m2 /day and MTX tab 20 mg/m/weekly for 2- 3 years. Standard protocols for treatment with 6MP usually
involve initial administration of low doses followed by gradual increase (Annex1).

2.7. 6-mercaptopurine

2.7.1 Drug discovery
In 1942 George Hitchings set out to develop a treatment for cancer. He wanted to create chemicals that were similar enough to normal nucleotides to be used in cells but different enough to interfere with DNA synthesis. Gertrude Elion joined Hitchings in his quest in 1944. By 1951 she was producing numerous chemicals that resembled normal purines, some of which prevented cell division (Emory University, 2008).

Mercaptopurine (Figure 2.2) is available in tablet form for oral administration. Each scored tablet contains 50 mg mercaptopurine and the inactive ingredients microcrystalline cellulose NF, lactose monohydrate NF, stearic acid NF, and colloidal silicon dioxide NF (Roxane Laboratories, 2006).

![Chemical structure of 6-mercaptopurine](Wikipedia, 2008).

Figure 2.2. Chemical structure of 6-mercaptopurine (Wikipedia, 2008).

Systematic (IUPAC) name 3, 7-dihydropurine-6-thione
2.8. Metabolism of 6-mercaptopurine (6MP)

6-MP is a prodrug, which requires activation before it can exert its cytotoxic effects. As an analogue of hypoxanthine, it can act as a substrate for hypoxanthine-guanine phosphoribosyltransferase (HGPRT) leading to the formation of 6-thioinosine monophosphate (TIMP). Sequential reactions involving inosine monophosphate dehydrogenase and guanine monophosphate synthetase yields thioguanosine monophosphate (TGMP). Subsequent reactions by phosphokinases yield 6-thioguanosine diphosphate and triphosphate (TGDP and TGTP). TGMP, TGDP, and TGTP are collectively termed thioguanine nucleotides (TGNs) (Figure 2.3). Incorporation of 6-TGTP into DNA is believed to trigger cell death, probably by a process that involves the mismatch repair pathway (Swann et al., 1996; Waters and Swann, 1997).

Two enzymes compete with HGPRT to reduce the intracellular levels of 6-TGNs. The first, xanthine oxidase, results in the formation of thiouric acid. Although this is an important route of inactivation of 6-MP, as demonstrated by the potentiation of 6-MP toxicity by the inhibitor allopurinol, xanthine oxidase levels do not appear to vary greatly between individuals (Guerciolini et al., 1991).

This is in contrast with the second enzyme, thiopurine methyltransferase (TPMT), which inactivates 6-MP through the formation of methyl mercaptopurine (Weinshilboum and Sladek, 1980). 6-TGNs have an average half-life of approximately 5 days (range 3–13 days) (Schwab and Klotz, 2001). And they can all be methylated by TPMT. 6-TIMP can be methylated by TPMT to form 6-methyl thioinosine 5’-monophosphate (6-MTIMP), 6-methyl thioinosine5’-diphosphate (6-MTIDP) and 6-methyl thioinosine 5’-triphosphate (6-MTITP). The last three metabolites listed are the so-called 6-methylmercaptopurine ribonucleotides (6-MMPRs). 6-TIMP can also be phosphorylated by monophosphate kinase to form 6-thioinosine5’-diphosphate (6-TIDP), then by diphosphate kinase to produce 6--thioinosine 5’-triphosphate (6-TITP), and ultimately back to 6-TIMP following an enzymatic reaction catalyzed by inosine triphosphate pyrophosphatase (ITPase). The metabolism of 6-TG is less complex than that of 6-MP and AZA (GlaxoSmitKline 2004).
2.9. Pharmacogenetics plays a role in drug toxicity and efficacy

Pharmacogenetics aims to use knowledge of these variations to ‘tailor’ therapy for improved response and reduced toxicity. The term "pharmacogenetics" represents the study of genetic factors that influence response to drugs and chemicals and was first termed in 1959 (Meyer, 2004). Most research so far has focused on single polymorphisms. A more comprehensive approach to predict treatment response will be to consider genetic variation in entire biological and pharmacological pathways. Genetic variations are the result of multiple mechanisms such as single nucleotide polymorphisms (SNPs) (over 90%), insertion, deletion, tandem repeats, and microsatellites. In an attempt to individualize therapy, pharmacogenetics is used in search for answers to the hereditary basis for individual differences in drug response. Some drugs require metabolism to be activated or inactivated. Some enzymes involved in the
biotransformation have polymorphic expression. Pharmacogenetics causes variability in the metabolism of MP (Figure 2.4), due to polymorphic variation in the activity of the TPMT enzyme (Lennard, 1992; Klemetsdal et al., 1992). TPMT deficiency is inherited as an autosomal recessive trait.

**Figure 2.4. Five stages of pharmacogenetics and pharmacogenomics in cancer therapy** (Huang and Ratain, 2009).

### 2.10. Thiopurine S-methyltransferase

Thiopurine methyltransferase (TPMT) is a cytoplasmic transmethylase present in prokaryotes and eukaryotes. Originally found in the kidney and liver of rats and mice, it was subsequently shown to be present in most human tissues, for example, heart, blood cells, placenta, pancreas, and intestine. Human TPMT has a molecular mass of 28 kDa, comprises 245 amino acids, and is not metal-dependent and TPMT is encoded by a 34 kb gene consisting of 10 exons and 9 introns and has been localized to chromosome 6p22.3 (Figure 2.5) (Krynetski et al., 1996; Szumlanski et al., 1996).

TPMT catalyses methylation of mercaptopurine, thioguanine, and thiopurine nucleotides and nucleosides (Deininger et al., 1994; Krynetski et al., 1995). The first studies of the enzyme that catalyzed this reaction were performed by Remy in the early 1960s using rodent tissue (Remy, 1963). However, it was not until the late 1970s that TPMT activity was first assayed and studied in human tissue (Weinshilboum et al., 1978), with the clearly stated goal of testing the
hypothesis that individual variation in this pathway for thiopurine biotransformation might be related to individual differences in drug toxicity and/or therapeutic efficacy. The first application of measurements of RBC TPMT activity involved pharmacogenetic experiments performed with large population samples and nuclear families (Weinshilboum and Sladek, 1980). Those studies demonstrated that the "trait" of level of RBC TPMT activity was controlled by a common genetic polymorphism.

Figure 2.5. Position of TPMT gene on chromosome 6 (Weizmann Institute of science, 2007).

The TPMT genotype is classified into three types: a wild type that has wild/wild alleles, a heterozygous type that has wild/mutant alleles, and a homozygous mutant type that has mutant/mutant alleles (Weinshilboum and Sladek, 1980). TPMT is also called S-adenosyl-L-methionine:thiopurine S-methyltransferase, thiopurine S-methyltransferase and Thiopurine methyltransferase the common name.

2.11. Molecular basis for altered TPMT activity

In humans, the molecular basis of the TPMT polymorphism is largely related to three common nonsynonymous coding single-nucleotide polymorphisms (Otterness, et al., 1997), each of which renders the protein unstable (Tai et al., 1997) and subject to enhanced ubiquitination and degradation (Tai et al., 1999; Wang et al., 2003). TPMT enzymatic activity is controlled by a common genetic polymorphism (Otterness et al., 1997). Thus, the homozygous deficiency in humans is characterized by almost undetectable levels of TPMT protein; heterozygotes have intermediate protein and activity levels and homozygous wild-type individuals have high levels of protein and activity (Szumlanski et al., 1996; Loennechen et al., 1998).
TPMT enzyme activity in the population is highly variable. Previous study suggested that about 90% of individuals have high/normal activity of more than 10 U/ml blood (reflecting two active alleles), about 10% have intermediate TPMT activity of between 5 and 10 U/ml blood (reflecting only one active allele) and 0.3% (about 1 person in 300) have low/absent activity due to two inactive alleles (Wang and Weinshilboum, 2006). The latter are defined as having less than 5 U/ml blood of TMPT activity and are commonly referred to as ‘poor TPMT metabolizers. To date, at least 23 TPMT alleles have been classified, including TPMT*1 (wild-type allele), which encodes the enzyme with normal activity. Three variant alleles TPMT*2 (a single nucleotide transversion in exon 5, 238G>C), TPMT*3A (two transition mutations, one in exon 7, 460G>A and the other in exon 10, 719A>G) and TPMT*3C (a single transition mutation in exon 10, 719A>G) are characterizing the three most common defective alleles (Weinshilboum and Sladek, 1980; Jones et al., 2007).

2.12. Genetic polymorphism and alleles of TPMT

These variants result from point mutations in the TPMT open-reading frame or at intron/exon splice sites. The wild-type allele, TPMT*1, encodes active enzyme. Molecular studies have identified 3 variant alleles that together account for >95% of reduced TPMT activity: TPMT*2 (238G>C), at codon 80 leading to an amino acid substitution (Ala>Pro), TPMT*3A (460G>A) at codon 154 (Ala>Thr) and (719A>G) at codon 240 (Tyr>Cys), and TPMT*3C (719A>G) (Figure 2.6) (Yates et al., 1997; Evans et al., 2004). Individuals with 2 variant alleles have low or no TPMT activity, while those with 1 variant allele have intermediate TPMT activity. Wild-type (TPMT*1) homozygotes, on the other hand, have normal enzyme activity. TPMT*3A and *3C appear to be common variant alleles associated with low enzyme activity (Krynetski and Evans, 2000).
Figure 2.6. Allelic variants of the TPMT gene

Boxes depict exons in the open reading frame of the human TPMT gene. The positions of the three point mutations detected by PCR-based assays are indicated (Coulthard et al., 1998).

2.13. Relationship between TPMT and toxicity

Variation in the expression and function of genes involved in drug metabolism has been associated with treatment response in children with ALL. For example, polymorphisms and the activity of TPMT influence response to this class of drugs (Evans and Relling, 2004; Stanulla et al., 2005). TPMT enzymatic activity usually shows a trimodal distribution of high, intermediate and very low activity among the population (Weinshilboum and Sladek, 1980; Krynetski and Evans, 1999). The enzyme is known to exhibit an autosomal codominant polymorphism with 89-94% of all individuals studied having a high activity, 6-11% an intermediate activity and 0.3% having a very low or non-detectable activity (Tai et al., 1996; McLeod et al., 2000).

A number of polymorphic variants of the TPMT gene encode enzymes with significantly reduced activity. Approximately 0.3% are homozygous for non-
functional TPMT alleles and the consequent absence of TPMT activity is associated with severe, and potentially fatal, thiopurine-induced haematological toxicity and bone marrow suppression (Marshall, 2003). This may be manifested by leukopenia, thrombocytopenia, or any combination of these. Pre-treatment screening for inactive TPMT alleles can identify individuals at risk of severe bone marrow toxicity. Substantial dose reductions are generally required for homozygous-TPMT deficiency patients (two non functional alleles) to avoid the development of life threatening bone marrow suppression (FDA, 2004). Heterozygosity for a low activity allele identifies patients in whom lower doses of thiopurine can be used safely. Although heterozygous patients with intermediate TPMT activity may have increased mercaptopurine toxicity, this is variable, and the majority of patients tolerate normal doses of purinethol. Even normal TPMT activity does not abolish the risk of myelosuppression, however close monitoring of haematological parameters is still required. For this reason, whether knowledge of TPMT status provides significant practical benefits have been questioned (Marshall, 2003). If a patient has clinical or laboratory evidence of severe toxicity, particularly myelosuppression, TPMT testing should be considered (FDA, 2004). A dose reduction of approximately 90% is required for patients with the homogeneous mutation and dose reductions of 40% may be required for patients with the heterogeneous mutant (Table 2.4) (COG, 2008).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Activity Level</th>
<th>Frequency in the population</th>
<th>Dose Adjustments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>High TPMT activity</td>
<td>89%*</td>
<td>Start at normal dose, may need to increase**</td>
</tr>
<tr>
<td>Heterogeneous Mutant</td>
<td>Intermediate TPMT activity</td>
<td>11%*</td>
<td>Start at 60% of normal dose**</td>
</tr>
<tr>
<td>Homogenous Mutant</td>
<td>TPMT deficiency</td>
<td>0.33%*</td>
<td>Start at &lt;10% of normal dose**</td>
</tr>
</tbody>
</table>

*Weinshilboum and Sladek, 1980
** Children’s Oncology Group, 2008
2.14. Ethnic variations in TPMT mutations

Although the overall prevalence of TPMT deficiency is similar between different ethnic groups, the frequency of variant alleles differs between different populations, for example, there is a higher representation of the TMPT*3A allele in South Asians compared with the high frequency of the TPMT*3C in East and West Africans (McLeod and Siva, 2002). The TPMT*1 (wild-type allele, 96%) and TPMT*3A (460G>A and 719A>G, 4%) alleles are the most common in Caucasians (Geary et al., 2003).

2.15. Difference between phenotype and genotype

TPMT phenotypes for patients who received a blood transfusion within 30 days prior to testing may reflect activity of the blood donor rather than the blood recipient. The TPMT enzyme is unstable. Blood specimens must be processed within three days of collection, and blood must be kept refrigerated. The TPMT enzyme can be inhibited by several common drugs. In contrast the genotype for TPMT cannot be inferred from TPMT activity (phenotype) (Arup Laboratories, 2008).

2.16. Frequency of TPMT allele distribution in different populations

Several studies aimed at determining the allelic variation of the TPMT in different populations. Some of these are summarized in Table 2.5.
Table 2.5: Summary of different studies of TPMT allele variation in different populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of alleles</th>
<th>*2</th>
<th>*3A</th>
<th>*3C %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>French</td>
<td>382</td>
<td>_</td>
<td>5.7</td>
<td>0.8</td>
<td>(McLeod, 2002)</td>
</tr>
<tr>
<td>British Caucasian</td>
<td>398</td>
<td>0.5</td>
<td>4.5</td>
<td>0.3</td>
<td>(Collie-Duguid, 1999)</td>
</tr>
<tr>
<td>Italian</td>
<td>412</td>
<td>0.4</td>
<td>3.9</td>
<td>1</td>
<td>(Rossi, 2001)</td>
</tr>
<tr>
<td>Norwegian</td>
<td>132</td>
<td>_</td>
<td>3.4</td>
<td>0.3</td>
<td>(Loennechen, 2001)</td>
</tr>
<tr>
<td>African-American</td>
<td>496</td>
<td>0.4</td>
<td>0.8</td>
<td>2.4</td>
<td>(Hon, 1999)</td>
</tr>
<tr>
<td>Caucasian-American</td>
<td>564</td>
<td>0.2</td>
<td>3.2</td>
<td>0.2</td>
<td>(Hon, 1999)</td>
</tr>
<tr>
<td>Japanese</td>
<td>384</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>(Hiratsuka, 2000)</td>
</tr>
<tr>
<td>Brazilian</td>
<td>408</td>
<td>2.2</td>
<td>1.5</td>
<td>1</td>
<td>(Boson, 2003)</td>
</tr>
<tr>
<td>South-east Asian</td>
<td>698</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>(Chang, 2002)</td>
</tr>
<tr>
<td>Turkish</td>
<td>212</td>
<td>0</td>
<td>0.9</td>
<td>0.9</td>
<td>(Tumer, 2007)</td>
</tr>
<tr>
<td>Swedish</td>
<td></td>
<td>0</td>
<td>3.7</td>
<td>0.4</td>
<td>(Haglund, 2004)</td>
</tr>
<tr>
<td>Mexican</td>
<td>218</td>
<td>0.9</td>
<td>3.2</td>
<td>1.4</td>
<td>(Taja-Chayeb, 2008)</td>
</tr>
<tr>
<td>Iranian</td>
<td>254</td>
<td>3.93</td>
<td>0.87</td>
<td>1.57</td>
<td>(Azad M, et al., 2009)</td>
</tr>
</tbody>
</table>

In our region the frequencies of TPMT risk alleles differed significantly among the Druze who showed fivefold and twofold higher frequencies than Jews and Moslems, respectively. Specifically, allelic frequencies of TPMT*3A were 0.73% 0.79% and 3.19% in Jews, Moslems and Druze, respectively. Although not found in Jews, TPMT*3C was found at an allelic, frequency of 1.05% and 0.75% in Moslems and Druze. TPMT*2 and TPMT*3B were not detected in any of the studied subpopulations studied (Efrati et al., 2009).
2.17. Prevalence of childhood acute lymphoblastic leukemia in Gaza Strip

All pediatric patients age between 0–12 years who have either suspected or confirmed acute leukaemia should be recently referred to Abd El Aziz Al Rantisi hospital Gaza, assessment, and the type of treatment given, will depend on the patient’s age and condition. In the past, assessment pediatric ALL patients were treated in AL-Nasser Hospital and European Gaza Hospital. Adult ALL patient with ages over 12 years are treated in Al Shifa hospital. The number of patients with childhood acute lymphoblastic leukemia admitted to AL-Nasser Hospital Gaza in 2006 was 10 patients, in 2007, 9 patients and in the European Gaza Hospital in 2006, 5 patients and in 2007, 5 patients. In 2008, 15 pediatric patient and in 2009, 10 patients were recorded in Abd El Aziz Al Rantisi hospital (Figure 2.7).

The peak incidence in children is at age 3-5 years according to Palestinian Ministry of Health hospital records. Incidence of ALL in children in Gaza strips 2 cases per 100000 children. Most patients diagnosed in the department of hematology as ALL, were referred to Telhashomir hospital for immunophenotyping and cytogenetic.

![Figure 2.7. Distribution of pediatric ALL patients in Gaza Strip from year 2006 to 2009.](image-url)
Chapter Three

MATERIALS AND METHODES

3.1. Study population

In this descriptive study, children suffering from and being managed for acute lymphoblastic leukemia in European Gaza hospital, AL-Nasser hospital and Abd El Aziz Al Rantisi hospital Gaza (56 patients) in the period between July 2008 to August 2009 were included (Convenient sample with definite time period). Other types of leukemia were excluded. All samples were processed and analyzed in the genetics laboratory at the Islamic University of Gaza.

3.2. Ethical Consideration

The objective of the study was explained to all participants and their families and their consent was taken. Permission was obtained from Helsinki committee and MOH for specimen collection and performing the study (Annex 2).

3.3. Materials

3.3.1. Chemicals and reagents

The chemicals and reagents used in this study are summarized in table 3.1.

Table 3.1. A list of chemicals and reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Hy lab</td>
</tr>
<tr>
<td>Wizard Genomic DNA purification Kit</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>100 bp DNA Lader</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>PCR Master Mix</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Restriction enzymes MwoI ,Accl</td>
<td>Biolabs</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Isopropyl Alcohol</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Agarose</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Ethidume bromide</td>
<td>Promega, USA</td>
</tr>
</tbody>
</table>
3.3.2. Disposables

The major disposables used in this study are listed in Table 3.2.

Table 3.2. List of disposables

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro Tubes, 1.5ml Capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>Micro Tubes, 0.2ml Capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>Micropipettes tips</td>
<td></td>
</tr>
<tr>
<td>Disposables Powder Free Gloves</td>
<td>Weihai Sun Genius - China</td>
</tr>
<tr>
<td>2.5 K3-EDTA Tube</td>
<td>Canelli, Italy</td>
</tr>
</tbody>
</table>

3.3.3. Major equipments used in the present study

The major equipments that were used are listed in Table 3.3.

Table 3.3. A list of major equipments used in the present study

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermocycler</td>
<td>Eppendrof, Germany</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Bio RAD, USA</td>
</tr>
<tr>
<td>Micocentrifuge</td>
<td>Centurrion Scientific LTD, UK</td>
</tr>
<tr>
<td>Water Bath</td>
<td>Pselecta, Span</td>
</tr>
<tr>
<td>UV-transilluminator</td>
<td>Scie – pals LTD, UK</td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>Nanodrop, USA</td>
</tr>
<tr>
<td>Microwave</td>
<td>LG, Korea</td>
</tr>
<tr>
<td>Refrigerator -70 ºC</td>
<td>Napco, Czech Republic</td>
</tr>
<tr>
<td>Refrigerator -20 ºC</td>
<td>LG, Korea</td>
</tr>
<tr>
<td>Vortex Mixer</td>
<td>Turbo Mixer, Georgia</td>
</tr>
<tr>
<td>Micropipette</td>
<td>Lab Mate, Poland</td>
</tr>
<tr>
<td>Safety Cabinet</td>
<td>Heraeus, Germany</td>
</tr>
</tbody>
</table>

3.4. Methods

3.4.1. Blood collection

Whole blood samples were collected from 56 patients in EDTA tubes. Approximately 2.5 ml venous blood samples were collected in each tube from each patient.
3.4.2. DNA extraction

DNA was extracted from blood samples using a commercial kit from promega. Briefly, RBCs from 300 µl blood samples were lysed using the provided buffer and the WBCs were pelleted by centrifugation for 20 seconds at 14000 rpm. The WBCs were then lysed using the nuclei lyses solution (provided) and proteins were precipitated using a provided protein precipitation buffer and centrifugation. The supernatant containing the nucleic acid was transferred to a new tube containing an equal volume of isopropanol and the DNA was pelleted by vortex mixing and subsequent centrifugation. The pelleted DNA was washed by 70 % ethanol and then resuspended in 100 µl of the provided alkaline hydration solution at 65 ºC.

3.4.3. TPMT genotyping

A total of 56 DNA samples from patients with ALL were analyzed. Total genomic DNA extracted from peripheral leucocytes was processed by PCR either immediately or stored at -20 ºC until being used. An allele-specific PCR was used to analyze the 238G>C (TPMT *2) mutation in exon 5, using sequence specific primers (Table 3.4). Two separate reactions were performed for each subject; one for the wild type genotype using (P2W) and (P2C) primers. And the other is mutation specific using (P2M) and (P2C). PCR amplifications were carried out in 20 µl final volumes containing 0.5 µM of each primer, 5 µl DNA, 3 µl H2O and 10µl 2X master mix. The cycling conditions consisted of initial denaturation at 94 ºC for 2 minutes followed by 35 cycles of 94 ºC for 30 second, 53 ºC for 30 second, and 72 ºC for 30 second, and a final extension at 72 ºC for 2 minutes.
Table 3.4 Description of primers and restriction enzyme and their generated DNA fragments.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence (5’ to 3’)</th>
<th>Location</th>
<th>Polymorphism</th>
<th>Type of mutation</th>
<th>PCR product</th>
<th>Restriction enzyme</th>
<th>Cut product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2W</td>
<td>GTATGATTTTATGCAAGGTTG TTAATAGGAACCACCTGGACAC</td>
<td>Exon 5</td>
<td>238G&gt;C</td>
<td>TPMT-2</td>
<td>254 bp</td>
<td>Allele specific</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2M</td>
<td>GTATGATTTTATGCAAGGTTTC</td>
<td></td>
<td></td>
<td></td>
<td>254 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P460F</td>
<td>GGGACGCTGCTCATTCTCTGCCTTACACCCAGGCTCTTG</td>
<td>Exon 7</td>
<td>460G&gt;A</td>
<td>TPMT-3A</td>
<td>338 bp</td>
<td>MwoI</td>
<td>114 bp and 224 bp</td>
<td>338 bp</td>
</tr>
<tr>
<td>P460R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P719F</td>
<td>AAGTGTTGGATTACAGGTTGCCTCAAAAACATGTCACTG</td>
<td>Exon 10</td>
<td>719A&gt;G</td>
<td>TPMT-3C</td>
<td>273 bp</td>
<td>AccI</td>
<td>273 bp</td>
<td>213 bp and 60 bp</td>
</tr>
<tr>
<td>P719R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

W, wild type-specific; M, mutant-specific; C, common primers.

The mutation (719A>G) was determined by polymerase-chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR amplifications were carried out in 20 μl final volume containing 0.5μM of the primers P719F and P719R (Table 3.4), 5 μl DNA, 3 μl H2O and 10μl master mix. The cycling conditions consisted of initial denaturation at 94 ºC for 2 minutes followed by 35 cycles of 94 ºC for 1 minute, 56 ºC for 1 minute, 72 ºC for 1 minute and final extension at 72 ºC for 2 minutes. The PCR products of exon 10 were digested with the restriction enzyme AccI to detect the A719G mutation. Eight micro liters of the PCR product were incubated with 10 U/ml of AccI enzyme and 1 μl of 10x reaction buffer. The samples were incubated for 1 hr at 37 ºC. All PCR products and digestion fragments were visualized on a 2.5% agarose gel and stained with ethidium bromide. The PCR products size and the digestion fragments in case of wild type or mutant are shown in (Table 3.4).

The 460G>A mutation was detected by PCR-RFLP analysis. PCR amplifications were carried out in 20 μl final volume containing 0.5 μM of the
primers P460F and P460R (Table 3.4), 5 µl DNA, 3 µl H2O and 10µl master mix. The cycling conditions consisted of initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute and final extension at 72°C for 2 minutes. The PCR products of exon 7 were digested with the restriction enzyme MwoI to detect the 460G>A mutation. Eight microliters of PCR product were incubated with 5 U/ml of MwoI enzyme and 1 µl of 10x reaction buffer. The samples were incubated for 1 hr at 60°C. All PCR products and digestion fragments were visualized on a 2% agarose gel stained with ethidium bromide. The PCR products size and the digestion fragments in case of wild type or mutant are shown in Table 3.4 (Zhang et al., 2004).

3.4.4. Data collection and analysis
Relevant medical information of the participants was collected from the patient's records in the hospitals. The collected data included age, duration of 6MP treatment, and complication after treatment with 6MP, type of leukemia, WBC, Platelets count, hemoglobin and living area. The genotyping results and other data were analyzed using the SPSS software (Version. 17). Frequency distribution, Chi-square test, t test were used with 95% confidence. Microsoft Excel 2003 software was used for figures.

Limitations
As a result of Israeli shelling of laboratories at the Faculty of Science at the Islamic University, all samples and the results were destroyed so I repeat sampling and testing. In previous results, one patient sample was found to be heterozygous mutant in exon 7, 460G>A and the other in exon 10, 719A>G.

I was unable to show the picture for the PCR product on agarose gel and digestion with enzyme. I tried to take another sample from the patient but the patient died.

Another problem facing researchers in Gaza Strip is that there is no health data base system available.
Chapter Four

RESULTS

4.1. Description of the study population

In this study, the sample included 56 pediatric patients suffering from ALL; 32 (57.1%) were male and 24 (42.9%) were females (Figure 4.1). Their ages at the time of diagnosis varied between 6 month to 12 years. The mean age was 4.4 ± 2.6 years (Figure 4.2). The majority of the samples were between the range of 3.7 - 5.1 years with 95% Confidence interval. All patients were diagnosed as ALL patients by blood film, bone marrow aspiration and complete blood cell count (CBC).

Figure 4.1. Distribution of patients by gender
Figure 4.2. Distribution of patients by age

In the present study the calculated incidence of ALL among children in Gaza strip during the specified period of samples collection were 2 patients per 100000 children. The incidence of ALL is higher in males than in females.
4.2. Management of patients

During the collection of the sample, most of the patients (75%) were given 6MP for different periods of time and 25% of them finished having the 6MP treatment.

Among patients who were receiving 6MP at the time of the sample collection (42 patients), 6 patients suffered from leucopenia, 5 patients from leucopenia and thrombocytopenia, 3 patients from leucopenia, thrombocytopenia and liver toxicity, 3 patients had only liver toxicity, one patient with neuroblastoma and one patient had mild brain atrophy (Table 4.1). Bone marrow transplantation was performed for 2 patients besides the 6MP treatment.

Table 4.1. Complications associated with 6MP treatment. Patients recorded at the time of the sample collection.

<table>
<thead>
<tr>
<th>Complications</th>
<th>Duration of treatment</th>
<th>Total No.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; One year</td>
<td>1-2 years</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>leucopenia</td>
<td>5</td>
<td>15.6</td>
<td>1</td>
</tr>
<tr>
<td>Leucopenia and thrombocytopenia</td>
<td>4</td>
<td>12.5</td>
<td>1</td>
</tr>
<tr>
<td>liver toxicity</td>
<td>2</td>
<td>6.3</td>
<td>1</td>
</tr>
<tr>
<td>mild brain atrophy</td>
<td>1</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>neuroblastoma</td>
<td>1</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>leucopenia, thrombocytopenia and liver toxicity</td>
<td>3</td>
<td>9.4</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>16</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

On comparing the duration of 6MP treatment period with the occurrence or non-occurrence of toxicity at the time of sampling there was no clear statistically significant relationship (p-value = 0.87) (Table 4.1).

The number of patients who take 6MP drug for less than one year with complications were more than the patients with one to two years of treatment.
The mean duration for the group with different adverse effect (42 patients) was 5.4 ± 5.8 months compared to 8.6 ± 6.5 months for those with no adverse effect (p-value = 0.105).

### 4.3. Genotyping of DNA samples

After the samples were collected, DNA was extracted and PCR amplified and the genotype for the most common three polymorphic alleles was determined by RFLP. Allele specific PCR was performed to detect TPMT*2 (238G>C) genotypes. As represented in Figure 4.3. PCR products yielded no heterozygous and homozygous mutated genotypes in the sample population.

![Electrophoresis patterns for TPMT*2 (238G>C) allele analyzed by allele-specific PCR.](image)

Lanes 1-4 show wild type specific allele amplification (254 bp) and lanes 5-8 show no amplification of the same samples using mutant specific primers. L, 100 bp DNA ladder; C: water negative control.

The PCR- RFLP method was performed to genotype mutations in exon 7, 460G>A and the other in exon 10, 719A>G. The PCR product was digested with the restriction enzyme MwoI to detect the 460G>A mutation. In the wild type the enzyme digestion products are two bands of 114 bp and 224 bp (Figure 4.4). The Accl enzyme was used to detect the 719A>G mutation. In wild type the enzyme doesn't digest the PCR product (Figure 4.5).
Figure 4.4. Electrophoreses pattern for detection 460G>A.

2% agarose gel stained with ethidium bromide showing the PCR product of a representative experiment L = 100 bp DNA ladder, C: is a water negative control; lanes 1 - 11 show the PCR product after digest with restriction enzymes MwoI to detect the 460G>A mutation, two band of 114 bp and 224 bp means wild type.

Figure 4.5. PCR product digest with restriction enzymes AccI to detect the 719A>G mutation.

2% agarose gel stained with ethidium bromide showing the Lanes 1-13 product undigested, wild type allele Product length 273(bp). C: is a water negative control.

The TPMT*3A variant was observed in only one sample allelic frequency = (0.89%) which was heterozygous for both the 460G>A and 719A>G loci and the remaining had the wild type allele. No polymorphism was detected at the TPMT*2 position and TPMT*3C in our study population (Table 4.2).
Table 4.2. Frequencies of TPMT alleles in 56 samples of Gaza pediatric patient with ALL

<table>
<thead>
<tr>
<th>Allele polymorphism</th>
<th>SNPs</th>
<th>Amino acid substitution</th>
<th>N0. (n =112)</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMT*1</td>
<td>Wild-Type</td>
<td>(Ala&gt;Pro)</td>
<td>111</td>
<td>99.11%</td>
</tr>
<tr>
<td>TPMT*2</td>
<td>238G&gt;C</td>
<td>(Tyr&gt;Cys)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPMT*3C</td>
<td>719A&gt;G</td>
<td>(Ala&gt;Thr), (Tyr&gt;Cys)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPMT*3A</td>
<td>460G&gt;A, 719A&gt;G</td>
<td></td>
<td>1</td>
<td>0.89%</td>
</tr>
<tr>
<td>Total of alleles tested</td>
<td></td>
<td></td>
<td>112</td>
<td>100%</td>
</tr>
</tbody>
</table>

The 11 cases of transient leucopoenia were resolved with dose reduction and one patient remained with leucopoenia and thrombocytopenia after dose reduction. Two patients did not respond well to steroid neither to chemotherapy. Allogenic bone marrow transplantation was done for two patients. There is no association between genotype and clinical and laboratory data obtained during chemotherapy maintenance phase. Fourteen patients developed adverse effects, characterized by rapidly developing severe myelosuppression. Adverse events consisted of leucopoenia and thrombocytopenia (WBC<3,000/μL). Thirteen patients were with normal TPMT genotype and one patient had a heterozygous mutated genotype.
Chapter Five

DISCUSSION

Since inherited decrease of TPMT activity results in potentially life-threatening clinical consequences for patients treated with thiopurine drugs, a need for estimating TPMT enzyme activity emerged. TPMT genotyping is highly sensitive and specific alternative to expensive TPMT enzyme activity determination (Kapoor et al., 2009). The genetic polymorphism of TPMT is one of the most developed examples of pharmacogenetics (Krynetski and Evans, 1998; McLeod et al., 2000). Over 23 polymorphisms in the TPMT gene have been associated with a decrease in enzyme activity (Schaeffeler et al., 2006). Approximately 90% of individuals having high activity, 10% having intermediate activity, and 0.3% having low or undetectable enzyme activity. TPMT activity has been shown to correlate with 6MP toxicity and therapeutic efficacy (Yong and Innocenti, 2007). For this reason, knowledge of the main defective Single Nucleotide Polymorphism frequencies in a population is essential to estimate the proportions of risk groups under 6MP therapy.

From this point of view, in the current study, the frequencies of three variant TPMT alleles (TPMT*2, TPMT*3C, TPMT*3A) accounting for more than 80% of all low activity cases world wide were determined in pediatric ALL patients in Gaza Strip.

Only the TPMT*3A allele was detected in one patient from 56 DNA samples of pediatric ALL patients in Gaza strip. The frequency of TPMT*3A allele is 0.89%. No TPMT*2 or *3C alleles were detected.

The overall prevalence of TPMT activity deficiency is similar between different ethnic groups. While the frequency of variant alleles differs between different populations. For example, there is a higher representation of the TMPT*3A allele in South Asians compared with the high carriage of TPMT*3C in East and West Africans (McLeod and Siva, 2002). Our study is in consistence with a study conducted in "Israeli" subpopulations that showed allelic frequencies of TPMT*3A of 0.73%, 0.79% and 3.19% in Jews, Moslems and Druze, respectively (Efrati et al., 2009).
In the same study, although not found in Jews, the TPMT*3C was found at an allelic, frequency of 1.05% and 0.75% in Moslems and Druze. TPMT*2 and TPMT*3B were not detected in any of the "Israeli" subpopulations studied. The frequency of TPMT*3A allele is also similar in Turkish population (0.9%) (Tumer, et al., 2007). In Iranian population the frequency of TPMT*3A is 0.87% (Azad et al., 2009). TPMT*3A and TPMT*3C were the only detected deficiency alleles in the Jordanian population with an allelic frequency of 0.59% and 0.30% respectively (Hakooz, 2010). On the other hand, the TPMT*3A allele frequency in Gaza strip (0.89%) was lower than the French (5.2%), Italian (3.9%) and Brazilian population (1.5%) (Rossi, 2001; Mcleod, 2002; Boson, 2003).

In the present study 14 patients developed mylotoxicity and they all had a normal TPMT genotype except one patient who had a heterozygous TPMT*3A allele characterized by rapidly developing severe myelosuppression (WBC<2,000/ìL). In the relationship between duration of 6MP and complications among pediatric ALL patients in Gaza Strip after one year and two years of treatment didn’t reach a statistically significant value (P-value =0.87). The researcher believe that this lack of correlation may be a result of the small population size.

It is note worthy to mention that not all cases of myelosuppression are due to a mutation in the gene coding for the TPMT enzyme and therefore, not all cases can be prevented by screening for TPMT status with either the enzymatic assay or genotype test (Marra et al., 2002). The presence of toxicity in a number of cases and the lack of common types of mutations confirms the existence of other types of mutations or other factors (Fankoury et al., 2007). This was obvious from our results as well. For example the inosine triphosphatase (ITPA) IVS2+21A→C variant alleles were significantly associated with lower platelet counts in ALL subjects (Hawwa et al., 2008). In addition, a part from genotype, environmental factors are also important in influencing TPMT activity and for the observed intra-individual variability in response seen in patients receiving thiopurines.

In Gaza pediatric hospitals ALL patients are treated according to BFM – 2002 protocol. In Gaza pediatric hospitals standard protocols for treatment with 6MP usually involve initial administration of low doses followed by gradual increase (Annex 1). But no TPMT or other metabolizing enzymes investigation.
Several previous studies showed that enzyme deficiency also confers a higher risk of developing therapy-related acute myeloid leukemia and radiation-induced brain tumors in patients receiving thiopurines (Evans, *et al.*, 2004). On the other hand, patients with high levels of enzyme activity might be at greater risk of relapse due to a decrease in exposure of leukemic cells to active drug metabolites (Stanulla *et al.*, 2005). And may require treatment with higher than standard doses, as well as very recent evidence that TPMT gene duplication can occur in leukemia cells with possible implications for drug response (Cheng *et al.*, 2005).
Chapter Six

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The present study is the first analysis of the polymorphisms of the TPMT gene in acute lymphoblastic leukemic childhood patients in Gaza strip.

TPMT*3A was the only deficiency alleles detected in the pediatric ALL patients in Gaza strip with an allelic frequency of 0.89%.

TPMT mutations are not associated with myelosuppression in ALL pediatric patients in Gaza strip.

Other types of mutation in TPMT gene my be responsible for myelotoxicity or factors other than TPMT polymorphisms may be responsible for the development of toxicity.

On light of our study and others, myelosuppression due to 6 mercaptopurine treatment is not always associated with TMPT gene mutations while the determination of the TPMT genotype may be useful for predicting myelosuppression in patients with TPMT homozygotes and heterozygotes.

6.2. Recommendations

Even in ALL patients with a wild TPMT genotype, clinicians should pay attention for the possible development of myelosuppression.

Further studies with more participants and analyzing more TPMT and/or other metabolizing enzymes alleles, will be needed to establish a nation wide pretreatment strategies among patients in Gaza Strip.
References:


Appendix

Annex 2

Palestinian National Authority  
Ministry of Health  
Helsinki Committee  

12/9/9

Name:
I would like to inform you that the committee has discussed your application about:
Thiopurine Methyltransferase Genotyping in Childhood Acute Lymphoblastic Leukemia in Gaza Strip

In its meeting on September 2009 and decided the Following:-
To approve the above mention research study.

Signature

Member

Member

Chairperson

Conditions:-
- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.