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Analyzing the Differential Gene Expression of p53, BCL2, BAX in T-Cell Acute Lymphoblastic Leukemia

A DISSERTATION PRESENTED
BY
RONAY ÇETIN
TO
THE FACULTY OF ENGINEERING AND NATURAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
BACHELOR OF SCIENCE
IN THE SUBJECT OF
GENETICS AND BIOENGINEERING
ADVISED BY ASST. PROF. YUK YIN NG

İSTANBUL BILGI UNIVERSITY
ISTANBUL, TURKEY
MAY 2015

This dissertation is dedicated to my parents.

Acknowledgments

I wish to express my sincere thanks to Asst. Prof. Yuk Yin NG, thesis supervisor, for all his supports and the continuos encouragement. I place on record my sincere thank you to Dr. Ozden Hatirnaz NG for her supervision, support and knowledge. I am extremely indepted to them for each step of the research. I am thankful to Assoc. Prof. Müge Aydın Sayitoğlu and Istanbul University Institute of Experimental Medicine to provide me all the necessary materials for the research. I am also grateful to Asst. Prof. Özgür Gül, lecturer in the Department of Genetics and Bioengineering. I am thankful to him for sharing expertise, valuable guidance, and encouragement.

I take this opportunity to express gratitude to my parents who have been a source of unceasing encouragement and inspiration for me during the challenges of undergraduate school and life.

I must acknowledge as well the many friends who played important roles along the journey, especially Doğancan Özturan, more than a housemate, Işık Kantarcıoğlu, and Emir Erkol. We faced with various challenges and we provide encouragement to each other at certain times when it seemed impossible to continue. Also loving thanks to my best friend Enes Buz. I will always appreciate all they have done.

Contents

List of figures

List of Tables

List of Abbreviations

BCL2 B-cell Lymphoma 2 Gene, page 9

ALL Acute Lymphoblastic Leukemia, page 3

BM Bone Marrow, page 1

DN Double Negative, page 4

DP Double Positive, page 4

HSC Hematopoietic Stem Cells, page 1

ISP Immature Single Positive, page 4

NK Natural Killer Cells, page 3

PCR Polymerase Chain Reaction, page 16

q-RT-PCR Quantitative Real Time Polymerase Chain Reaction, page 16

SP Single Positive, page 4

T-ALL T-Cell Acute Lymphoblastic Leukemia, page 3

TCR T Cell Receptors, page 4

UPL Universal Probe Library, page 16

Thesis advisor: Asst. Prof. Yuk Yin Ng Ronay Çetin

Analyzing the Differential Gene Expression of p53, BCL2, BAX in T-Cell Acute Lymphoblastic Leukemia

Abstract

Acute Lymphoblastic Leukemia is a severe disease and it is the most common type of childhood leukemia. T-cell Acute Lymphoblastic Leukemia occurs in the malignant transformation of T-cells in the thymus and approximately 15-18% of childhood Acute Lymphoblastic Leukemia patients belong to T-Cell Acute Lymphoblastic Leukemia. Several genes that are involved in T-cell development have relations with many pathways that have important roles in leukemogenesis. We have identified the expression levels of p53, BAX, and BCL2. We studied 30 T-cell Acute Lymphoblastic Leukemia childhood patients with quantitative real time polymerase chain reaction. p53, tumor suppressor gene, and BAX are pro-apoptotic genes while BCL2 inhibits apoptosis. p53 was found to be significantly upregulated in patient samples when compared with control thymocytes. Upregulation of p53 might lead the cells to malignant transformation.

No man ever steps in the same river twice.

Heraclitus

1 Introduction

1.1 Hematopoiesis

Mature blood cells have limited lifespan and they must be replaced. Hematopoiesis is defined as the formation of cellular blood components. Blood is highly regenerative tissue. Approximately one trillion (10^{12}) blood cells are rising daily in adult human bone marrow (BM) [1] . Production of the blood cells is accomplished by the differentiation and proliferation of pluripotent hematopoietic stem cells (HSC). The major site of hematopoiesis is BM in humans [2].

All blood cells are divided into two origins such as myeloid and lymphoid [3] (Figure.1.1.1).

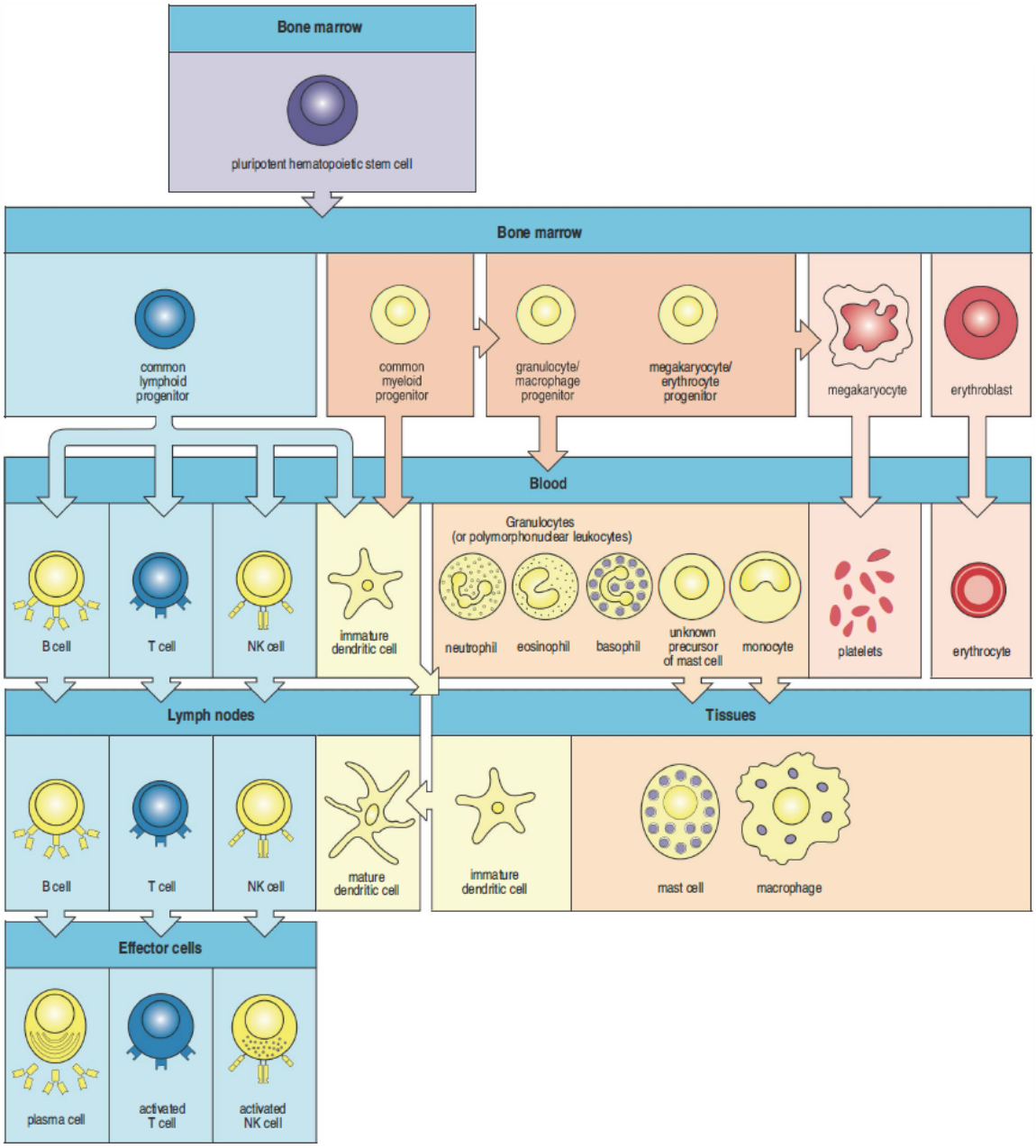


Figure 1.1.1: Development of cellular elements of blood; arise from hematopoietic stem cell in the bone marrow [4]

1.2 Production of white Blood Cells (Lymphocytes)

Lymphocytes are essential in immune system. They are derived from common lymphoid progenitors. The lymphoid lineage is consisting of T-cells, B-cells, and natural killer cells (NK) [5]. B and T lymphocytes are primary immune cells of the body. B cells are responsible for antibody production and they originated from bone marrow. T cell progenitors are also produced in the bone marrow however these progenitors leave the bone marrow and mature in the thymus [6].

1.3 Leukemia

Hematopoiesis is a tightly controlled process. Aberrant changes in blood cell development lead to blood cell diseases including leukemia. Leukemia may be acute or chronic. In acute leukemia the disease development occurs fast. On the other hand in chronic leukemia the process is much slower and may not show symptoms for years. Acute leukemia is formed by a rapid increase in the number of immature blood cells. In these cells the bone marrow is unable to produce healthy blood cells. Leukemia also may be lymphocytic or myelogenous (Figure.1.3.1). Lymphocytic leukemia affects white blood cells [7].

1.4 Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is the malignant disorder of lymphoid cells. It affects adults and children. ALL is mostly seen in children whose ages between 2 and 5. The cure rate for the ALL is higher than 80% for children [8]. The most common form of childhood leukemia is recognized as ALL. Lymphoblasts are overproduced in the bone marrow and continuously multiply in the patient. ALL can be generated either from T-cell or B-cell lymphocytes. Most cases of ALL involve B cells. [9]

Approximately 15-18% of children with ALL have T-Cell Acute Lymphoblastic Leukemia (T-ALL). Boys are affected in this type of leukemia more than girls. Also T-ALL affects older children more than B-ALL. It could also spread to cerebrospinal fluid at early stages of the disease [10].

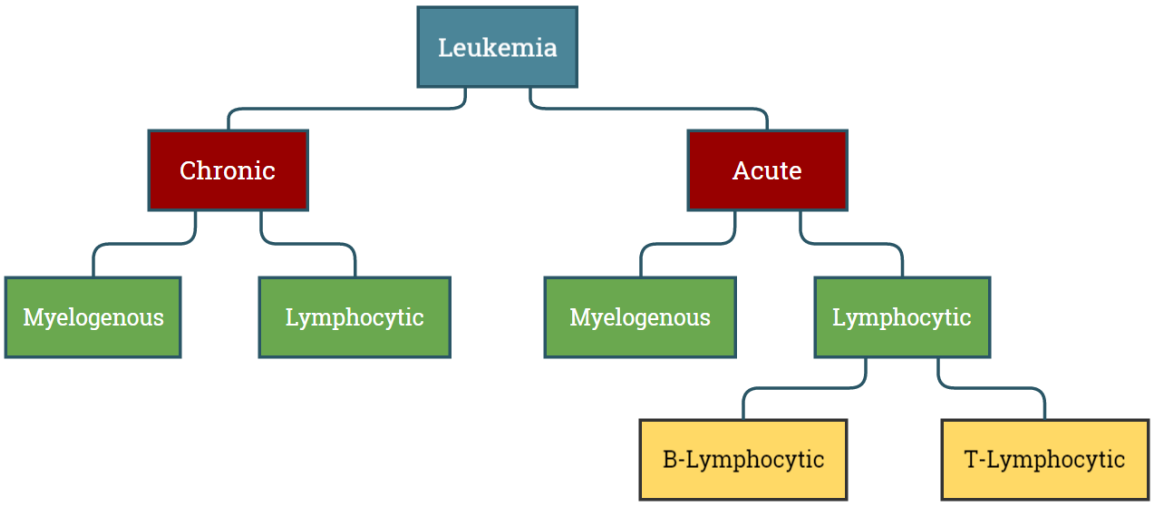


Figure 1.3.1: Classification of leukemia based on its speed of progression and the type of cells involved

1.5 Human T-Cell Development

Progenitors of the T cells migrate from the bone marrow into the thymus. Depending on the CD4 and CD8 coreceptor expression, thymocytes are divided into subsets such as double positive (DP) , double negative (DN), and single positive (SP) (Figure.1.5.1). At the DN stage the expression of CD4 and CD8 are both negative in the cells. The DN stage is divided into three distinct groups in human. $CD34^{+}CD38^{-}CD1a^{-}$ represents the earliest stage in the maturation and the $CD34^{+}CD38^{+}CD1a^{-}$ and $CD34^{+}CD38^{+}CD1a^{+}$ stages follow first stage. In the development stages immature single positive (ISP) follows DN cells. ISP is positive for CD4 coreceptor. At DP stage, next step after ISP, the expression of CD4 and CD8 are positive. Then DP thymocytes mature into SP T cells. SP cells express functional T cell receptors (TCR) and leave the thymus [11].

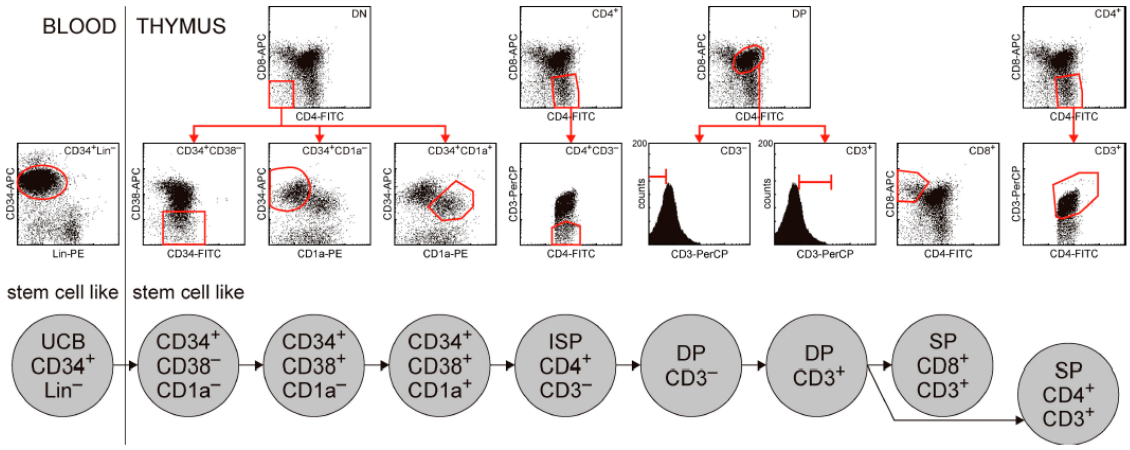


Figure 1.5.1: Human T cell development stages [12]

1.6 Cell Cycle

The cell cycle is a conserved mechanism in eukaryotic cells replication. Cell division is divided into four processes: cell growth, DNA replication, distribution of chromosomes, and cell division. In eukaryotes cell cycle consist of two basic parts such as mitosis and interphase. The period between mitoses is called interphase. Approximately 95% of the time in cell cycle is spent in interphase. In this phase chromosomes are decondensed and distributed in the nucleus. Besides the DNA is synthesized in interphase but only a portion of it. DNA synthesize occurrs in four discrete phases(Figure.1.6.1). The M phase is followed by cytokines and it corresponds to mitosis. G1 phase that is a time interval between mitosis and DNA replication (gap1) follows M phase. During the G1 phase DNA is not replicated but the cell is active and it incessantly grows. During DNA replication S phase takes place. When DNA synthesis is completed, G2 phase (gap2) begins. During G2 phase cell growth continues and proteins are started to synthesize [13].

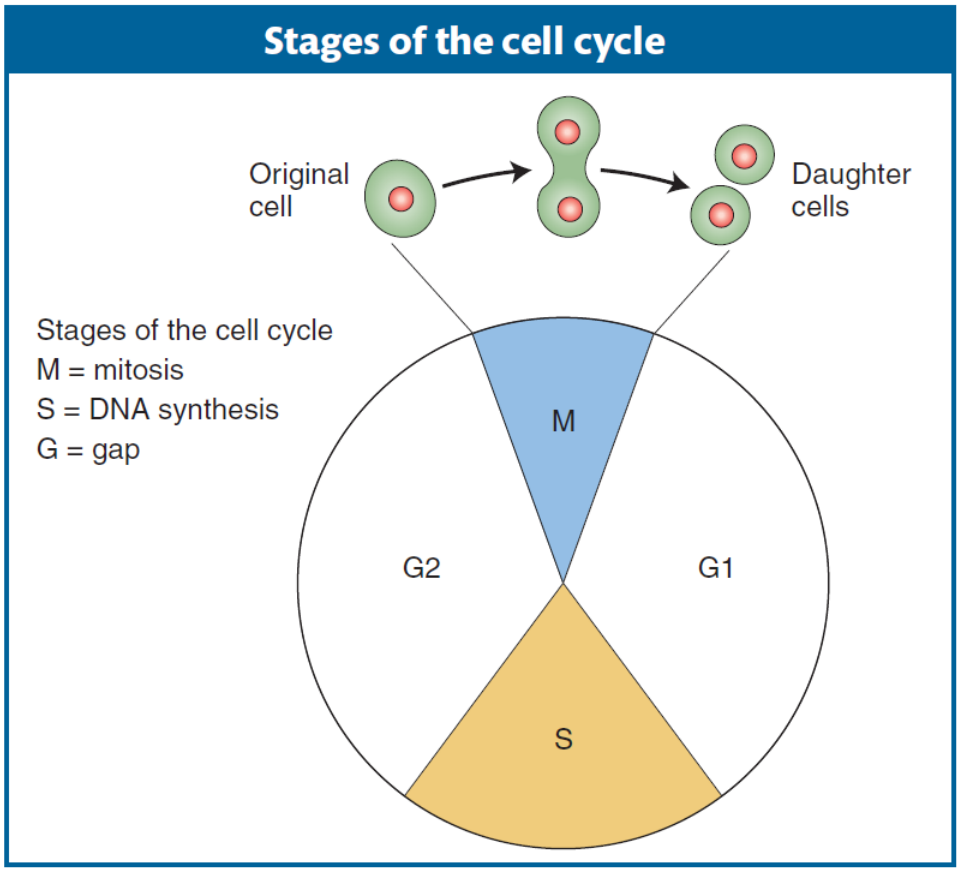


Figure 1.6.1: The stages of cell cycle [14]

1.7 Apoptosis

Apoptosis is a highly conserved mechanism for eukaryotic cells to commit suicide. Mostly apoptosis is determined as process of programmed cell death. Organisms can eliminate defective and unwanted cells by apoptosis. Elimination of cells by apoptosis occurs during normal development. When the apoptosis is regulated abnormally, it contributes to numerous disorders such as cancer, autoimmune diseases, viral infection, stroke, AIDS, anemia, and neurodegenerative disorders [15].

1.8 P53 IN CELL CYCLE CONTROL

p53 is a tumor suppressor gene. Deletion or mutation of p53 gene increases cancer risk. Under cellular stress or DNA damage the level of p53 protein is upregulated by post-transcriptional mechanisms. Then p53 upregulates the expression of genes that block the cell cycle, and allow the DNA damages before the DNA is replicated [16] (Figure.1.8.1). Thus p53 blocks the harmful mutations. In case of extensive DNA damages, proapoptotic genes are induced by p53 and results with cell death. So the damaged cells are eliminated [17].

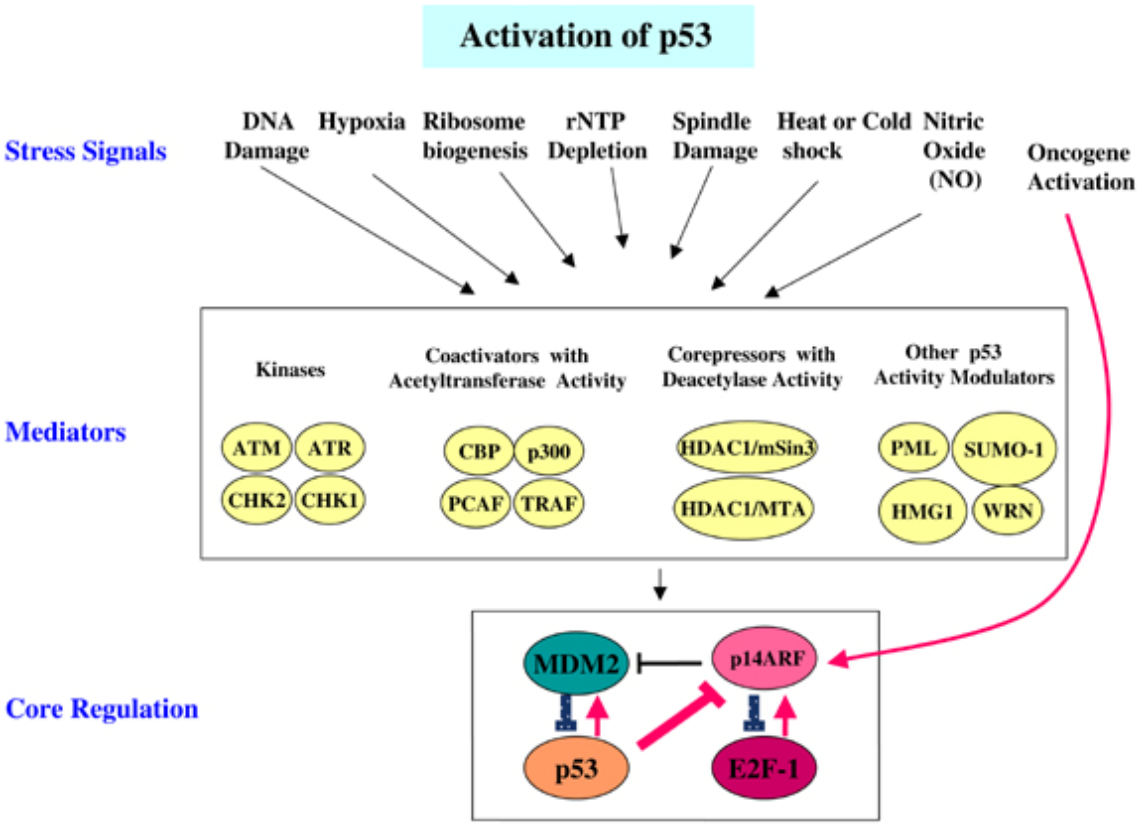


Figure 1.8.1: Upstream pathway of p53 [18]

The upstream mediators detect and interpret the upstream signals after varied stress signals activating the pathway. Then p53 is regulated through its interaction with several proteins.

Under several stress signal p53 is activated to regulate apoptosis, cell cycle arrest, senescence, and DNA repair processes (Figure.1.8.2). p53 is an efficient mechanism for reducing cancer susceptibility but on the other hand it create a risk on longevity by accelerating the aging. Regulation of p53 pathway is not completely known. Understanding the p53 pathway is important to overcome the problems about cancer and senescence [19].

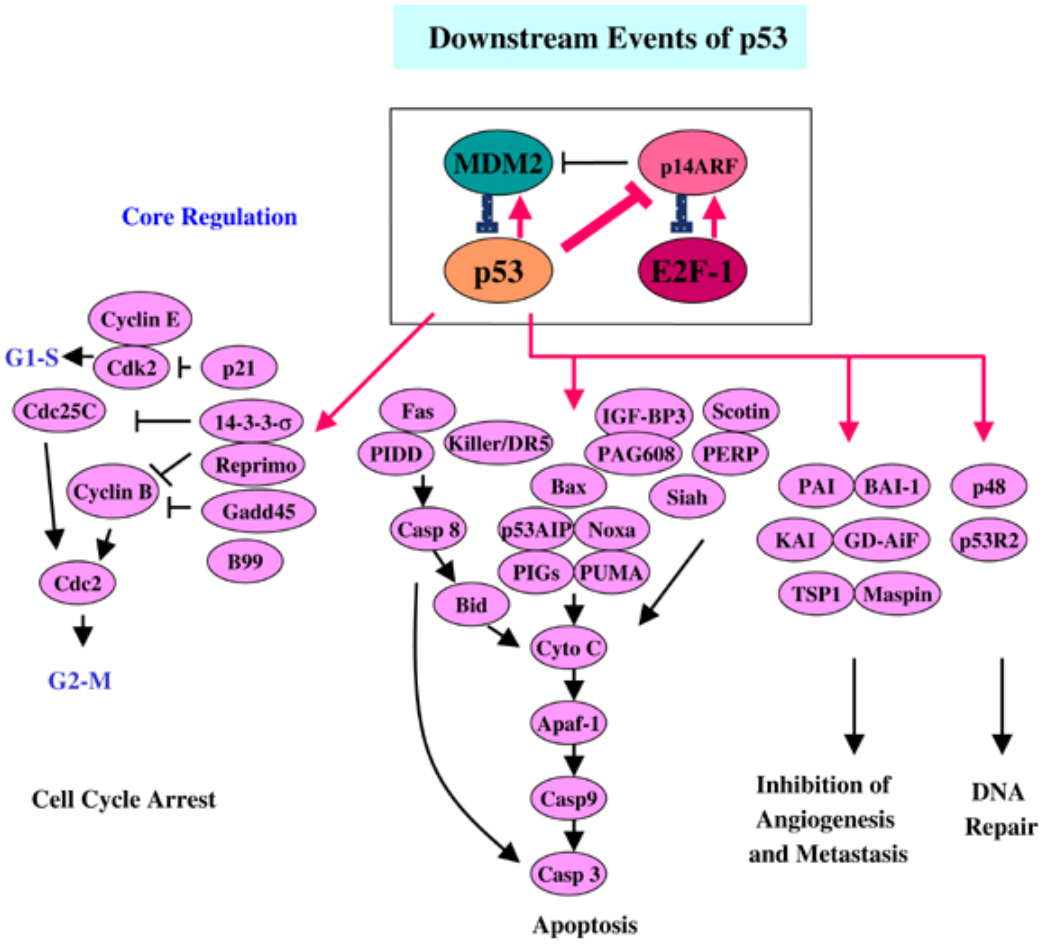


Figure 1.8.2: Downstream pathway of p53 [18]

The downstream events such as transcriptional activation or protein-protein interactions are occurred after regulation of p53 with several proteins that modulate its stability. Finally cell cycle arrest, apoptosis or DNA repair take part.

1.9 *BCL2* in Cell Cycle Control

BCL2 (B-cell lymphoma 2 gene) family members play a role in cell cycle control and apoptosis regulation. Relationships between cell death and cell cycle have already known. *BCL2* is an oncogene that inhibits the apoptosis but vice versa interestingly it is antiproliferative [20].

BCL2 gene was first determined in follicular B-cell lymphoma. Apoptosis and deregulation of *BCL2* have linked with the treatment failure and pathogenesis in ALL by numerous studies. Aberrant gene expression of *BCL2* affects the survival capacity of B cell progenitors and also promotes leukemogenesis. Levels of *BCL2* influence the leukemic cells' sensitivity to therapy. Also regulation of *BCL2* is a key event in apoptosis in ALL [21].

1.10 *Bax* in Cell Cycle Control

BAX gene is homologous to *BCL2* in sequence and it can repress the *BCL2* gene's ability to block apoptosis. *BAX* mostly withstand with the *BCL2* activity, it induces cell death. When the *BCL2* is excess, *BCL2* homodimers are formed or *Bcl2-Bax* heterodimers are formed and cell survival signal is generated. When the *Bax* is high, *Bax* homodimers are formed and cell death signal is generated [22] (Table.1.10.1).

1.11 Relationships Between p53, *Bcl2*, and *Bax*

Stimulation of the *BAX* gene by p53 induces programmed cell death. Overexpression of *BCL2* gene may block p53 dependent apoptosis. If the expression of *BAX* is not enough in the cell, upregulation of *BAX* by p53 may be required for induce apoptosis. If the *BAX* level is high, *BAX* mediated cell death might not be necessary and p53 dependent apoptosis may be revealed. Another possibility for apoptosis is that p53 promotes cell death by repressing survival factors, in that case *Bcl2* [22](Table.1.11.1).

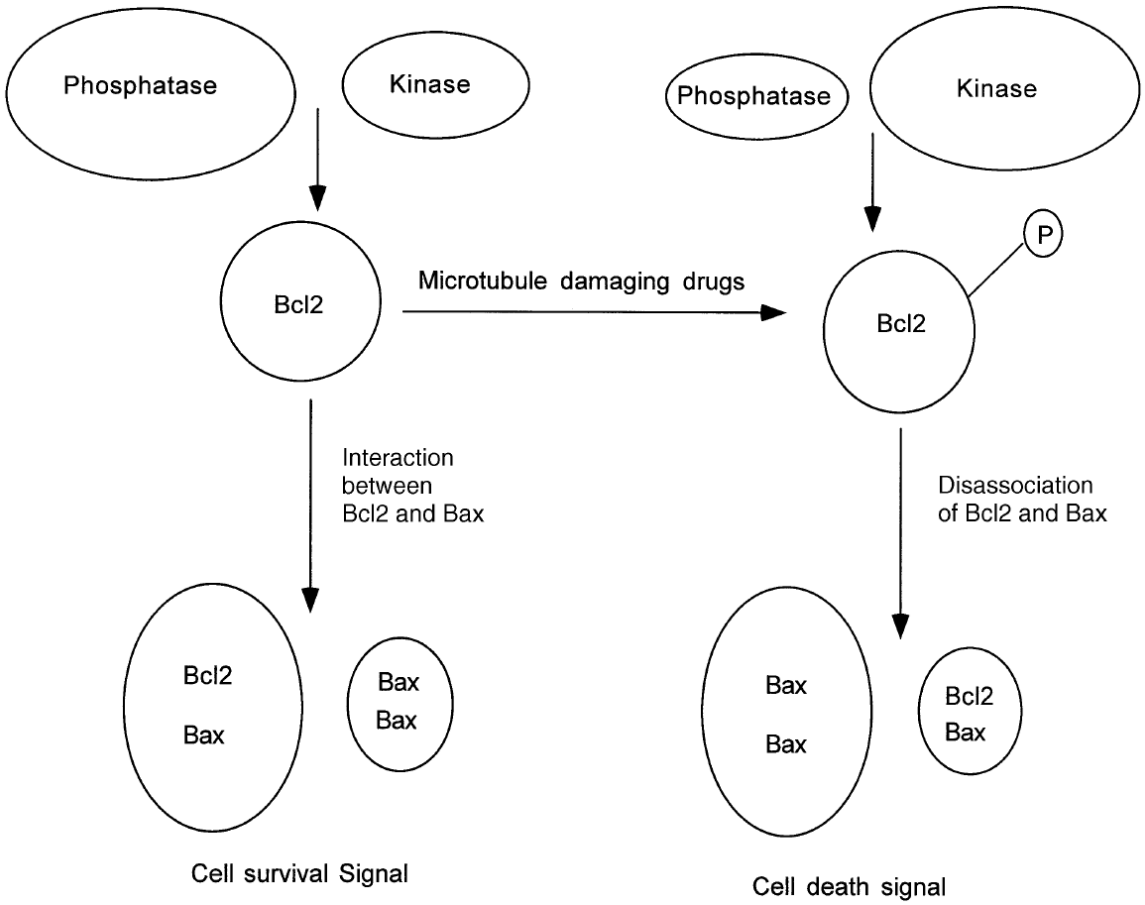


Table 1.10.1: *Bax*, *Bcl2* dependent, p53 independent apoptosis [22]

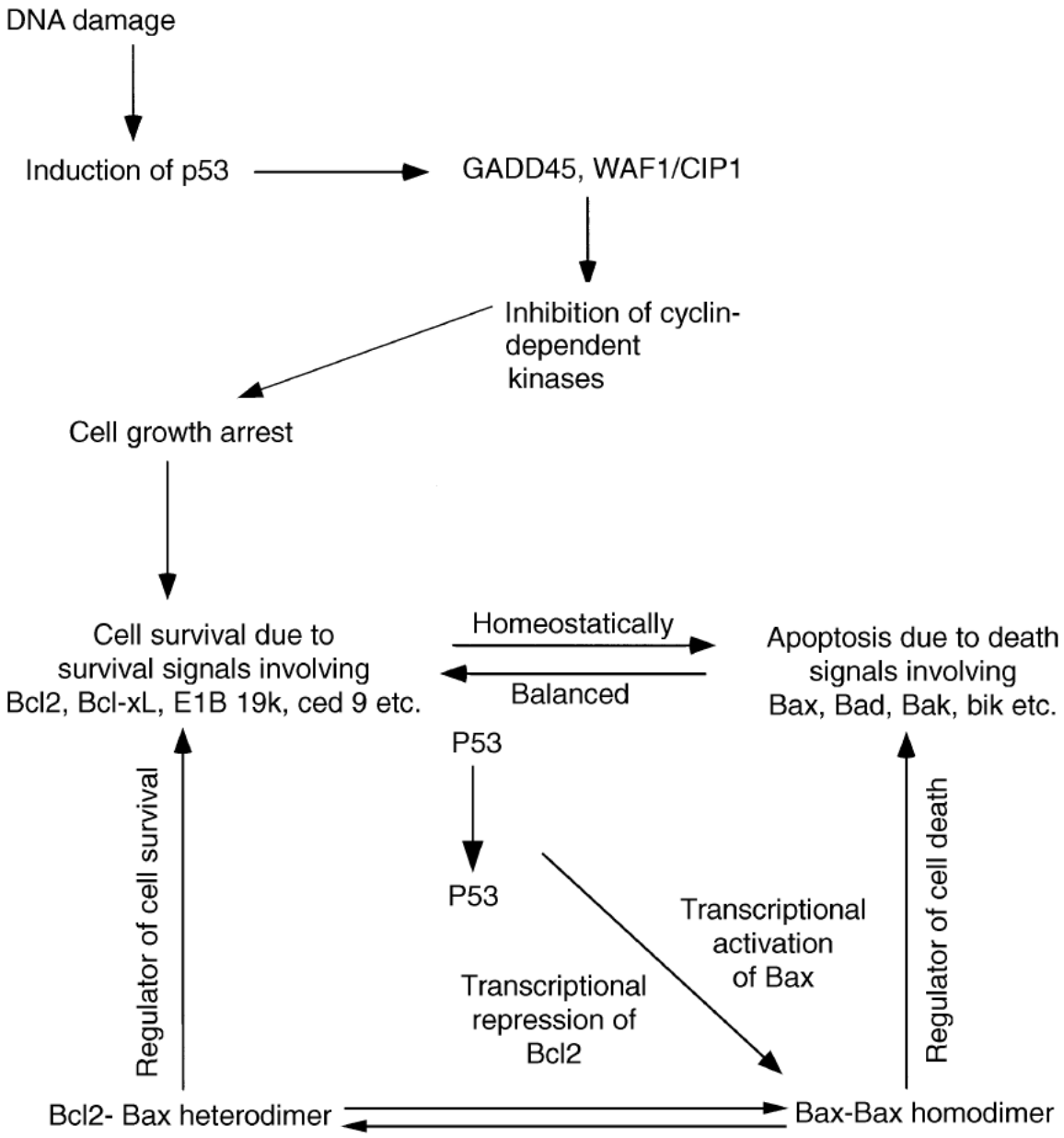


Table 1.11.1: Control of cell cycle and apoptosis by p53, *Bcl2*, and *Bax* [22]

I cannot teach anybody anything, I can only make them think.

Socrates

2

Materials and Methods

2.1 Materials

2.1.1 Patient Samples

Thirty childhood T-ALL patient samples were used in this study. The thymus samples were collected at the time of diagnosis. The samples that were archived after the diagnostic test at Istanbul University Institute of Experimental Medicine were used. Twenty one boys and nine girls were enrolled in the study. The mean age was 10.5 ranging between 1-16.

Clinical features, age, sex, and lineage, of the patients that have been used in that project are shown in Table 2.1.1.

Patient Samples	Age	Sex	Lineage
T6	11	M	Immature
T14	10	M	Unknown
T17	16	M	Mature
T18	12	F	Cortical
T21	9	M	Cortical
T30	1.3	F	Immature
T32	12	M	Unknown
T35	12	M	Mature
T42	3	F	IMB/further
T53	8.1	M	Mature
T58	14	M	Immature
T66	10	F	Mature
T68	3.5	M	Cortical
T70	16	F	Mature
T71	16	M	Mature
T73	12	F	Immature
T100	2.5	M	-
T101	10	M	Immature
T105	0.8	M	-
T112	15	M	Cortical
T114	9	F	Mature
T124	3	F	-
T127	9	M	-
T130	15	M	-
T131	4	M	-
T134	8	M	-
T136	5	M	-
T137	11	M	-
T138	12	M	-
T139	9	F	-

Table 2.1.1: Clinical Features of T-ALL Patients

2.1.2 Healthy Samples

Healthy thymocyte subsets were used as control. The samples were provided by Istanbul University, Institute of Experimental Medicine. Total thymus and 5 thmocyte subsets that represent different stages of T-ALL disease such as DP total, DP 3^+, DP 3^-, SP4, SP8 are used as a healthy control.

2.1.3 cDNA Synthesis

RNA (500 ng)
10X Ca Buffer
$MgCl_2$ (0.2 M)
DTT (100 mM)
(dN)6 (100 OD/mL)
Oligo (dT)15 (100 ug/mL)
dNTP mix (20 mM)
Milli Q DEPC
RNAsin (40 U/µl)
Superscript II (200 U/µl)

2.1.4 Quantitative Real Time PCR

Master Mix
UPL Primers
UPL Probe
96-well PCR plates

2.2 Methods

2.2.1 RNA Isolation

RNA isolation was performed by using total RNA isolation kit (GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich). Firstly cells were lysed and lysate cells are transferred into the GenElute Filtration Column for removing cellular debris and shears DNA. Column was centrifuged at maximum speed for 2 minutes. 70% ethanol solution was added to the filtered lysate after discarding the filtration column. Then 700 µL of the lysate/ethanol mixture was transferred into the GenElute Binding Column and centrifuged at maximum speed for 15 seconds. Column was washed with 500 µL of Wash Solution 1 with centrifugation at maximum speed for 15 seconds. Same centrifugation was repeated for second wash with 500 µL Wash Solution 2. Column was washed with Wash Solution 2 at maximum speed for 2 minutes for third wash. Then binding column was transferred in a new collection tube and 50 µL Elution Solution was pipette into the binding column. Column was centrifuged at maximum speed for 1 minute. Finally purified RNA was in the flow through and it was ready to use.

2.2.2 RNA Quantification

Spectrophotometer (NanoDrop 2000, Thermo Scientific) was used to determine the concentration and quality of RNA. 1 µL RNA was used for measurements and spectrophotometric measurements of RNA was done under 230, 260, and 280 nm wavelengths. The ratio of absorbance at 260 nm and 280 (260/280) nm is used for the purity of RNA. A ratio around 2.0 is generally accepted as pure for RNA. 260/230 ratio is used as secondary measure of nucleic acid purity. 2.0-2.2 range is accepted as pure for RNA. RNA molecules that comply with the criteria were used for cDNA synthesis.

2.2.3 cDNA Synthesis

cDNA synthesis was performed by SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies Corporation). Each RNA sample was diluted to provide 500 ng RNA in 10 µL. 0.5µg/10µL template RNA was incubated at 65 °C for 10 minutes. RNA samples placed on ice immediately. cDNA reaction mix was done as follows:

10X Ca Buffer	2.0 µL
$MgCl_2$ (0.2 M)	0.5 µL
DTT (100 mM)	2.0 µL
(dN)6 (100 OD/mL)	0.5 µL
Oligo (dT)15 (100 µg/mL)	2.0 µL
dNTP mix (20 mM)	1.0 µL
Milli Q DEPC	0.5 µL
RNAsin (40 U/µL)	0.5 µL
Superscript II (200 U/µL)	1.0 µL

Table 2.2.1: cDNA Reaction Mix

10 µL cDNA reaction mix was added per tube and mixed and total volume of the cDNA reaction was 20 µL. Samples were incubated at 42 °C for 50 minutes and then 99 °C for 3 minutes. Finally cDNA samples were stored at -70 °C to -20 °C to use afterwards.

2.2.4 Analyzing Gene Expression Levels

Expression levels of the genes were determined by using Universal Probe Library (UPL) probes by quantitative real time polymerase chain reaction (q-RT-PCT) method by using Light Cycler 96 (Roche). Three main products are required for q-RT-PCR such as primers, probe, and Master Mix. Primers and probes which are specific for primers and emit fluorescence were chosen by using UPL assay design center. Master Mix consists of dNTPs, buffer, and $MgCl_2$ that are required for q-RT-PCR reaction. 96-well plate was used for each PCR . Each sample was run as duplicate per patient and healthy samples. Two µL cDNA, 5 µL Master Mix, 1 µL primers, 0.2 µL probe, and 1.8 µL dH_2O were used per well. Conditions for PCR is done by 95 °C for 10 minutes for pre-incubation; 95 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 1 seconds for 45 cycles for amplification. PCR amplification is done for 45 cycles. Melting curve analysis was performed to ensure amplification specificity. ΔΔCt method was used to calculate the relative cDNA expression level [23].

2.2.5 Genes that were Examined within the Project

In consequence of literature study, 3 different anti- or pro-apoptotic genes (*p53*, *BCL2*, *BAX*) were chosen within the project. Cyclophilin gene that is expressed in all cells of an organism under each condition was chosen as a housekeeping gene.

Primer	Length	Position	Tm	GC%	Sequence	UPL Probe
Left	19	1143-1161	59°C	53	ccccagccaaagaagaaac	58
Right	19	1201-1219	59°C	53	aacatctcgaagcgctcac	58

Table 2.2.2: Primers for p53

Primer	Length	Position	Tm	GC%	Sequence	UPL Probe
Left	19	1998-2016	59°C	58	agtacctgaaccggcacct	75
Right	20	2052-2071	60°C	55	gccgtacagttccacaaagg	75

Table 2.2.3: Primers for *BCL2*

Primer	Length	Position	Tm	GC%	Sequence	UPL Probe
Left	18	509-526	59°C	61	caagaccagggtggttgg	55
Right	18	575-592	59°C	56	cactcccgccacaaagat	55

Table 2.2.4: Primers for *BAX*

2.3 Statistical Analysis

cDNA was synthesized from RNA that was isolated from patient and healthy samples and Ct values were measured by q-RT-PCR. ΔCt values were measured by subtracting Ct value of housekeeping gene from Ct value of target gene ($Ct_{targetgene} - Ct_{housekeepinggene}$). $2^{-[\Delta Ct_{target} - \Delta Ct_{housekeeping}]}$ was calculated for relative expression results. Results were analyzed by comparing patient samples and healthy samples for each gene and also healthy samples were compared between each other, as a result of these analysis charts were generated.

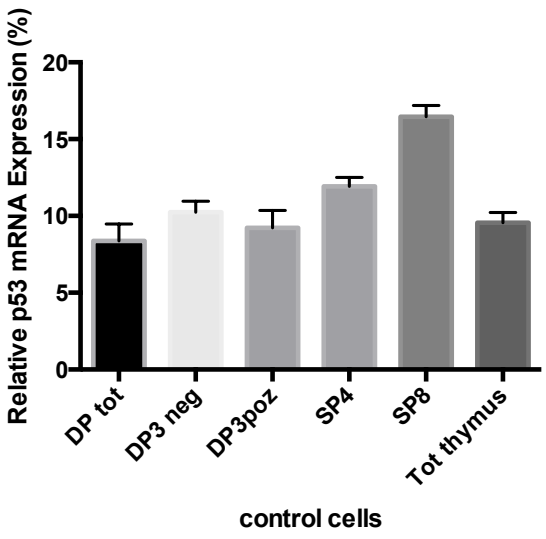
Kind Words Can Be Short And Easy To Speak, But Their Echoes Are Truly Endless.

Mother Teresa

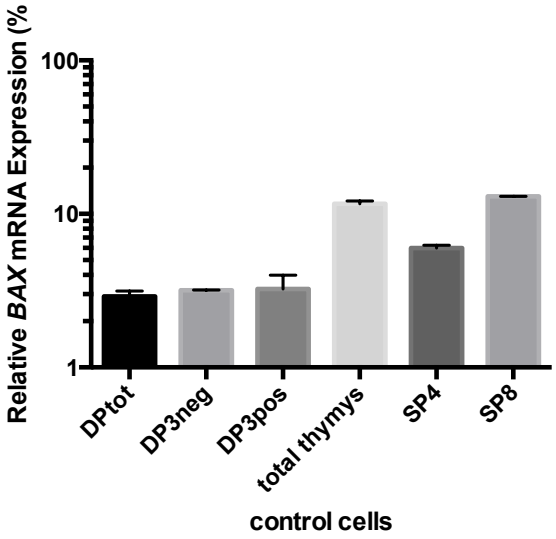
3 Results

3.1 Analysis of Relative Quantification of Gene Expressions

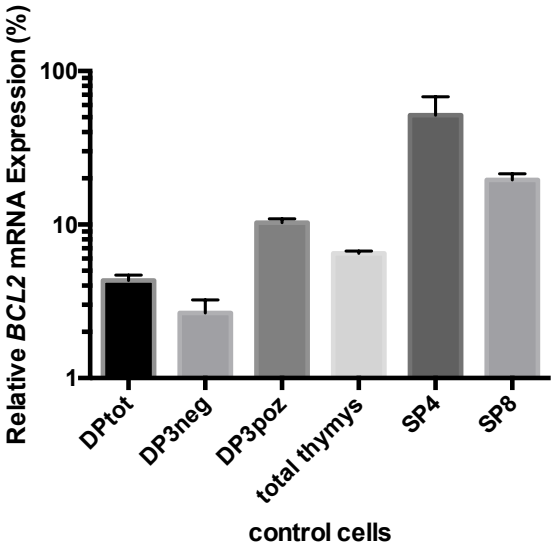
Here we studied *p53*, *BCL2*, and *BAX* genes both in T-ALL patients and healthy thymocyte subsets. Expression levels for different stages of T-cell development are shown in Figure.3.1.1. Relative p53 mRNA expression is increasing consecutively during the different developmental stages. Early loss and late return of *BCL2* expression occurs during T-cell development. *BAX* expression is also increased at he last stages of the T-cell development.



(a) p53 in healthy samples

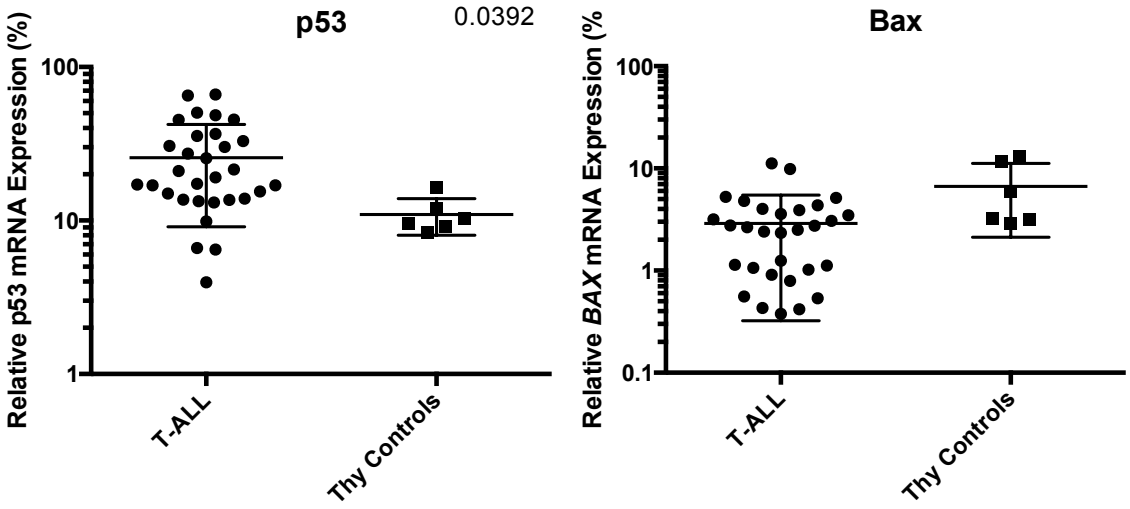


(b) *Bax* in healthy samples



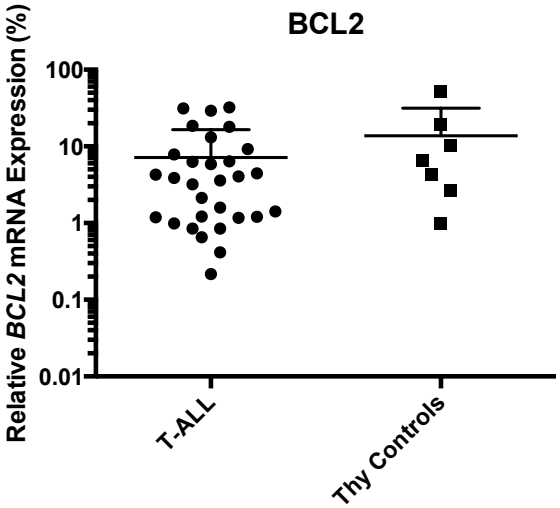
(c) *Bcl2* in healthy samples

Figure 3.1.1: Relative mRNA Expressions of p53, BCL2, and BAX Genes in Thymocyte Subsets



(a) p53

(b) *Bax*



(c) *Bcl2*

Figure 3.1.2: Relative mRNA Expressions of p53, BCL2, and BAX Genes in T-ALL and Healthy Thymocytes

Relative mRNA expressions for patient samples and healthy controls were compared for *p53*, *BCL2*, and *BAX* and differences in expression levels are demonstrated. p53 expression in T-ALL patient samples was higher than healthy control samples and the difference was statistically significant with a p value of 0.0392. No significant difference was observed for *BAX* and *BCL2* in T-ALL patients when compared to controls (Figure.3.1.2). Expression levels of the *p53*, *BCL2*, and *BAX* was compared with the clinical features of patients and there is no any relationship was found.

Experience Is Not What Happens To You; It's What You Do With What Happens To You.

Aldous Huxley

4 Discussion

Acute Lymphoblastic Leukemia is the most common type of childhood leukemia. Oncogenes and tumor suppressor genes have important roles in leukemogenesis [9]. We have studied the expression levels of *p53*, *BAX*, and *BCL2* that have crucial roles in apoptosis and cell cycle arrest on mRNA level by q-RT-PCR.

SnoN/SKIL has previously been identified as a direct activator of p53 and it accelerates tumorigenesis. Moreover *SnoN* is the negative regulator of TGF-ß [19]. Expression level of the *SnoN* was identified in subsets of the thymocytes and it was found that lowest expression of *SnoN* is observed in DN stage, expression is increasing in DP stage, the highest expression level is observed in SP stages. Besides that *SnoN* expression was demonstrated that it is downregulated in T-ALL patient samples [24]. In our project we have demonstrated the mRNA expression level of p53 in T-ALL patient samples and in healthy thymocytes and thymus. p53 encodes a tumor suppressor protein that responds to various cellular stresses to regulate expression of target genes by inducing cell cycle arrest, apoptosis, senescence, or DNA repair. The average p53 expression is

significantly upregulated in T-ALL patient samples when compared with healthy samples (p=0.0392). The lowest expression level for p53 is observed in DP stage among thymocytes samples and it is increasing during T-cell development. These two results are supported by previously published microarray study [24].

BAX is the pro-apoptotic member of the *BCL2* gene family. The protein encoded by *BAX* gene forms a heterodimers with *BCL2* and functions as an apoptotic activator. Expression of *BAX* gene is regulated by indirectly p53 gene. Since we observed elevated level of p53 in T-ALL samples, we expected also elevated levels of *BAX*. Unfortunately there is no significant change in the expression of *BAX* according to q-RT-PCR data.

Apoptosis is regulated by several pathway groups that include various genes. *BCL2* is one of the most common gen families that play role on apoptosis. *BCL2* controls the cell cycle, it inhibits apoptosis and also it is antiproliferative [20]. Expression level of *BCL2* gene was determined by q-RT-PCR and significant expression change in T-ALL patients is not observed. In the T-cell development stages, expression of all three genes reaches maximum level in SP stage, last stage of the development.

Analyzing of the mRNA expression levels of p53, *BAX*, and *BCL2* might be used for diagnosis in clinic. Currently, the studies on T-ALL and apoptosis pathways are limited in literature. Future studies on TALLare increased on this area it is going to be easier to understanding the pathways that have a part in leukemogenesis. Analyzing other members of the apoptosis pathway, whole genome analysis with more patient samples, detecting protein products of our target genes by immunohistochemistry or western blot, studying epigenetic mechanisms that control gene expression can be done as a future aim of the project.

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