

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**REGULATION OF PI3K/AKT SIGNALLING PATHWAY IN
PEDIATRIC ACUTE MYELOID LEUKEMIA (AML): A
NOVEL TUMOUR-SUPPRESSOR PHLPP**

**M.Sc. Thesis by
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**Department : Advanced Technologies
Programme: Molecular Biology – Genetics and Biotechnology**

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**PI3K/ AKT SİNYAL İLETİ YOLAĞININ PEDIATRİK AKUT
MYELOİD LÖSEMİDEKİ REGÜLASYONU: YENİ BİR
TÜMÖR BASKILAYICI GEN PHLPP**

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ABBREVIATIONS

AML	: Acute Myeloid Leukemia
HSC	: Haematopoietic Stem Cell
LCS	: Leukemic Stem Cell
FAB	: French-American-British
PI3K	: Phosphoinositide-3-kinase
Akt	: Mammalian homolog of the transforming viral oncogene v-Akt
PKB	: Protein kinase B
PtdIns (3,4,)P2	: Phosphatidylinositol 3,4 bisphosphate
PtdIns (3,4,5)P3	: Phosphatidylinositol-3,4,5-triphosphate
PKC-1	: Phosphoinositide-dependent protein kinase-1
VEGF	: Vascular Endothelial Growth Factor
VEGFR	: VEGF Receptor
IGF1	: Insulin-like Growth Factor-1
IGFR	: IGF Receptor
ERα	: Estrogen Receptor Alpha
ERβ	: Estrogen Receptor Beta
mTOR	: The mammalian target of rapamycin
PHLPP	: Human PH domain and Leucine rich repeat Protein Phosphatase
PTEN	: Phosphatase and tensin homologue deleted on chromosome 10
PH	: Pleckstrin Homology
LRR	: Leucine Rich Repeat
PP2C	: Protein Phosphatase 2C
PDZ	: is named after the founding members of this protein family (PSD-95, DLG and ZO-1).
Abl	: Abelson
B2M	: Beta-2-microglobulin
CYC	: Cyclophilin
SDS	: Sodium Dodecyl Sulfate
PCR	: Polymerase Chain Reaction
DHPLC	: Denaturing High Performance Liquid Chromatograph

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REGULATION OF PI3K/AKT SIGNALLING PATHWAY IN PEDIATRIC ACUTE MYELOID LEUKEMIA (AML): A NOVEL TUMOUR-SUPPRESSOR PHLPP

SUMMARY

The constitutive activation of PI3K/Akt signaling pathway has been implicated in both pathogenesis and progression of Acute Myeloid Leukemia (AML). Upregulated PI3K/Akt pathway has been found to be associated with shorter overall survival in AML cases, which makes this pathway an important target for development of novel therapeutic strategies against AML. Two key proteins control the termination of Akt signaling: PTEN, a lipid phosphatase, that prevents activation by removing the second messenger that activates Akt, and PH domain and leucine rich repeat protein phosphatase, PHLPP, which inactivates Akt by direct dephosphorylation of the hydrophobic motif Ser473. In a number of studies, it was demonstrated that certain tumors were insensitive to inhibitors of PI3 kinase and to the overexpression of PTEN. Besides, it has been previously shown that PHLPP levels are markedly reduced in a number of colon cancer and glioblastoma cell lines as well as in chronic lymphocytic leukemia patients with deletion 13q14 and colon tumours. Considering these findings, the important role of PHLPP in Akt pathway regulation as a negative regulator and tumour suppressor emerges while making PHLPP an attractive target for development of innovative anti-cancer strategies. Human PHLPP contains four major functional domains; an amino-terminal PH domain, a leucine-rich repeat region (LRR), a PP2C-like catalytic core and a PDZ binding motif. So far, there are no described mutations in PHLPP gene. In this study, the architecture of PHLPP gene variations and the expression of four major functional domains of PHLPP gene together with PI3K/Akt pathway genes; Akt-1, PTEN and caspase-3 were examined. Mutation screenings of 11 exons covering the four domains of PHLPP gene were performed in pediatric AML patients by DHPLC analysis. Mutation detection was accomplished by direct sequencing. The following sequence variations were found: **exon2**; 59insA(5.2%), 60T>G(2.8%), 77C>A(2.8%), 109A>T, **exon3**; 289C>A(7.8%), 352C>A(7.8%), 343insA(2.8%), **exon5**; 599insA (47%), **exon14**; 1980T>C(5.2%), 1992T>C(5.2%), **exon17**; 3280C>A(13.1%), 3302insA(13.1%), 3303T>C(13.1%), 3407insA(5.2%) and 3611insC (7.8%). The expression studies were also performed in pediatric AML patients and in CD33+ healthy bone marrow cells by qRT-PCR. The results revealed that Akt was up-regulated in pediatric AML patients (OR=4.4 95%CI=0.04-2.9, p=0.06). PTEN, PHLPP and caspase-3 were found to be decreased in pediatric AML patients compared to CD33+ healthy bone marrow cells (3 times (p>0.05), 10 times (p>0.05) and 3 times (p>0.05) respectively). In order to characterize PHLPP gene, the expression of its four major domains (PH domain, LRR, PP2C-like catalytic core, and PDZ binding motif) were studied. Expressions of PH domain, PP2C-like catalytic core domain and PDZ binding domain were detected in both, pediatric AML patients and control group. Interestingly, expression of LRR domain in AML patients was not detected. PHLPP mRNA covering the region between exon 2 to exon 17 was amplified by conventional PCR in pediatric AML samples lacking LRR domain expression, three different transcript variants varying between 1800-5000 bps were identified. Direct sequencing results revealed a single nucleotide change in exon 5 at position 55 in those patients. Additionally, further studies were performed on the expression of four major functional PHLPP domains in various

tumour tissues (colon, stomach, pancreas and breast tumours). LRR domain expression could not be determined neither in pediatric AML samples nor in tumour samples. Moreover, PP2C like catalytic core expression was found to be lost in tumour samples. Recently, the loss of PHLPP expression in colon tumour tissues was also reported by Liu *et al.* (2008). Our results are in concordance with recent findings. Furthermore, PHLPP mRNA transcript variants different than those observed in pediatric AML samples were detected in tumour samples. Western blot analysis results also confirmed the loss of intact PHLPP expression in pediatric AML patients and tumour samples. This is the first study evaluating sequence variations together with the expression of PHLPP functional domains. It can be proposed that PHLPP gene might act as a tumour suppressor in AML leukomogenesis and tumorigenesis. This can provide an important guidepost for the development of appropriate diagnostic and therapeutic tools. The role of epigenetic regulation in cancer might explain the presence of PHLPP transcript variants in pediatric AML and different tumour tissues. Therefore, the possible underlying mechanisms need to be further studied.

PI3K/AKT SİNYAL İLETİ YOLAĞININ PEDIATRİK AKUT MYELOİD LÖSEMİDEKİ REGÜLASYONU: YENİ BİR TÜMÖR BASKILAYICI GEN PHLPP

ÖZET

Upregüle edilmiş fosfoinositid-3-kinaz (PI3K)/Akt yolağının, Akut Myeloid Lösemi (AML) patogeneğinde ve hastalığın ilerlemesinde rol oynadığı bilinmektedir. Ayrıca, bu yolağın upregülasyonunun AML vakalarındaki genel sağ kalımı belirgin bir şekilde azalttığına gösterilmesi bu sinyal ileti kaskadını AML'ye karşı yeni terapötik stratejiler geliştirilmesinde, önemli bir hedef haline getirmiştir. Akt sinyal ileti yolunun terminasyonu, Akt defosforilasyonu ile gerçekleşmektedir ve bundan başlıca iki protein sorumludur. Bu proteinler, PTEN ve yeni tanımlanmış bir protein fosfataz olan PHLPP'dir. PTEN, Akt aktivasyonunu, Akt'yi aktifleyen ikincil mesajcıyı ortadan kaldırarak engellerken, PHLPP, Akt inaktivasyonunu, Akt'nin hidrofobik motifini direkt olarak defosforile ederek gerçekleştirir. Yapılan çalışmalar sonucu, belli tümörlerin PTEN'in çok fazla eksprese olmasına veya PI3 kinaz inhibitörlerine duyarlı olmadığı bildirilmiştir. Ayrıca PHLPP' nin ekspresyon seviyesinin kolon kanseri ve glioblastoma hücre soylarında, 13q14 delesyonu taşıyan kronik lenfoid lösemi hastalarında ve kolon tümöründe azaldığı önceki çalışmalarda gösterilmiştir. Bu sonuçlar yeni bir tümör supresör olarak önerilen PHLPP'nin Akt sinyal ileti yolundaki rolüne önem kazandırmakta ve PHLPP' yi yeni anti-kanser stratejilerinin geliştirilmesi için ilginç bir hedef haline getirmektedir. PHLPP, amino-terminal PH bölgesi, lösin yönünden zengin tekrar bölgesi (LRR), PP2C-benzeri katalitik merkez ve PDZ bağlanma bölgesi olmak üzere dört temel fonksiyonel bölge içerir. PHLPP geni için tanımlanmış mutasyon henüz bulunmamaktadır. Bu çalışmada, PHLPP gene varyasyonları ve dört temel fonksiyonel PHLPP gen bölgesi ekspresyonları PI3K/Akt sinyal ileti yolağı genlerinden; Akt-1, PTEN ve kaspaz-3 gen ekspresyon seviyeleri ile birlikte incelenmiştir. Dört temel bölgeyi kodlayan 11 ekzonun pediatrik AML hastalarındaki mutasyon taraması DHPLC analizi ve direkt dizileme yöntemi ile gerçekleştirilmiştir. Bulunan PHLPP geni varyasyonları şunlardır: ekzon 2; 59insA (5.2%), 60C>T (2.8%), 77C>A (2.8%) ve 109A>T, ekzon 3; 289C>A (7.8%), 352C>A (7.8%) ve 343insA (2.8%), ekzon 5; 599insA (47%), ekzon 14; 1980T>C (5.2%) ve 1992T>C (5.2%), ekzon17; 3280C>A (13.1%), 3302insA (13.1%), 3303T>C (13.1%), 3407insA (5.2%) ve 3611insC (7.8%). Ekspresyon çalışmaları pediatrik AML hastaları ve kontrol grubunu oluşturan, sağlıklı bireylere ait CD33+ kemik iliği hücrelerinde qRT-PCR yöntemi ile gerçekleştirilmiştir. Sonuçlar, Akt sinyal ileti yolağının hasta grubunda upregüle olduğunu göstermiştir (OR=4.4 95% CI= 0.04-2.9, p=0.06). Bunun yanı sıra, PTEN, PHLPP ve kaspaz-3'ün ekspresyonunun pediatrik AML hastalarında CD33+ sağlıklı kemik iliği hücrelerindeki göre daha az olduğu tespit edilmiştir (sırasıyla 3 kat (p>0.05), 10 kat (p>0.05) ve 3 kat (p>0.05)). PHLPP'nin dört temel bölgesinin ekspresyon çalışmaları sonucunda ise, PH, PP2C-benzeri katalitik merkez ve PDZ bağlanma bölgelerinin hasta ve kontrol gruplarında ekspresyonlarının olduğu bulunmuştur. Ancak LRR bölgesinin ekspresyonu hastalarda gözlemlenmemiştir. Bunun yanı sıra LRR bölgesi ekspresyonunun görülmediği hasta örneklerine ait PHLPP mRNA' sının ekzon 2 ve ekzon 17 bölgeleri arası standart PCR yöntemi ile çoğaltılmış ve boyları 1800-5000 bp arasında değişen üç farklı transkript varyantı tespit edilmiştir. Bu hastalara ait örneklerde direkt dizileme yöntemi ile ekzon 5 pozisyon 55' te tek baz değişikliği bulunmuştur. Ayrıca dört temel fonksiyonel PHLPP bölgesi ekspresyonuna kolon,

mide, pankreas ve meme tümörü dokularında da bakılmıştır. LRR bölgesinin ekspresyonunun tümör dokularında da olmadığı tespit edilmiştir. Pediatrik AML hasta örnekleriyle gerçekleştirilmiş ekspresyon çalışmalarının sonuçlarından farklı olarak, PHLPP'nin PP2C-benzeri katalitik bölgesinin ekspresyonunun tümör dokularında olmadığı bulunmuştur. Liu ve arkadaşlarının (2008) yaptıkları yeni çalışmada, kolon tümörü dokularında PHLPP ekspresyonunun olmadığı belirlenmiştir. Bu sonuçlar bizim çalışmamızın bulgularıyla da tutarlılık göstermektedir. PHLPP transkriptinin çoğaltılmasına yönelik yapılan çalışmalar sonucunda ise tümör dokularında, pediatrik AML hastalarında gözlemlenenlerden farklı PHLPP transkript varyantları görülmüştür. Western blot analizi sonuçları da eksiksiz PHLPP ekspresyonunun pediatrik AML hastalarına ait örneklerde ve tümör dokularında olmadığını göstererek gen ekspresyon çalışma sonuçlarını konfirme etmiştir. Bu çalışma PHLPP geninin sekans varyasyonlarının ve beraberinde ekspresyon seviyelerinin araştırıldığı ilk çalışmadır. Çalışmanın sonuçları, PHLPP geninin AML lökomogenezinde ve tümörigenezde bir tümör supressör olarak rol oynayabileceğini ve bunun kanserin diagnoz ve tedavisine yönelik yöntemler geliştirilmesinde kullanılabileceğini düşündürmektedir. Pediatrik AML hasta örneklerinde ve farklı tümör dokularında tespit edilen PHLPP transkript varyantlarının varlığı, kanser oluşumu ve ilerleyişinde rol oynayan epigenetik regülasyonlar ile açıklanabilir. Bu bağlamda ileriki çalışmalarda farklı transkript varyantlarının oluşumu altında yatan mekanizmalar araştırılmalıdır.

1. INTRODUCTION

1.1 Haematopoiesis

Blood contains many types of cells with very different functions, ranging from the transport of oxygen to the production of antibodies. All blood cells, however, have certain similarities in their life history. They all have limited life-spans and are produced throughout the life of animal [1]. The production of blood cells is an enormous task. The normal adult produces about 2.5 billion erythrocytes, 2.5 billion platelets and 1.0 billion granulocytes per kilogram of body weight daily. This number can be increased dramatically in response to various stresses, such as anaemia, bleeding or immunologic challenge. Since mature haematopoietic cells have a limited lifespan and no capacity for self-renewal, all of the circulating cells in the bloodstream are continually replaced by pluripotent haematopoietic stem cells (HSCs). These cells differentiate to form the mature cells of the haematopoietic system through a process termed haematopoiesis [2]. The multipotent stem cell normally divides infrequently to generate either more multipotent stem cells, which are self-renewing, or committed progenitor cells, which are limited in the number of times that they can divide before differentiating to form mature blood cells. The progenitors become more specialized in the range of cell types that they can give rise to as they go through their divisions (Figure 1.1) [1].

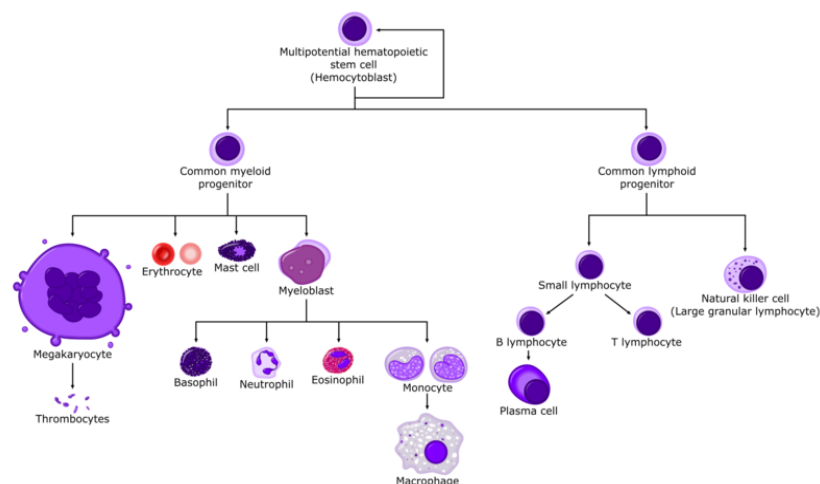


Figure 1.1 : Haematopoiesis: A schematic representation of the pathways leading to the production of the mature cells of the haematopoietic system [1].

The maintenance of the haematopoietic system requires a flexible control mechanism that is able to keep the correct cellular balance. To sustain this balance it would respond to various physiological stresses such as bleeding or infection. Therefore the control system requires both positive signals to initiate cellular production and negative feedback to keep the levels of cellular output in check. In order to maintain the appropriate balance of cellular production, haematopoiesis is controlled at two main levels: (i) through a series of humoral regulators known as cytokines and growth factors that are secreted by various cell types into the bloodstream and may act at distant sites in the body; and (ii) locally by cells in the microenvironment of the marrow known as stromal cells and haematopoietic cells. Disruption of the control of hemopoiesis can result in hemoproliferative disorders or leukemia, the malignant transformation of blood cells, that are often characterised by uncontrolled proliferation and deficient cellular differentiation [3].

1.2 Leukomogenesis

It has been hypothesized that carcinogenesis, and for that matter, leukomogenesis, arises from neoplastic stem cells. Leukemia stem cells (LSC) exhibit characteristics similar to those of normal HSCs. Like normal HSCd, LSCs are quiescent and have self-renewal and clonogenic capacity. They are thought to arise from normal stem cells through the accumulation of oncogenic insults [4], [5]. It has been also suggested that LSCs may arise from differentiated progenitor cells that have reacquired the capacity for self-renewal [6-8]. Like normal HSCs, LSCs give rise to differentiated daughter cells that lose their self-renewal capacity. However, defects in their cellular machinery usually eliminate their ability to differentiate fully into morphologically and phenotypically mature cells. As a result, the leukemic population consists of undifferentiated and variably differentiated leukemia cells. The degree of differentiation of leukemia cells has traditionally formed the basis of the morphologic classification of leukemias [9, 10]. It is also to mention that, since LSCs are quiescent like normal HSCs, they do not respond to cell cycle specific cytotoxic agents used to treat leukemia and so contribute to treatment failure. These cells may undergo mutations and epigenetic changes, further leading to drug resistance and relapse. It has been also suggested that mature leukemia cells may acquire LSC characteristics, thereby evading chemotherapeutic treatment and sustaining the disease. Ongoing studies are likely to identify the molecular mechanisms responsible for LSC characteristics and lead to novel strategies for eradicating leukemia.

The studies done to understand the molecular mechanisms underlying the malignant transformation of many cell types have shown that mutations or deregulation of a wide spectrum of molecules acting at cellular sites ranging from the cell surface to the nucleus are implicated in tumorigenesis [11]. Moreover, results of studies combining random mutagenesis screens and targeted amino acid substitutions in cellular genes, have revealed several mechanisms by which the qualitative properties of receptor signalling or the normal ligand-control of receptor function can be altered in leukemogenesis [12].

1.3 Acute Myeloid Leukemia

Leukemias have traditionally been classified and treated on the basis of phenotypic characteristics, such as morphology and cell-surface markers, and cytogenetic aberrations.

Acute Myeloid Leukemia (AML) is accepted as a heterogeneous group of malignant hematopoietic disorders. It has been recognized that like solid tumours, AML consists of a heterogeneous population of cells with a small percentage of noncycling, quiescent cells. AML is further characterized by uncontrolled proliferation of clonal neoplastic cells and accumulation of blasts with an impaired differentiation program in the bone marrow (Figure 1.2). These blast cells can be found blocked at various maturation steps and are resistant to cell death [13].

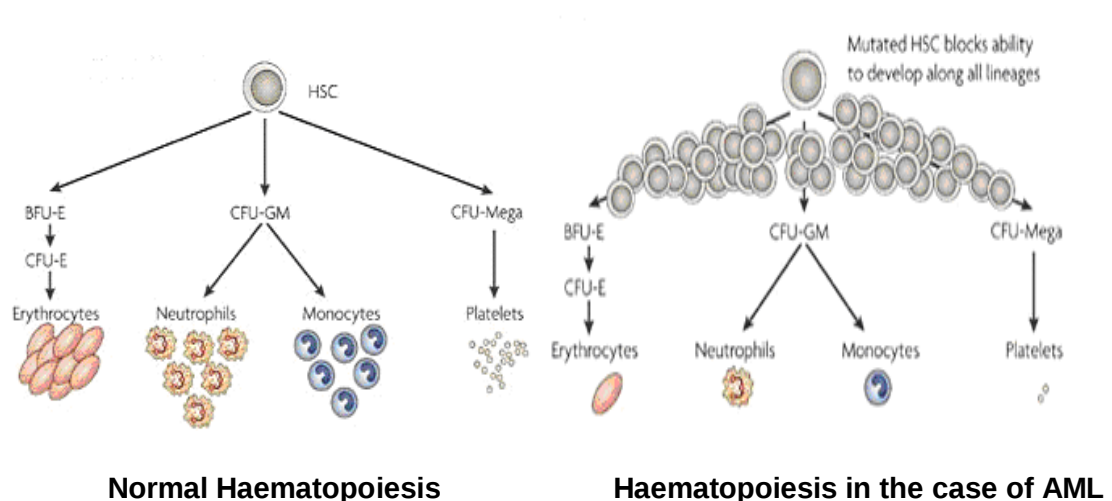


Figure 1.2 : Normal haematopoiesis versus haematopoiesis in AML [14].

AML has 9 subgroups according to French-American-British (FAB) Classification which is based on the type of cell from which the leukemia developed and how mature the cells are (Table 1.1). In this manner, microscopic images of the leukemia cells after routine staining builds up largely the basis of this classification.

Table 1.1 : French-American-British (FAB) Classification of Acute Myeloid Leukemia (AML)

FAB subtype	Name	Cytogenetic remarks	Approximate % of adult AML patients	Prognosis compared to average for AML
M0	Undifferentiated acute myeloblastic leukemia	t(10;11)	5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation	Trizomy 11	15%	Average
M2	Acute myeloblastic leukemia with maturation	t(8;21)	25%	Better
M3	Acute promyelocytic leukemia	t(15;17)	10%	Best
M4	Acute myelomonocytic leukemia	t(8;16)	20%	Average
M4eos	Acute myelomonocytic leukemia with eosinophilia	inv(16)	5%	Better
M5	Monocytic leukemia	11q23	10%	Average
M6	Acute erythroid leukemia	t(3;5)	5%	Worse
M7	Acute megakaryoblastic leukemia	—	5%	Worse

Overall incidence of AML has been reported to be stable or slowly increasing over the last 15-20 years. Despite considerable advances in the diagnosis of the different AML subtypes and progress in therapeutic approaches, current chemotherapies produce only initial remission, so that most patients will relapse and die from the disease. Therefore, there remains a need for new, rationally designed, minimally toxic, effective therapies for AML [13]. In this manner, new therapeutic targets has to be determined to eradicate AML.

A ‘two hits’ model has suggested that AML development requires multiple genetic changes that deregulate different cell programs [15]. Transcription factor fusion proteins such as AML1/ETO, PML-RAR α , CBF β /MYH11 or MLL/AF9 block myeloid cell differentiation by repressing target genes, thus providing one necessary event for leukemogenesis [16,17]. As second necessary event, disordered cell growth and upregulation of cell survival genes, is proposed. Mutations in growth regulatory genes such as FLT3, Ras and c-Kit are common in AML patients. These two classes of molecular events are highly interdependent. Changes in the transcriptional control in hematopoietic cells modify the arrays of signal transduction effectors available for growth factor receptors, whereas activating mutations in signal transduction molecules induce alterations in the activity and expression of several transcription factors that are essential for normal myeloid differentiation [18-20].

1.4 Phosphoinositol-3-kinase PI3K/Akt Pathway Human Cancer

Carcinogenesis is the result of a disturbed balance between cell division and growth on one hand, and programmed cell death (i.e., apoptosis) on the other. This balance is impaired when proteins and signalling pathways regulating cell growth, differentiation and development undergo oncogenic changes .

The phosphoinositide-3-kinase (PI3K)/Akt signaling network is crucial to widely divergent physiological processes that include cell cycle progression, differentiation, transcription, translation and apoptosis. Activation of this signalling pathway promotes cell survival and cell growth. As mentioned before, the balance between cell survival and apoptosis critically controls normal cell growth. Akt/Protein kinase B (PKB) regulates this balance through a phosphorylation cascade that primarily alters the function of transcription factors that regulate pro- and antiapoptotic genes. Upon activation, Akt delivers survival signals by inhibiting pro-apoptotic molecules such as Bad, Caspase-9, I κ B kinase (IKK) and forkhead transcription factors. The PI3K/Akt signalling pathway is targeted by genomic abnormalities including amplification, mutation and rearrangement more frequently than any other pathway in human cancer, with the possible exception of the p53 and retinoblastoma pathways. Activation of PI3K/Akt signaling results in disturbance of control of cell proliferation and apoptosis, ensuing in competitive growth advantage for tumor cells [21-23]. Constitutive activation of PI3K/Akt pathway has been implicated in the both the pathogenesis and the progression of a wide variety of neoplasias. Furthermore, PI3K/Akt axis upregulation is asserted to play a major role not only in tumour development but also in the tumours potential response to cancer treatment [24].

Overexpression of Akt/PKB was demonstrated to be an early event in colorectal carcinogenesis [25]. Furthermore, in gastric carcinomas high levels of Akt expression was found [26]. It has been also shown that overexpression of Akt contributes to the malignant phenotype of a subset of human pancreatic cancers [27]. Also, in breast tumours phosphorylated Akt in active form was associated with larger tumours and a reduced disease-free survival [28]. In addition to Akt expression levels contributing to carcinogenesis, downstream of PI3K are other molecules regulating this pathway, such as PI-3,4,5-P₃ phosphatases. PTEN (Phosphatase and tensin homologue deleted on chromosome 10; also referred to as MMAC1, mutated in multiple advanced cancers 1) is a phosphatase with dual activity on lipids and proteins. It was originally identified as a tumour-suppressor gene. Its main physiologic lipid substrate is PI(3,4,5)P₃, i.e., the PI3K product.

PTEN dephosphorylates PI(3,4,5)P3 and in this way acts as a negative regulator for PI3K induced signalling [24]. Studies on PTEN overexpression in different cell lines suggested that it acts as a tumour suppressor by inhibiting cell growth and enhancing cellular sensitivity for apoptosis and anoikis. PTEN has been reported to be frequently mutated in advanced stages of several human tumours, notably in glioblastoma and prostate cancers [29, 30]. In addition, PTEN mutations in germ cell lines result in the rare hereditary syndrome referred to as Cowden's disease, which is associated with a higher risk for development of malignant tumours, notably breast cancer [31]. Since, PTEN negatively regulates Akt activation through PI(3,4,5)P3 dephosphorylation, PTEN activity loss or impairment leads to permanent PI3K/Akt pathway activation.

1.5 PI3K/Akt Pathway in Acute Myeloid Leukemia

Studies showed that PI3K/Akt signaling is frequently activated in AML patient blasts and strongly contributes to proliferation, survival and drug resistance of these cells [32-35]. Moreover, both the disease-free survival and overall survival has been reported to be significantly shorter in AML cases with upregulated PI3K/Akt pathway [36]. Upregulation of the PI3K/Akt network in AML may be due to several reasons, including FLT3, Ras or c-Kit mutations, which are common in AML patients. In vitro studies including small molecules designed to selectively target key components of this signal transduction cascade have shown that apoptosis is induced and/or conventional drug sensitivity is markedly increased in AML blasts. Considering these findings, PI3K/Akt pathway is suggested to represent a valid target for innovative therapeutic treatments of AML patients and inhibitory molecules are currently being developed for clinical use either as single agents or in combination with conventional therapies.

1.6 Activation of PI3K/Akt Pathway:

The large family of PI3K lipid kinases in mammalian cells has been categorized into 3 classes, referred to as I, II and III. This classification is based on molecular structure and substrate specificity. Class I PI3Ks are the best understood ones and are key players of multiple intracellular signaling networks that integrate a wide variety of signals, engaged by many polypeptide growth factors. Growth factor receptors drive activation of class I PI3Ks either directly or via associated tyrosine kinases, heterotrimeric G proteins or Ras. Class I PI3K preferred in vivo substrate is phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P2), which is phosphorylated to

yield phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P₃). The adaptor/regulatory subunits act to localize PI3K to the plasma membrane by the interaction of their Src homology 2 (SH2) domains with phosphotyrosine residues in activated receptors. They also serve to stabilize p110 and to limit its activity. Insulin and some growth factors preferentially signal through p110 β [37].

Akt, a serine/threonine protein kinase also known as protein kinase B (PKB), is the mammalian homolog of the transforming viral oncogene v-Akt that causes murine T-cell lymphoma. Akt belongs to the cAMP-dependent, cGMP-dependent protein kinase C (AGC) family of protein kinases. Akt is known to include 3 closely related, highly conserved isoforms encoded by the following distinct genetic loci: Akt-1/ α , Akt-2/ β and Akt-3/ γ . Akt-1 is ubiquitously expressed at high levels with the exception of the kidney, liver and spleen. Akt-2 expression varies between different organs, with higher expression levels in the skeletal muscle, intestinal organs and reproductive tissues. Akt-3 is not detected in several tissues where Akt-1 and Akt-2 are abundantly expressed, but it is relatively highly expressed in the brain and testis.

Akt is activated by growth factors and other stimuli that cause generation of the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns P₃) through activation of PI3K. Akt contains an NH₂-terminal pleckstrin homology (PH) domain, which interacts with the phosphorylated lipid products of PI3K (mainly PtdIns (3,4,5)P₃ and, to a lesser extent, phosphatidylinositol 3,4 bisphosphate (PtdIns (3,4,)P₂)) synthesized at the plasma membrane. This lipid product places Akt to the membrane by engaging its PH domain, which results in a conformational change and enables the activation loop of the kinase to be phosphorylated on Thr308 by phosphoinositide-dependent protein kinase-1 (PDK-1, which also requires 3-phosphorylated inositol lipids for activation and plasma membrane translocation) and at Ser 473 in the C-terminal hydrophobic motif by a kinase (often referred to as PDK-2). Candidate PDK-2s include integrin-linked kinase, DNA-dependent protein kinase and mitogen-activated protein kinase-kinase 2. After phosphorylation, Akt is locked in an active conformation and is released into the cytosol and nucleus where it phosphorylates substrates such as forkhead (FoxO) family members of transcription factors, proapoptotic factors BAD and caspase-9, nuclear factor- κ B (NF- κ B), and glycogen synthase kinase 3 β (GSK3 β). AKT also promotes cell growth by phosphorylating targets such as TSC2 (a negative regulator of mTOR) and mTOR itself. [38, 39].

Akt activity is modulated by a complex network of regulatory proteins that interact with the PH domain, or the kinase domain or the C-terminal of Akt [40]. One of these

proteins is heat-shock protein-90 (HSP-90), a molecular chaperone that forms a complex with the co-chaperone Cdc37. This complex binds a variety of proteins, including tyrosine and serine/ threonine protein kinases [41]. The HSP-90/Cdc37 complex interacts with the Akt kinase domain. Therefore, small molecules capable of disrupting such an interaction may represent valid drugs to block Akt function.

It should also be mentioned that PI3K/Akt signaling can be upregulated by many forms of cellular stress including heat shock, low pH, ultraviolet light, ischemia, hypoxia, hypoglycemia and oxidative stress [42]. Stress-induced PI3K/Akt upregulation is to be viewed as a compensatory protective mechanism which cells activate for escaping death.

1.7 Mechanism of PI3K/Akt Signalling Pathway Activation in AML

It has been highlighted that constitutive activation of PI3K/Akt signaling is a common feature of AML. From 50 to 70% of patients with AML display phosphorylation of both Thr 308 and Ser 473 Akt [43].

In about 15–25% of AML cases, N-Ras or K-Ras gene point mutations have been detected. These mutations cancel Ras intrinsic GTPase activity and lead to constitutive Ras activation with a consequent stimulatory effect on the PI3K/Akt pathway. It has been shown that Ras can activate the PI3K/Akt axis either by itself or through the Raf/MEK/ERK pathway. Furthermore, up to 20–25% of AML patients harbor internal tandem duplication (ITD) of the juxtamembrane domain of FLT3. This mutation results in ligand-independent dimerization of FLT3 and constitutive upregulation of its tyrosine kinase activity, ensuing in stimulation of downstream signaling pathways, including PI3K/Akt. Importance of FLT3-ITD in causing PI3K/Akt upregulation of mouse myeloid precursors is demonstrated by a study in which overexpression of FLT3-ITD cDNA resulted in constitutive activation of Akt, which phosphorylated and inhibited the transcription factor FoxO3 [44]. Besides, about 80% of AML patients have blast cells that express c-Kit, another class III receptor tyrosine kinase for the stem cell factor (SCF) ligand [45]. Mutations in the extracellular or intracellular portions of c-Kit are known for activating PI3K/Akt. As to PTEN, it was observed that PTEN phosphorylation was present in approximately 75% of AML patients. PTEN phosphorylation was significantly associated with Akt phosphorylation and with shorter overall survival [46]. It was demonstrated that phosphorylation at the C-terminal regulatory domain of PTEN stabilizes the molecule, but makes it less active towards its substrate, PtdIns (3,4,5)P₃ so interfering with Akt phosphorylation [47]. Furthermore, PTEN expression has been

shown to be low or absent in some AML patients [34]. However, the level of PTEN expression did not always correlate with the degree of Akt phosphorylation [48].

Other possible activation mechanisms of the PI3K/Akt cascade in AML cells have been proposed to include vascular endothelial growth factor (VEGF), which is a powerful angiogenic molecule for hematological malignancies. It behaves as a critical regulator of endothelial cell survival, motility and proliferation [49]. AML blasts synthesize and secrete VEGF and have demonstrable VEGF receptors, VEGFR-1 and VEGFR-2 respectively [50]. Using human leukemic cell lines as experimental models, it has been shown that VEGF resulted in PI3K dependent Akt phosphorylation [51]. In addition, angiopoietins were detected to activate PI3K through an autocrine mechanism in AML blasts and human acute leukemia cell lines. Therefore, it was suggested that at least in some AML cases, upregulation of the PI3K/Akt axis might be due to an autocrine and/or paracrine production of angiogenic factors, such as VEGF and angiopoietins [52]. Besides, it was shown that activation of PI3K/Akt signaling was also dependent on autocrine secretion of insulin-like growth factor-1 (IGF-1). It is found that the growth of the AML blast cells is increased in vitro in response to IGF-1 stimulation [53]. Multiple large case-control studies have reported positive associations between high circulating levels of IGF-1 and risk for different types of cancer [54]. Regarding malignant hematopoietic disorders, the role of IGF-1 in promoting proliferation, survival and drug resistance of multiple myeloma cells through PI3K/Akt signaling is well established [55]. Finally, interactions between very late antigen (VLA)-4 ($\alpha_4\beta_1$ integrin) on leukemic cells and fibronectin on bone marrow cells has been shown to activate PI3K/Akt signal transduction network [56].

Whatever the reason might be for PI3K/Akt activation, it is very important to consider that results of different studies have highlighted that upregulation of PI3K/Akt axis is present in the AML cell population, where it exerts a powerful prosurvival effect. This finding indicates that therapeutical targeting of the PI3K/Akt pathway has the potential for eradicating AML.

1.8 Downstream Targets of PI3K/Akt Pathway

1.8.1 Antiapoptotic Targets of PI3K/Akt Pathway

Akt promotes cell survival by phosphorylating transcription factors that control the expression of pro- and anti-apoptotic genes. Akt either negatively affects factors that promote death gene expression or positively regulates factors inducing survival

genes. So, as a prototypic kinase, Akt promotes cellular survival to apoptotic insults. Akt enhances survival by directly phosphorylating key regulatory proteins of the apoptotic cascades. Akt phosphorylates Bad, a proapoptotic member of the Bcl-2 family, at Ser 136. This phosphorylation event promotes Bad sequestration in the cytosol, thereby preventing Bad from interacting with either Bcl-2 or Bcl-XL at the mitochondrial membrane. The final effect is inhibition of apoptosis [57]. A similar negative regulation has been demonstrated for Yes-associated protein, whose phosphorylation by Akt leads to repression of p53 related transcription factor p73 and reduced expression of the proapoptotic protein Bax [58].

Most signals that lead to apoptosis do so by activating interleukin-1 β converting enzyme (ICE)-like proteases termed caspases. Apoptosis is ultimately carried out by caspases that are common mediators both through the receptor mediated pathway containing members of the tumour necrosis factor (TNF) family and death receptors (APO-1/FAS and others), and the mitochondrial-mediated pathway involving BCL2, BCL-XL and cytochrome c release from the mitochondria and can be mediated by cytosolic BAX [59]. Caspases are synthesized as proenzymes. Cleavage at specific aspartate residues converts the proenzymes into biologically active cysteine proteases. The activated caspases abolish the effect of substrates that protect cellular integrity, such as the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP), and thereby induce apoptotic cell death. The activation of at least one caspase appears to be an essential step in cellular apoptosis. Consistent with this scheme, caspase 3 (CPP32, pICE, or Yama) has been found to be involved in leukemic cell apoptosis [60]. In addition, in previous studies it has been shown that IGF-I activated Akt resulted in inhibited caspase 3 activation, and these effects were reversed by the PI3K inhibitors [61].

Furthermore, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) is an important mediator of apoptosis in cells exposed to a variety of noxious stimuli, including chemotherapeutic drugs [62]. Akt interferes with SAPK/JNK signaling and negatively regulates it. In another mechanism towards apoptosis, Akt promotes phosphorylation and nuclear translocation of Mdm2, an E3 ubiquitin ligase which mediates ubiquitinylation and proteasome-dependent degradation of the p53 tumor suppressor protein, [63, 64] thereby downregulating p53 and antagonizing p53-mediated cell cycle checkpoint. In that wise, in some AML cases, p53 is regulated through PI3K/Akt dependent signaling and this pathway is proposed being a mechanism to promote resistance to cytotoxic agents [65].

Akt also alters intracellular localization of FoxO family of transcription factors, formerly referred to as forkhead transcription factors, via phosphorylation. In the absence of Akt activation, FoxO proteins are predominantly localized in the nucleus where they are able to promote transcription of proapoptotic target genes such as Fas ligand (Fas-L) and Bim [66, 67]. In addition to downregulating FoxO activity, Akt is capable of upregulating nuclear factor-kappa B (NF- κ B), which is deeply involved in the regulation of cell proliferation, apoptosis and survival [68, 69]. The survival-promoting activity of NF- κ B is mediated by its ability to induce expression of antiapoptotic proteins which oppose caspase activation. NF- κ B function is regulated through its association with the inhibitory cofactor I- κ B, which sequesters NF- κ B. Phosphorylation of I- κ B by upstream kinases, referred to as IKKs, promotes its degradation via the ubiquitinproteasome pathway. This, in turn, allows NF- κ B nuclear translocation and upregulation of target genes [70]. Akt phosphorylates directly and activates IKK α and, more importantly, it is believed to be essential for IKK-mediated destruction of I- κ B [71].

1.8.2 PI3K/Akt Targets Acting on Cell Cycle Regulation

Akt targets p27Kip1, a direct inhibitor of cyclin-dependent kinase (cdk) 2, one of the cdks responsible for the activation of E2F1 transcription factors that promote DNA replication. When phosphorylated by Akt on Thr 157, p27Kip1 mainly localizes to the cytoplasm where it cannot exert its inhibitory effect, so that cell proliferation is enhanced [71]. A direct relationship between cytoplasmic localization of p27Kip1 and PI3K/Akt activation has been demonstrated in HL60 cells [72]. Moreover, cytoplasmic localization of p27Kip1 in AML blasts with upregulated Akt activity was demonstrated to be significantly associated with shorter disease-free and overall survival [73, 74]. Cyclin D1 levels were also found to be upregulated through PI3K/Akt signaling in leukemic cell lines [72]. This might depend on Akt-mediated inhibition of glycogen synthase kinase 3 β (GSK3 β), because cyclin D1 phosphorylation by GSK3 β results in its destabilization. Enhanced cell proliferation could also be a consequence of nuclear exclusion of FoxO factors, because these transcription factors, once in the nucleus, upregulate expression of three target genes which lead to G1/S arrest, p27Kip1, p21Waf/Cip1 and the retinoblastoma family member p130 [75-77]. Moreover, FoxO factors can also promote cell cycle arrest by repressing the expression of cyclin D1 and D2, two positive cell cycle regulators [78, 79].

1.8.3 PI3K/Akt Targets Playing Role in Metabolism

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase which regulates translation in response to nutrients/growth factors by phosphorylating components of the protein synthesis machinery, including p70S6 kinase (p70S6K, a ribosomal kinase) and eukaryotic initiation factor (eIF)-4E binding protein (4EBP)-1. Phosphorylation of 4EBP-1 results in release of the translation initiation factor eIF-4E, allowing eIF-4E to participate in assembly of a translational initiation complex [80]. Furthermore, mTOR acts as a checkpoint sensor indicating to cells that there are sufficient nutrients available to proceed through the cell cycle [81, 82]. Therefore, mTOR regulates a variety of steps involved in protein synthesis, but in particular favors the production of key molecules such as c-Myc, cyclin D1 and ribosomal proteins [83]. p70S6K, which can also be directly activated by PDK-1, phosphorylates the 40s ribosomal protein, S6, leading to active translation of mRNAs [84]. By controlling protein synthesis, p70S6K and 4E-BP1 also regulate cell growth and hypertrophy, which are important processes for neoplastic progression. Therefore, even more distal steps in the PI3K/Akt pathway may have the potential to be exploited for cancer treatment. Akt-mediated regulation of mTOR activity is a complex multistep process. Akt inhibits tuberous sclerosis 2 (TSC2 or hamartin) function through direct phosphorylation [85]. Tuberous sclerosis 2 is a GTPase activating protein (GAP) that functions in association with the putative TSC1 (or tuberin) to inactivate the small G protein Rheb (Ras homolog enriched in brain) [86]. Tuberous sclerosis 2 phosphorylation by Akt represses GAP activity of the TSC1/TSC2 complex, allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates the protein kinase activity of mTOR when complexed with the Raptor (regulatory-associated protein of mTOR) adaptor protein and mLST8. The mTOR/Raptor/mLST8 complex is sensitive to rapamycin and, importantly, inhibits Akt via a negative feedback loop which involves p70S6K [87]. The association of Akt with mTOR further includes the mTOR/Rictor (rapamycin-insensitive companion of mTOR)/mLST8 complex, which displays rapamycin-insensitive activity. Akt directly phosphorylates and activates mTOR, which is the only known example of Akt-mediated phosphorylation resulting in substrate activation [88]. Besides, mTOR was found to be phosphorylated in AML blasts, along with its two downstream substrates, p70S6K and 4EBP-1, in a PI3K/Akt-dependent fashion [34].

Another Akt substrate important for metabolic function is GSK3 β , which phosphorylates and inactivates glycogen synthase in response to insulin stimulation. When phosphorylated by Akt on Ser 9, GSK3 β is downregulated [89]. Glycogen

synthase kinase 3 β is shown to be phosphorylated in AML cells with upregulated Akt function [46].

Activation of PI3K/Akt pathway through mitogenic growth factor receptors and its effects on cell proliferation through downstream targets are represented in Figure 1.3 given below.

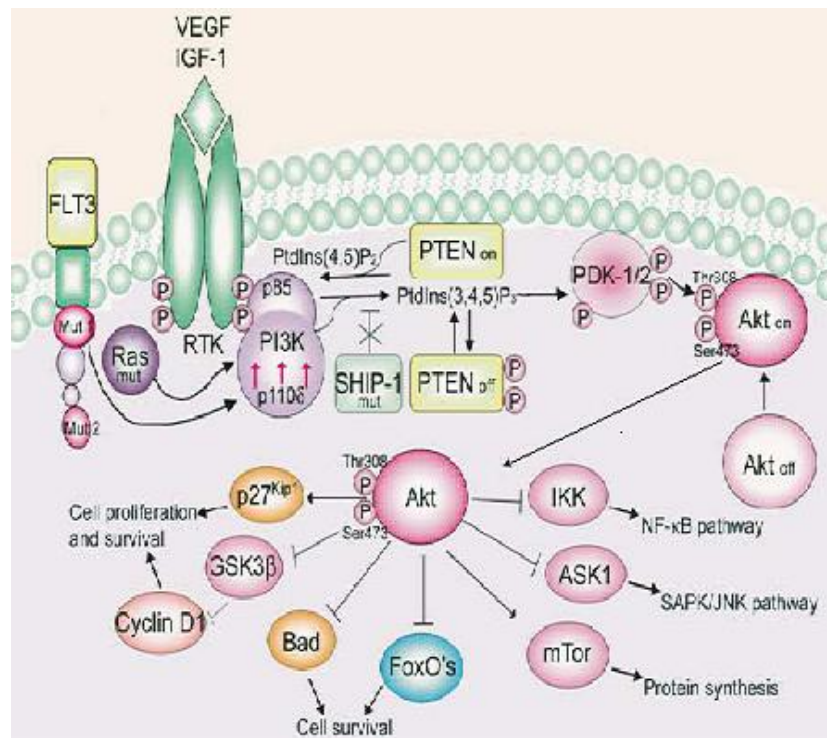


Figure 1.3 : Activation of PI3K/Akt pathway in AML and its downstream targets [38].

1.9 Negative Regulation of the PI3K/Akt Pathway

The termination of Akt signaling is under the control of two key proteins: PTEN, a lipid phosphatase, that prevents activation by removing the second messenger that activates Akt, and PHLPP, a protein phosphatase, that inactivates Akt by direct dephosphorylation of the hydrophobic motif (Figure 1.4) [90].

PTEN (Phosphatase and TENsin homolog deleted on chromosome 10) is a dual specificity lipid and protein phosphatase that preferentially removes the 3-phosphate mainly from PtdIns (3,4,5)P₃ but is also active on PtdIns (3,4,)P₂, thereby antagonizing PI3K/Akt signaling network [91]. PTEN-inactivating mutations or silencing occur in a wide variety of human cancers (including glioblastoma, melanoma, prostatic and endometrium carcinomas) and this results in Akt upregulation [92]. Therefore, PTEN is a tumor suppressor acting upstream of Akt [93]. Two other phosphatases, SHIP-1 and SHIP-2 (for SH domain-containing

inositol phosphatases), are capable of removing the 5-phosphate from PtdIns (3,4,5)P₃ to yield PtdIns (3,4,)P₂. Whereas SHIP-1 is predominantly expressed in hematopoietic cells, SHIP-2 is more ubiquitous. However, their role on Akt function is not well understood, and in some cases they could not reverse Akt activation, something PTEN can do [94]. Protein phosphatase 2A (PP2A), which is rapidly emerging as a new oncosuppressor, [95] is capable of directly dephosphorylating and downregulating Akt, [96,97] whereas it was also indicated that Ser 473 phospho-Akt is dephosphorylated by a PP2C family phosphatase, referred to as PHLPP, another candidate tumour suppressor [90].

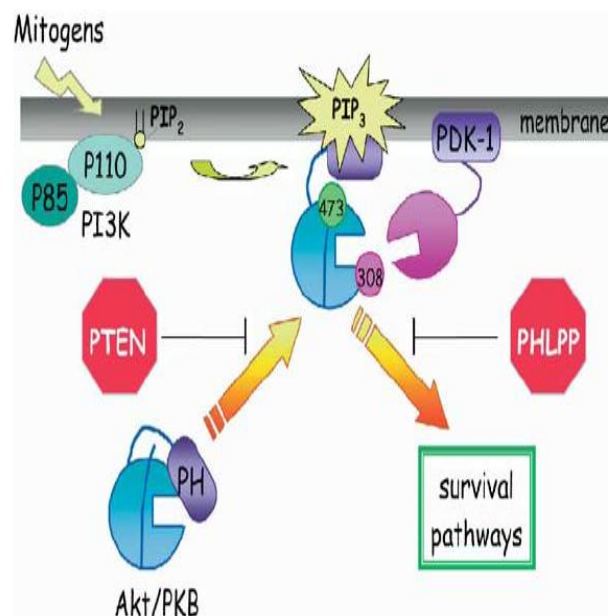


Figure 1.4 : Negative regulation of Akt by two tumour suppressors; PTEN and PHLPP [90].

Human PH domain and leucine rich repeat protein phosphatase, PHLPP, is a 1205 residue protein and weighs 133564 Da. PHLPP is predicted to contain an amino-terminal pleckstrin homology (PH) domain, a leucine-rich repeat region (LRR), a PP2C-like catalytic core, and a PDZ binding motif. The PDZ binding motif is asserted to be crucial for the biological function of PHLPP. It was shown that deletion of the 3 carboxy-terminal residues encoding this motif inhibits the ability of PHLPP to dephosphorylate Akt, to promote apoptosis, and to suppress tumors. PDZ domains can occur in one or multiple copies and are nearly always found in cytoplasmic proteins. They bind either the carboxyl-terminal sequences of proteins or internal peptide sequences. In most cases, interaction between a PDZ domain and its target is reported to be constitutive. However, agonist-dependent activation of cell surface receptors is sometimes required to promote interaction with a PDZ

protein. PDZ domain proteins are frequently associated with the plasma membrane, a compartment where high concentrations of phosphatidylinositol 4,5-bisphosphate (PIP2) are found. Direct interaction between PIP2 and a subset of class II PDZ domains has been demonstrated. Furthermore, the PDZ binding motif is conserved in lower organisms such as *C. elegans* and *Drosophila* unlike the PH domain, which is supposed to be added later in evolution and is not required for function. In general terms, PH domain is a domain of about 100 residues that occurs in a wide range of proteins involved in intracellular signalling or as constituents of the cytoskeleton. The function of this domain is not clear, but several putative functions have been suggested including; binding to the beta/gamma subunit of heterotrimeric G proteins, binding to lipids, e.g. phosphatidylinositol-4,5-bisphosphate, binding to phosphorylated Ser/Thr residues like Akt1 phosphorylated at Ser473 and Thr308, and attachment to membranes by an unknown mechanism. More to PHLPP functional domains, PP2C-like catalytic core refers to protein phosphatase 2C, which is a Mn^{++} or Mg^{++} dependent protein serine/threonine phosphatase. LRR are short sequence motifs present in a number of proteins with diverse functions and cellular locations. These repeats are usually involved in protein-protein interactions [90]. A significant role for LRR domain of LRRC4, a putative tumour suppressor, has been reported in glioma cell proliferation. In the LRR cassette domain of LRRC4 the third LRR motif of the core LRR has been found to play a crucial role as a “proliferation-inhibition switch”. Moreover, it is proposed that LRRC4 requires a functional LRR cassette domain to inhibit proliferation of glioma cells in vitro by modulating the extracellular signal-regulated kinase/protein kinase B/nuclear factor- κ B pathway [98]. Human PHLPP gene is located on 18q21.33 and comprises 17 exons. Coding sequence for PH domain expands exon2, exon3, and exon4; for LRR exon5, exon6, and exon 7; for PP2C exon 14, exon15, exon16, and exon 17; and coding sequence for PDZ is included in exon 17 (Figure 1.5) [90].

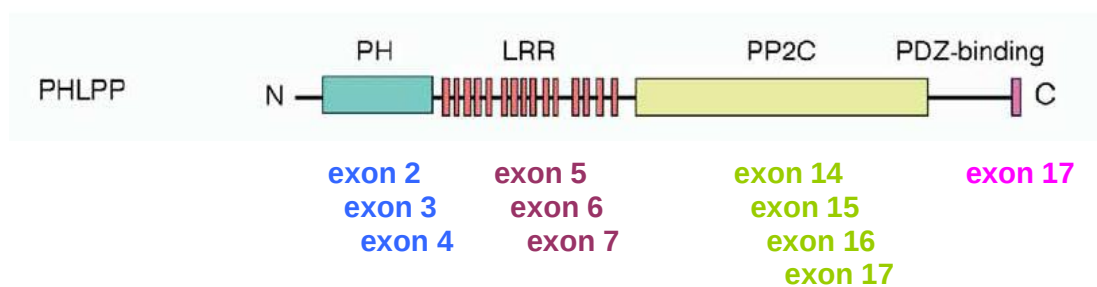


Figure 1.5 : Coding regions for four major functional PHLPP domains [90].

PTEN has proven to be an archetypal tumor suppressor by its effects on the Akt signaling pathway. Nonetheless, there are abundant examples of Akt

phosphorylation being elevated in cancer cell lines having wild type PTEN. Thus, it is clear that other mechanisms causing elevation of Akt phosphorylation contribute to tumour progression. Gao *et al.* (2005) showed that PHLPP levels are markedly reduced in a number of colon cancer and glioblastoma cell lines. Moreover, reintroduction of PHLPP into a glioblastoma cell line that is wild type in PTEN decreased the growth rate of these cells by ~ 50%. The magnitude of this growth-suppressive effect was similar to that observed after reintroduction of PTEN into glioblastoma cell lines defective in PTEN [99]. In addition, it has been reported that subcutaneous injection of glioblastoma cells transfected with PHLPP dramatically reduced the ability of these cells to induce tumors [90]. Recently, Liu *et al.* (2008) found loss of PHLPP expression in colon tumour tissues [100]. Also, PHLPP RNA was reported to be absent in ~ 50% chronic lymphocytic leukemia cases with deletion 13q14 [101].

To conclude, as it was demonstrated in a number of studies that certain tumors were insensitive to inhibitors of PI3 kinase and to the overexpression of PTEN, importance of PHLPP in AKT pathway regulation as a negative regulator and tumour suppressor emerges.

1.10 Aim of the Study

Acute myeloid leukemia (AML) is one of the most common and deadly forms of hematopoietic malignancies. The overall incidence of AML has been stable or slowly increasing over the last 15–20 years. The current chemotherapies produce only initial remission which emphasize the need for new, rationally designed, minimally toxic, effective therapies for AML. PI3K/Akt signaling is frequently activated in AML patient blasts and strongly contributes to proliferation, survival and drug resistance of these cells. The overactivated PI3K/Akt pathway represents potential therapeutic targets for AML. The inactivation of Akt is under the control of two key proteins: PTEN, a lipid phosphatase, that prevents activation by removing the second messenger that activates Akt, and PHLPP, a protein phosphatase, that inactivates Akt by direct dephosphorylation of the hydrophobic motif S473. Since there are abundant examples of elevated Akt phosphorylation level in cancer cell lines having wt PTEN, the role of PHLPP, referred to a novel tumor-suppressor, in Akt signaling emerges and makes PHLPP an attractive target for the development of novel anticancer strategies.

This study aims to determine the role of Akt signaling pathway in pediatric AML and to demonstrate the importance of PHLPP and PTEN in the regulation of Akt

signaling pathway. Human *PHLPP* contains an amino-terminal PH domain, a leucine-rich repeat region (LRR), a PP2C-like catalytic core and a PDZ binding motif. So far, there are no described mutations in *PHLPP* gene. In this study, it is aimed to understand the architecture of *PHLPP* gene variations in pediatric AML patients. Molecular screening was performed for 11 exons covering the four domains of *PHLPP* gene in pediatric AML patients. The screening for the presence of a mutation was performed by Denaturing High Performance Liquid Chromatography analysis and mutation detection was accomplished by direct sequencing. To indicate the role of *PHLPP* in upregulation of Akt pathway in AML patients, first Akt-1 expression was studied in pediatric AML patients as well in controls. Since upregulated Akt signalling is expected to reduce the caspase-3 gene expression, caspase-3 expression was also analysed in both patient and control group. To understand the importance of PTEN and *PHLPP* in Akt upregulation, their expression levels in patients were compared to their expression levels in controls. Also expression analysis of the exons covering the four functional domains of *PHLPP* was performed for molecular characterisation of *PHLPP* gene. In gene expression studies, expression levels of selected housekeeping genes -*abelson*, *beta-2-microglobulin*, *cyclophilin*- were studied in patients and controls to normalize the results. Expression of Akt, pAkt (Ser473), pAkt (Thr308), PTEN, and *PHLPP* was also checked at protein level to confirm the results of gene expression studies. For this purpose total proteins isolated from pediatric AML patients were studied via Western Blot Analysis. The phosphorylated Akt was studied with Ser473 and Thr308 specific antibodies separately, since *PHLPP* directly dephosphorylates Akt at Ser473 residue and so the role of *PHLPP* and PTEN in Akt dephosphorylation might be demonstrated. Since there is no previous data for molecular genetic characterisation of *PHLPP* gene, gene expression studies were performed in different tumour tissues (stomach, colon, pancreas, and breast) to obtain comparative data.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Equipments

The laboratory equipment used in this study is listed in Appendix A.

2.1.2. Chemicals, Enzymes and Markers

The chemicals, enzymes and markers used are given in Appendix B together with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C.

2.1.3 Used Kits

The kits used and their suppliers are given in Appendix D.

2.1.4 Case and Control Group

Study group is composed of 40 pediatric Acute Myeloid Leukemia (AML) patients newly diagnosed according to the French American British (FAB) classification. Diagnosis was based on peripheral blood and bone marrow examination for morphology, cytochemistry and immunophenotypic studies. The bone marrow samples of the patients used in this study were collected for diagnostic purposes in the first place. The age of the patients ranged from 17 months to 14 years with a median age of 8 years. There were 16 females and 22 males. Further information about patient group is listed in the table 2.1 given below.

Control group used in gene expression studies was composed of CD33+ cells isolated from bone marrow samples of 5 healthy donors. In mutational analysis, control group was consisting of peripheral blood samples of 50 healthy controls.

Human pediatric bone marrow was obtained from AML patients and control group after obtaining informed consent from the volunteers. The appropriate standards for human experimentation were followed, and the experimental design of this study has been reviewed and approved by the Ethics Review Committee of the Medical Faculty of Istanbul University.

Table 2.1 : List of translocations, FLT3-ITD,FLT3-D835 mutations and Vascular Endothelial Growth Factor Receptor 1 (FLT-1), Vascular Endothelial Growth Factor Receptor 2 (KDR), Estrogen Receptor Alpha (Er α), and Estrogen Receptor Beta (Er β) expressions in pediatric AML patient group.

Patients no	Lineage	T(15;17)	T(8;21)	inv(16)	FLT3-ITD	FLT3-D835	FLT-1	KDR	ER α	ER β
1	AML	-	-	-	-	-	-	+	+	-
2	AML	-	-	-	+	-	+	+	-	-
3	AML	-	-	-	-	-	-	-	+	-
4	AML	-	-	-	-	-	-	-	+	-
5	AML-M3	+	-	-	-	-	-	+	+	-
6	AML	+	-	-	-	-	+	-	+	-
7	AML	-	-	-	-	-	-	+	+	-
8	AML M3	-	-	-	-	-	+	+	+	-
9	AML M2	-	-	-	-	-	-	-	+	-
10	AML M1	-	+	-	-	-	+	+	-	-
11	AML M3	-	-	-	-	-	+	-	-	-
12	AML M3	+	-	-	-	-	-	-	-	-
13	AML	+	ND	-	-	-	-	-	-	-
14	AML M3	+	ND	-	+	-	+	+	-	-
15	AML M2	-	ND	-	+	-	+	+	-	-
16	AML M3	-	ND	-	-	-	-	-	-	-
17	AML	-	ND	-	-	-	+	+	-	-
18	AML M7	-	ND	-	-	-	+	-	-	-
19	AML	-	ND	-	-	-	-	-	-	-

Table 2.1 : (continued) List of translocations, FLT3-ITD, FLT3-D835 mutations and Vascular Endothelial Growth Factor Receptor 1 (FLT-1), Vascular Endothelial Growth Factor Receptor 2 (KDR), Estrogen Receptor Alpha (ER α), and Estrogen Receptor Beta (ER β) expressions in pediatric AML patient group.

Patients no	Lineage	T(15;17)	T(8;21)	inv(16)	FLT3-ITD	FLT3-D835	FLT-1	KDR	ER α	ER β
20	AML	-	ND	ND	-	-	+	+	-	-
21	AML	-	ND	ND	-	+	-	-	+	-
22	AML	ND	ND	ND	-	-	+	-	+	-
23	AML M3	+	ND	ND	-	-	+	-	+	-
24	AML M3	ND	ND	ND	-	-	+	-	+	-
25	AML M3	ND	ND	ND	+	-	-	-	+	-
26	AML M0	-	-	-	-	-	+	+	-	-
27	AML M2	-	-	-	-	-	+	-	-	-
28	AML M7	-	-	-	-	-	+	-	+	-
29	AML M0	-	-	-	-	-	+	+	+	-
30	AML M2	-	-	-	-	-	+	+	+	-
31	AML M3	-	-	-	-	-	-	-	+	-
32	AML M1	-	-	-	-	-	+	+	+	-
33	AML M3	-	-	-	-	-	-	+	+	-
34	AML M5	-	-	-	-	-	+	+	+	-
35	AML M3	-	-	-	-	-	+	-	-	-
36	AML M3	-	-	-	+	-	-	+	+	-
37	AML M3	-	-	-	-	-	-	+	+	-
38	AML M3	-	-	-	+	-	-	-	-	-

2.1.5 Tumour Tissues

Breast, stomach, colon and pancreas tumour tissue (10 of each) samples were used in gene expression studies.

2.2 Collection and Storage of Blood Samples and Tumour Tissues

The bone marrow samples and peripheral blood samples are collected from the patients who came to the Medical Faculty of Istanbul University, Department of Pediatric Hematology and Oncology. The bone marrow and peripheral blood samples were collected in vacuum tubes containing EDTA. The samples were kept at +4 °C for short term storage (1/2 day).

Tumour tissues were stored at -80°C for longer terms of storage (several months).

2.3 Isolation of Bone Marrow and Peripheral Blood Leukocytes by Ficoll Gradient Centrifugation

Leukocytes were isolated via applying Ficoll density gradient centrifugation. A volume of 2.5 ml Ficoll-Histopaque (Sigma) was put in a 10 ml glass tube. Subsequently, 5 ml of freshly drawn (maximum ½ day of storage at +4 °C) blood or bone marrow sample was poured into the tube. The mixture was centrifuged at 2000 x rpm for 20 minutes at room temperature. Fractions were collected from the top with a Pasteur pipette (plasma and leukocytes). Leukocytes were washed twice with phosphate-buffered saline (PBS). For each washing step 3.5 ml of PBS was added onto the leukocyte pellet and the mixture was centrifuged at 2000 x rpm for 5 minutes at room temperature. For long term storage (several months) leukocyte pellet was homogenized in 600 µl of solution D in a sterile tube and stored at -80 °C. Solution D contains Buffer RLT, which immediately inactivates DNases and RNases as well as proteases to ensure isolation of intact DNA, RNA, and proteins.

2.4 CD33+ Cell Isolation on Magnetic Bead Columns

CD33+ cells were fractioned from pediatric bone marrow samples obtained from 5 healthy donors. Firstly, leukocytes were isolated from 5 ml of freshly drawn bone marrow sample by Ficoll Gradient Centrifugation as indicated above. Leukocyte pellet obtained after 2 washing steps with PBS was resuspended in 80 µl of MACS buffer (MACS BSA stock solution diluted with 1:20 autoMACS Rinsing solution). 20 µl of CD33 MicroBeads (MACS) was added to this suspension and the mixture was

incubated at +4 °C for 15 min in order to magnetically label CD33+ cells. Then, the cells were washed by adding 2 ml of MACS buffer to the mixture and a following centrifugation step was accomplished at 2000 x rpm for 5 min at room temperature. The supernatant was pipetted off completely and the cells were resuspended in 500 µl of MACS buffer. After the steps of magnetic labeling, magnetic separation was applied by using MiniMACS Column and MACS Separator (Miltyeni Biotec). The column was placed in the magnetic field of a MACS Separator. Next, the column was prepared by rinsing it with 500 µl of MACS buffer. The cell suspension was loaded onto the column. The magnetically labeled CD33+ cells were retained on the column and unlabeled cells which passed through were collected. The column was washed 3 times by adding 500 µl of MACS buffer each time once the column reservoir was empty. After 3 washing steps, the column was removed from the separator and placed on a sterile collection tube. 500 µl of MACS buffer was added onto the column. The fraction with the magnetically labeled cells was immediately flushed out by firmly applying the plunger supplied with the column. The purity of CD33+ cells eluted from the MACS column were checked by FACS scanning and CD33+ cell suspension in MACS buffer used in final elution was centrifuged at 10,000 x rpm for 5 min at room temperature. The supernatant was pipetted off and the CD33+ cell pellet was resuspenden in 350 µl of solution D and stored at -80 °C.

2.5. Flow Cytometric Analysis of the Sorted Cells

Flow cytometric analysis was performed to ensure the purity of the isolated CD33+ cells. Before analysis, the enriched CD33+ cells were labeled with FITC-conjugated anti-CD33 beads. Flow cytometric analysis was accomplished by BD FACSCalibur™ Flow Cytometer (Becton Dickinson).

2.6 DNA, RNA, and Protein Isolation from Leukocytes

For simultaneous purification of DNA, RNA, and protein from leukocytes stored in solution D, AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany) was used. Leukocytes in solution D were thawed and homogenized in Buffer RLT, which immediately inactivates DNases and RNases as well as proteases to ensure isolation of intact DNA, RNA, and proteins. The homogenate was then centrifuged at 14,000 x rpm for 2 min at room temperature. The supernatant was then passed through an AllPrep DNA spin column which in combination with the high-salt buffer, allowing selective and efficient binding of genomic DNA. In this step DNA spin column was placed on a 2 ml collection tube and centrifugation was performed at

12,000 x rpm for 30 sec. The DNA spin column was put onto a new 2 ml collection tube and stored at +4 °C for further steps of DNA elution.

For RNA purification, ethanol was added to the flow-through from the AllPrep DNA spin column in order to provide appropriate binding conditions for RNA (250 µl of ethanol was added for 350 µl of solution D used for storage of leukocytes). The sample was then applied to a RNeasy spin column, where total RNA binds to the membrane and contaminants are washed away. RNeasy spin column was put onto a new 2 ml collection tube and centrifuged at 12,000 x rpm for 15 sec. The flow-through was transferred to a 2 ml tube for protein purification. 700 µl of Buffer RW1 was added to the RNeasy spin column and the column was centrifuged at 12,000 x rpm for 15 sec to wash the spin column. The flow-through was discarded. 500 µl of Buffer RPE was added to the RNeasy spin column and the column was centrifuged at 12,000 x rpm for 15 sec. The flow-through was discarded. This step was repeated twice. The RNeasy spin column was placed in a new 2 ml collection tube and centrifugation was carried out at 14,000 x rpm for 1 min. Afterwards, the RNeasy spin column was placed in a new 1,5 ml collection tube and 30 µl of RNase-free water was added to the spin column membrane. The column was let at room temperature for 1 min and then centrifuged at 12,000 x rpm for 1 min to elute total RNA. The flow-through was put into the column and the last centrifugation step was repeated in order to increase RNA yield.

For total protein precipitation, 600 µl of Buffer APP was added to the flow-through from first centrifugation step of total RNA purification, where Buffer APP is an aqueous protein precipitation solution. The mixture was mixed vigorously by using vortex and let at room temperature for 10 min. The precipitated proteins are pelleted by centrifugation at 14,000 x rpm for 10 min. The supernatant was carefully decanted via pipetting it off. 500 µl of 70% ethanol was added to the protein pellet and this was followed by a centrifugation at 14,000 x rpm for 2 min. The supernatant was removed by using a pipet. Then, the protein pellet was dried at room temperature for 10 min. Intact total proteins were redissolved in 100 µl of Buffer ALO, which is compatible with SDS-PAGE. The suspension was incubated at 95 °C for 5 min to completely dissolve and denature the protein. Next, the sample was cooled to room temperature and centrifuged at 14,000 x rpm for 1 min to pellet any residual insoluble material. The supernatant was collected and stored in a sterile 2 ml tube for further SDS-PAGE and Western Blotting studies.

To purify genomic DNA, 500 µl of Buffer AW1 was added to the AllPrep DNA spin column stored for DNA elution in previous steps. After addition of Buffer AW1, the

column was centrifuged at 10,000 x rpm for 15 sec. The flow-through was discarded and the DNA spin column was placed in a new 2 ml collection tube. 500 µl of Buffer AW2 was added to the column and centrifuged at 14,000 x rpm for 2 min. The column was placed in a new 2 ml collection tube and centrifuged at 14,000 x rpm for 1 min. Next, the column was placed in a new 1,5 ml collection tube and 100 µl of Buffer EB was added to the column membrane. After incubation at room temperature for 1 min, the column was centrifuged at 10,000 x rpm for 1 min to elute the DNA. The flow-through was added to the column membrane and following incubation at room temperature for 1 min, the column was centrifuged at 10,000 x rpm for 1 min to elute further DNA.

The isolated stock of total DNA, RNA, and dissolved protein were quantified via NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and kept at -80 °C for longer terms (several months) of storage.

2.7. RNA Isolation from Leukocytes

To purify high yields of total RNA to use in quantitative Real Time PCR studies, total RNA was isolated via using QIAamp RNA Blood Mini Kit (Qiagen) from low number leukocyte samples stored in solution D. Isolation was performed according to suppliers manual.

Each of the leukocyte samples stored in 500 µl of solution D was transferred directly into a QIAshredder spin column (Qiagen) in a 2 ml collection tube and centrifuged at 14,000 x rpm for 2 min. The column was discarded and homogenized lysate was saved. 1 volume (500 µl) of 70% ethanol was added to the homogenized lysate and mixed by pipetting. Then, the sample was transferred into a new QIAamp spin column in a 2 ml collection tube and centrifuged at 10,000 x rpm for 15 sec. The column was placed into a new 2 ml collection tube and 700 µl of Buffer RW1 was added to the column. Next, the column was centrifuged at 10,000 x rpm for 15 sec. The column was placed in a new 2 ml collection tube and 500 µl of Buffer RPE was put into the column. The column was centrifuged at 10,000 x rpm for 15 sec and the supernatant were discarded. Then, 500 µl of Buffer RPE was added to the column membrane and the column was centrifuged at 14,000 x rpm for 3 min. The column was placed in a new 2 ml collection tube and centrifuged at 14,000 x rpm for 1 min to eliminate the chance of possible Buffer RPE carryover. In the final step, the column was transferred into a 1.5 ml microcentrifuge tube and 30 µl of RNase-free water was added directly onto the column membrane. To elute total RNA, the column was centrifuged at 10,000 x rpm for 1 min. The purified total RNA was

quantified via NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and kept at -80 °C for longer terms (several months) of storage.

2.8 DNA, RNA, and Protein Isolation from Tumour Tissues

To subsequently purify DNA, RNA, and protein from tumour tissues, AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany) was used. 30 mg of tumour tissue sample was firstly homogenized in 600 µl of TRIzol Reagent (Invitrogen). The homogenized samples were put into 1,5 ml microcentrifuge tubes and centrifuged at 14,000 x rpm for 10 min. The supernatant was transferred into AllPrep DNA spin column (Qiagen) and the purification steps were carried on as indicated in section 2.6.

2.9 Quantification and Qualification of DNA

The isolated stock of total DNA via AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany) was quantified by applying NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). For each measurement 1µl of stock DNA was applied. As the blank solution Buffer EB (Qiagen) was used. The concentration and purity of the isolated DNA were calculated by using the absorbance values measured at 260, 280 and 320 nm. The concentration of the DNA was calculated with the equation 2.1 and the purity of the DNA samples were calculated with the equation 2.2 given below:

$$DNA\ Concentration\ (ng / \mu L) = (A_{260} - A_{320}) \times 50 \times Dilution\ Factor \quad (2.1)$$

$$DNA\ Purity = \frac{(A_{260} - A_{320})}{(A_{280} - A_{320})} \quad (2.2)$$

In order to have a set of DNA samples that have the same DNA concentration (50 ng/µL), dilutions from the stock DNA are prepared (working solutions).

2.10. Quantification and Qualification of RNA

The concentration of RNA was determined by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) measurement of the absorbance at 260 nm (A_{260}). An

absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml of water (in pH 7 conditions). For each measurement 1 µl of isolated RNA stock diluted in 30 µl of RNase-free water was applied. As the blank solution RNase free water was used. The concentration of the sample was calculated according to the formula 2.3 given below:

$$\text{Concentration } (\mu\text{g/ml}) = 40\mu\text{g} \times A_{260} \times \text{Dilution factor} \quad (2.3)$$

Also measurement was performed at A_{280} to check DNA contamination. The purity of RNA was estimated via calculating the A_{260}/A_{280} ratio.

Qualification of purified RNA was made via RNA Gel Electrophoresis. For this purpose 1% agarose gel was prepared by adding 0.5 g of agarose into 50 ml of 1x TAE buffer. The mixture was heated in a microwave oven to melt the agarose and then cooled to 65 °C at room temperature. 2 µl (0.4 µg/mL) of ethidium bromide was added to the mixture. All components were mixed and gel was poured onto gel support. Gel was left to polymerize at room temperature. Before loading the samples the gel tank was filled with 1x TAE Gel Running Buffer. 5 µl of RNA was mixed with 1 µl of 6x Loading Dye (Fermentas) and loaded onto the agarose gel in TAE buffer. The RNA samples were run at 90 V. The detection was accomplished via UV lamp.

2.11 Quantification of Proteins

2.11.1 Measurement by NanoDrop™ 1000 Spectrophotometer

The protein concentration was first checked by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) measurement of the absorbance at 280 nm (A_{280}).

2.11.2 Bradford Assay

For more accurate determination of protein concentration Bradford Assay was performed prior the use of the proteins by SDS-PAGE and Western Blot Analysis.

Use of Coomassie Blue Dye in a colorimetric reagent for the detection and quantification of total protein was first described by Dr. Marion Bradford in 1976. In the acidic environment of the reagent, protein binds to the coomassie dye, which results in a spectral shift from the reddish/brown form of the dye (absorbance max. at 465 nm) to the blue form of the dye (absorbance max. at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, thus it is the optimal wavelength to measure the blue color from the coomassie dye-protein complex.

Besides, the blue color can be measured at any wavelength between 575 nm and 615 nm. Bradford protein assay has been associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. Free amino acids, peptides and low molecular weight proteins do not produce color with coomassie dye reagents.

The assay is performed at room temperature. The measurement was accomplished at 595 nm by using a 96 well plate. For measurement in a working range 100-1500 µg/ml, protein samples of 10 µl were prepared from protein stocks in a 1:10 ratio, using distilled water as diluent. Bovine serum albumin (BSA) dilutions in a range of 28 µg-1,25 µg/ 20 µl diluted with distilled water were used as protein standards. 5 µl of BSA standards and protein samples were loaded in a 96 well plate. 200 µl of Bradford Reagent was added onto each well and homogenized via pipetting. After incubation at room temperature for 5 min, the absorbance values were measured at 595 nm by Microplate reader 3550-UV (Biorad).

2.12 Mutation Analysis

2.12.1 Template DNA

Purified DNA samples from 38 pediatric AML patients and 30 healthy controls were used in mutational analysis of PHLPP gene coding regions for four major functional domains.

2.12.2 Primer Design

The coding regions for four major functional domains of PHLPP gene were targeted for mutational analysis (Fig. 2.1). For this purpose, exonic sequences coding for PH-domain, Leucine-rich Repeat (LRR) domain, PP2C-like catalytic core, and PDZ-binding core were selected and primers were designed to amplify the coding regions.

Coding region for PH-domain encompasses exon 2, 3, and 4; for LRR-domain exon 5, 6, and 7; for PP2C-like catalytic core exon 14, 15, 16, and 17; for PDZ binding region exon 17. Primer sequences to amplify coding regions for four functional domains were designed using the Primer3 software (available online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers with length

ranging from 18 to 22 bp and having a G/C content of 40-60 % were chosen as optimal, avoiding self-complementarity within a primer and formation of primer dimers. To avoid dimerization, the primer sets were analyzed for hairpin, heterodimer, and self dimer formation via Primer Design Tools on the www.cybergene.se website.

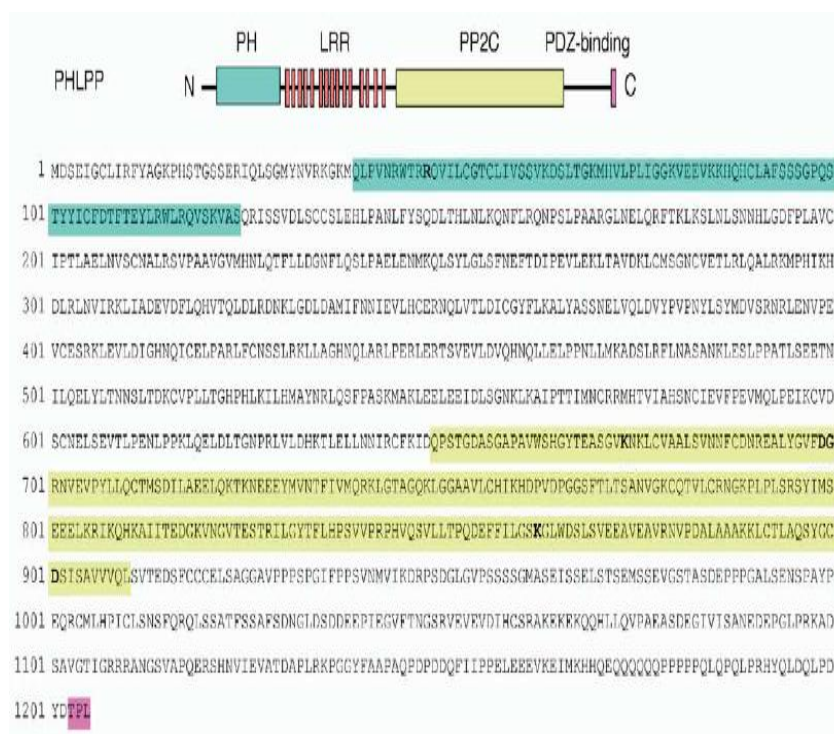


Figure 2.1 : The functional domains of PHLPP gene [90].

The primers designed to amplify exonic regions coding for functional domains are listed below in table 2.2.

Table 2.2 : Oligonucleotide primers designed for use in mutational analysis

Exonic region	Oligonucleotide Sequence used to amplify the exonic region
Exon2F	5'-TATGTAGATGACCAGGAA-3'
Exon2R	5'-GACCAACGCCACTACACTT-3'
Exon3F	5'-TTCTGCTTCTCTCTGTTTGC-3'
Exon3R	5'-AGAAGCTTGAGTGGATTCTCTG-3'
Exon5F	5'-CCCCATTTCTCTTTAGTTG-3'
Exon5R	5'-CTACGTCTCATGTGCCACACC-3'

Table 2.2 : (continued) Oligonucleotide primers designed for use in mutational analysis

Exonic region	Oligonucleotide Sequence used to amplify the exonic region
Exon6F	5'- CGGTCTACAGGTTATGGAAGTTG-3'
Exon6R	5'- CAATATAAGAACAGCCACTCA-3'
Exon6.1F	5'- CGGTCTTGCACTTATGGAAGTTG-3'
Exon6.1R	5'- CATTCCAAGAAAGACCCAGA-3'
Exon6.2F	5'- CTGGGTCTTTCTTAACAATG-3'
Exon6.2R	5'- GCTTGTTTGCCTTAAGGTTA-3'
Exon7F	5'- TATTAGGCTTGCGCCATTTA-3'
Exon7R	5'- CCATTAGAGCAACTTTGATG-3'
Exon14F	5'- ACGCAGTTAGAAATGGTAAGT-3'
Exon14R	5'- CCTCACTGATGGGGAACTTG-3'
Exon15F	5'- CTCAGCACTCGAAGTGTGGTT-3'
Exon15R	5'- AGCAGGTATTGCAGGAAGTCT-3'
Exon16F	5'- AGAGCAGTCAGTGCTTTGAT-3'
Exon16R	5'- CCTGTAACTCACCTCAGTG-3'
Exon17AF	5'- TCTGTGCACCTACAGTCGAG -3'
Exon17AR	5'- CCTTGATGTCCATGTTCACT -3'
Exon17A.1F	5'- CCCTTCTTAGCCTCTGTGG-3'
Exon17A.1R	5'- TAGGCAGAAAGGGTTGTG-3'
Exon17A.2F	5'- CTACACCTAACCTCCATCCC-3'
Exon17A.2R	5'- CCTTGATCATTCATGTTCACT-3'
Exon17BF	5'- AGTCCTGGCAACTTTCCTC-3'
Exon17BR	5'- CTGCTGTTTGAACCTTCTCCT-3'
Exon17CF	5'- AGGAGAAGGACTAACAGCAG-3'
Exon17CR	5'- TCATAGTGGCAAGTCGTAAT-3'

2.12.3 Amplification of Functional Domains by Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used to amplify the target genomic DNA sequences on PHLPP gene which are coding for PH, LRR, PP2C-like catalytic core, and PDZ binding domains. For this purpose the isolated genomic DNAs of the study and control group were used as templates. A standard mixture of PCR (except from the primers) was used to amplify the related sequences of all 11 exonic regions.

Table 2.3 : Standard PCR mix

Ingredient	Stock Concentration	Volume	Final Concentration
Taq Buffer	10X	2 μ L	1X
MgCl ₂	25 mM	2 μ L	1.875 mM
Forward Primer	10 pmol/ μ L	1,5 μ L	0.5 μ M
Reverse Primer	10 pmol/ μ L	1,5 μ L	0.5 μ M
dNTP mix	2 mM	2 μ L	40 μ M
Taq Polymerase	5 U/ μ L	0,15 μ L	0.05 U/ μ L
dH ₂ O	–	19,85 μ L	–
Template DNA	50 ng/ μ L	2 μ L	100 ng
FINAL	32 μ L		

The performance of the PCR reaction is affected from the purity of the template DNA as well as the amplicon size (length) and content (GC%).

2.12.4 PCR Cycle Conditions

The PCR cycle conditions were modified in order to obtain the optimum amplicon in expected size. General PCR cycle conditions are listed in Table 2.4 given below. Annealing temperature was spesific for each primer. PCR reaction were carried out in Techne 512 Thermo Cycler (Thermo Scientific).

Table 2.4 : General PCR cycle conditions

Cycle Number	Degree	Time	Phase
1	95 °C	10 min	Initial Denaturation
35	94 °C	1 min	Denaturation
	Variable	1 min	Annealing
	72 °C	1 min	Elongation
1	72 °C	10 minutes	Final Extension
1	4°C	∞	Final Hold

2.12.5 PCR Optimization

For amplification of the specific product in expected size and to increase the reactions efficiency, PCR conditions were optimized via changing the annealing temperature, Mg^{2+} concentration in the reaction mixture and changing the template DNA amount used for amplification. To optimize the annealing temperature, PCR reactions prepared with the same reaction mix were run at different annealing temperatures. Since Mg^{2+} is the cofactor of Taq DNA polymerase, the amplification efficiency changes within a scale of low to high concentrations of Mg^{2+} . Frequent observations about the effect of Mg^{2+} concentration on PCR include the decrease in amplicon amount with low concentration, and unspecific amplification of non-target regions with high concentrations. In order to obtain the appropriate amount of Mg^{2+} for a PCR reaction, PCR mixtures were prepared where only the Mg^{2+} concentration in the reaction differed.

2.12.6 Agarose Gel Electrophoresis of PCR Products

Due to the size of the PCR products ranging between 195 to 360 bp, 2% agarose gels were prepared and used in this study. For this purpose, agarose gels were composed of 1 g agarose, 2 μ l (0.4 μ g/mL) of ethidium bromide, and 50 mL of 1X TAE buffer, which is diluted from 10X stock TAE. 8 μ L of PCR product was mixed with 1 μ L 6X loading dye (Fermentas) and loaded into the wells. Gene ruler 1 kb marker (Fermentas) was used as DNA ladder. The gels were run in 1X TAE buffer, at 90V with power supplier, for at least 30 minutes. The gels are observed under UV light.

2.12.7 Denaturing High Performance Liquid Chromatography (DHPLC) Analysis

DHPLC is one of the most sensitive and accurate methods used in identification of unknown mutations. It allows fast and reliable scanning of genes or gene regions. Due to its high sensitivity, it detects mutations that can not be find out via gel based or capillary electrophoresis based systems and it also shows somatic mutation sites which can not be detected in sequencing systems. DHPLC technique is frequently applied to diagnose cancer mutations due to the large length of cancer related genes and low frequency of their mutations. Also DHPLC system is accepted as a pre-sequencing technique.

When single chain DNA carrying a mutation or a polymorphism, is hibridized with a wild type single chain DNA molecule, heteroduplexes will be formed. The melting temperature of these heteroduplexes are lower than those of homoduplexes. In the application of DHPLC, wild-type and mutant alleles are hybridized @ 95°C and cooled to 25°C for 45 minutes. Semi melted or partly denatured heteroduplexes are disassociated before the homoduplexes, which allows their detection.

In DHPLC system, an amphipatic ion (triethyl-amonium asetat, TEAA) in mobile phase, and an organic solvent (Asetonitril) is used. The positively charged amonium ion of TEAA binds to DNA whereas its hydrophobic ethil groups are attached to the column material (steady phase). Hence, a bridge will be formed between column material and nucleic acid. The detachment of nucleic acids bound to the column is achieved at a spesific temperature (T_m) via increasing asetonitril concentration which disrupts the interaction between TEAA and the column. Nucleic acids will then be separated from the column as the weak ones will disassociate first, and will be detected via UV detection.

24 μ L of PCR product was loaded into DHPLC plate, and the specific melting temperatures for DHPLC analysis were determined via WAVE software.

2.12.8 Direct Sequencing

Samples and controls were selected for direct sequencing analysis according to the DHPLC results. Selected samples carrying different peak patterns than normals were commercially sequenced by Macrogen (South Korea).

2.13 Akt-1, PTEN, Caspase 3, and PHLPP Gene Expression Studies

In gene expression studies RNA samples of 35 pediatric AML patients and CD33+ cells obtained from bone marrows of 5 healthy pediatric donors were used.

2.13.1 Gene Expression of PHLPP Functional Domains

The PHLPP exons coding for 4 major functional domains were targeted for gene expression studies. For this purpose, exonic sequences coding for PH-domain, LRR domain, PP2C-like catalytic core, and PDZ-binding core were selected and primers were designed to amplify those regions Figure 2.2.

Coding region for PH-domain encompasses exon 2, 3, and 4; for LRR-domain exon 5, 6, and 7; for PP2C-like catalytic core exon 14, 15, 16, and 17; for PDZ binding region exon 17. Primer sequences to amplify coding regions for 4 functional domains were designed using the Primer3 software. To avoid dimerization, the primer sets were analyzed for hairpin, heterodimer, and self dimer formation via Primer Design Tools on the www.cybergene.se website.

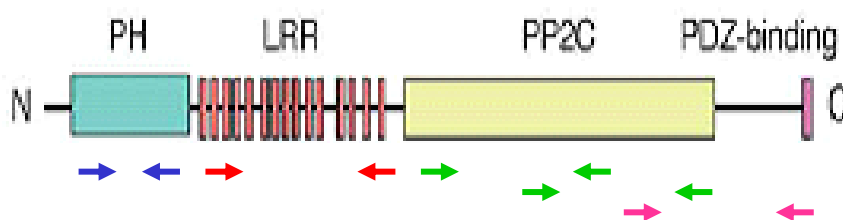


Figure 2.2 : Primer design for *PHLPP* gene expression domains: targeting exons coding for four major functional domains of *PHLPP* gene.

2.13.2 Akt-1, PTEN, and Caspase 3 Gene Expression Studies

Furthermore, primers were selected to study gene expression of Akt-1 gene together with its downstream target caspase-3 gene and 2 negative regulators of Akt pathway, the tumour suppressor PTEN and PHLPP. To normalize the results of the gene expression studies, house-keeping genes Abelson (ABL), beta-2-microglobulin (B2M), and cyclophilin (CYC) were used in gene expression studies.

Selected primers to amplify exonic regions coding for functional domains of PHLPP, Akt, PTEN, caspase-3, house-keeping genes; ABL, B2M, CYC- are listed below in Table 2.5.

Table 2.5 : Primers used in gene expression studies

Primer	Oligonucleotide sequence
PTENF	5'-CAAGCAGATGTTTGAACTATTCCAATG-3'
PTENR	5'-CCTTAACCTGGCAGACCACAA-3'
CASP3F	5'-ACATCCGGTGTCTATAAATACC-3'
CASP3R	5'-CACAATTCGACTGGATGAAC-3'
Akt1F	5'-ATGAGGAACGTGGCTATTGTGAAG-3'
Akt1R	5'-GAGGCAATCAGCCACAGTCTGGATG-3'
PH F	5'- AGTCATGGTATGTGGGACCT-3'
PH R	5'- GTGAGGTGATGGCTGTAGAA -3'
LRR F	5'- TGATGCAGGACTTACAGACA-3'
LRR R	5'- ACCAGTTGAATCCTTTCACA -3'
PP2C1 F	5'- AGCCCTGACGGTGAATAA-3'
PP2C1 R	5'- CACCAGTTCCAAGTTTCCTT-3'
PP2C2 F	5'- TTGGCCTTGACCTCTGCTAA -3'
PP2C2 R	5'- CTTGCCATCTTCAATGATAA-3'
PDZ F	5'-GGAAGAGGAGGTGGAAGAAA-3'
PDZ R	5'- TCATAGTGGCGTGGCTTAAT-3'

In gene expression studies, pediatric AML patient group included 35 samples and as controls CD33+ cells isolated from bone marrows of 5 healthy pediatric donors were used.

Gene expression studies were also performed for 40 tumour tissue samples including 10 colon, 10 stomach, 10 pancreas, and 10 breast cancer tumour tissues.

2.13.3 Reverse Transcriptase Reaction: First- Strand cDNA Synthesis

After qualification and quantification of isolated RNA, reverse transcriptase reaction was performed using First Strand cDNA Synthesis Kit from FERMENTAS® including: DEPC water, dNTP mix 10mM, 5x Reaction Buffer, Oligo(dT)₁₈ primer (0.5 µg/µl), Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT), and RiboLock (Ribonuclease Inhibitor).

First, 5µg of total RNA was mixed with 1µl of Oligo(dT)₁₈ primer, and DEPC water was added till the total volume reached 11µl on ice. The contents of the reaction tube was mixed and micro-centrifuged on ice. The tube was incubated at 70°C for 5 min, chilled on ice, and micro-centrifuged. Later, 4 µl of 5x reaction buffer, 1 µl RiboLock, and 2 µl of dNTP mix were added on ice. The tube was incubated at 37°C for 5 min. After that, 2 µl of M-MuLV RT was added, and the tube was incubated at 37°C for 60 min. The reaction was stopped via heating at 70°C for 10 min. The newly synthesized cDNAs are immediately placed on ice and stored at -20 °C for later usage.

2.13.4 Amplification of One Strand cDNA and Quantification of the Amplicon

2.13.4.1 Standard PCR and Agarose Gel Electrophoresis

The target sequence in one strand cDNA synthesized via reverse transcriptase reaction from total RNA isolate was amplified via standard PCR reaction using sequence specific primer pairs for β-actin housekeeping gene under same cycling conditions as aforementioned for standard PCR reaction.

The amplicon of each PCR reaction was checked via 2% agarose gel electrophoresis.

2.13.4.2 Quantitative Real Time PCR (qRT-PCR)

The mRNA expression level of target genes in pediatric AML patients and in control group was quantified by real-time PCR analysis using Roche Light Cycler 2.0 instrument and the SYBR® Premix Ex Taq™ kit (TaKaRa Biotechnology) as a

fluorescent reporter. The following PCR cycle parameters were used: 95°C for 10 sec, 40 cycles at 95°C for 5 sec, 60°C for 34 sec.

The optimized reaction ingredients are given in table 2.6 and the Real-Time PCR, cycle conditions are given in the table 2.7 below.

Table 2.6 : Real-Time PCR mixture

Ingredient	1X volume	Final Concentration
dH ₂ O	2.5 µL	–
SYBR® Premix Ex Taq™	5 µL	75 nM
Forward Primer (10 µM)	0.25 µL	250 nM
Reverse Primer (10 µM)	0.25 µL	250 nM
cDNA template	2 µL	–

Table 2.7 : Real-Time PCR Cycling Conditions

Cycle Number	Temperature	Time	Phase
1	95°C	10 min	Initial Denaturation
35	95°C	10 sec	Denaturation
	specific for each primer	10 sec	Annealing
	72°C	15 sec	Elongation
1	95°C		Melting Curve Analysis
	70°C	15 sec	
	95°C		
1	40		Cooling

Furthermore, specificity of the amplicons were checked by melting curve analysis set by the melting temperatures specific for primers. For each sample specific melting curve pattern was expected to be observed. Cycle conditions for melting curve analysis are given in the table 2.7 above. To ensure amplicon specificity,

amplification products were loaded on 2% agarose gel and the product sizes were compared with the expected size for each specific product.

2.13.5 Relative Quantification of the Samples

The relative quantification of the samples is done by $2^{-\Delta\Delta C_T}$ method. The ΔC_T values for PHLPP, Akt 1, PTEN, and caspase 3 were calculated via subtracting the C_T values of each sample from the previously obtained average C_T values for control genes *abelson*, $\beta 2$ -microglobulin and cyclophilin ($C_{T \text{ Average of Control Genes}} - C_{T \text{ Case}}$). These obtained ΔC_T values are then placed as the power of 2 ($2^{\Delta C_T}$). The resulting values give the relative expression levels of each sample, normalized with the values obtained from the control samples.

2.14 Amplification of PHLPP Transcripts

Reverse transcriptase PCR was applied to amplify the whole mRNA transcript of PHLPP gene encompassing 3600 bp. Total transcript of PHLPP gene was studied with cDNAs obtained from 35 pediatric AML, 40 different tumour (10 breast, 10 stomach, 10 colon, and 10 pancreas cancer tissues), and 5 healthy control samples. To amplify the 3600bp mRNA product Expand Long Taq kit (Roche) was used.

RT-PCR reaction ingredients are given in table 2.8 and long RT-PCR cycle conditions are given in the table 2.9 below.

Table 2.8 : Long RT-PCR reaction ingredients

Ingredient	1X volume	Final Concentration
dH ₂ O	41 μ L	–
Roche Expand Long Taq Buffer1	5 μ L	75 nM
dNTPs (10mM)	2.5 μ L	200nM
Forward Primer (10 μ M)	0.25 μ L	250 nM
Reverse Primer (10 μ M)	0.25 μ L	250 nM
cDNA template (neat cDNA)	1 μ L	
Total reaction MasterMix	50 μ L	

Table 2.9 : Long RT-PCR cycle conditions

Cycle Number	Temperature	Time	Phase
1	92°C	2 min	Initial Denaturation
35	92°C	15 sec	Denaturation
	specific for each primer	30 sec	Annealing
	68°C	4 min	Elongation
1	68°C	7 min	Final Extension
1	4°C	∞	Final Hold

The PCR products were loaded on to an 1% agarose gel prepared with 1 g of agarose, 100 ml of 1xTAE buffer, and 3.6 µl ethidium bromide (10mg/ml). 10 kb GeneRuler DNA ladder (Fermentas) was used to determine fragment lengths.

2.15 Purification of Long RT-PCR Products from Agarose Gel

Long RT-PCR products including PHLPP transcript variants in different sizes were excised from agarose gel and purified by the use of High Pure PCR Product Purification Kit (Roche) according to the suppliers manual.

DNA band of interest was cut from agarose gel using an ethanol-cleaned razor blade on a UV light box. The excised agarose gel slice was placed in a sterile 1.5 ml microcentrifuge tube. The gel mass was determined by first pre-weighting the tube, and then reweighting the tube with the excised gel slice. 300 µl of Binding Buffer was added for every 100 mg agarose gel slice to the microcentrifuge tube. Then, agarose gel slice was dissolved in order to release the DNA. The microcentrifuge tube was vortexed for 15-30 sec to resuspend the gel slice in the Binding Buffer. The suspension was incubated for 10 min at 56°C. The tube was vortexed briefly every 2-3 min during incubation. After the agarose gel slice was completely dissolved, 150 µl of isopropanol was added for every 100 mg agarose gel slice to the tube. The tube was vortexed. One High Pure Filter Tube was inserted into the Collection Tube. The entire contents of the microcentrifuge tube was transferred into the upper reservoir of the Filter Tube. The tube was centrifuged at 14,000 x rpm for 1 min. The flowthrough solution was removed and the Filter Tube was put into the same Collection Tube. 500 µl of Wash Buffer was added to the upper reservoir. The

tube was centrifuged at 14,000 x rpm for 1 min. The flowthrough solution was removed and the Filter Tube was reconnected with the same Collection Tube. 200 µl of Wash Buffer was added onto the tube. Next, the tube was centrifuged at 14,000 x rpm for 1 min. The flowthrough solution was discarded. The Filter Tube was combined with a clean 1.5 ml microcentrifuge tube. 50 µl of Elution Buffer was added to the upper reservoir of the Filter Tube. The purified DNA was eluted into the microcentrifuge tube by a centrifugation step at 14,000 x rpm for 1 min. The purified products were then loaded onto the 2% Agarose gel for the quality check. Products in expected size and in good quality were commercially sequenced by Macrogen (South Korea).

2.16 Expression Studies at Protein Level

Isolated total proteins from pediatric AML patients, tumour samples, and healthy controls were analysed for PHLPP (sc-46452), total Akt 1/2/3 (sc-8312), pAkt (Ser473) (sc-7985-R), pAkt (Thr308) (sc-16646-R), and PTEN (sc-6817-R) presence. First and compatible secondary antibodies conjugated with Alkaline Phosphatase were purchased from supplier SantaCruz.

2.16.1 SDS-PAGE Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) allows separation of proteins according to their sizes.

%15 Separating gel was prepared with 2.5 ml of %30 acrylamide, 1.30 ml of 1.5 M Tris pH 8.8, 50 µl of %10 SDS, 50 µl of %10 APS, 2 µl of TEMED, and 1.10 µl of dH₂O. Separating gel was transferred into SDS gel cassette via pipetting. A thin layer of isopropanol was added onto the gel in the cassette. The gel was let at room temperature for 30 min for polymerization. Next, the isopropanol in the cassette was discarded with a tissue paper. %5 stacking gel was prepared via mixing; 330 µl of %30 acrylamide, 250 µl of 0.5 M Tris pH 6.8, 20 µl of %10 SDS, 20 µl of %10 APS, 2 µl of TEMED, and 1.4 ml of dH₂O. The stacking gel was added onto the polymerized separating gel in the cassette and left 30 min at room temperature for polymerization. 5 µl of 0.4 ng/µl protein samples was mixed with 2x loading buffer and denatured at 95°C for 5 min. 5 µl of each sample was loaded onto SDS-PAGE gel. As protein ladder, SeeBlue Plus2 Prestained Protein Ladder (Invitrogen) was used. 7µl of protein ladder was loaded onto the SDS-PAGE gel. Electrophoresis was accomplished in Tri-glycine running buffer at 100 V, 250mA for 90 min. The content of the running gel is given in the below table 2.10.

Table 2.10 : Tris-glycine running buffer

Ingredient	Concentration	Amount used in solution
Tris-HCl	0,125M	3g
Glycine	0,192M	14,4g
%10 SDS	%0,1	10ml
dH ₂ O		1 l

2.16.2 Western Blot Analysis

The protein samples separated on SDS-PAGE were transferred to a PVDF membrane and analysed for presence of target proteins. Transfer was performed in a wet blotting system at 20V, 100mA for 2.5 hours (Transfer buffer: 3 g of Tris 25mM, 14.4 g Glycine 192mM, 200 ml of %20 Methanol, 0.05 g of %0.05 SDS, and 1l of dH₂O). PVDF membrane was washed twice with TBS buffer at room temperature each for 10 min. [TBS buffer: 2.4 g of NaCl, 0.06 g of KCl, 0.9 g of Tris, and 300 ml of dH₂O (pH of the buffer was set to pH 7.4)]. Next, the membrane was incubated at 4°C overnight with blocking buffer which included 30 ml TBS and 900 mg of non-fat dry milk. Next day, the membrane was washed with TTBS and TBS washing buffers at room temperature each for 10 min (TTBS buffer: 90µl of % 0.05 Tween20 and 180 ml TBS). Then, the membrane was first incubated with first antibody blocking solution at 4°C o/n. The first antibody blocking solution included 5µl of First antibody (200µg/ml), 300 mg of non-fat dry milk, and 10 ml of TBS buffer. After treating the membrane with first antibody, the membrane was washed again twice with TTBS buffer and once with TBS buffer at room temperature each for 10 min. Secondary antibody blocking buffer was composed of 2 µl of AP conjugated secondary antibody (1:5000), 300 mg of non-fat dry milk (%3), and 10 ml of TTBS. The membrane was incubated in secondary antibody blocking buffer for 1.5 hour at room temperature. Next, the membrane was washed twice with TTBS and once with TBS buffer at room temperature each treatment was for 10 min. Lastly, the membrane was incubated in NCBT/BCIP solution until the protein bands were detectable (200µl NCBT/BCIP stock solution + 10ml NBT/BCIP buffer; NCBT/BCIP substrate buffer: 3 g of 0.1 M Tris-HCl pH 9.5, 1.461 g of 0.1 M NaCl₂, 2.541 g of 0.05 M MgCl₂.6H₂O, and 250 ml of dH₂O).

3. RESULTS

3.1 Standard PCR Amplification and Visualisation of PHLPP Coding Regions for Four Major Functional Domains

Polymerase chain reaction was accomplished to specifically amplify the target genomic DNA sequences on PHLPP gene which are coding for PH, LRR, PP2C-like catalytic core, and PDZ binding domains. Coding region for PH-domain encompasses exon 2, 3, and 4; for LRR-domain exon 5, 6, and 7; for PP2C-like catalytic core exon 14, 15, 16, and 17; for PDZ binding region exon 17. All PCR products were loaded on agarose gel. PCR amplification results of the coding regions are shown in Figure 3.1.A-G.

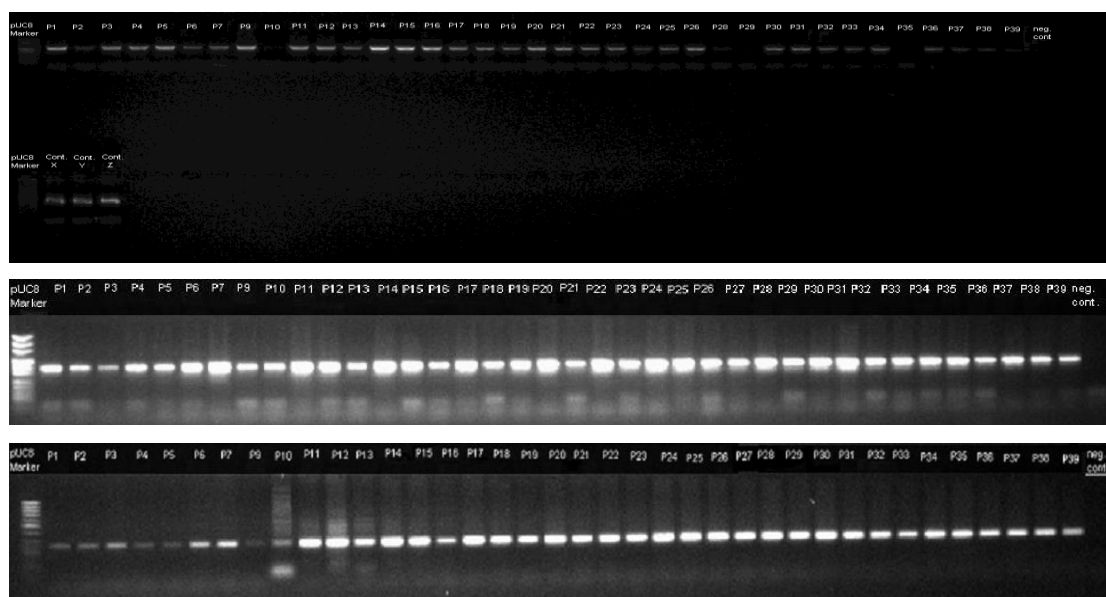


Figure 3.1 : Agarose Gel Electrophoresis of PHLPP coding regions for four major functional domains: **(A)** Exon 17 Standard PCR amplification results of exon 17 coding for PP2C-like catalytic core and PDZ binding motif. **(B)** Exon 16 Standard PCR amplification results of exon 16 coding for PP2C-like catalytic core. **(C)** Exon 15 Standard PCR amplification results of exon 15 coding for PP2C-like catalytic core. **(D)** Exon 7 Standard PCR amplification results of exon 7 coding for LRR domain. **(E)** Exon 6 Standard PCR amplification results of exon 6 coding for LRR domain. **(F)** Exon 5 Standard PCR amplification results of exon 5 coding for LRR domain. **(G)** Exon 3 Standard PCR amplification results of exon 3 coding for PH domain.

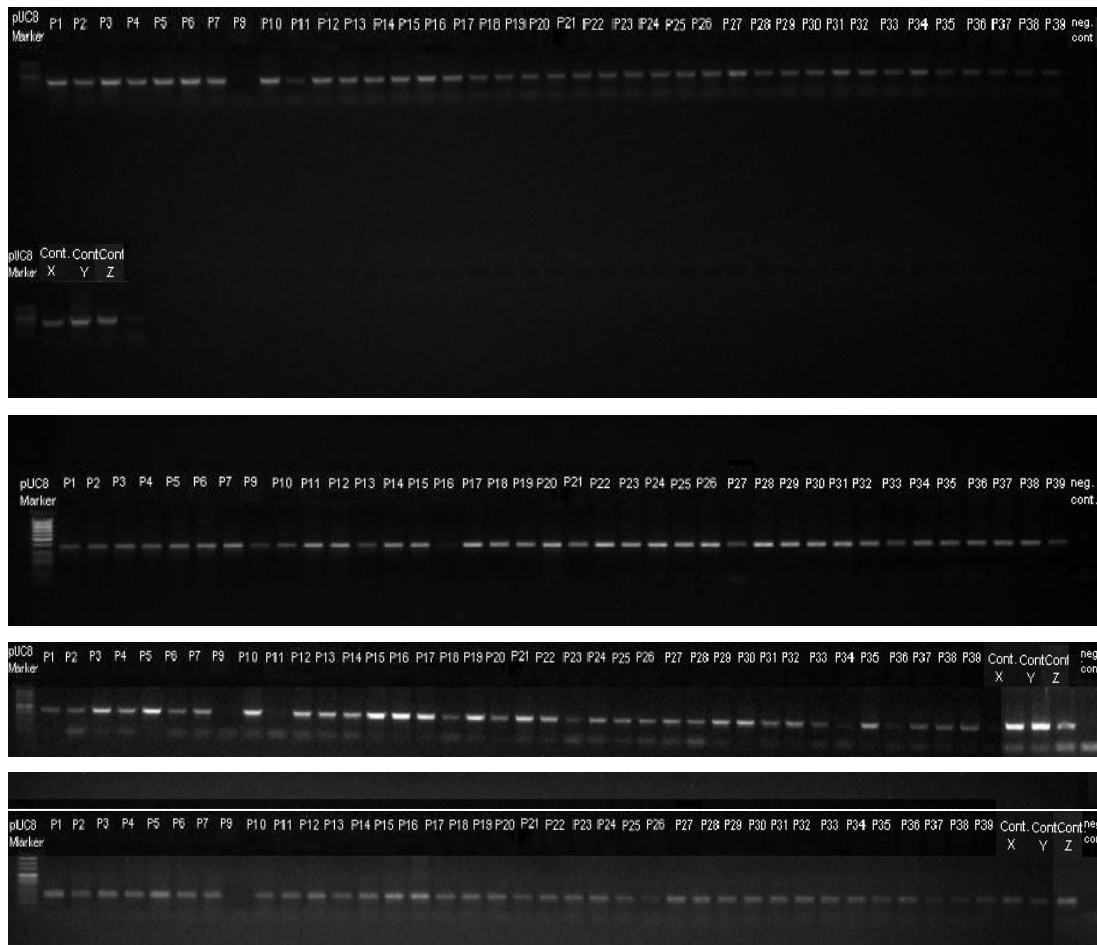


Figure 3.1 : (continued) Agarose Gel Electrophoresis of PHLPP coding regions for four major functional domains: **(A)** Exon 17 Standard PCR amplification results of exon 17 coding for PP2C-like catalytic core and PDZ binding motif. **(B)** Exon 16 Standard PCR amplification results of exon 16 coding for PP2C-like catalytic core. **(C)** Exon 15 Standard PCR amplification results of exon 15 coding for PP2C-like catalytic core. **(D)** Exon 7 Standard PCR amplification results of exon 7 coding for LRR domain. **(E)** Exon 6 Standard PCR amplification results of exon 6 coding for LRR domain. **(F)** Exon 5 Standard PCR amplification results of exon 5 coding for LRR domain. **(G)** Exon 3 Standard PCR amplification results of exon 3 coding for PH domain.

3.2 DHPLC Analysis of PHLPP Functional Domains

Targeted PHLPP genomic sequences coding for 4 major functional domains; PH domain, LRR region, PP2C-like catalytic core and PDZ binding motif were analysed for any sequence variations by the use of DHPLC analysis.

The melting peak patterns observed in DHPLC analysis are given in Figures 3.2 - 3.10. Melting peak patterns of pediatric AML patient samples different from those observed in controls were considered to represent possible sequence variations. Following the analysis, samples with double melting peaks were selected for further sequence analysis by direct sequencing.

DHPLC results of exon 2 coding for PH domain are given in the Figure 3.2. Two different melting peak patterns observed in pediatric AML patient samples which were not detected in controls.

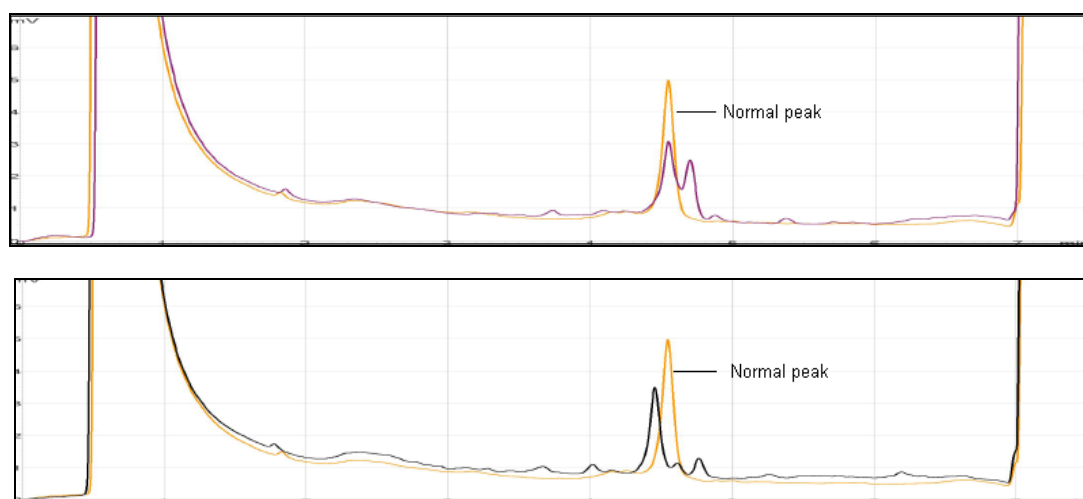


Figure 3.2 : DHPLC results of exon 2 coding for PH domain.

In Figure 3.3, DHPLC result of exon 3 coding for PH domain is shown. The melting peak pattern of pediatric AML patient samples was different from that observed in controls.

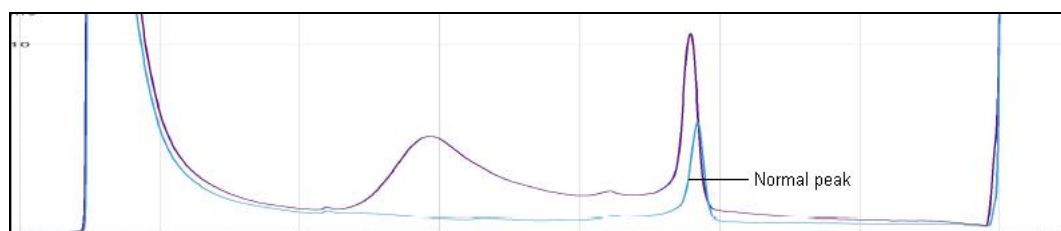


Figure 3.3 : DHPLC results of exon 3 coding for PH domain.

In DHPLC analysis of exon 5 coding for LRR domain, the melting peak observed in pediatric AML samples was varying from the melting peak pattern of controls.

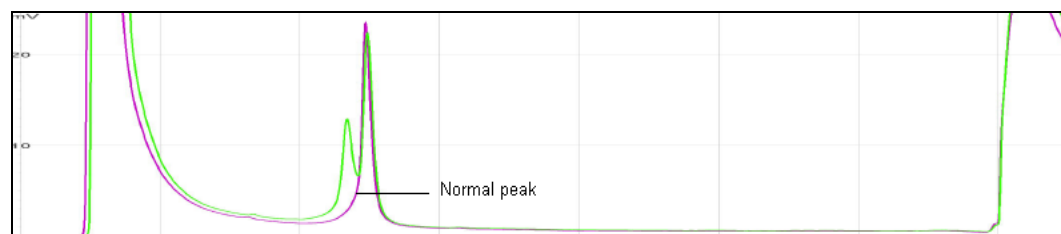


Figure 3.4 : DHPLC results of exon 5 coding for LRR domain.

DHPLC results of exon 6 coding for LRR domain are given in the Figure 3.5. Two different melting peak patterns observed in pediatric AML patient samples were not detected in controls.

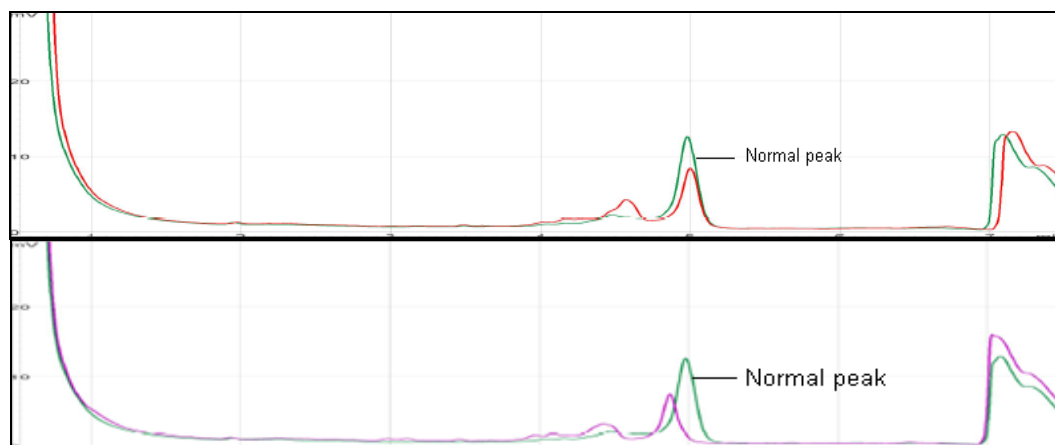


Figure 3.5 : DHPLC results of exon 6 coding for LRR domain.

In DHPLC analysis of exon 14 coding for PP2C-like catalytic core domain, in pediatric AML samples, two melting peak patterns different from each other as well as from that observed in control samples were identified. This result is given in Figure 3.6.

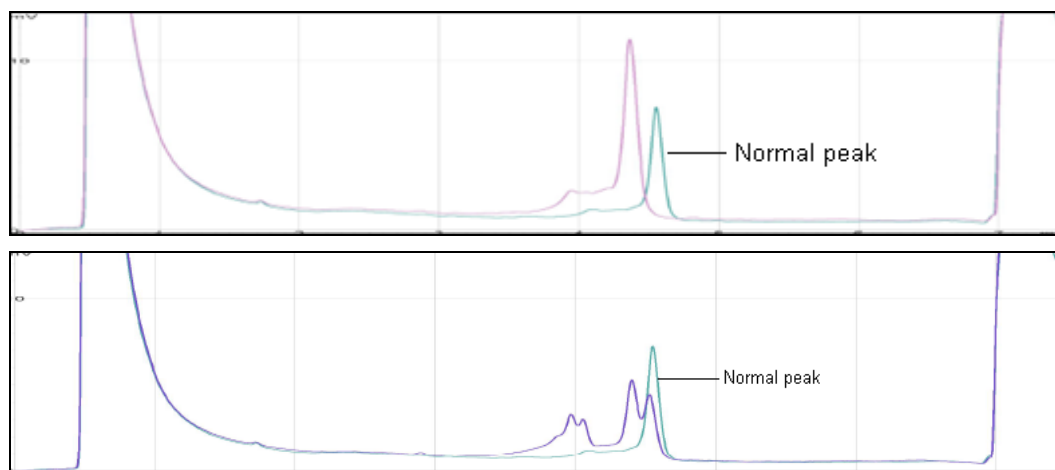


Figure 3.6 : DHPLC results of exon 14 coding for PP2C-like catalytic core domain.

The results of DHPLC analysis of exon 15 coding for PP2C-like catalytic core domain given in Figure 3.7 indicated that the melting peak pattern observed in pediatric AML samples was different from that observed in controls.

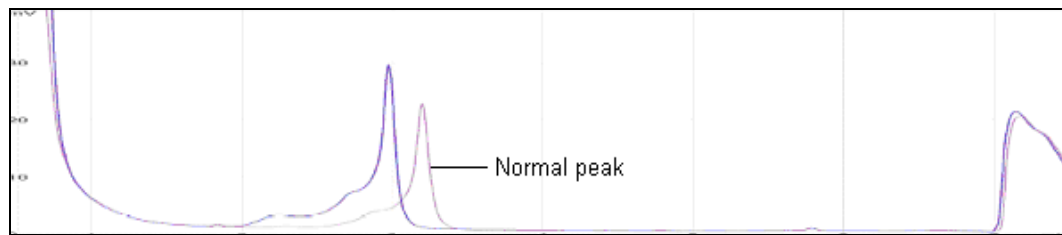


Figure 3.7 : DHPLC results of exon 15 coding for PP2C-like catalytic core domain.

In pediatric AML patient samples, DHPLC analysis of exon 16 coding for PP2C-like catalytic core domain resulted in detection of three different melting peak patterns which were varying from the melting peak pattern observed in controls (Figure 3.8).

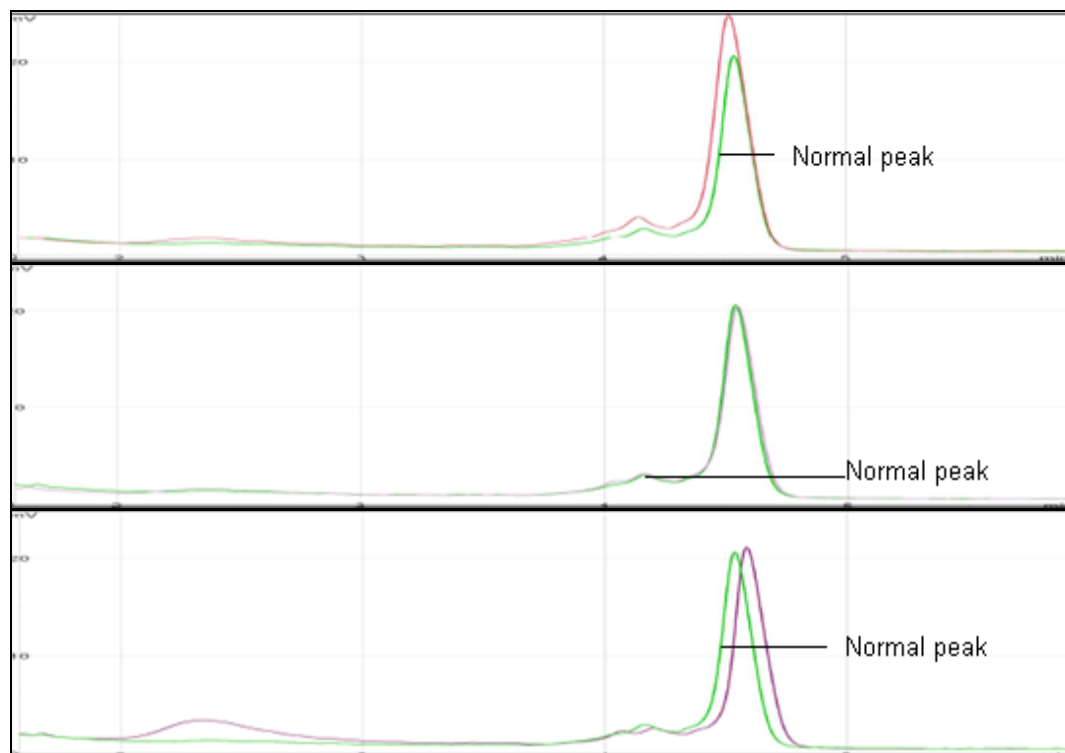


Figure 3.8 : DHPLC results of exon 16 coding PP2C-like catalytic core domain.

DHPLC results of exon 17 regions coding for PP2C-like catalytic core domain are given in the Figure 3.9. The melting peak patterns detected in pediatric AML patient samples were different from each other as well as from that observed in controls.

The results of DHPLC analysis of exon 17 coding for PDZ binding motif are given in Figure 3.10. Two different melting peak patterns observed in pediatric AML samples were varying from that observed in controls.

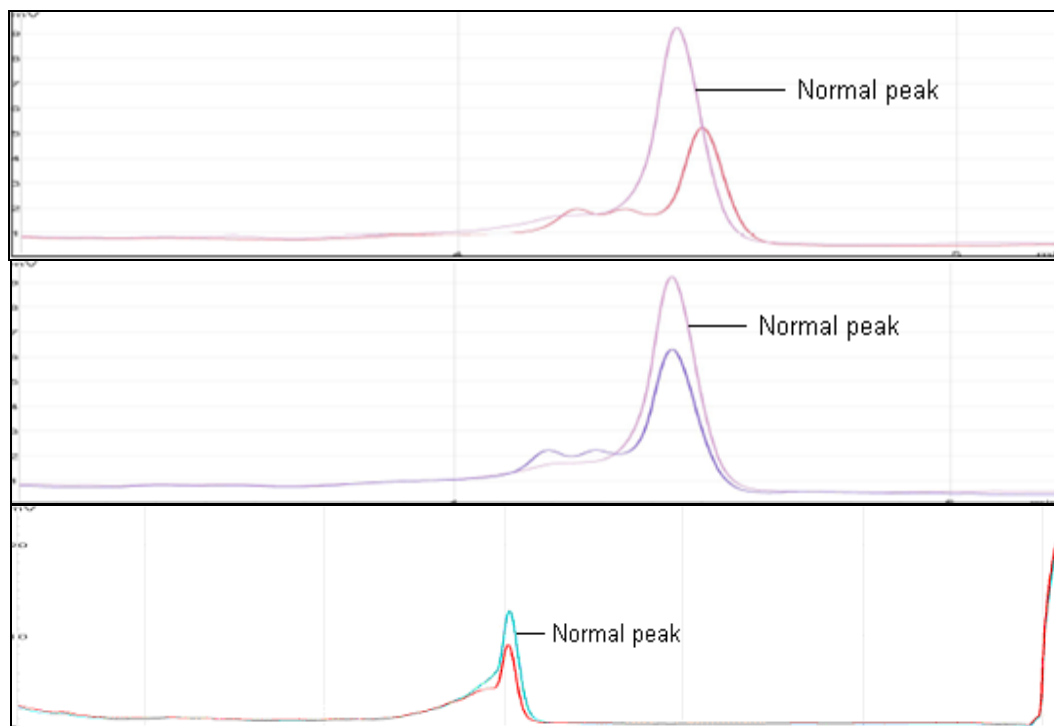


Figure 3.9 : DHPLC results of exon 17 coding for PP2C-like catalytic core domain.

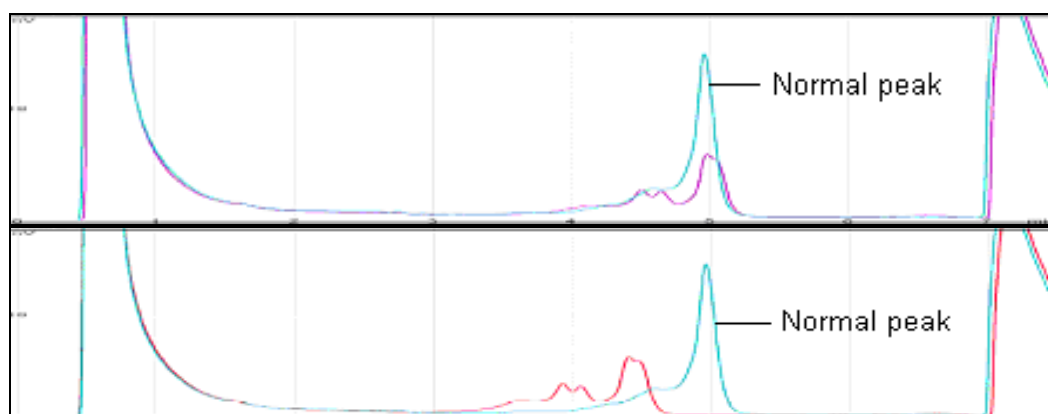


Figure 3.10 : DHPLC results of exon 17 coding for PDZ binding motif.

3.3 Direct Sequencing Results of Pediatric AML Samples Selected Following DHPLC Analysis

Pediatric AML samples selected according to DHPLC mutation analysis results were commercially sequenced. Result of sequencing studies were analysed via ChromasPro tool online available at <http://www.technelysium.com.au/ChromasPro.html> (Figure 3.11). Sequence variations found in pediatric AML samples are listed in table 3.1 given below.

59 insA (aaG-gAgt)

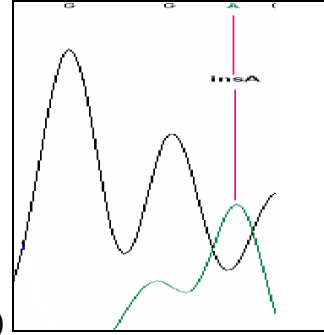


Figure 3.11 : Sequence analysis via ChromasPro tool.

Table 3.1 : Sequence Variations in Coding Regions of *PHLPP* Gene

	Exon	Base pair change	Amino acid change	Polarity change	Charge change	Frequency (%)	Reference
PH	Exon 2	59insA	G→E	non-polar→polar	neutral→acidic	%5.2	This study
	Exon 2	60T>G	—	—	—	%2.8	This study
	Exon 2	77C>A	K→Q	—	neutral→basic	%2.8	This study
	Exon 2	109A>T	K→STOP	—	—	%2.8	This study
	Exon 3	289C>A	P→T	non-polar→polar	—	%7.8	This study
	Exon 3	352C>A	Q→K	—	basic→neutral	%7.8	This study
LRR	Exon 3	343insA	W→STOP	—	—	%2.8	This study
	Exon 5	599insA	S→R	—	neutral→basic	%47	This study
	Exon 14	1980T>C	—	—	—	%5.2	This study
	Exon 14	1992T>C	—	—	—	%5.2	This study
PP2C	Exon 17	3280C>A	L→M	—	—	%13.1	This study
	Exon 17	3302insA	—	—	—	%13.1	This study
	Exon 17	3303T>C	—	—	—	%13.1	This study
	Exon 17	3407insA	—	—	—	%5.2	This study
	Exon 17	3611insC	—	—	—	%7.8	This study

3.4 Gene Expression Studies In Pediatric AML Samples

35 pediatric AML patients and 5 healthy controls bone marrow samples were used for gene expression studies. CD33+ cells of the controls were isolated by MACS Magnetic Bead Columns as described in section 2.4 and they were pooled together to be used as positive controls. The gene expression studies were performed by qRT-PCR as described in section 2.13.

3.4.1 Gene Expression Analysis of PHLPP Domains in pediatric AML Patients

The expression levels of the 4 major functional domains (PH domain, LRR, PP2C-like catalytic core, and PDZ binding motif) were studied in order to characterize PHLPP gene. Expression in PH domain, PP2C-like catalytic core domain and PDZ binding domain in AML patients and in control group were detected. Expression of PH domain and PP2C-like catalytic core were significantly lower in pediatric AML patients compared to controls. Remarkably, expression of LRR domain in pediatric AML patients was not detected (Figure 3.12).

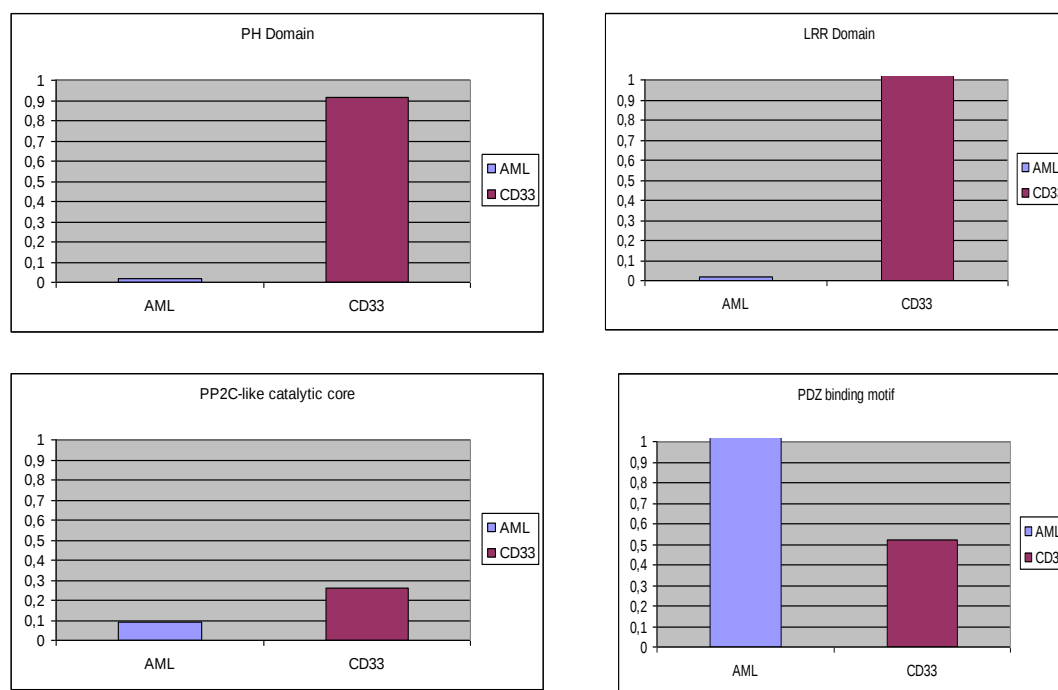


Figure 3.12 : Gene expression analysis of PHLPP domains: **(A)** PH domain Expression levels. **(B)** LRR domain expression levels. **(C)** PP2C-like catalytic core expression levels. **(D)** PDZ binding motif expression levels.

3.4.2 Akt-1, Caspase 3 and PTEN Expression in pediatric AML Patients

Akt was found to be up-regulated in pediatric AML patients (OR=4.4 95%CI=0.04-2.9, $p=0.06$). Gene expression study results showed that caspase-3 expression was decreased 3 times ($p>0.05$) in pediatric AML patients compared to controls, which confirmed overactivated Akt in pediatric AML patients as caspase-3 is a downstream target of Akt and as a pro-apoptotic factor it is expected to be expressed lower in the case of upregulated Akt activation. PTEN and PHLPP expressions were also found to be decreased in pediatric AML patients compare to CD33+ healthy bone marrow cells (3 times ($p>0.05$), 10 times ($p>0.05$) and respectively) (Figure 3.13).

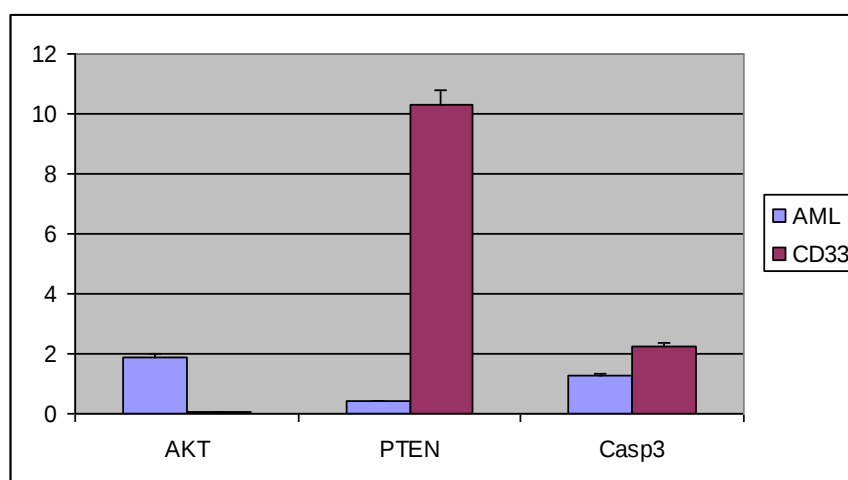


Figure 3.13 : Gene expression analysis Akt, PTEN, Caspase-3.

3.5 Amplification of PHLPP Transcripts by RT-PCR in pediatric AML patients

As described in section 3.4.2 the gene expression studies revealed diminished level of expressions of PHLPP domains. And, the significant loss of LRR domain expression in pediatric AML patients was further studied towards defining the possible transcript variants. Pediatric AML patients with very low levels of LRR domain expression (LRR +) and patients lacking LRR domain expression (LRR -) were chosen to be used in Reverse Transcriptase RT-PCR analysis for possible transcript amplifications as described in section 2.14. This analysis would reveal all the possible PHLPP transcripts in pediatric AML patients. In 15 pediatric AML samples having low levels of LRR domain expression, a single specific band representing total transcript was observed at 3600 bps as expected. However, in 20 pediatric AML samples lacking LRR domain expression, three different transcript variants varying between 1800-5000 bps were detected (Figure3.14).

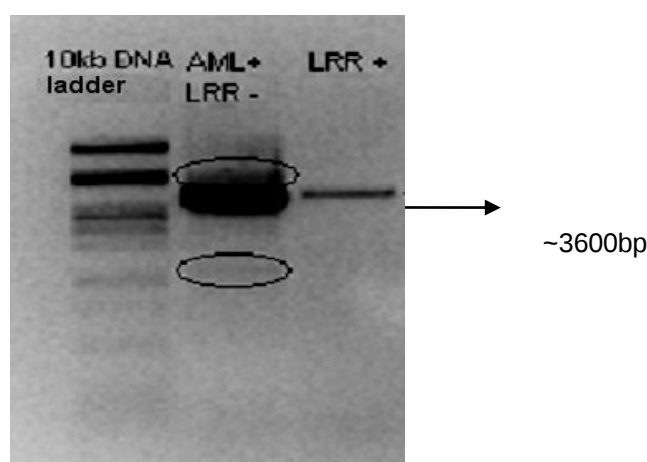


Figure 3.14 : PHLPP RT-PCR of total transcript.

Each band was excised from the agarose gel. They were purified by High Pure PCR Product Purification Kit (Roche) according to the suppliers manual as described in section 2.15. Those purified bands were then sequenced. The middle band showed a single nucleotide change in exon 5 at position 55 in AML LRR- patients (Figure 3.15). The upper and the lower bands could not be sequenced due to the low product amount.

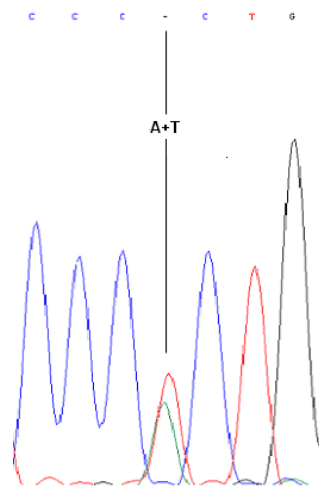


Figure 3.15 : Direct sequencing results of pediatric AML samples showing three different transcript variants in RT-PCR studies for total PHLPP mRNA amplification.

3.6 RT-PCR of LRR Domain Upstream Region

Single exons (exon 2, 3, 4, and 5) located upstream of LRR domain coding sequence were amplified via RT-PCR in order to understand the underlying mechanisms of LRR domain expression loss in pediatric AML patients. The results would possibly reveal the preliminary data about the possible location of transcriptional regulation site in this region. Results were given in the Figure 3.16.

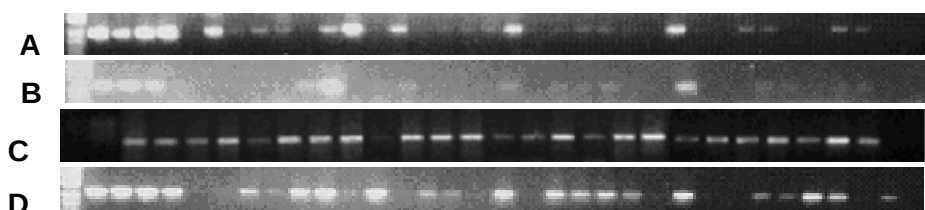


Figure 3.16 : RT-PCR results of LRR upstream regions: **(A)** RT-PCR results of Exon2. **(B)** RT-PCR results of Exon3. **(C)** RT-PCR results of Exon4. **(D)** RT-PCR results of Exon5.

Expression levels of exon 2 and exon 3 were lower than those of exon 4 and exon 5.

3.7 Gene Expression Studies of Four Major Functional PHLPP Domains in Different Tumour Tissues

The expression of 4 major functional domains (PH domain, LRR, PP2C-like catalytic core, and PDZ binding motif) were also studied in 10 colon, 10 stomach, 10 pancreas, and 10 breast tumour tissues. Expression levels were determined as described in 2.13.3.

Expressions of LRR domain (exon 5, exon 6 and exon 7) and PP2C-like catalytic core (exon 15 and exon 16) were found to be diminished in colon, stomach, pancreas, and breast tumour tissues (Figure 3.17 and Figure 3.18).

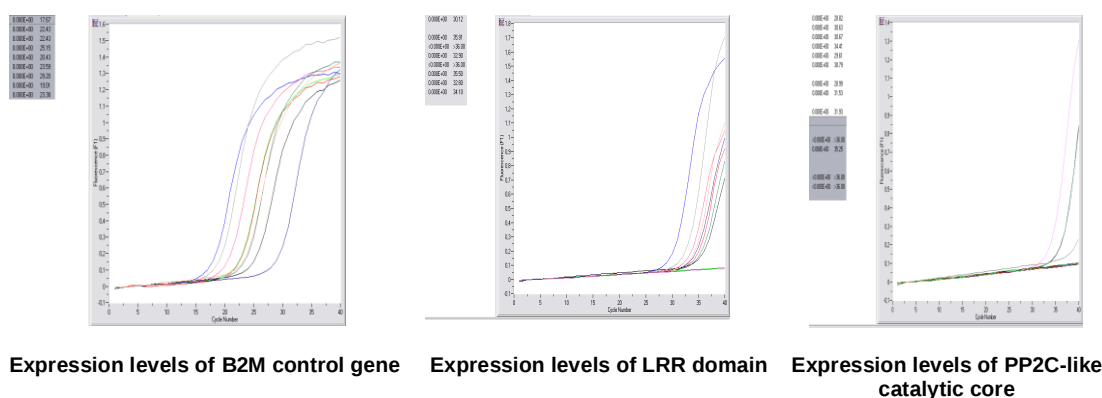


Figure 3.17 : Comparison of expression levels of beta-2-microglobulin (B2M) control gene, LRR domain, and PP2C-like catalytic core in different tumour tissues.

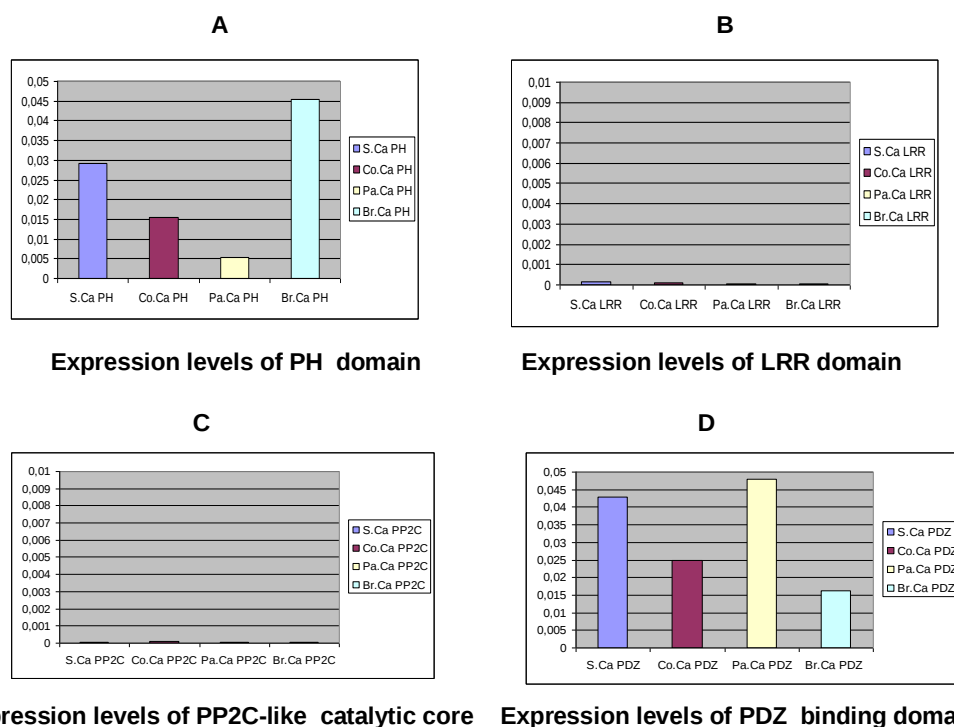


Figure 3.18 : Expression levels of four major PHLPP functional domains in different tumour tissues.

PH domain expression was found to be highest in breast cancer tissues followed by stomach and colon tumour tissues respectively. High level of PDZ binding domain expression was observed in pancreas tumour tissues followed by stomach, and colon tumour tissues respectively. In pancreas and breast tumour tissues expression levels of PH and PDZ domains were negatively correlated.

3.8 RT-PCR of PHLPP Transcripts in Tumour Tissues

In order to determine the transcript variants, the region covering exon 2 to exon 17 was amplified via Reverse Transcriptase (RT)-PCR in tumour tissues as described in section 2.14. Transcript variants different from those in pediatric AML were detected in tumour samples (Figure 3.19).

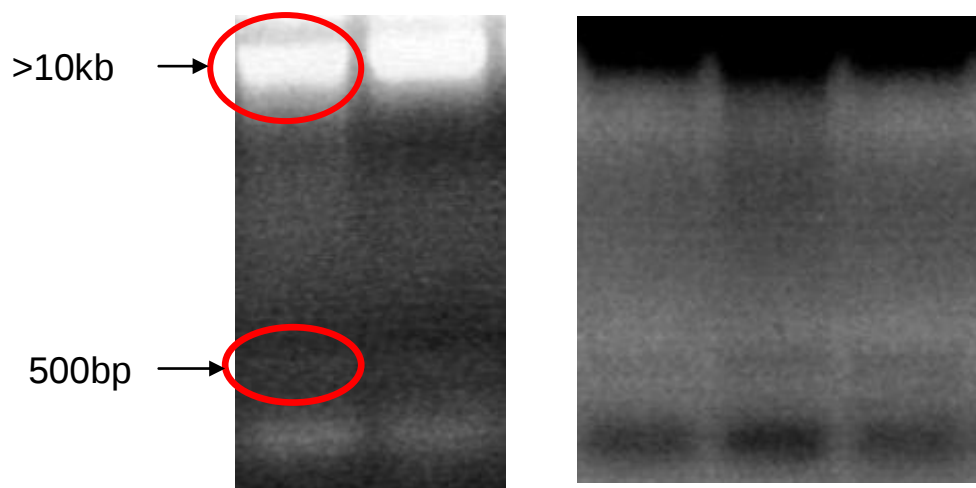


Figure 3.19 : PHLPP transcript variants in tumour tissues.

3.9 Western Blot Studies

The expression of total Akt 1/2/3, phospho-Akt (Ser 473) specific, phospho-Akt (Thr 308) specific, and two negative regulators of Akt signalling pathway; tumour suppressors PTEN and PHLPP, were studied at protein level in pediatric AML patients samples by the use of Western Blot Analysis as described in section 2.15.2.

Pediatric AML samples were analyzed by Western blotting employing antibodies for Akt, pAkt Ser473, pAkt Thr308. Akt and phosphorylated Akt at two specific sites were detected in Western blot analysis as shown in Figure 3.20.A and 3.20.C-D. A well known tumour suppressor PTEN was also detected at protein level in pediatric AML samples (Figure 3.20.B). However, another tumour suppressor, PHLPP protein expression was found to be lost in pediatric AML patients as shown in Figure 3.20.E.

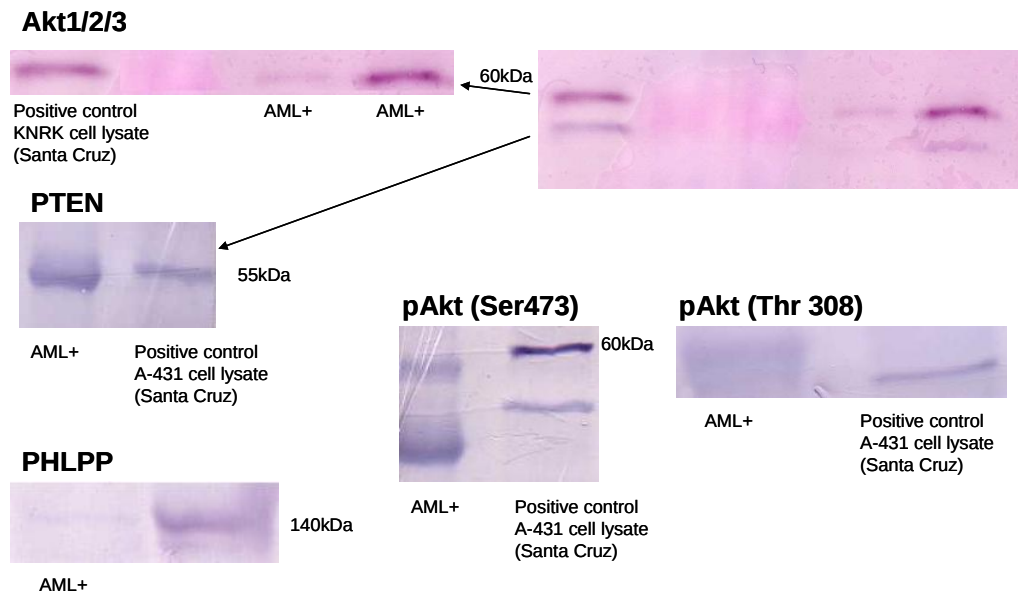


Figure 3.20 : Western Blot Analysis of pediatric AML patients. **(A)** Total Akt1/2/3 was detected in pediatric AML patient samples as well as commercial positive control. **(B)** PTEN expression was observed in pediatric AML patients and in commercial positive control. **(C)** Phosphorylated Akt specific at Ser473 was observed both in pediatric AML samples and commercial positive control. **(D)** Phosphorylated Akt specific at Thr308 was observed both in pediatric AML samples and commercial positive control. **(E)** PHLPP expression was not detected in pediatric AML samples but in commercial positive control.

4. DISCUSSION AND CONCLUSION

PI3K/Akt signalling pathway has been reported to be frequently activated in AML. Studies showed that upregulated PI3K/Akt signalling strongly contributes to proliferation, survival and drug resistance of AML blasts. Moreover, both the disease-free survival and overall survival has been reported to be significantly shorter in AML cases with upregulated PI3K/Akt pathway [32-35].

Since the molecular analysis of PHLPP gene has not been elucidated, in this study it is aimed to identify sequential variations in the PHLPP coding regions for four major functional domains together with their expression levels.

To accomplish the mutational analysis, Denaturing High Performance Liquid Chromatography (DHPLC) method was applied since it is suggested to be one of the most sensitive and accurate methods used in identification of unknown mutations. It allows to detect mutations that can not be identified via gel or capillary electrophoresis based systems and also it shows somatic mutation sites which can not be detected in sequencing systems. DHPLC technique is frequently applied to diagnose cancer mutations due to the large length of cancer related genes and low frequency of their mutations. Also DHPLC system is accepted as a pre-sequencing technique. Sequence variations found in PHLPP coding regions for four major functional domains included single base changes which might result in amino acid substitution and related charge change at protein level. Those changes might also affect the protein structure and function. Due to the sequence variations, the function of PHLPP, a novel tumour suppressor on Akt signalling pathway, might be impaired. This might further lead to upregulation of Akt pathway in pediatric AML. Nearly half of pediatric AML patients were carrying 599insA (S200R) in coding region for LRR domain. It is known that LRR domain is involved at protein-protein interaction, so the changes at amino acid charges might lead structural and functional changes at PHLPP protein phosphatase. There are also sequence variants found in pediatric AML patients but not in healthy controls in coding regions for PH domain exon2; 59insA (5.2%), 60T>G (2.8%), 77C>A (2.8%), 109A>T (2.8%), exon3; 289C>A

(7.8%), 352C>A (7.8%), 343insA (2.8%), and PP2C-like catalytic core exon14; 1980T>C (5.2%), 1992T>C (5.2%), exon17; 3280C>A (13.1%), 3302insA (13.1%), 3303T>C (13.1), 3407insA (5.2%) and 3611insC (7.8%) , which might also result in charge and polarity change at protein level. It is known that PH domain binds to phosphorylated Ser/Thr residues. A structural change in this domain due to sequential variations might disrupt the interaction of PHLPP directly with phosphorylated Akt at Ser473 residue. Variations at PP2C-like catalytic core of PHLPP and regarding charge and polarity changes at protein level might interfere with PHLPP function as a protein phosphatase.

The expression studies were performed in 35 pediatric AML patients and in healthy controls; peripheral blood samples and CD33+ bone marrow cells by qRT-PCR. The results revealed that Akt was up-regulated in AML patients that were examined (OR=4.4 95%CI=0.04-2.9, p=0.06). PI3K/Akt signalling pathway has been reported to be frequently activated in AML [32-35]. However, there is no previous data reporting Akt expression levels in pediatric AML patients. Moreover, constitutive activation of PI3K/Akt pathway has been implicated in the both the pathogenesis and the progression of a wide variety of neoplasias. Overexpression of Akt/PKB was demonstrated to in colorectal carcinogenesis [25], in gastric carcinomas [26], pancreatic cancers [27], and also in breast tumours [28]. PTEN, PHLPP and caspase-3 were found to be decreased in pediatric AML patients compared to CD33+ healthy bone marrow cells (3 times (p>0.05), 10 times (p>0.05) and 3 times (p>0.05) respectively). In previous studies, PTEN mRNA expression was found to be lower in AML patients compared to normal controls (60.0 % vs 90.%, p<0.05) [102]. In this study the difference in PTEN expression levels between pediatric AML patients and controls was found higher compared to this reference, where PTEN expression was studied in adult AML patient group. The expression of caspase 3, an apoptosis related gene; was found to be lower in pediatric AML patients than in control group. The decrease in expression levels were as expected because when activated Akt inhibits downstream pro-apoptotic targets. Both cleaved and uncleaved caspase 3 levels reported to be higher in AML blasts and adult AML patients. Moreover, high levels of uncleaved caspase 3 was associated with decreased survival. Conversely, high levels of cleaved caspase 3 denoted improved survival and correlated with the inactivation of the DNA-repair enzyme poly (ADP-ribose) polymerase [60].

Gene expression studies revealed that the expression of PH domain and PP2C-like catalytic core were diminished whereas LRR domain expression was lost in pediatric AML patients. LRR domain is known to be involved at protein-protein interaction. It has also been reported that in the LRR cassette domain of LRRC4, a putative tumour suppressor gene, the third LRR motif of the core LRR plays a crucial role as a “proliferation-inhibition switch”. Moreover, it is proposed that LRRC4 requires a functional LRR cassette domain to inhibit proliferation of glioma cells in vitro by modulating the extracellular signal-regulated kinase/protein kinase B/nuclear factor- κ B pathway [98]. LRR domain might also be crucial for appropriate function of PHLPP as a Akt pathway off-switch. The insertion found in exon 5 in the LRR coding region in nearly half of the patients and the gene expression studies indicating LRR domain loss in patients might lead one to speculate that impaired LRR domain function might affect PHLPP function as a protein phosphatase. Recently, Ouillet *et al.* also studied PHLPP expression in Chronic Lymphocytic Leukemia (CLL) patients with deletion 13q14 and found the loss of PHLPP expression in those patients [101]. Interestingly, expression level of PDZ binding motif was found to be higher in patient group than in controls. Different level of expressions determined in functional domains of PHLPP might be due to the epigenetic regulations. Further analysis performed by RT-PCR studies for amplification of PHLPP mRNA from exon 2 to exon 17 in pediatric AML patients lacking LRR domain showed three different PHLPP transcript variants at different sizes. However, pediatric AML samples having very low level of LRR domain expression revealed only one specific mRNA transcript at expected size. Direct sequencing results showed a single nucleotide change in exon 5 at position 55 in pediatric AML samples lacking LRR expression. Upstream LRR domain (exon 2, exon 3, exon 4 and exon 5) was investigated in order to identify the susceptible regions carrying possible regulatory sites affecting LRR domain expression in pediatric AML patients. It was detected that expression levels of exon 2 and exon 3 were lower than those of exon 4 and exon 5. This result might indicate that upstream exon 4 is the possible location of transcriptional regulatory site.

Expression studies of four major functional PHLPP domains in tumour tissues (colon, stomach, pancreas and breast) showed diminished level of LRR domain expression and loss of PP2C like catalytic core expression. Liu *et al.* has recently

found loss of PHLPP expression in colon cancer [100]. This result is in concordance with our data. The loss of PHLPP confirms it being a tumour suppressor gene in tumourigenesis and leukemogenesis. Furthermore, PHLPP mRNA transcript variants different from those in pediatric AML patients were detected in tumour samples. The role of epigenetic regulation in cancer progression might explain the PHLPP transcript variants in different sizes observed in pediatric AML and tumours.

Expression studies at protein level by Western Blot Analysis confirmed the results of the gene expression studies. Total Akt 1/2/3 was present in pediatric AML patients. Also pAkt (Ser 473) and pAkt (Thr 308) were detected in pediatric AML patient samples. One of the two tumour suppressors acting negatively on Akt pathway, PTEN, was also found to be expressed at protein level in AML patients, whereas PHLPP expression could not be detected at Western Blot Analysis. Commercial antibody used in PHLPP detection was raised against a peptide mapping at the N-terminus of PHLPP. In this study, sequence variations in coding regions for N-terminal PH domain of PHLPP were found, which might further lead to a structural change at protein level hindering proper detection of PHLPP with the antibody used. Antibodies for western blot analysis of PHLPP might be raised against different PHLPP regions to visualise the protein or possible truncated forms of the protein.

To sum up, this is the first study evaluating sequence variations together with the expression of PHLPP gene in pediatric AML and solid tumours. Considering the results of this study, it can be proposed that PHLPP gene might act as a tumor suppressor in AML leukomogenesis and this can provide an important guidepost for the development of diagnostic tools for acute leukemia. Further functional studies are needed to determine the impacts of sequential variations to PHLPP protein structure and function. Also, to decide if the sequential variations are mutations or polymorphisms populations studies in larger number can be accomplished. The 599insA (S200R) in coding region for LRR domain observed in %47 of pediatric AML patients should be further examined in order to determine the role of LRR domain in PHLPP and also its effect in cancer progression. The role of epigenetic regulation in cancer might explain the presence of PHLPP transcript variants in pediatric AML samples and different tumour tissues. Therefore, the possible underlying mechanisms need further be studied.

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APPENDICES

APPENDIX A : Laboratory Equipment

APPENDIX B : Chemicals, Enzymes and Markers

APPENDIX C : Solutions and Buffers

APPENDIX D : Used Kits

APPENDIX A

LABORATORY EQUIPMENT

Balances

Schimadzu

Precisa

Centrifuges

Eppendorf

Allegra 25R Centrifuge Beckman
Coulter

Sigma

Minicentrifuge

Beckman coulter

Electrophoresis Equipments

Stratagene

Gel Documentation System

BIO-RAD

Mini-Vertical Gel System

BioWorld

Orbital Shaker

Forma

Thermomixer

Eppendorf

Microplate Reader

Biorad

Spectrophotometer

NanoDrop™ 1000
Spectrophotometer, Thermo
Scientific

MiniMACS Column

Miltenyi Biotec

MACS Separator

Miltenyi Biotec

pH meter

Mettler Toledo MP220

Real time PCR

Roche Lightcycler 1.2

Thermo cycler

Techne 512 Thermo Cycler,
Thermo Scientific

dHPLC

Wave System

Flow Cytometer

Becton Dickinson BD
FACSCalibur Flow Cytometer

Vortex

Kermanlar

Pipettes

Gilson Pipetman 1000 µl

Thermo Finnpiquette 10 µL, 200 µl

Laminar Air Flow Cabin

Thermo Scientific

Fridge and Freezers (+4°C, -20°C, -80°C)

Heraeus Sepatech, Bosch,
Sanyo, Arcelik

Water Distillation System

Millipore

APPENDIX B

CHEMICALS

Acrylamide	Merck
Agarose	Sigma
Antibodies for Western Blot	Santa Cruz

Studies:

Akt1/2/3 (H-136) sc-8312
p-Akt1/2/3 (Ser 473)-R sc-7985-R
p-Akt1/2/3 (Thr 308)-R sc-16646-R
PTEN (C-20)-R sc:6817-R
SCOP (N-12) sc-46452
Donkey anti-goat IgG AP sc-2022
Donkey anti-rabbit IgG AP sc-2315

Positive controls for Western	Santa Cruz
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Blot Studies:

Y79 nuclear extract sc-2126
KNRK Whole Cell Lysate sc-2214
A-431 + EGF Whole Cell Lysate sc-2202

B-Mercaptoethanol	Sigma
Bromophenol Blue	Sigma
Buffer D	Wave System
dNTP	Fermentas
DTT (Dithiothreitol)	Fermentas
EDTA	Sigma
Ethanol	Merck
	Riedel-de Haën
Ethidium Bromide	Sigma

Ficoll	Sigma Histopaque
Glacial Acetic Acid	Merck
Glycerol	Sigma
Glycine	Merck
Isopropanol	Fluka
KCl	Carlo Erba
MgCl₂	Fermentas
NaCl	Carlo Erba
NaH₂PO₄	Riedel-de Haën
Na₂HPO₄·7H₂O	Riedel-de Haën
NaOH	Riedel-de Haën
PVDF membrane	Roche
Primers	Integrated DNA Technologies, Inc. XX IDT
Random Primer	Fermentas
Sodium dodecyl sulfate (SDS)	Merck
Syringe Wash Solution	Wave System
TEAA Buffer A	Wave System
TEAA Buffer B	Wave System
TEMED	Merck
Tris Base	Merck
	Sigma
2-mercapto-ethanol	Merck
10X PCR Buffer	Fermentas
5X Reverse Transcriptase Buffer	Fermentas
6X Sample Loading Dye	Fermentas

ENZYMES

Expand Long Taq Polymerase

Roche

Reverse Transcriptase

Fermentas

SYBR Premix Ex Taq

Takara

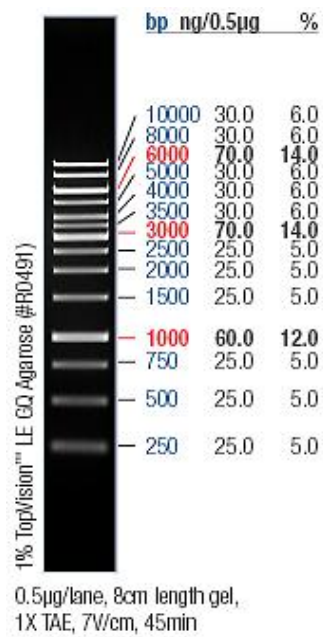
Taq DNA Polymerase

Fermentas

MARKERS

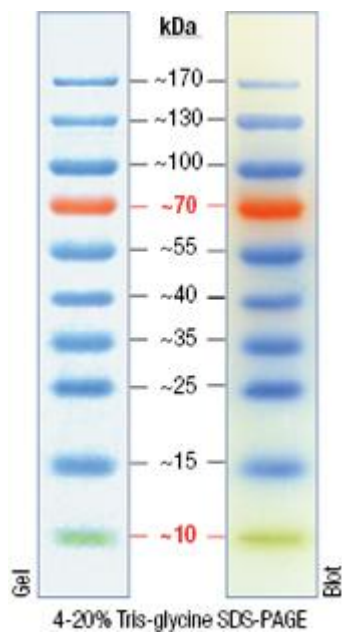
Gene Ruler™ 1 kb DNA Ladder

Fermentas



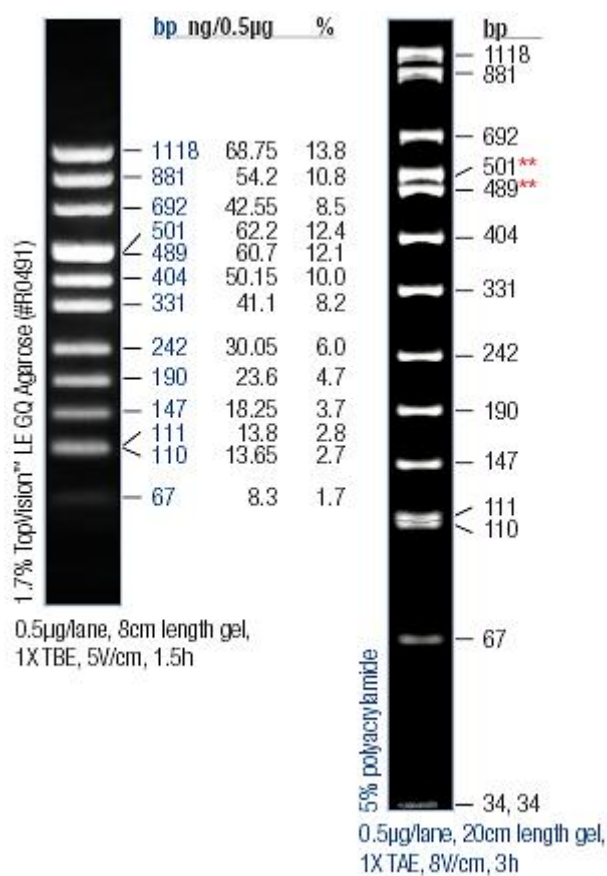
PageRuler™ Prestained Protein Ladder

Fermentas



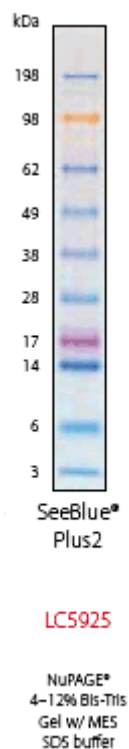
pUC Mix Marker, 8

Fermentas



SeeBlue Plus2 Prestained Protein Ladder

Invitrogen



APPENDIX C

SOLUTIONS and BUFFERS

Ethidium Bromide

Ethidium bromide dilution of a final concentration 10mg/ml was prepared with dH₂O and EtBr stock.

Solution D

100 µl of β-Mercaptoethanol was added to 10 ml of Buffer RLT (erythrocyte lysis buffer).

SDS Sample Buffer (10 ml, 2X)

		Final Concentration
Tris-HCl pH 6.8, 0.5 M	2.5 ml	0.12 M
SDS (10%)	4 ml	%4
Glycerol (100%)	2 ml	%20
Bromophenol blue	5 mg	% 0.05
DTT (dithiothreitol)	231 mg	0.15
dH ₂ O added to 100 ml.		

TAE (Tris-Acetate-EDTA) Buffer (500 ml, 50X)

Tris base	121 g
Glacial Acetic Acid	28.5 ml
EDTA	50 ml (0.5 M, pH 8.0)

dH₂O was added to 500 ml and the pH was adjusted to 8.0

Tris-glycine Running Buffer

		Final Concentration
Tris base	3 g	0.125 M
Glycine	14.4 g	0.192 M
SDS (10%)	10 ml	% 0.1
dH ₂ O added to 1 l.		

Transfer Buffer

		Final Concentration
Tris base	3 g	125 mM
Glycine	14.4 g	192 mM
Methanol	200 ml	%20
SDS	0.05 g	% 0.05
dH ₂ O added to 1 l.		

TBS

NaCl	2.4 g
KCl	0.06 g
Tris	0.9 g

dH₂O was added to 300 ml and the pH was adjusted to 7.4.

TTBS

Tween20	90 µl
TBS	180 ml

BCIP/ NBT Substrate Buffer

Tris-HCl pH 9.5, 0.1 M	3 g
NaCl ₂	1.461 g
MgCl ₂ .6H ₂ O	2.541 g

dH₂O was added to 250 ml.

10X Phosphate Buffered Saline (PBS)

NaCl	90 g
Na ₂ HPO ₄ .7H ₂ O	26.8 g
NaH ₂ PO ₄	3.2 g

dH₂O was added to 1 l and the pH was adjusted to 7.2 after every 1/10 dilution.

APPENDIX D

USED KITS

DNA, RNA, Protein Isolation	Qiagen, AllPrep Mini Kit
RNA Isolation	Qiagen, RNA Mini Blood Kit
cDNA synthesis kit	Fermantas, RevertAid™ First Strand cDNA Synthesis Kit
Real-Time PCR	Takara, SYBR Premix Ex Taq
High Pure PCR Product Purification Kit	Roche

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Tekiner, T. A., Atalar, F., Karabay, A., Anak, S., and Ozbek, U. A novel tumor suppressor in pediatric acute myeloid leukemia progression : PHLPP. 8th National Medical Genetics Congress 2008, Canakkale, Turkey, May 2008. (Oral presentation- Best presentation award)

Tekiner, T. A., Atalar, F., Karabay, A., Anak, S., and Ozbek, U. PHLPP gene is mutated in pediatric acute myeloid leukemia patients and might act as a tumor suppressor gene. European Human Genetics Conference 2008, Barcelona, Spain, June 2008. (Poster presentation & published in European Journal of Human Genetics, Volume 16, Supplement 2, May 2008.)

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