Exploring the genetic association of ABC transporters with Acute lymphoblastic leukemia

A Major Project dissertation submitted

in partial fulfilment of the requirement for the degree of

Master of Technology

In

Biomedical Engineering

Submitted by

Sakshi Sharma

(DTU/13/M.Tech/385)

Delhi Technological University, Delhi, India

Under the supervision of

Dr. Pravir Kumar

Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road,
Delhi-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"Exploring the genetic association of ABC transporters with acute lymphoblastic leukemia"**, submitted by **SAKSHI SHARMA (DTU/13/M.Tech/385)** in partial fulfilment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

Date: 14 July 2015

Dr. Pravir Kumar
Associate Professor
(Project Mentor)
Department of Bio-Technology
Delhi Technological University

Dr. D Kumar
Professor, HOD
Department of Bio-Technology
Delhi Technological University

Sir Ganga Ram Hospital

CERTIFICATE OF EXTERNAL GUIDE

This is to certify that the thesis entitled "**Exploring the genetic association of ABC transporters with Acute lymphoblastic leukemia**" submitted by **Sakshi Sharma** to Delhi Technological University, Delhi, for the degree of **Master of Technology in Biomedical Engineering,** is her original work, based on the results of the experiments and investigations carried out by her at the Department of Research during the study period January to June 2015 under the co-guidance of Dr. Pravir Kumar and Dr. Veronique Dinand.

The above said work has not been previously submitted for the award of any degree, diploma or fellowship in any Indian or foreign University.

Date: 13 July 2015

Place: New Delhi

Dr. Veronique Dinand

Chief Scientific Officer

Department of Research

Sir Ganga Ram Hospital

New Delhi – 110 060

Sir Ganga Ram Hospital Marg, Rajinder Nagar, New Delhi 110060, INDIA
Phones : +91-11-25750000, 42254000 Fax : +91-11-25861002 Email : gangaram@sgrh.com Website : www.sgrh.com

DECLARATION

I declare that my major project dissertation entitled "Exploring the genetic association of ABC transporters with acute lymphoblastic leukemia" submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at Department of Research, Sir Ganga Ram Hospital, New Delhi.

Date: 14 July 2015 SAKSHI SHARMA

Place: Delhi

ACKNOWLEDGEMENT

I express my profound gratitude to **Dr. N.K. Ganguly**, Chairman, Department of Research, Sir Ganga Ram Hospital, New Delhi for granting me permission to pursue the dissertation work and avail the facilities for carrying out the work in the prestigious institute.

I express my sincere gratitude to **Dr. Veronique Dinand**, Chief Scientific officer, Department of Research, Sir Ganga Ram Hospital for the scholarly guidance, generous encouragement and suggestion throughout the course of my dissertation work.

I would like to achnowledge my heartiest thanks to **Dr. Pravir Kumar**, Associate Professor and **Dr. D. Kumar**, HOD, Department of Biotechnology, Delhi Technological University whose continuous encouragement made me work honestly and to the best of my ability.

I am extremely grateful to Dr. Vibha Taneja and other members for giving me all the practical and theroritical knowledge which came in hand during my course of project.

I express my hearty indepthness to **Abhishek Vats**, **Meenakshi Verma**, **Neha Katyal**, **Saurabh Jha**, **Neeraj Jha**, **Dhiraj**, Research Scholars for their personal guidance throughout my dissertation which helped me complete this project successfully.

Finally my heartfelt appreciation goes to all, who have directly or indirectly contributed in completing this work.

SAKSHI SHARMA
2K13/BME/01

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PBS- Phosphate Buffered Saline

PBMC- Peripheral Blood Mononuclear Cells

AML-Acute Myeloid Leukemia

ALL-Acute Lymphoblastic Leukemia

PCR-Polymerase Chain reaction

EDTA-Ethylene Diamine Tetra Acetate

CCDS- Consensus Coding Sequence

GAPDH-Glyceraldehyde 3-phosphate dehydrogenase

ACTB- Beta actin

HSP70- Heat Shock Protein 70 kDa

GRP78-Glucose related protein 78kDa

ABCC1- ATP-binding cassette transporter family C member 1

ABCC2- ATP-binding cassette transporter family C member 2

ABCC3- ATP-binding cassette transporter family C member 3

ABCC4- ATP-binding cassette transporter family C member 4

ABCC5- ATP-binding cassette transporter family C member 5

ABCB1- ATP-binding cassette transporter family B member 1

ABCG2- ATP-binding cassette transporter family G member 2

BCRP- Breast cancer resistance protein

LRP- Lung resistance protein

SYBR- Synergy Brands Inc

ROX- 6-Carboxyl-X-Rhodamine

NCBI- National Center for Biotechnology Information

UV- Ultra Violet

BP- Base Pair

ATP- Adenosine triphosphate

RT- Reverse Transcriptase

RNA- Ribonucleic acid

FP- Forward Primer

RP- Reverse Primer

RT- Reverse transcriptase

ER- endoplasmic reticulum

IRE1- inositol requiring enzyme 1

ATF6- activating transcription factor 6

PERK- PKR-like ER kinase

Exploring the genetic association of ABC transporters with Acute lymphoblastic leukemia

SAKSHI SHARMA

Delhi Technological University, Delhi, India

1. ABSTRACT

Acute lymphoblastic leukemia, a malignant illness of white blood cells is one of the most significant blood cancers with 80% of cases in children between 2-5 years of age. Despite the long term survival rate, which is more than 85% due to the success in the treatment of the disease, the difficulty arises as a result of resistance in chemotherapy which poses a major challenge. One of the major factor for this resistance has been found to be ATP-binding cassette transporters. ABC transporters are engaged in energy dependent transportation of xenobiotic and other toxic compounds, as well as anti-cancer drugs. In various cancers including lymphoblastic and myeloid leukemia, these transporters have been shown to extrude the drugs resulting in resistance to chemothereutic drugs. It is important to evaluate the role of ABC transporters in drug response in ALL. The aim of this study was to understand whether the gene expression of ABC transporters (ABCC1, ABCC5 and ABCB1) are independent predictors of treatment success and to find out the significant association between ABC transporters and ALL. Role of two molecular chaperons (HSP70 and GRP78) in childhood ALL was also examined to find out possible relation.

2. INTRODUCTION

Cancer is the second most important leading cause of death and is caused by damage to DNA present in the cell that leads to uncontrolled cellular proliferation giving rise to a lump or mass of tissue called as Tumor or Neoplasm and there after spreads throughout the body. This occurs either by increasing chemical signals that cause growth or by interrupting chemical signals that control growth (Jemal et al., 2011).There are many types of cancers which includes- carcinoma, sarcoma, melanoma, lymphoma and leukemia. Leukemia is a cancer of blood and does not form solid tumors.

Acute lymphoblastic leukemia (ALL) is a disease of blood and bone marrow and it is considered as the most common type of cancer in children. ALL can occurs in both adults and children.There are approximately 882 million people in Indian out of which estimated children that develop ALL every year is around six thousand (Chandy, M. 2006). Over 85% success rate has been achieved in treatment of children as compared to adults which is only 40%.The anti cancer drugs and chemotherapy is give as treatment against the disease.

ATP-binding cassette (ABC) transporters are a type of ATP-dependent uniport pumps. They are membrane-bound ubiquitous proteins. ABC transporters are expressed mostly in the liver, blood-brain barrier, intestine, blood-testis barrier, kidney and placenta. ABC proteins transport a large number of endogenous substrates, including inorganic anions, peptides, proteins, metal ions, sugars, amino acids, drugs, hydrophobic compounds and metabolites present across the plasma membrane.

Cancerous cells over a period of time become resistant to drugs which are structurally unrelated. This is known as multi drug resistance. Multidrug resistance (MDR) hampers the success of cancer pharmacotherapy which is a serious problem. ATP-binding cassette (ABC) transporters overexpression is a major mechanism resulting in MDR thus declining the intracellular drug concentration by extruding it out of cell.

The cure rate for ALL disease over the past decades has relatively improved, but the ALL treatment failure due to multidrug resistance (MDR) phenomenon remains the most important reason. Multidrug transport proteins contribute majorly to chemo resistance through efflux of anticancer drugs from cancerous cells (Fletcher et al., 2010).

The incorporation of new knowledge about the mechanisms of tumor resistance to antineoplastic drugs may contribute towards increasing the chances of cure, either through the development of new drugs, or by means of strategies that may modulate or reverse the resistance (Szabó et al., 2000).

3. REVIEW OF LITERATURE

3.1 CANCER

Cancer is an uncontrolled growth of any cell leading to pathological distruption of normal body function. These cells may interfere with the nervous system, digestive system or circulatory system.Cancer may arise due to DNA damage, inability of a cell to undergoprogrammed cell death, fusion of two or more genes or oncogene activation.

The damage to the cell may be caused by environmental factors such as radiation, drugs or chemicals. There are a wide variety of cancer causing agents. It includes environmental carcinogens such as pesticide, tobacco chemicals in food and chemicals from industries (kavlock et al., 1996, Soto et al., 2010), genotoxic carcinogens such as 2-acetamide fluorine, N-nitroso-diethylamine (Hayashi et al., 1992, Lee et al., 2013); human carcinogens such as Bile acids (Bernstein et al., 2009, Ajouz et., 2014, Bernstein et al., 2005, Costarelli et., 2009); chemical carcinogens such as parabens (Wogan et al., 2004, Darbre et al., 2014, Khanna et al., 2014) and occupational carcinogens such as organic dust , metals , combustion products, mineral oils, radon , solar radiation(Clapp. 2008, Fernandez et al., 2012, Van Tongeren et al., 2012). The generally accepted environmental risk factor for Acute Leukemia examined by epidemiological studies include exposure to environmental carcinogens and ionizing radiations. Among suspected risk factors, exposure to pesticides, low frequency magnetic fields and infections are notable (Mezei et al., 2014).

Restriction of a tumor to a particular place showing limited growth is termed as Benign ; if the tumor starts to invade nearby tissues via lymphatic system or circulatory system (blood and lymph) then the term malignant is used for that tumor and the process is described as Metastasis. For metastasis to take place down regulation of cell adhesion molecules that are responsible for cell to cell attachment occurs (sarkar et al., 2013).

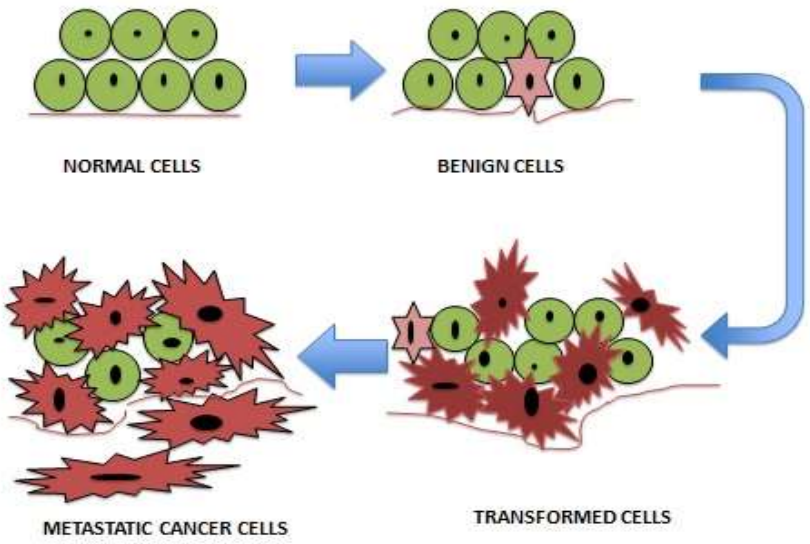


Figure 1- Transformation of a normal cell into cancerous cell

3.2 ACUTE LYMPHOBLASTIC LEUKEMIA

ALL is a form of cancer that arises when a large number of immature lymphocytes are produced by the bone marrow thus it is a cancer of bone marrow and blood. There are four types of hematologic malignancies including leukemia, lymphoma, and multiple myeloma, among them ALL is found to have higher frequency in children (Campos-Sanchez et al., 2011). In this type of cancer excess lymphoid lineage hematopoietic precursors arises thereby crowding and then leads to the death of healthy cells. ALL is a malignancy of B or T lymphoid cells. Genetic alteration in these cells results in blocking of lymphoid differentiation thus resulting in uncontrolled cellular proliferation (Zuckerman et al., 2014). ALL occurs when humans are exposed to chemicals and radiations. Higher levels of radiation exposure are known risk factors for development of leukemia (Belson et al., 2007).

Current diagnosis standards of ALL are detailed in 2008 WHO classification of lymphoid neoplasm and integrate the study of genetics/cytogenetics, cell morphology and immunophenotype (Vardiman et al., 2009).

Around 80% event-free survival rate has been achieved in case of childhood ALL. This success in part has been achieved due to risk-stratified therapy implementation. However, there are still 15-20% children newly diagnosed with ALL in which relapse of disease occur, as there is an inadequate traditional risk assessment (Vrooman et al., 2009). Selection of treatment that is, drug dosage and number of drugs to be given takes place by considering efficient prognostic factors like age, leukocyte count at diagnosis, translocation of chromosomes.

3.3 ABC TRANSPORTERS

These transporters are transmembrane proteins functioning as pump and extruding toxins and drugs out of the cell. A large number of subrates move in and out of the cell with the help of ABC transporters. These transporters are also involved in transport in intracellular compartment. It is an energy dependent process and requires ATP hydrolysis to transport a substrate across the membrane. The transport occurs against the concentration gradient. (Stefkova et al., 2004). They are also involved in other processes such as DNA repair and translation of RNA.

The human genome contains 49 *ABC* genes, arranged in eight subfamilies based on structural organization and sequence similarity (Dean and Rzhetaky 2001) and named via divergent evolution and this family has members that play pivotal roles in many cellular processes (Vasiliou et al., 2009). This superfamily also includes membrane proteins which are involved in extruding variety of substances across cell membranes. These transporters are involved in developing resistance to multiple drugs.

3.4 MULTIPLE DRUG RESISTANCE

ABC transporters in humans reside in the plasma membrane and has a protective function in the body. It extrudes of large number of toxic compounds or xenobiotics through active ATP dependent process. A multifactorial phenomenon MDR is attributable to ABC transporters (Gillet et al., 2004; Shukla et al., 2012). Tumor cells make use of the same proteins to fight against various anti-cancer agents thus making the cell resistant to it (Chauhan et al., 2012). These ABC transporters develop resistance to chemotherapy in various kinds of cancers which includes lymphoblastic leukemia and myeloid leukemia (Steinbach et al., 2006; Abedi et al., 2013).

MDR arises when the molecular pathways of cells are altered (Hlavac et al., 2013). This alteration in molecular pathways can be through DNA damage repair, decreased apoptosis, loss of drug transport protein of the cells, from the surface, decrease in absorption of water soluble drugs (Aberuyi et al., 2013). Disturbance in transporter mediated drug delivery to cancer cells or genetic and epigenetic changes in transporters of cancer cells which affects the drug sensitivity are responsible for cell becoming resistant to anti-cancer drugs.

Three major groups of efflux ABC transporters exists that are involved in cancer MDR. This includes classical P-glycoprotein (also called as ABCB1, MDR1, P-gp, P-170); multidrug resistance associated proteins (also called as MRPs, ABCC subfamily), and the breast cancer resistance protein (also called as BCRP, ABCG2). Their overexpression limits the anticancer drugs exposure (Choi and Yu 2014).

3.5 ROLE OF ABC TRANSPORTERS IN ACUTE LYMPHOBLASTIC LEUKEMIA

Increased drug excretion from the cells by ABC transporters is responsible for generation of drug resistance in ALL patients. Polymorphisms in many different metabolic pathways have been demonstrated in single gene studies to influence the outcome of ALL. Challenges arise in establishing the generalizability of observations and interpreting complex gene-gene interactions in multigene pathways (Mehta et al., 2004).

Several MDR-related efflux pumps have been characterized, including P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), lung resistance protein (LRP) and breast cancer resistance protein (BCRP). MDR1 (P glycoprotein) and BCRP extrude large hydrophobic, positively charged molecules from the cells. The members of the MRP family has the ability to extrude both uncharged hydrophobic molecules and anionic compounds that are water-soluble (Glavinas et al., 2004). The MRPs are known to extrude cancer drugs such as methotrexate, doxorubicin, etoposide, vincristine, 6-mercaptopirine (Kool et al., 1999, Borst et al., 2002, Jedlitschky et al., 2002, Belinsky et al., 2002).

P-gp expression and/or activity has been associated with unfavourable outcome in pediatric ALL patients. LRP might contribute to drug resistance in B-lineage ALL, but larger studies are needed to confirm these results (Swerts et al., 2006).

The relationships between MDR-1, MRP and BCRP expression, resistance to treatment and survival among children with ALL are still unclear (den Boer et al., 1998; Norgaard and Hokland *et al*., 2000). These transporters are discussed below:

3.6 ABCB1

Also called as P-glycoprotein (P-gp) is one of the most studied gene for drug resistance. ABCB1 was first reported in 1976 and is the first ABC transporter to be identified. Evidence of its association with drug resistance was first found in Chinese hamster ovary cell line, which was being selected for colchicine resistance but also showed resistance to puromycin and daunomycin (Juliano and Ling 1976). It was first purified in 1979 and was named P-gp (Riordan and Ling. 1979). It is a 170-kDa glycoprotein located on chromosome 7 at q21 and has 28 exons encoding 1280 amino acid protein product (Gottesman et al., 1995).

It is composed of 12 hydrophobic transmembrane domains which determines the substrate characteristics and 2 nucleotide binding domains which is responsible for ATP binding and hydrolysis, thus driving substrate transport (Hyde et al., 1990).

The protein Pgp has been found to alter the cell defense mechanism thus has been said to be associated with development of cancer such like colorectal carcinoma and hepatocarcinoma therefore mutation in gene might also lead to leukemia (Wang. 2004, Hattori et al., 2006).

Dooge et al in 2002 reported an elevated risk of death in leukemic relapse patients with high P-gp expression (Dooge et al., 2002). Similar results were reported by El-Ghaffar et al in 2006 (Abd El-Ghaffar et al., 2006).

3.7 ABCG2/BCRP

ABCG2, is known as a ‘ABC half transporter’ tan it functions in cell membrane as a homodimer, It is an important member of multi drug resistance protein. When a single nucleotide mutation occurs from arginine to glycine or threonine at position 482 it has been seen that it results in wider recognition of drug and increased catalytic activity of the protein. (Cervenak et al., 2006). BCRP overexpression has been found to be associated with drug resistance in ALL (Fronkova et al., 2008). A study in 2003 by Plasschaert et al showed a higher expressions in patients with B-cell ALL than T-cell ALL.

3.8 MRP FAMILY

There are total 12 members in the MRP family. These are divided into MRP members which are short that is they have two nucleotide binding domains and two membrane spanning domains. These include MRP4, MRP5, MRP8, and MRP9. The long MRPs have additional membrane spanning domain and include MRP1, MRP2, MRP3, MRP6, and MRP7.

There are a variety of chemotherapeutic drugs with which ALL patients are treated. These transporters contribute to absorption, dispersion and elimination of drugs for cancer. These MRPs have functional activity which overlap each other (Deeley et al., 2006).

3.8.1 ABCC1

It was the first member to be identified in the MRP family. MRP1 is described as one of the main gene responsible for drug resistance. Higher mRNA expression of this particular transporter has been linked to poor survival rate in ALL (El-Sharnouby et al., 2009, Plasschaert SL), AML (Schaich et al., 2005), neuroblastoma (Norris et al., 1996, Haber et al., 2006). Mahjoubi et al reported in their sudy that high MRP 1 expressions are associated with poor clinical outcomes (Mahjoubi et al., 2008).

3.8.2 ABCC2

It is also known as canalicular multispecific organic anion transporter (Yan et al., 2014).The mRNA expression are found to be higher in adult and child ALL and AML (Steinbach et al., 2003). It is found to be responsible in methotrexate elimination (Rau et al., 2006).

3.8.3 ABCC3

Higher mRNA expression of this gene has been linked to poor overall survival and event free survival in ALL and AML but gave a better outcome in neuroblastoma (Henderson et al., 2011). It also leads to higher resistance to cancer drug methotrexate. Steinbach et al reported a 10 fold higher expression in patients with T-ALL in 2003 (Steinbach et al., 2003).

3.8.4 ABCC4

This transports a variety of drugs but affects the disposition of key drugs used in ALL treatment like- 6- mercaptopurine and methotrexate (Chen et al., 2002, Russel et al., 2008, Janke et al., 2008). Wide variation has been seen in childhood ALL for MRP4 expression (sampath et al., 2002).

3.8.5 ABCC5

It has been found to have a role in cancer development.

3.9 MOLECULAR CHAPERONES

Chaperones are a complex network of proteins which work to prevent protein aggregation and assist in protein folding. This activity is necessary so as the proteins can gain their functional activity by folding into a three dimensional structure. Molecular Chaperones include heat shock proteins that help other proteins to cope up with stress-induced denaturation (Feder and Hofmann 1999). This is highly conserved stress response mechanism that cells use to protect themselves from damage. It involves upregulation of chaperone and heat shock protein expression to prevent damage and aggregation of proteins (Graner et al., 2007).

3.9.1 HSP70

Heat shock proteins, induced in stressful conditions are highly conserved molecular chaperons protecting the cells from toxic conditions. Under stress the levels of HSPs increase depending on the type of stress. The main function of HSP 70 is assisting the folding of misfolded proteins and also newly synthesized proteins. It also functions to transport proteins across cell membranes. It refolds denatured proteins by binding to ATP and undergoing a conformational change (Beckmann et al., 1990, Shi et al., 1992).

Structure of HSP70 consists of 45kDa ATPase Domain with high sequence conservation at the N-terminal and a 25kDa substrate binding domain at C terminal. ATP hydrolysis and binding is important for HSP70 protein chaperone activity. This ATPase cycle is majorly controlled by nucleotide exchange factors and J- domain proteins. The ATP bound state of HSP70 has lower affinity and exchanges fast with the substrate while ADP bound state has high affinity for HSP70 and low exchange rate for substrate (Mayer et al., 2005). HSP70 is an important apoptosis regulator and can regulate all main pathways of apoptosis. The cells in later phase of apoptosis can also be rescued by this protein (Jaattela et al., 1998).

Xiao et al. in their study in 1996 showed that the expression of HSP70 in the blood cells was much lower in almost all patients than that of normal blood cells indication that the gene might get down regulated in acute lymphoblastic leukemia (Xiao et al., 1996). Fujita et al. in 1996 also reported a lower expression of HSP70 in ALL (Fujita et al., 1996). In recent study by Kiser et al in 2011 on adult leukemia HSP70 has been found to be aberrantly expressed. (Kiser et al., 2011). Sedlackova et al. found no significant difference in expression of HSP70 (Sedlackova et al., 2011). It has been reported by Xavier et al that the patients with lower expression of HSP70 has been shown to have a significantly longer overall survival than those patients with higher expression of HSP70 thus showing poor prognosis (Xavier et al., 2005).

3.9.2 GRP78

Also known as BiP, MIF2 and is a member of Heat Shock protein 70 family. Mainly localized in the lumen of endoplasmic reticulum plays a role in protein folding and assembly in endoplasmic reticulum (Bortolozzi et al., 2014).

Several studies revealed that ER stress activation has been used by chemotherapeutics to drive cell death. Thus cell therapy can be generated by promoting the prodeath function of ER stress or by its prosurvival activity inhibition (Kim et al., 2008, Tabas et al., 2011). A process called as Unfolding Protein Response is activated by cells in response to ER stress and unfolded protein accumulation. It works to restore the stability of ER through protein synthesis attenuation or chaperone upregulation leading to folding of proteins by integrating several signaling pathways.

Chaperon Glucose Response Protein (GRP8) acts as a main UPR activator and inhibits receptors of ER membrane – inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK) (Xu et al., 2005). These receptors are activated when misfolded proteins gets accumulated and GRP78 is released from the ER membrane to target these misfolded proteins for degradation. Protein synthesis is blocked by PERK which is followed by restoring of ER homeostasis by ATF6 (Szegezdi et al., 2006). Endoplasmic reticulum associated protein degradation system (ERAD) ubiquitinates the misfolded proteins followed by proteasome degradation thus decreasing the toxicity caused due to aggregation of proteins (Vembar et al., 2008).

According to the work carried out by Uckun et al. in 2011, GRP78 is expressed abundantly in relapsed acute lymphoblastic leukemia cases and also contribute in chemotherapy resistance. Targeting it can induce apoptosis in acute lymphoblastic leukemia cells which are resistant to chemotherapy. And therefore identified GRP78 as a potential biomarker for chemoresistance (Uckun *et al*., 2011). In 2014 Kharabi et al. reported higher levels of GRP78 in plasma cells specifically during B-cell development at Pre-B cell receptor checkpoint. And also that pre-B ALL have vulnerability to ER stress (Kharabi et al., 2014). Tsai et al reported in 2015 that GRP78 plays a critical role in oncogenic signalling pathway and is emerging as a target for anti cancer therapy (Tsai et al., 2015).

4. METHODOLOGY

Inclusion Criteria

Newly diagnosed and relapsed cases of B-cell to T-cell acute lymphoblastic leukemia with age <18 years were included in this study. Bone marrow stem cell transplant donor for matched sibling donor transplant were included as healthy controls.

Exclusion Criteria

Patients with other hematological malignancies, such as acute myeloblastic leukemia (AML), chronic leukemia and lymphoma, solid tumors and patients on steroids were not included in this study.

Sample collection

Bone marrow samples were collected in EDTA vials from patients with newly diagnosed or relapsed acute lymphoblastic leukemia and from healthy controls after giving written informed consent. Bone Marrow samples were also taken from healthy controls. No additional invasive procedure was carried out to draw bone marrow from these individuals.

Primer Designing

Consensus CCDS sequence of the study genes were taken from NCBI database. CCDS is a nucleotide sequence without introns and UTR regions. Website called Primer 3 was used to design real time PCR primers. The conditions for primer selection were:

	Min	Opt	Max
Primer Size	19	20	22
Primer Tm	57	60	62
Primer GC%	50	55	60

Table 1 – Primer selection conditions

General considerations taken into account before selecting primers is as follows:

- Optimum length of the primer should be 20-24 base pairs
- Tm difference between the primers should not be more than 2°C
- GC content should not be more than 50%
- No hairpin or self-dimers are formed by the primers

A list of all possible primers wase generated by Primer 3 after entering the sequence. All primer pairs were checked using the UCSC genome browser in-silico PCR tool. A primer pair was selected which included alternate exons or lie on the boundary. And the selected primer pairs were ordered from Sigma. CCDS sequence of study genes along with the location of primer pair is given below:

ABCC1 CCDS sequence

GTCACCACGTACTTGAACTGGCTGGTTCGGATGTCATCTGAAATGGAAACCAACATCGTGGCCGTG
GAGAGGCTCAAGGAGTATTCAGAGACTGAGAAGGAGGCGCCCTGGCAAATCCAGGAGACAGCTC
CGCCCAGCAGCTGGCCCCAGGTGGGCCGAGTGGAATTCCGGAACTACTGCCTGCGCTACCGAGAG
GACCTGGACTTCGTTCTCAGGCACATCAATGTCACGATCAATGGGGGAGAAAAGGTCGGCATCGT
GGGGCGGACGGGAGCTGGGAAGTCGTCCCTGACCCTGGGCTTATTTCGGATCAACGAGTCTGCCG
AAGGAGAGATCATCATCGATGGCATCAACATCGCCAAGATCGGCCTGCACGACCTCCGCTTCAAG
ATCACCATCATCCCCCAGGACCCTGTTTTGTTTTCGGGTTCCCTCCGAATGAACCTGGACCCATTCA
GCCAGTACTCGGATGAAGAAGTCTGGACGTCCCTGGAGCTGGCCCACCTGAAGGACTTCGTGTCA
GCCCTTCCTGACAAGCTAGACCATGAATGTGCAGAAGGCGGGGAGAACCTCAG

ABCC2 CCDS sequence

TTCTAGGTCCAATGGCAGGCATCTGAAGTCCCTGAGAAACTCCTTGAAAACTCGGAATGTGAATAG
CCTGAAGGAAGACGAAGAACTAGTGAAAGGACAAAAACTAATTAAGAAGGAATTCATAGAAACT
GGAAAGGTGAAGTTCTCCATCTACCTGGAGTACCTACAAGCAATAGGATTGTTTTCGATATTCTTC
ATCATCCTTGCGTTTGTGATGAATTCTGTGGCTTTTATTGGATCCAACCTCTGGCTCAGTGCTTGGA
CCAGTGACTCTAAAATCTTCAATAGCACCGACTATCCAGCATCTCAGAGGGACATGAGAGTTGGA
GTCTACGGAGCTCTGGGATTAGCCCAAGGTATATTTGTGTTCATAGCACATTTCTGGAGTGCCTTTG
GTTTCGTCCATGCATCAAATATCTTGCACAAGCAACTGCTGAACAATATCCTTCGAGCACCTATGA
GATTTTTTGACACAACACCCACAGGCCGGATTGTGAACAGGTTTGCCGGCGATATTTCCACAGTGG
ATGACACCCTGCCTCAGTCCTTGCGCAGCTGGATTACATGCTTCCTGGGGATAATCAGCACCCTTG
TCATGATCTGCATGGCCACTCCTGTCTTCACCATCATCGTCATTCCTCTTGGCATTATTTATGTATCT
GTTCAGATGTTTTATGTGTCTACCTCCCGCCAGCTGAGGCGTCTGGACTCTGTCACCAGGTCCCCAA
TCTACTCTCACTTCAGCGAGACCGTATCAGGTTTGCCAGTTATCCGTGCCTTTGAGCACCAGCAGC
GATTTCTGAAACACAATGAGGTGAGGATTGACACCAACCAGAAATGTGTCTTTTCCTGGATCACCT
CCAACAG

ABCC3 CCDS sequence

ATGGACGCCCTGTGCGGTTCCGGGGAGCTCGGCTCCAAGTTCTGGGACTCCAACCTGTCTGTGCAC
ACAGAAAACCCGGACCTCACTCCCTGCTTCCAGAACTCCCTGCTGGCCTGGGTGCCCTGCATCTAC
CTGTGGGTCGCCCTGCCCTGCTACTTGCTCTACCTGCGGCACCATTGTCGTGGCTACATCATCCTCT
CCCACCTGTCCAAGCTCAAGATGGTCCTGGGTGTCCTGCTGTGGTGCGTCTCCTGGGCGGACCTTTT
TTACTCCTTCCATGGCCTGGTCCATGGCCGGGCCCCTGCCCCTGTTTTCTTTGTCACCCCCTTGGTG
GTGGGGGTCACCATGCTGCTGGCCACCCTGCTGATACAGTATGAGCGGCTGCAGGGCGTACAGTCT
TCGGGGGTCCTCATTATCTTCTGGTTCCTGTGTGTGGTCTGCGCCATCGTCCCATTCCGCTCCAAGA
TCCTTTTAGCCAAGGCAGAGGGTGAGATCTCAGACCCCTTCCGCTTCACCACCTTCTACATCCACTT
TGCCCTGGTACTCTCTGCCCTCATCTTGGCCTGCTTCAGGGAGAAACCTCCATTTTTCTCCGCAAAG
AATGTCGACCCTAACCCCTACCCTGAGACCAGCGCTGGCTTTCTCTCCCGCCTGTTTTTCTGGTGGT
TCACAAAGATGGCCATCTATGGCTACCGGCATCCCCTGGAGGAGAAGGACCTCTGGTCCCTAAAG
GAAGAGGACAGATCCCAGATGGTGGTGCAGCAGCTGCTGGAGGCATGGAGGAAGCAGGAAAAGC
AGACGGCACG

ABCC4 CCDS sequence

GAGTAAAACTGCAACTTTCACGGATGCCAGGATCAGGACCATGAATGAAGTTATAACTGGTATAA
GGATAATAAAAATGTACGCCTGGGAAAAGTCATTTTCAAATCTTATTACCAATTTGAGAAAGAAG
GAGATTTCCAAGATTCTGAGAAGTTCCTGCCTCAGAGGGATGAATTTGGCTTCATTTTTCAGTGCA
AGCAAAATCATCGTGTTTGTGACCTTCACCACCTACGTGCTCCTCGGCAGTGTGATCACAGCCAGC
CGCGTGTTCGTGGCAGTGACGCTGTATGGGGCTGTGCGGCTGACGGTTACCCTCTTCTTCCCCTCA
GCCATTGAGAGGGTGTCAGAGGCAATCGTCAGCATCCGAAGAATCCAGACCTTTTTGCTACTTGAT
GAGATATCACAGCGCAACCGTCAGCTGCCGTCAGATGGTAAAAAGATGGTGCATGTGCAGGATTT
TACTGCTTTTTGGGATAAGGCATCAGAGACCCCAACTCTACAAGGCCTTTCCTTTACTGTCAGACCT
GGCGAATTGTTAGCTGTGGTCGGCCCCGTGGGAGCAGGGAAGTCATCACTGTTAAGTGCCGTGCTC
GGGGAATTGGCCCCAAGTCACGGGCTGGTCAGCGTGCATGGAAGAATTGCCTATGTGTCTCAGCA
GCCCTGGGTGTTCTCGGGAACTCTGAGGAGTAATATTTTATTTGGGAAGAAATACGAAAAGGAAC
GATATGAAAAAGTCATAAAGGCTTGTGCTCTGAAAAAG

ABCC5 CCDS sequence

ATGAAGGATATCGACATAGGAAAAGAGTATATCATCCCCAGTCCTGGGTATAGAAGTGTGAGGGA
GAGAACCAGCACTTCTGGGACGCACAGAGACCGTGAAGATTCCAAGTTCAGGAGAACTCGACCGT
TGGAATGCCAAGATGCCTTGGAAACAGCAGCCCGAGCCGAGGGCCTCTCTCTTGATGCCTCCATGC
ATTCTCAGCTCAGAATCCTGGATGAGGAGCATCCCAAGGGAAAGTACCATCATGGCTTGAGTGCTC
TGAAGCCCATCCGGACTACTTCCAAACACCAGCACCCAGTGGACAATGCTGGGCTTTTTTCCTGTA
TGACTTTTTCGTGGCTTTCTTCTCTGGCCCGTGTGGCCCACAAGAAGGGGGAGCTCTCAATGGAAG
ACGTGTGGTCTCTGTCCAAGCACGAGTCTTCTGACGTGAACTGCAGAAGACTAGAGAGACTGTGG
CAAGAAGAGCTGAATGAAGTTGGGCCAGACGCTGCTTCCCTGCGAAGGGTTGTGTGGATCTTCTGC
CGCACCAGGCTCATCCTGTCCATCGTGTGCCTGATGATCACGCAGCTGGCTGGCTTCAGTGGACCA
AATTTTCAGGATGGCTGTATTCTGCGGTCAGAATGA

ABCG2 CCDS sequence

CCACAGAGATCATAGAGCCTTCCAAGCAGGATAAGCCACTCATAGAAAAATTAGCGGAGATTTAT
GTCAACTCCTCCTTCTACAAAGAGACAAAAGCTGAATTACATCAACTTTCCGGGGGGTGAGAAGAA
GAAGAAGATCACAGTCTTCAAGGAGATCAGCTACACCACCTCCTTCTGTCATCAACTCAGATGGGT
TTCCAAGCGTTCATTCAAAAACTTGCTGGGTAATCCCCAGGCCTCTATAGCTCAGATCATTGTCAC
AGTCGTACTGGGACTGGTTATAGGTGCCATTTACTTTGGGCTAAAAAATGATTCTACTGGAATCCA
GAACAGAGCTGGGGTTCTCTTCTTCCTGACGACCAACCAGTGTTTCAGCAGTGTTTCAGCCGTGGA
ACTCTTTGTGGTAGAGAAGAAGCTCTTCATACATGAATACATCAGCGGATACTACAGAGTGTCATC
TTATTTCCTTGGAAAACTGTTATCTGATTTATTACCCATGAGGATGTTACCAAGTATTATATTTACC
TGTATAGTGTACTTCATGTTAG

HSP70 CCDS sequence

ATGTCGGTGGTGGGCATAGACCTGGGCTTCCAGAGCTGCTACGTCGCTGTGGCCCGCGCCGGCGGC
ATCGAGACTATCGCTAATGAGTATAGCGACCGCTGCACGCCGGCTTGCATTTCTTTTGGTCCTAAG
AATCGTTCAATTGGAGCAGCAGCTAAAAGCCAGGTAATTTCTAATGCAAAGAACACAGTCCAAGG
ATTTAAAAGATTCCATGGCCGAGCATTCTCTGATCCATTTGTGGAGGCAGAAAAATCTAACCTTGC
ATATGATATTGTGCAGTTGCCTACAGGATTAACAGGTATAAAGGTGACATATATGGAGGAAGAGC
GAAATTTTACCACTGAGCAAGTGACTGCCATGCTTTTGTCCAAACTGAAGGAGACAGCCGAAAGT
GTTCTTAAGAAGCCTGTAGTTGACTGTGTTGTTTCGGTTCCTTGTTTCTATACTGATGCAGAAAGAC
GATCAGTGATGGATGCAACACAGATTGCTGGTCTTAATTGCTTGCGATTAATGAATGAAACCACTG
CAGTTGCTCTTGCATATGGAATCTATAAGCAGGATCTTCCTGCCTTAGAAGAGAAACCAAGAAATG
TAGTTTTTGTAGACATGGGCCACTCTGCTTATCAAGTTTCTGTATGTGCATTTAATAGAGGAAAACT
GAAA

GRP78 CCDS sequence

GTGGTTGAAAAGAAAACTAAACCATACATTCAAGTTGATATTGGAGGTGGGCAAACAAAGACATT
TGCTCCTGAAGAAATTTCTGCCATGGTTCTCACTAAAATGAAAGAAACCGCTGAGGCTTATTTGGG
AAAGAAGGTTACCCATGCAGTTGTTACTGTACCAGCCTATTTTAATGATGCCCAACGCCAAGCAAC
CAAAGACGCTGGAACTATTGCTGGCCTAAATGTTATGAGGATCATCAACGAGCCTACGGCAGCTG
CTATTGCTTATGGCCTGGATAAGAGGGAGGGGGAGAAGAACATCCTGGTGTTTGACCTGGGTGGC
GGAACCTTCGATGTGTCTCTTCTCACCATTGACAATGGTGTCTTCGAAGTTGTGGCCACTAATGGA
GATACTCATCTGGGTGGAGAAGACTTTGACCAGCGTGTCATGGAACACTTCATCAAACTGTACAAA
AAGAAGACGGGCAAAGATGTCAGGAAAGACAATAGAGCTGTGCAGAAACTCCGGCGCGAGGTAG
AAAAGGCCAAACGGGCCCTGTCTTCTCAGCATCAAGCAAGAATTGAAATTGAGTCCTTCTATGAA
GGAGAAGACTTTTCTGAGACCCTGACTCGGGCCAAATTTGAAGAGCTCAACATGGATCTGTTCCGG
TCTACTATGAAGCCCGTCCAGAAAGTGTTGGAAGATTCTGATTTGAAGAAGTCTGATATTGAT

GENE		SEQUENCE	LENGTH	Tm	GC%	HAIRPIN	PRODUCT SIZE
ABCC1	FP	GGACTTCGTTCTCAGGCACA	20	59.97	55	0	250
	RP	GTCCAGGTTCATTCGGAGGG	20	60.11	60	0	
ABCC2	FP	TCCAGCATCTCAGAGGGACA	20	59.96	55	0	223
	RP	GGAAATATCGCCGGCAAACC	20	59.97	55	0	
ABCC3	FP	CAAGATGGTCCTGGGTGTCC	20	60.04	60	0	225
	RP	CAGACCACACACAGGAACCA	20	59.82	55	0	
ABCC4	FP	GGCTGACGGTTACCCTCTTC	20	60.11		0	198
	RP	GAGTTGGGGTCTCTGATGCC	20	60.11	60	0	
ABCC5	FP	CGCACAGAGACCGTGAAGAT	20	60.11	55	0	234
	RP	AAAGCCCAGCATTGTCCACT	20	60.18	50	0	
ABCB1	FP	GAGGTGAAGAAGGGCCAGAC	20	60.04	60	0	240
	RP	CCGGCTGTTGTCTCCATAGG	20	60.18	60	0	
ABCG2	FP	TCCCCAGGCCTCTATAGCTC	20	59.88	60	0	168
	RP	AGTTCCACGGCTGAAACACT	20	59.82	50	0	
HSP70	FP	CTTCCAGAGCTGCTACGTCG	20	60.52	60	0	196bp
	RP	TGCTCGGCCATGGAATCTTT	20	60.03	50	0	
GRP78	FP	GCGGAACCTTCGATGTGTCT	20	60.39	55	0	158bp
	RP	CCTGACATCTTTGCCCGTCT	20	60.04	55	0	

Table 2- List of primer pairs

PRIMER RECONSTITUTION

Lyophilized 100µM primers (STOCK)

Nuclease free water was added to the primer pair as mentioned on the data sheet provided by the sigma company to reconstitute 100µM primer stock. Reconstituted Primers were vortexed for 15 minutes followed by short spin. An aliquot of 50 µl was made for each primer pair. The primers were stored at -80°C.

Further 10 µM and 0.5 µM working solution of the primers were made for PCR and Real time PCR respectively. To avoid contamination UV crosslinked Nuclease free water, pipettes, tips, stands and eppendorfs were used.

For 10µM primers

100µM stock primer	: 10µl
NFW	: 90µl
Total	: 100µl

For 0.5µM Primers (Primer Pair)

Forward Primer (10µM):	2.5µl
Reverse Primer (10µM):	2.5µl
NFW	: 45µl
Total	: 50µl

Checking the primers through PCR

Gradient PCR was done by varying the annealing temperature in the range from 55-65°C followed by checking the amplification through agarose gel electrophoresis to select the best annealing temperature. The best amplification temperature was selected as 60°C. The constituents for one PCR reaction of 25 µl is as follows:

Taq buffer (1x)	2.5µl
$Mgcl_2$ (25 µM)	2.0µl
dNTP	0.6µl
Forward Primer	1.0µl
Reverse Primer	1.0µl
Nuclease free water	15.8µl
Taq polymerase	0.2µl
cDNA (2mcg/20 µl)	2.0µl
Total	25 µl

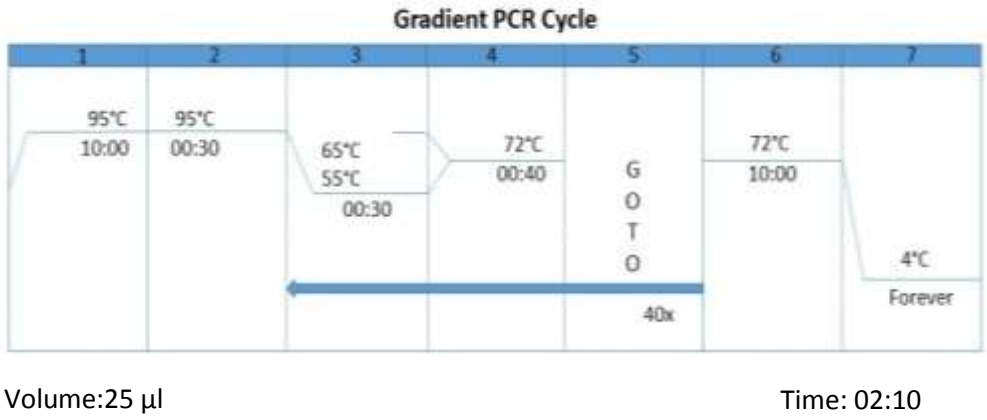


Figure 2- Gradient PCR reaction cycle

Primer Optimization

Different concentrations of primers ranging from 0.25-0.8 µM were used to get best results. Primers concentration giving best amplification results in real time PCR using a control cDNA (kept for standardization only) was selected. 0.5 µM primer concentration was used further for the work. . The constituents for one real time PCR reaction of 10 µl is as follows

SYBR green	5µl
cDNA (2mcg)	0.5µl
Primer pair (0.5µM)	1.0µl
Nuclease free water	3.5µl
	10 µl

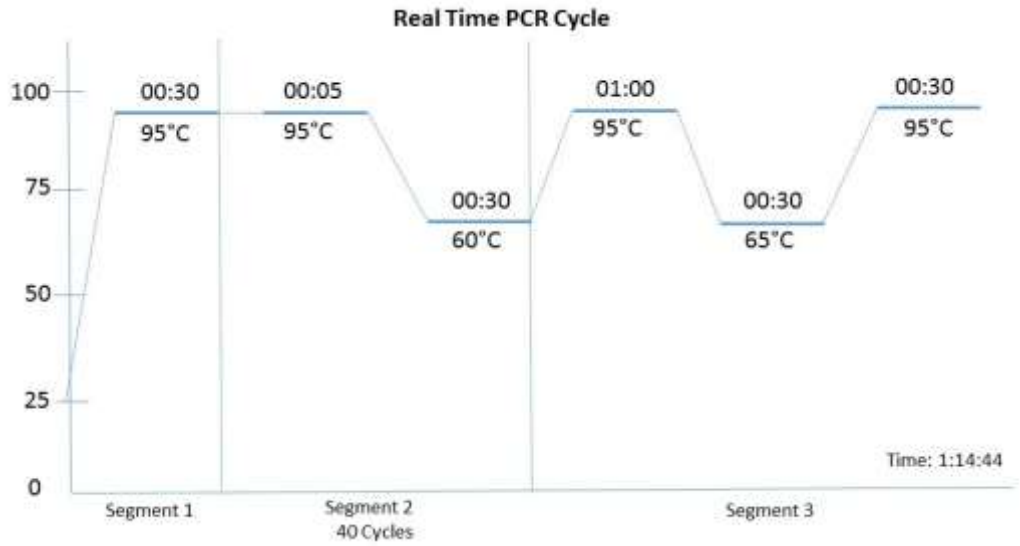


Figure 3- Reaction cycle for real time PCR (primer optimization)

RNA isolation using RNAzol

2ml bone marrow was collected in an EDTA vacutainer and was diluted with 4 ml of PBS (1X) buffer and mixed by inverting the tube. Diluted bone marrow was then gently overlaid onto a falcon (15 ml) containing 4 ml Histopaque and centrifuged at 1200 rpm for 35 minutes at room temperature. The buffy coat/PBMC were separated and transferred to another falcon. Three volumes of PBS was added to the isolated PBMCs and mixed by tilting. Centrifugation was done at 2000 rpm for 10 minutes at room temperature. The supernatant was discarded; the pellet was re-suspended in 1 ml PBS (1x) and transferred into an eppendorf followed by centrifugation at 1500 rpm for 10 minutes at room temperature. The supernatant was removed completely and 1ml RNAzol (SIGMA-ALDRICH) was added to the pellet and rigorous pipetting was done to completely mix the pellet. Vortexing was done for 2minutes after adding 400 µlof nuclease free water. The eppendorf was then incubated at room temperature for 15 minutes. Centrifugation was done thereafter at 4°C at 12000 g for 15 minutes. The supernatant (600 µl) was transferred into 2 eppendorfs and equal amount of isopropanol was added and mixed with pipetting.

Incubation was done for 15 minutes at room temperature and centrifuged at 12000 g for 10 minutes at room temperature. The supernatant was discarded from both the eppendorfs and 800 µlof 75% of ethanol was added to first eppendorf and the pellet along with the ethanol was transferred to the second eppendorf and centrifuged at 7500g for 3 minutes at room temperature. Second ethanol (75%) wash was given after discarding the supernatant followed by centrifugation at 7500 g for 3 minutes at room temperature. Ethanol was removed and the pellet was air dried till it became transparent. Elution was done in 30 µl of nucleus free water. The RNA was then stored at -80°C till further use.

Quantification and checking the quality of RNA on agarose gel

Gel electrophoresis was done to check the quality of RNA and Nano Drop (C1000) was used to quantify the RNA concentration isolated from blood.

RNA to cDNA conversion

RNA to cDNA conversion was done using RT kit (Applied Biosystems) and 2 reactions of 20 µl each of 2µg cDNA was made. The constituents for one reaction is as follows:

RT Buffer	2µl
dNTP	0.8µl
Random primer	2µl
Reverse transcriptase	1µl
RNase Inhibitor	1µl
RNA	(calculated according to the concentration for 2mcg)
Nuclease free water	(to make up the volume)
	20 µl

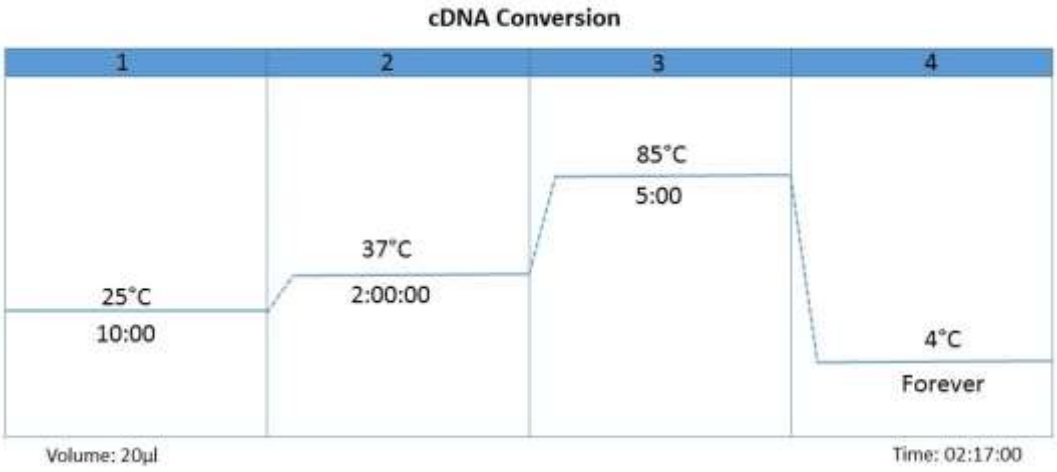


Figure 4- cDNA conversion reaction cycle

cDNA quality check

cDNA was checked for normal amplification through real time PCR. Endogenous primer pair GAPDH was used for the quality check. Non amplifying cDNA samples were not processed further. The constituents for one real time reaction is as follows:

SYBR green	5µl
cDNA(2mcg)	0.5µl
Primer pair (0.5µM)	1.0µl
Nuclease free water	3.5µl
	10 µl

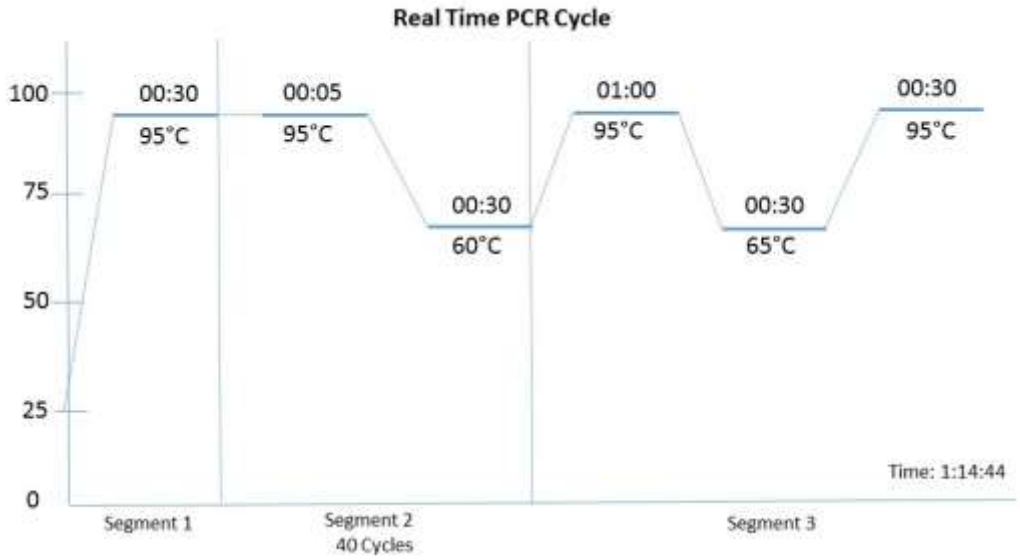


Figure 5- Reaction cycle for real time PCR (cDNA check)

Real time PCR

Real time PCR was dome using SYBR Green (Applied Biosystems). Real time expression was obtained by using Agilent technologies Stratagene MX3005P real time PCR machine. SYBR Green an intercalating dye binds to the double stranded DNA at the minor groove. SYBR green binds to the amplified product and amplification plot and SYBR dissociation curve is formed. Both amplification and dissociation curves are analyzed to check any non specific product. ROX dye was selected as a reference dye. The constituents for one real time reactions is as follows:

SYBR green	5µl
cDNA(2mcg)	0.5µl
Primer pair (0.5µM)	1.0µl
Nuclease free water	3.5µl
	10 µl

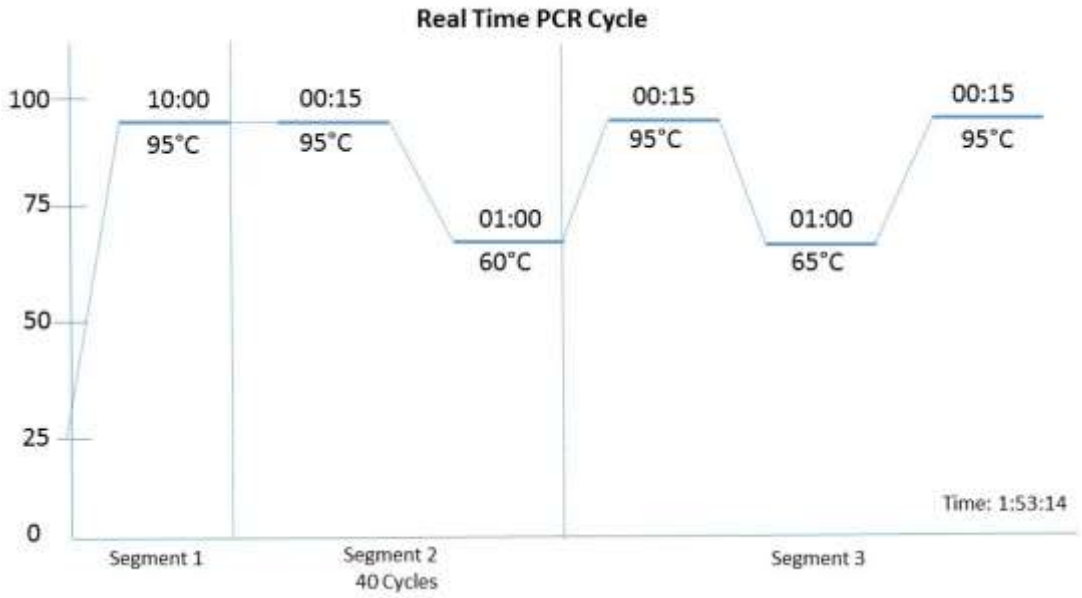


Figure 6- Reaction cycle for real time PCR for ABC transporters

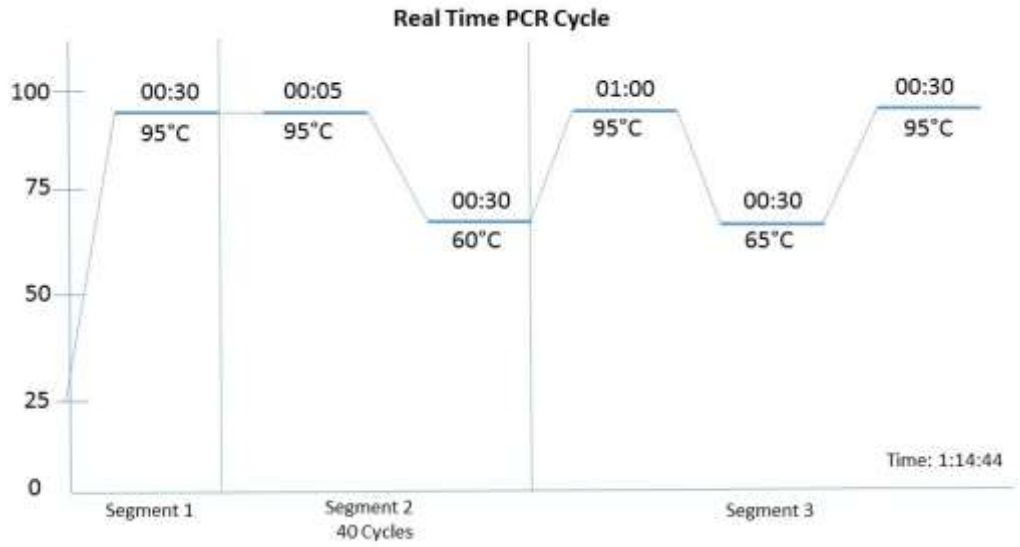


Figure 7- Reaction cycle for real time PCR for HSP70 and GRP78

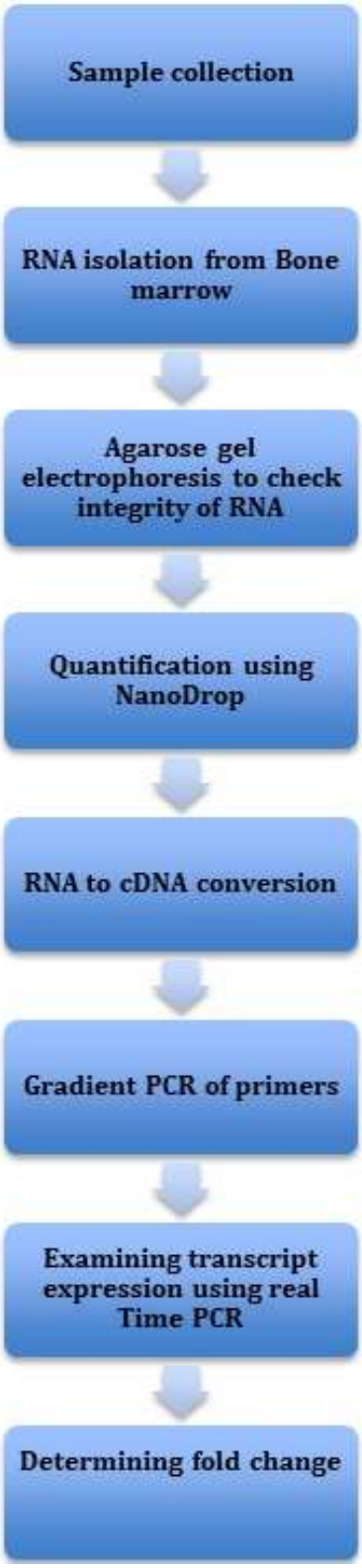


Figure 8- Work Flow

5. RESULTS

RNA extraction from Bone marrow

Good quality RNA from bone marrow of 6 acute lymphoblastic leukemia and 6 controls were extracted. The elution of RNA was done in 30 µl nuclease free water. Integrity of RNA was visualized by agarose gel electrophoresis.

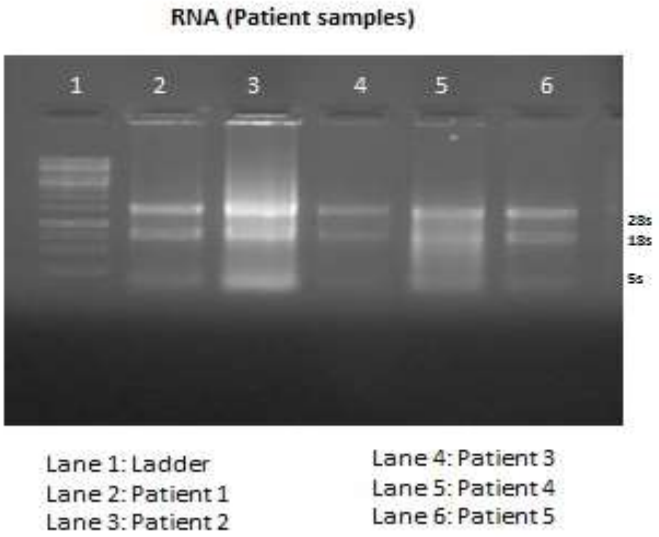


Figure 9- RNA bands on gel of 5 ALL patients

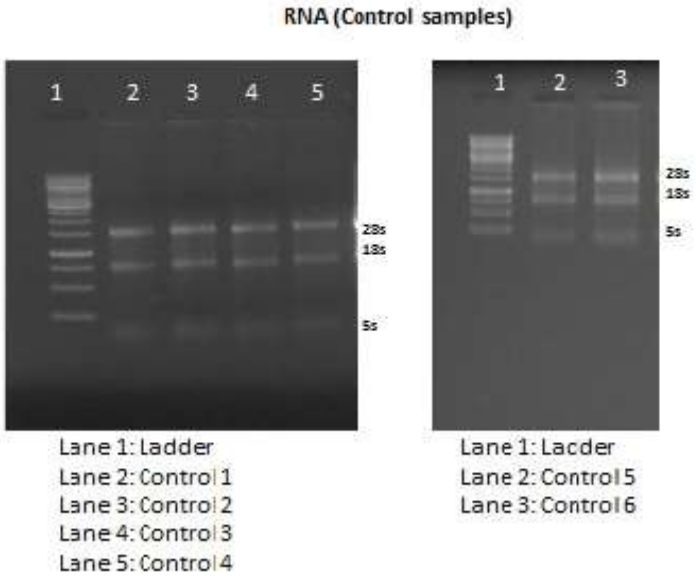


Figure 10- RNA bands on gel of 6 controls

Quantification of RNA was done using ND 1000 nanodrop spectrophotometer. RNA concentration varied between 400-1200 ng/ µl. $A_{260/280}$ and $A_{260/230}$ ratios were above 1.8 which showed that high purity of RNA (absence of protein contamination and absence of phenolic contamination respectively).

RNA to cDNA conversion

RNA was converted to cDNA by using high capacity reverse transcriptase kit from applied biosystems. Two 20µl reactions were made which contained 2000ng of RNA each.

cDNA quality check

Quality of cDNA synthesized from RNA was checked using real time PCR. GAPDH primers were used to check cDNA from 6 patients and 6 controls. The amplification was visualized in real time using SYBR green amplification and dissociation curves. The non amplified cDNA was not used further in the study.

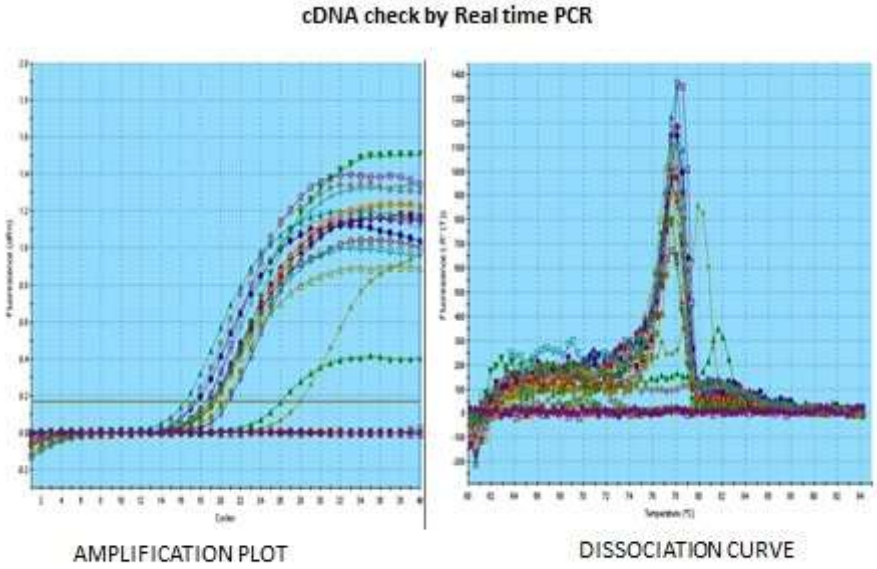


Figure 11- Amplification and dissociative curves of cDNA checked through real time PCR

Primer check using gradient PCR

Gradient PCR was used to check all the primer pairs and to find the best annealing temperature for the amplification of all the genes. The temperature gradient used was from 55-65°C.The results were visualized using agarose gel electrophoresis. Results showed that the primers of genes ABCC1, ABCC2, ABCC5, ABCB1 and ABCG2 showed good amplification at temperature 60°C while primers of genes ABCC3, ABCC4 did not show any

amplification at any temperature. Primers for ABCC3 and ABCC4 were not used further for the study.

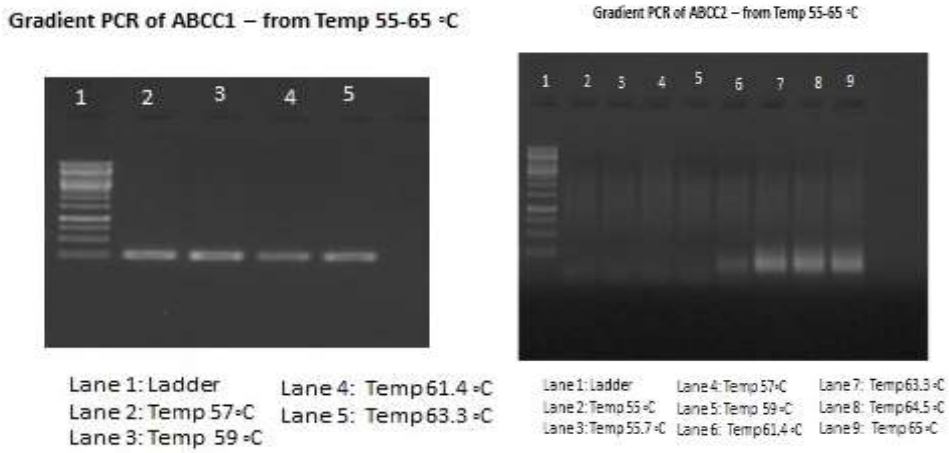


Figure 12-cDNA amplification after gradient PCR of ABCC1 (left) and ABCC2 (right)

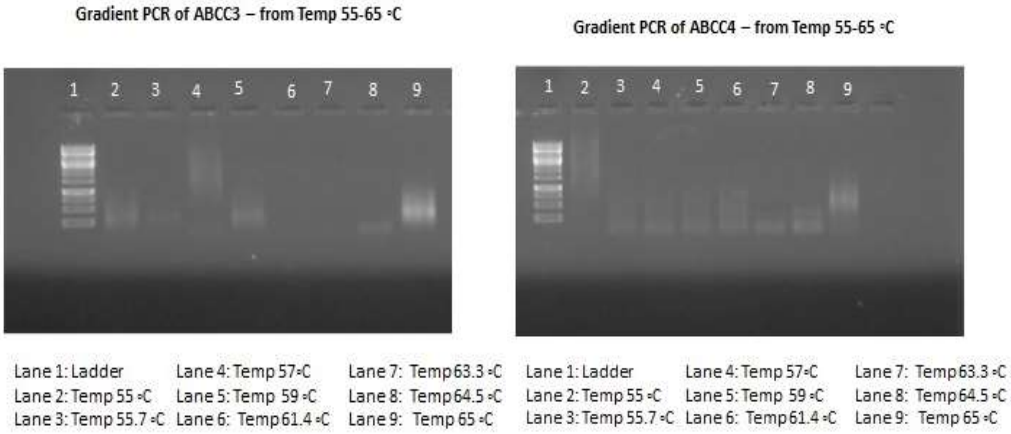


Figure 13- cDNA amplification after gradient PCR of ABCC3 (left) and ABCC4 (right)

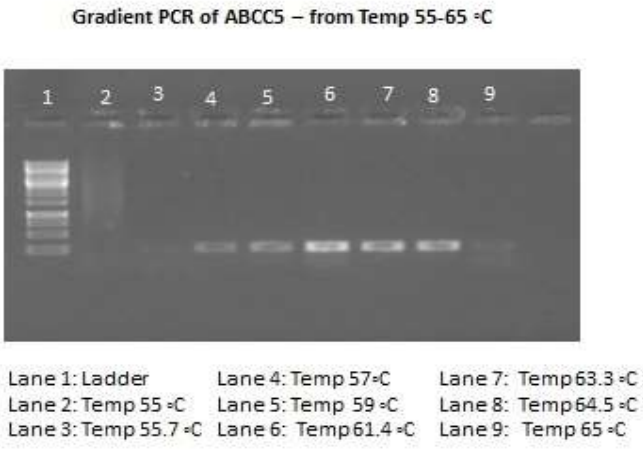


Figure 14- cDNA amplification after gradient PCR of ABCC5

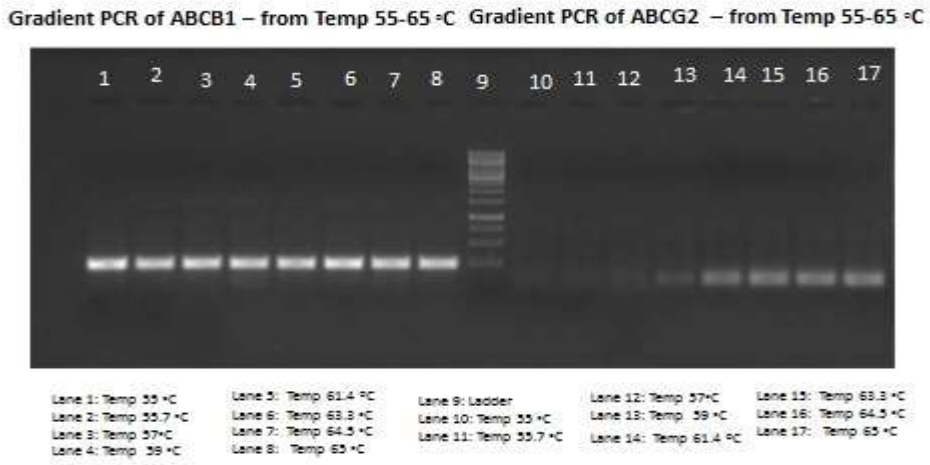


Figure 15- cDNA amplification after gradient PCR of ABCB1 (left) and ABCG2 (right)

Primer check using real time PCR

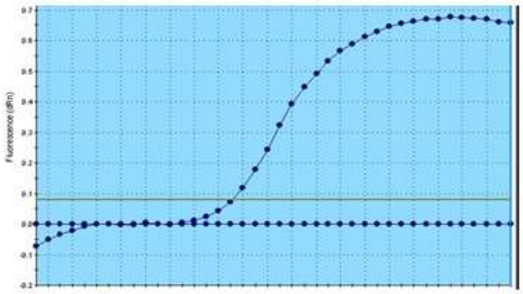
Primers were further checked using real time PCR. Real time PCR results showed the reaction cycle used worked for HSP 70 and GRP78 primers and showed specific binding and best Ct. But did not work for ABC transporters. The reaction cycle was altered for ABC transporters and showed single peak i.e. specific binding for ABCC1, ABCC5, ABCB1 but Ct values of ABCC2 and ABCG2 were very high (above 30) therefore these values were neglected and both the primers were not used further.

Real time PCR

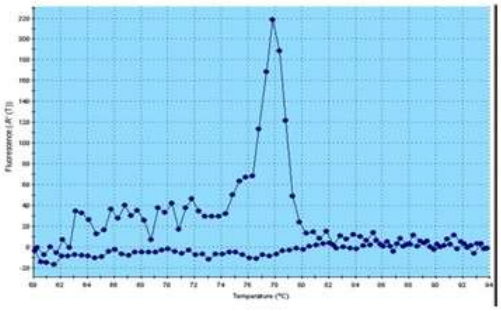
Real time expression was analysed by SYBR green dissociation curve to check any non specific products. GAPDH and beta-Actin primers were used as endogenous genes. Plate was set up using 2 ALL patients and 2 controls at a time.

For each individual cDDA, endogenous genes were run in triplicates and study genes ABCC1, ABCC5, ABCB1, HSP70 and GRP78 were run in duplicates. Non template controls were also put for each gene. The results showed tight Ct (ie difference in Ct values of duplicates/triplicates were less than 1) for all the study genes. No Ct was observed in case of non template control showing absence of non specific product. A representative amplification plot and dissociation curve is shown below for each of the genes to demonstrate the specificity.

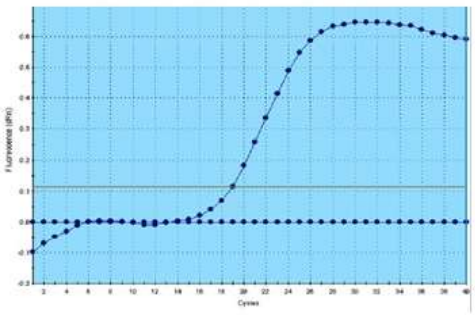
GAPDH - Amplification Plot



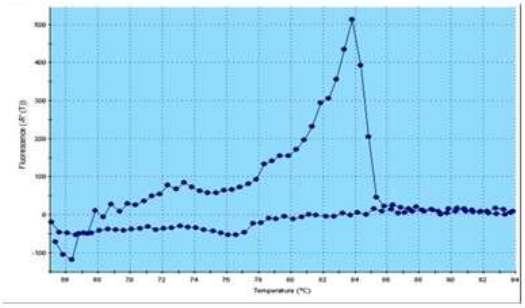
GAPDH - Dissociation curve



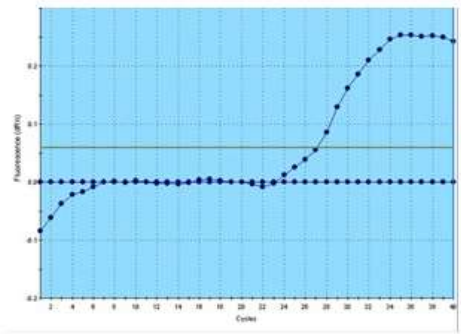
ACTB - Amplification Plot



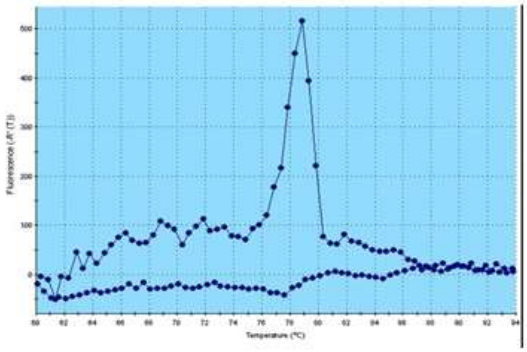
ACTB - Dissociation curve



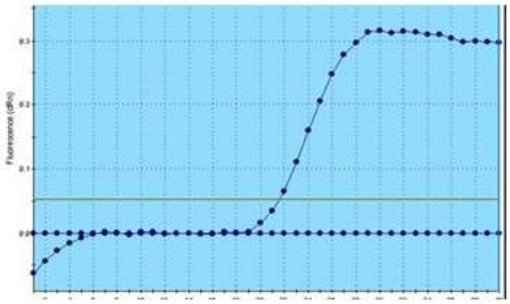
ABCC1 - Amplification Plot



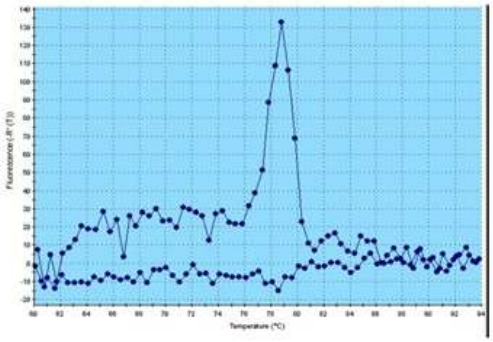
ABCC1 - Dissociation curve



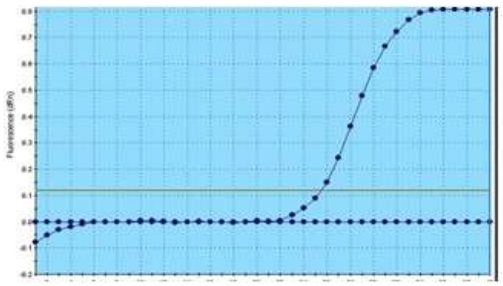
ABCC5 - Amplification Plot



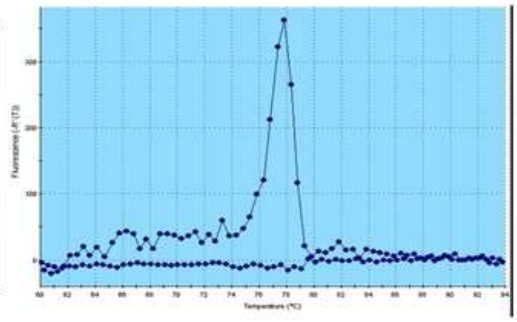
ABCC5 - Dissociation curve



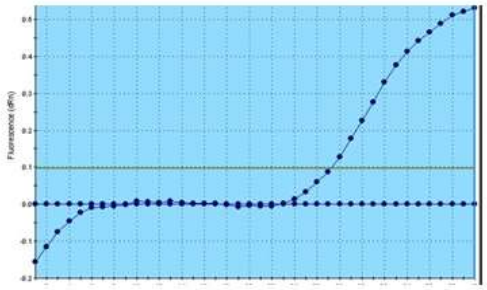
ABCB1 - Amplification Plot



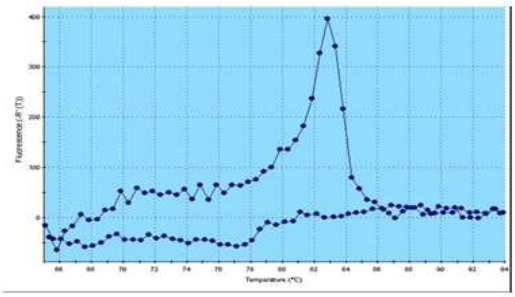
ABCB1 - Dissociation curve



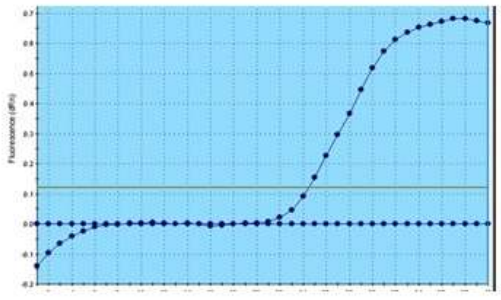
HSP70 - Amplification Plot



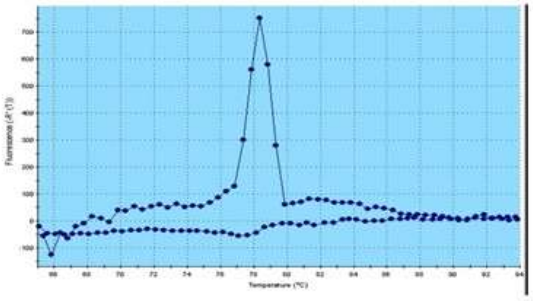
HSP70 - Dissociation curve



GRP78 - Amplification Plot



GRP78 – Dissociation Curve



Calculation of fold change with an example

There are two methods of calculating fold change. In method I, we subtracted controls' average individual fold change value from patients' individual fold change average value for a specific gene. In method II, we subtracted average controls average individual fold

change value from individual patient's fold change value. Both the methods are explained below:

Method I

Well	Well Name	Ct (dRn)	Average	Delta Ct	Individual fold change
A1	ACTB C1	18.78			
A2	ACTB C1	18.05			
A3	ACTB C1	18.78	18.5366667		
A7	HSP70 C1	27.55			
A8	HSP70 C1	27.41	27.48	8.9433333	0.00203137
C1	ACTB P1	18.3			
C2	ACTB P1	17.87			
C3	ACTB P1	18.4	18.19		
C7	HSP70 P1	29.45			
C8	HSP70 P1	30.38	29.915	11.725	0.00029541
E1	ACTB C2	17.68			
E2	ACTB C2	17.45			
E3	ACTB C2	17.65	17.5933333		
E7	HSP70 C2	26.41			
E8	HSP70 C2	26.55	26.48	8.8866667	0.00211274
G1	ACTB P2	20.34			
G2	ACTB P2	20.25			
G3	ACTB P2	20.88	20.49		
G7	HSP70 P2	29.76			
G8	HSP70 P2	29.11	29.435	8.945	0.00202902

Table 3- Individual fold change of two ALL patients and controls

Average Ct value was calculated for every replicate as shown in the table above. Formulas used are explained below:

Delta Ct = Ct (target) – Ct (endogenous)

Individual fold change = $2^{-\text{delta Ct}}$

	Patient individual fold change		**Control Individual fold change**		
	P1	P2	C1	C2	Control Average
HSP70	0.00029541	0.00202902	0.00203137	0.00211274	0.00207206

Table 4- Fold change with average of patient and average of control

	P1	**Control Average**	**FOLD CHANGE**	**Less than 1**
HSP70	0.000295409	0.002072055	0.14256797	7.014198231

Table 5- Fold change with P1 and Control Average

	P2	**Control Average**	**FOLD CHANGE**	**Less than 1**
HSP70	0.002029022	0.002072055	0.979231448	1.021209033

Table 6- Fold change with P2 and Control average

Method 2

	P1	**P2**	**Patient Average**	**C1**	**C2**	**Control Average**
HSP70	0.000295409	0.002029022	0.001162215	0.002031367	0.002112743	0.002072055

Table 7- Calculating patient and control individual fold change average

$$\text{Fold change} = \frac{\text{Patients Individual fold change average}}{\text{Controls Individual fold change average}}$$

Upregulation is shown in +Y axis and downregulation is shown in –Y axis. Fold change values which are less than 2 shows downregulation. In order to show down regulation in –Y axis, we use the formula:

Down Regulation = -1/value less than 1

	Patient Average	Control Average	Fold Change	Less than 1
HSP70	0.001162215	0.002072055	0.560899709	-1.782849918

Table 8- Method 2 for calculating fold change

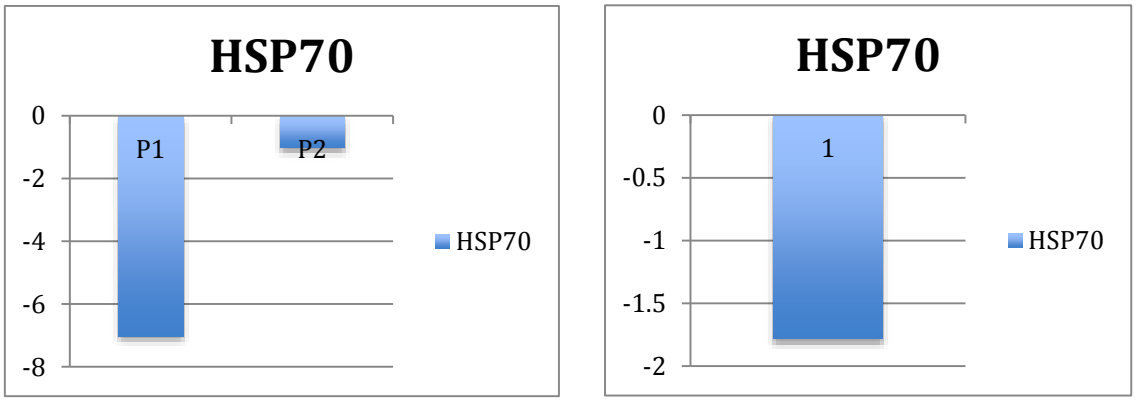


Figure 16- Fold change of HSP70 obtained from method1 and method2 (EXAMPLE)

Real time data analysis

As the reaction conditions used for ABC transporters and Heat shock proteins were different so that data was analysed separately.

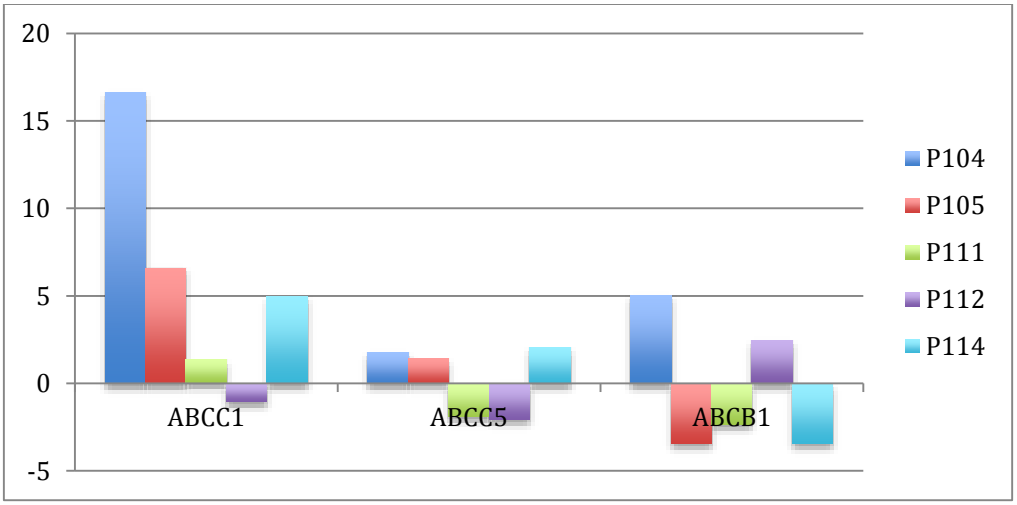


Figure 17- Fold change in ABC transporters genes normalized with GAPDH (method1)

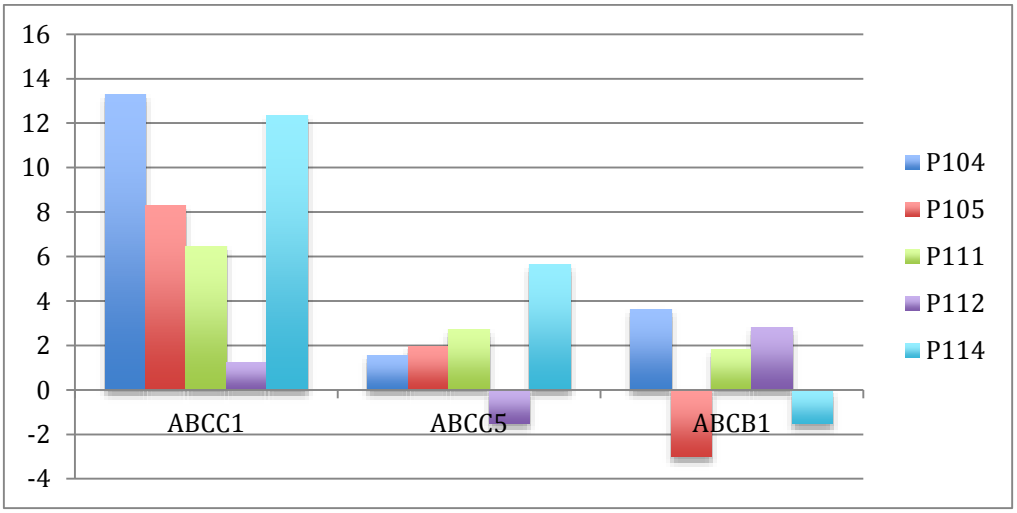


Figure 18- Fold change in ABC transpoters genes normalized with ACTB (method 1)

The transcript level of all the gens were analysed by normalizing separately with GAPDH and ACTB as endogenous controls.

On normalizing with GAPDH an upregulation of gene ABCC1 by 5 fold was observed in two ALL patients P105 and P114 and upregulation by 16 fold was obsverved in P104. No change in expression levels was observed in P111 and P112.

Levels of ABCC5 were upregulated by 2 fold in one patient P114 and downregulated by 2 fold in P111 and P112 while P104 and P105 showed no fold change.

Gene ABCB1 showed more than 2.5 fold upregulation in two patients (P104, P112). While three patients (P105, P111 and P114) showed downregulation by more than 2 fold.

On normalization with ACTB as endogenous control more than 12 fold upregulation was observed in ABCC1 gene in two patients P104, P114 and more than 6 fold upregulation in P105 and P111. No fold change was observed in P112.

Gene ABCC5 showed 5 fold upregulation in one patient P114 and 2 fold change was observed in P105 and P111. No fold change was observed in P104 and P112.

Gene ABCB1 was upregulated by 2 fold in two patients P104, P111 and P112. Downregulation of gene was observed in P105 by 3 fold. No fold change was observed in P114

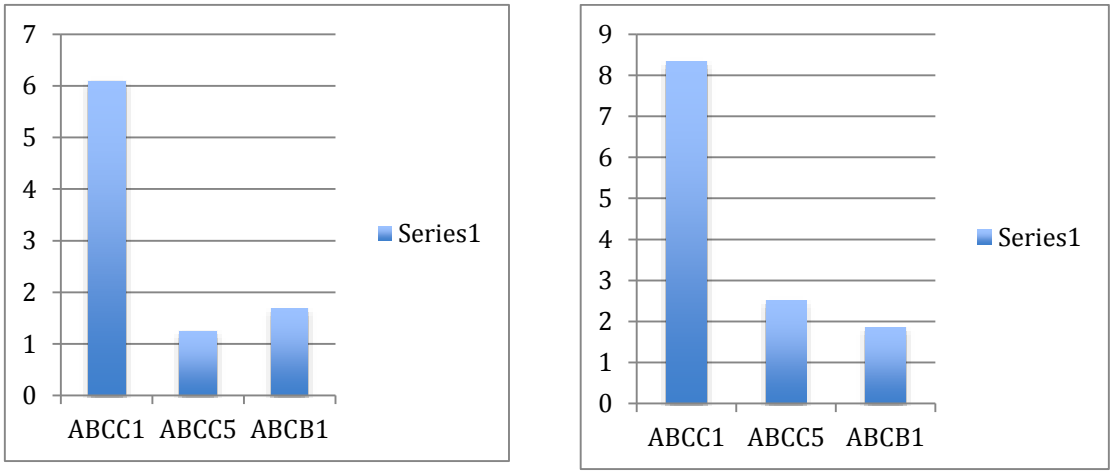


Figure 19- Fold change of ABC transporter genes with GAPDH and ACTB (method 2)

On averaging the patient data more than 6 fold upregulation was observed in ABCC1 with GAPDH normalization and ACTB normalization respectively, wheras ABCC5 showed no fold change with GAPDH but 2.5 fold change was observed with ACTB as endogenous control.

In ABCB1 gene a fold change of more than 1.5 was observed with both GAPDH normalization and ACTB normalization.

Figure 20- Fold change of HSP70 and GRP78 normalized with ACTB (method 1)

Since the reaction conditions for HSP70 and GRP78 were different therefore they were analysed separately. ACTB was used as an endogenous control and gene HSP70 showed 10 fold downregulation in one patient (P105) and more than 3 fold downregulation in wsa observed in two patients (P104 and P112). Upregulation of 3 fold was observed in P114.

Gene GRP78 showed 5 fold increase in one patient P114 and a 1.5 fold downregulation in P112. No fold change was observed in P104, P105.

Figure 21- Fold change of HSP70 and GRP78 normalized with ACTB (method 2)

On averaging the patient data it was observed that HSP70 did not show any fold change whereas a 2 fold upregulation was observed in GRP78 gene.

6. DISCUSSION AND FUTURE PERSPECTIVE

ABCC1, ABCC5 and ABCB1 are ABC transporters responsible for transporting drugs and other molecules such as ions, proteins, peptides etc. out of the cell. This function of ABC transporters are being used by cancer cells to extrude out anti cancer drugs from inside the cell making the cell resistant to these drugs. Multidrug resistence thus hinders the effectiveness of the drug.

Upregulation of ABCC1 and ABCC5 was observed in children with ALL in this study indicating that there is a high risk of multidrug resistance. It is important to follow these cases during the course of treatment to see development of any resistance or relapse after the end of treatment. Interestingly, upregulation of these genes have been reported in a study from Iran (Mahjoubi et al., 2012) and shown to be associated with poor outcome and prediction of relapse (Plasschaert et al., 2005). Our results coincide with their data and encourages us to extend it to larger sample set and corelate with treatment response.

ABCB2 gene particulaly showed upregulation in relapsed case of ALL (P104) but minimal upregulation was observed in newly diagnosed cases indicating a correlation between this gene and relapse of disease. It can be concluded that the expression of this gene is usually less in initial phase of the disease.

As this is an ongoing study the increase in sample size would help us to evaluate the role of these genes as potential biomarkers for predicting relapse and resistance to anti leukemic drugs.

No conclusive results were observed for molecular chaperon GRP78. But downregulation of HSP70 was seen.

7. REFERENCES

Abd El-Ghaffar HA; Aladle DA; Farahat SE; Abd El-Hady N (2006). P-glycoprotein (P-170) expression in acute leukemias. Hematology. **11**(1):35-41.

Abedi M; S. Rahgozar (2013). P-glycoprotein 170; Its clinical importance and pathophysiological role in cancer. Journal of Isfahan Medical School. **31**(228):274–293.

Abedi, M, S. Rahgozar (2012). The mechanisms of drug resistance in Acute lymphoblastic leukemia. The 6th Annual Congress of Iranian Blood and pediatric cancer Society. 61.

Aberuyi, N; S. Rahgozar (2013). New approach of multi-drug resistance in acute lymphoblastic leukemia. 3th National Congress of Hematology. **3**:51.

Ajouz H; Mukherji D; Shamseddine A (2014). Secondary bile acids: an underrecognized cause of colon cancer. World J Surg Oncol. **12**:164.

Albrecht, C; E. Viturro (2007). The ABCA subfamily—gene and protein structures, functions and associated hereditary diseases. Pflügers Archiv-European Journal of Physiology. **453**(5):581–589.

Beckmann RP; Mizzen LE; Welch WJ (1990). Interaction of Hsp70 with newly synthesized proteins: Implications for protein folding and assembly. Sceince **248**:850-4

Belinsky MG; Chen ZS; Shchaveleva I; Zeng H; Kruh GD (2002). Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6,ABCC6). Cancer Research. **62**(21):6172.

Belson M; Kingsley B; Holmes A (2007). Risk factors for acute leukemia in children: a review. Environ Health Perspect.;**115**(1):138-45.

Bernstein H; Bernstein C; Payne CM; Dvorak K (2009). Bile acids as endogenous etiologic agents in gastrointestinal cancer. World J Gastroenterol. **15**(27):3329-40.

Bernstein H; Bernstein C; Payne CM; Dvorakova K; Garewal H (2005). Bile acids as carcinogens in human gastrointestinal cancers. Mutat Res. **589**(1):47-65.

Borst P; Evers R; Kool M; Wijnholds J (2000). A family of drug transporters: the multidrugresistance-associated proteins. Journal of the National Cancer Institute.

Bortolozzi R; Viola G; Porcù E; Consolaro F; Marzano C; Pellei M; Gandin V; Basso G (2014). A novel copper(I) complex induces ER-stress-mediated apoptosis and sensitizes

B-acute lymphoblastic leukemia cells to chemotherapeutic agents. Oncotarget. **5**(15):5978-91.

Campos-Sanchez E; Toboso-Navasa A; Romero-Camarero I; Barajas-Diego M; Sanchez-García I; Cobaleda C (2011). Acute lymphoblastic leukemia and developmental biology: a crucial interrelationship. Cell Cycle.**10**(20):3473-86.

Cervenak J; Andrikovics H; Ozvegy-Laczka C; Tordai A; Német K; Váradi A; Sarkadi B (2006). The role of the human ABCG2 multidrug transporter and its variants in cancer therapy and toxicology. Cancer Lett.; **234**(1):62-72.

Chandy M (1995). Childhood acute lymphoblastic leukemia in India: an approach to management in a three-tier society. Med Pediatr Oncol. **25**(3):197-203.

Chauhan PS; Bhushan B; Singh LC; Mishra AK; Saluja S; Mittal V; Gupta DK; Kapur S (2012). Expression of genes related to multiple drug resistance and apoptosis in acute leukemia: response to induction chemotherapy. Exp Mol Pathol. **92**(1):44-9.

Chen ZS; Lee K; Walther S; Raftogianis RB; Kuwano M; Zeng H; Kruh GD (2002). Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res. **62**(11):3144-50.

Choi YH; Yu AM (2014). ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des. **20**(5):793-807

Clapp RW; Jacobs MM; Loechler EL (2008). Environmental and occupational causes of cancer: new evidence 2005-2007. Rev Environ Health. **23**(1):1-37.

Coelho, A.C; P.C. Cotrim (2013). The Role of ABC Transporters in Drug-Resistant Leishmania, in Drug Resistance in Leishmania Parasites. Springer

Costarelli V (2009). Bile acids as possible human carcinogens: new tricks from an old dog. Int J Food Sci Nutr. 60 Suppl 6:116-25.

Darbre PD; Harvey PW (2014). Parabens can enable hallmarks and characteristics of cancer in human breast epithelial cells: a review of the literature with reference to new exposure data and regulatory status. J Appl Toxicol. **34**(9):925-38.

de Grouw EP; Raaijmakers MH; Boezeman JB; van der Reijden BA; van de Locht LT; de Witte TJ; Jansen JH; Raymakers RA(2006). Preferential expression of a high number of ATP binding cas sette transporters in both normal and leukemic CD34+CD38- cells. Leukemia. **20**(4):750-4.

Dean M; Rzhetsky A; Allikmets R (2001). The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. **11**(7):1156-66.

Deeley RG; Westlake C; Cole SP (2006). Transmembrane transport of endo- and xenobiotics bymammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev. **86**:849–99.

den Boer ML; Pieters R; Kazemier KM; Janka-Schaub GE; Henze G; Veerman AJ (1998). The modulating effect of PSC 833, cyclosporin A, verapamil and genistein on in vitro cytotoxicity and intracellular content of daunorubicin in childhood acute lymphoblastic leukemia. Leukemia. **12**(6):912-20.

Dhooge C; De Moerloose B; Laureys G; Ferster A; De Bacquer D; Philippe J; Leroy J; Benoit Y (2002). Expression of the multidrug transporter P-glycoprotein is highly correlated with clinical outcome in childhood acute lymphoblastic leukemia: results of a long-term prospective study. Leuk Lymphoma. **43**(2):309-14.

El-Sharnouby JA; Abou El-Enein AM; El Ghannam DM; El-Shanshory MR; Hagag AA; Yahia S; Elashry R (2010). Expression of lung resistance protein and multidrug resistance-related protein (MRP1) in pediatric acute lymphoblastic leukemia. J Oncol Pharm Pract. **16**(3):179-88.

Feder ME; Hofmann GE (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol. **61**:243-82.

Fernandez RC; Driscoll TR; Glass DC; Vallance D; Reid A; Benke G; Fritschi L (2012). A priority list of occupational carcinogenic agents for preventative action in Australia. Aust N Z J Public Health. **36**(2):111-5.

Fletcher JI; Haber M; Henderson MJ; Norris MD (2010). ABC transporters in cancer: more than just drug efflux pumps. Nat Rev Cancer.; **10**(2):147-56.

Fronkova E; Mejstrikova E; Avigad S; Chik KW; Castillo L; Manor S; Reznickova L; Valova T; Zdrahalova K; Hrusak O; Jabali Y; Schrappe M; Conter V; Izraeli S; Li CK; Stark B; Stary J; Trka J (2008). Minimal residual disease (MRD) analysis in the non-MRD-based ALL IC-BFM 2002 protocol for childhood ALL: is it possible to avoid MRD testing? Leukemia. **22**(5):989-97.

Fujita M; Nagai M; Murata M; Kawakami K; Irino S; Takahara J (1997). Synergistic cytotoxic effect of quercetin and heat treatment in a lymphoid cell line (OZ) with low HSP70 expression. Leuk Res. **21**(2):139-45.

Gillet JP; Efferth T; Steinbach D; Hamels J; de Longueville F; Bertholet V; Remacle J (2004). Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. Cancer Res.; **64**(24):8987-93

Glavinas H; Krajcsi P; Cserepes J; Sarkadi B (2004). The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv.; **1**(1):27-42.

Gottesman MM; Hrycyna CA; Schoenlein PV; Germann UA; Pastan I (1995). Genetic analysis of the multidrug transporter. Annu Rev Genet. **29**:607-49.

Gottesman; M.M, Fojo; S.E. Bates (2002). Multidrug resistance in cancer: role of ATP–dependent transporters. Nature Reviews Cancer. **2**(1):48–58.

Graner MW; Cumming RI; Bigner DD (2007). The heat shock response and chaperones/heat shock proteins in brain tumors: surface expression, release, and possible immune consequences. J Neurosci. **27**(42):11214-27.

Haber M; Smith J; Bordow SB; Flemming C; Cohn SL; London WB; Marshall GM; Norris MD (2006). Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma. J Clin Oncol. **24**(10):1546-53.

Hattori H; Suminoe A; Wada M; Koga Y; Kohno K; Okamura J; Hara T; Matsuzaki A (2007). Regulatory polymorphisms of multidrug resistance 1 (MDR1) gene are associated with the development of childhood acute lymphoblastic leukemia. Leuk Res. **31**(12):1633-40.

Hayashi Y (1992). Overview of genotoxic carcinogens and non-genotoxic carcinogens. Exp Toxicol Pathol. **44**(8):465-71.

Henderson MJ; Haber M; Porro A; Munoz MA; Iraci N; Xue C; Murray J; Flemming CL; Smith J; Fletcher JI; Gherardi S; Kwek CK; Russell AJ; Valli E; London WB; Buxton AB; Ashton LJ; Sartorelli AC; Cohn SL; Schwab M; Marshall GM; Perini G; Hlavac V; Brynychova V; Vaclavíkova R; Ehrlichova M; Vrana D; Pecha V; Kozevnikovova R; Trnkova M; Gatek J; Kopperova D; Gut I; Soucek P (2013). The expression profile of ATP-binding cassette transporter genes in breast carcinoma. Pharmacogenomics. **14**(5):515-29.

Hyde SC; Emsley P; Hartshorn MJ; Mimmack MM; Gileadi U; Pearce SR; Gallagher MP; Gill DR; Hubbard RE; Higgins CF (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature. **346**(6282):362-5.

Jaattela M; Wissing D; Kokholm K; Kallunki T; Egeblad M (1998). Hsp70 exerts its anti‐apoptoticfunction downstream of caspase‐3‐like proteases. Embo J.**17**:6124‐34.

Janke D; Mehralivand S; Strand D; Godtel-Armbrust U; Habermeier A; Gradhand U; Fischer C; Toliat MR; Fritz P; Zanger UM; Schwab M; Fromm MF; Nurnberg P; Wojnowski L; Closs EI; Lang T (2008). 6-mercaptopurine and 9-(2-phosphonyl-methoxyethyl) adenine (PMEA) transport altered by two missense mutations in the drug transporter gene ABCC4. Hum Mutat. **29**(5):659-69.

Jedlitschky G; Burchell B; Keppler D (2000). The multidrug resistance protein 5 functions asan ATP-dependent export pump for cyclic nucleotides. The Journal of Biological Chemistry. **275(**39):30069-74.

Jemal A; Bray F; Center MM; Ferlay J; Ward E; Forman D (2011). Global cancer statistics. CA Cancer J Clin.. **61**(2):69-90.

Juliano RL; Ling V (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. **455**(1):152-62.

Kaiser M; AKühnl K; Reins J; Fischer S; Ortiz-Tanchez J;; Schlee C Mochmann LH; Heesch S; Benlasfer O; Hofmann WK; Thiel E ; Baldus CD (2011). Antileukemic activity of the HSP70 inhibitor pifithrin-μ in acute leukemia. Blood Cancer Journal. **1**:e28

Kavlock RJ; Daston GP; DeRosa C; Fenner-Crisp P; Gray LE, Kaattari S; Lucier G; Luster M; Mac MJ; Maczka C; Miller R; Moore J; Rolland R; Scott G; Sheehan DM; Sinks T; Tilson HA (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect. **4**:715-40.

Khanna S; Dash PR; Darbre PD (2014). Exposure to parabens at the concentration of maximal proliferative response increases migratory and invasive activity of human breast cancer cells in vitro. J Appl Toxicol. **34**(9):1051-9.

Kharabi Masouleh B; Geng H; Hurtz C; Chan LN; Logan AC; Chang MS; Huang C; Swaminathan S; Sun H, Paietta E; Melnick AM; Koeffler P; Müschen M (2014). Mechanistic rationale for targeting the unfolded protein response in pre-B acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. **111**(21):E2219-28.

Kim I; Xu W; Reed JC (2008). Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov. **7**:1013–30.

Kool M; van der Linden M; de Haas M; Scheffer GL; de Vree JM; Smith AJ; Jansen G; Peters GJ; Ponne N; Scheper RJ; Elferink RP; Baas F; Borst P (1999). MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A. **96**(12):6914-9.

Lee SJ; Yum YN; Kim SC; Kim Y; Lim J; Lee WJ; Koo KH; Kim JH; Kim JE; Lee WS; Sohn S; Park SN; Park JH; Lee J; Kwon SW (2013). Distinguishing between genotoxic and non-genotoxic hepatocarcinogens by gene expression profiling and bioinformatic pathway analysis. Sci Rep. **3**:2783.

Liu Y; Yin Y; Sheng Q; Lu X; Wang F; Lin Z; Tian H; Xu A; Zhang J (2014). Association of ABCC2 -24C>T polymorphism with high-dose methotrexate plasma concentrations and toxicities in childhood acute lymphoblastic leukemia. PLoS One. **9**(1):e82681.

Lucie Sedlackova; Martin Spacek; Ernst Holler; Zuzana Imryskova; Ilona Hromadnikova (2010). Heat-shock protein expression in leukemia. Tumor Biology. **32**(1): 33-44

Mahjoubi F; Akbari S (2012). Multidrug resistance-associated protein 1 predicts relapse in Iranian childhood acute lymphoblastic leukemia. Asian Pac J Cancer Prev. **13**(5):2285-9.

Mahjoubi F; Golalipour M; Ghavamzadeh A; Alimoghaddam K (2008). Expression of MRP1 gene in acute leukemia. Sao Paulo Med J.**126**(3):172-9.

Mayer MP; Bukau B (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci. **62**(6):670-84.

Mehta PA; Davies SM (2004). Pharmacogenetics of acute lymphoblastic leukemia. Curr Opin Hematol.; **11**(6):434-8.

Mezei G; Sudan M; Izraeli S; Kheifets L (2014). Epidemiology of childhood leukemia in the presence and absence of Down syndrome. Cancer Epidemiol. **38**(5):479-89.

Norgaard JM; Hokland P (2000). Biology of multiple drug resistance in acute leukemia. Int J Hematol. 72(3):290-7. Review. Erratum in: Int J Hematol 2001 Jan;73(1):132..

Norris MD (2011). ABCC multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux. J Natl Cancer Inst. **103**(16):1236-51

Norris MD; Bordow SB; Marshall GM; Haber PS; Cohn SL; Haber M (1996). Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma.New Eng J Med. **334**:231–8.

Plasschaert SL; de Bont ES; Boezen M; vander Kolk DM; Daenen SM; Faber KN; Kamps WA; de Vries EG; Vellenga E (2005). Expression of multidrug resistance-associated proteins predicts prognosis in childhood and adult acute lymphoblastic leukemia. Clin Cancer Res. **11**(24 Pt 1):8661-8.

Plasschaert SL; van der Kolk DM; de Bont ES, Kamps WA; Morisaki K; Bates SE; Scheffer GL; Scheper RJ; Vellenga E; de Vries EG (2003). The role of breast cancer resistance protein in acute lymphoblastic leukemia. Clin Cancer Res. **9**(14):5171-7.

Raaijmakers M (2007). ATP-binding-cassette transporters in hematopoietic stem cells and their utility as therapeutical targets in acute and chronic myeloid leukemia. Leukemia.; **21**(10):2094–2102.

Rau T; Erney B; Gores R; Eschenhagen T; Beck J; Langer T (2006). High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. Clin Pharmacol Ther. **80**(5):468-76.

Riordan JR; Ling V (1979). Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. J Biol Chem. **254**(24):12701-5.

Russel FG; Koenderink JB; Masereeuw R (2008). Multidrug resistance protein 4

(MRP4/ABCC4): a versatile efflux transporter for drugs and signaling molecules. Trends Pharmacol Sci. **29**:200-207.

Sampath J; Adachi M; Hatse S; Naesens L; Balzarini J; Flatley RM; Matherly LH; Schuetz JD (2002). Role of MRP4 and MRP5 in biology and chemotherapy. AAPS PharmSci. **4**(3):E14.

Sarkar S; Horn G; Moulton K; Oza A; Byler S; Kokolus S; Longacre M (2013). Cancer development, progression, and therapy: an epigenetic overview. Int J Mol Sci. **14**(10):21087-113.

Schaich M; Soucek S; Thiede C; Ehninger G; Illmer T (2005). MDR1 and MRP1 gene expression are independent predictors for treatment outcome in adult acute myeloid leukaemia. Br J Haematol. **128**:324–32.

Shi Y; Thomas JO (1992). The transport of proteins into nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol Cell Biol.**12**:2186-92.

Shukla S; Chen ZS; Ambudkar SV (2012). Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance. Drug Resist Updat.;**15**(1-2):70-80.

Soto AM; Sonnenschein C (2010). Environmental causes of cancer: endocrine disruptors as carcinogens. Nat Rev Endocrinol.. **6**(7):363-70.

Stefková J; Poledne R; Hubácek JA (2004). ATP-binding cassette (ABC) transporters in human metabolism and diseases. Physiol Res. **53**(3):235-43.

Steinbach D; Gillet JP; Sauerbrey A; Gruhn B; Dawczynski K; Bertholet V; de Longueville F; Zintl F; Remacle J; Efferth T (2006). ABCA3 as a possible cause of drug resistance in childhood acute myeloid leukemia. Clin Cancer Res. **12**(14 Pt 1):4357-63.

Steinbach D; Lengemann J; Voigt A; Hermann J; Zintl F; Sauerbrey A (2003). Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia. Clin Cancer Res. **9**:1083–6.

Steinbach D; Wittig S; Cario G; Viehmann S; Mueller A; Gruhn B; Haefer R; Zintl F; Sauerbrey A (2003). The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. Blood. **102**(13):4493-8.

Swerts K; De Moerloose B; Dhooge C; Laureys G; Benoit Y; Philippé J (2006). Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia. Eur J Cancer. **42**(3):295-309.

Szabó D; Keyzer H; Kaiser HE; Molnár J. Reversal of multidrug resistance of tumor cells (2000). Anticancer Res. **20**(6B):4261-74.

Szegezdi E; Logue SE; Gorman AM; Samali A (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep. **7**:880–5

Tabas I; Ron D (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol.**13**:184–90

Thomas X; Campos L; Le QH; Guyotat D (2005). Heat shock proteins and acute leukemias. Hematology. **10**(3):225-35.

Tsai YL; Zhang Y; Tseng CC; Stanciauskas R; Pinaud F; Lee AS (2015). Characterization and mechanism of stress-induced translocation of 78-kilodalton glucose-regulated protein (GRP78) to the cell surface. J Biol Chem. **290**(13):8049-64.

Uckun FM; Qazi S; Ozer Z; Garner AL; Pitt J Ma H; Janda KD (2011). Inducing apoptosis in chemotherapy-resistant B-lineage acute lymphoblastic leukaemia cells by targeting HSPA5, a master regulator of the anti-apoptotic unfolded protein response signalling network. Br J Haematol. **153**(6):741-52.

Van Tongeren M; Jimenez AS; Hutchings SJ; MacCalman L; Rushton L Cherrie JW (2012). Occupational cancer in Britain. Exposure assessment methodology. Br J Cancer. **1**:S18-26.

Vardiman JW; Thiele J; Arber DA; Brunning RD; Borowitz MJ; Porwit A; Harris NL; Le Beau MM; Hellstrom-Lindberg E; Tefferi A; Bloomfield CD (2009). The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood.; **114**(5):937-51.

Vasiliou V; Vasiliou K; Nebert DW (2009). Human ATP-binding cassette (ABC) transporter family. Hum Genomics. **3**(3):281-90.

Vembar SS; Brodsky JL (2008). One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Molecular Cell Biol. **9**:944–957

Vrooman LM; Silverman LB (2009). Childhood acute lymphoblastic leukemia: update on prognostic factors. Curr Opin Pediatr.; **21**(1):1-8.

Wang BL; Zhai HY; Chen BY; Zhai SP; Yang HY; Chen XP; Zhao WT; Meng L (2004). Clinical relationship between MDR1 gene and gallbladder cancer. Hepatobiliary Pancreat Dis Int. **3**(2):296-9.

Wogan GN; Hecht SS; Felton JS; Conney AH; Loeb LA (2004). Environmental and chemical carcinogenesis. Semin Cancer Biol. **14**(6):473-86.

Xiao K; Liu W; Qu S; Sun H; Tang J (1996). Study of heat shock protein HSP90α, HSP70, HSP27 mRNA expression in human acute leukemia cells. Journal of Tongji Medical University. **16**(4): 212-216

Xu C; Bailly-Maitre B; Reed JC (2005). Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest. **115**:2656–64

Zuckerman T; Rowe JM (2014). Pathogenesis and prognostication in acute lymphoblastic leukemia. F1000Prime Rep. **6**:59.