Expression and Function of Adaptor Protein TAPP2 in Normal and Leukemia B Cells

by

Jennifer Costantini

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirement of the degree of

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Abstract

Phosphatidylinositol 3-kinase (PI3K) is involved in regulating many cellular processes that are important for B cell signaling and survival. PI3Ks generate lipid second messengers such as PI(3,4)P2 and PIP3. Adaptor proteins, TAPP1 and TAPP2, and enzymes SHIP1, SHIP2 and PTEN, are involved in regulating PI3K signal transduction. PTEN has been previously established to be involved in lymphomas and other cancers through regulating levels of PIP3 and PI(3,4)P2. However, the specific role of PI(3,4)P2 which is enhanced by SHIP and preferentially binds to TAPP1 and TAPP2, has not been studied in B cell leukemias. My hypothesis is that the PI(3,4)P2 signaling pathway is deregulated in B cell leukemias resulting in functional consequences that contribute to malignant transformation of these cells. To determine the expression of these components of the PI3K pathway in resting lymphocytes, activated lymphocytes, and transformed B cells, I determined their protein expression through the use of Western Blots. Protein samples were isolated from normal B cells and chronic lymphocytic leukemia cells. Function of the PI(3,4)P2 pathway was determined using TAPP2 over-expression or siRNA knockdown of TAPP1 and TAPP2.

In my first results section, I optimized the conditions for expression studies and was able to show results suggesting over-expression of TAPP2 and SHIP proteins in a subset of CLL. I generated evidence that ZAP70 is expressed in some of our CLL patients, and ZAP70 expression seems to correlate positively with expression of the TAPP2. I was able to show that a subset of CLL samples has little or no expression of PTEN, consistent with previous studies. Fluorescence microscopy studies confirmed over-expression of TAPP2

in a subset of CLL cells. These protein expression results suggest that CLL patients have abnormal expression levels of the PI(3,4)P2 signaling components, potentially contributing to formation of malignancies and clinical characteristics of the CLL patients. In my second results section, I optimized the conditions for siRNA knockdown and tested the role of TAPP1 and TAPP2 in adhesion in BJAB cells. I generated evidence indicating that TAPP2 protein levels were substantially reduced through specific siRNA treatment resulting in decreased adhesion. In contrast, TAPP2 over-expression led to increased adhesion and cell spreading, and increased accumulation of F-actin at the cell periphery. We have also generated evidence from over-expression studies that TAPP2 regulates chemotaxis in transformed B cell lines. Together these results indicate that TAPP proteins may be important in linking PI3K signaling to rearrangements of cellular cytoskeleton required for cell adhesion and motility. In preliminary studies on CLL cells, I was unable to see evidence of correlation between CLL adhesion or chemotaxis function and TAPP2 expression; likely due to the small sample size and variable expression of other factors affecting these cellular functions. Together my results provide the first evidence for expression and potential functional roles of the TAPPs in transformed B cells; further studies will be required to determine the functional importance of TAPPs in CLL and other cancers.

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Abbreviations

AID	activation-induced cytidine deaminase
AMV RT	avian myeloblastosis virus reverse transcriptase
Bam32	B lymphocyte adaptor molecule of 32 kDa
BCR	B cell antigen receptor
BLNK	B cell linker
BM	Bone Marrow
bp	base pair
BSA	bovine serum albumin
CD	Conserved domain
cDNA	complementary deoxyribonucleic acid
CLL	chronic lymphocytic leukemia
C-terminal	carboxyl terminal
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
g	gram
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal center
HBSS	Hank's Buffered Salt Solution
HRP	horseradish peroxide
ICAM	intracellular adhesion molecule
Ig	Immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine activation motif
ITIM	immunoreceptor tyrosine inhibition motif
kDa	kilodalton
LN	Lymph node
LPA	lysoposphatidic acid
MDM2	murine double minute 2
2-ME	2-mercaptoethanol
$MgCl_2$	Magnesium Chloride
MMAC1	mutated in multiple advanced cancers
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUPP1	multi-PDZ-domain protein 1
NaCl	sodium chloride
NFκB	Nuclear factor kappa B
NLC	Nurse-like cells

N-terminal	amino terminal
NP40	Nonidet-p40
PB	Peripheral Blood
PBS	phosphate buffered saline
PDK	3-phosphoinositide-dependent kinase
PDZ	post-synaptic density protein PDS-95/SAP90, Drosophila septate Junctionprotein ZO-1
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PI(3,4)P2	phosphatidylinositol (3,4)-bisphosphate
PI(4,5)P2	phosphatidylinositol (3,4)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PLCγ	phospholipase C gamma
PMA	phorbol 12-myristate 13-acetate
PKB/Akt	protein kinase B
PTEN	phosphatase and tensin homolog deleted from chromosome 10
PTPL1	protein tyrosine phosphatase L1
PVDF	polyvinylidene difluoride
rcf	relative centrifugal force
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per min
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency disease
SDF-1	Stromal Cell-derived factor 1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SHIP	SH2 domain-containing polyinositol-phosphate phosphatase
SHM	somatic hypermutation
siRNA	small interfering ribonucleic acid
TAPP	Tandem PH domain containing protein
TBST	tris-buffered saline tween-20
TEMED	Tetramethylethylenediamine
ug	microgram
uL	microliter
V	variable region of Ig heavy chain
ZAP70	zeta-associated protein 70

Introduction

1. PI3K Pathway

a) Kinases and Isoforms

Phosphoinositide 3-kinase (PI3K) activities are found in all eukaryotic cells and play a role in many cellular functions such as cell growth, proliferation, differentiation, motility and survival (1). As well, they also play a role in intracellular trafficking events such as autophagy and phagasome formation. PI3K enzymes can be divided into 4 classes. Class I PI3K enzymes are approximately 110kDa and are found primarily in the cytosol of resting cells (1). It is not until these cells are stimulated that they are recruited to the membrane via interaction with receptors or adaptor proteins (1). Class I PI3K enzymes can be further subdivided into 2 groups of heterodimeric enzymes, IA and IB. Class IA contains 3 catalytic isoforms, p110 α, p110β and p110δ (2-6). These isoforms are all capable of binding to any one of these 5 regulatory subunits, p85α, p55α, p50α, p85β and p55γ (2-6). Regulatory subunits, p85α and p85β are longer isoforms that contain SH3 and 3 proline-rich regions in their N-terminal which allow them to make additional protein-protein interaction (5).

PI3K enzymes are known to be activated by tyrosine kinase associated receptors such as co-stimulatory receptors. These receptors contain a phosphorylated YXXM motif which is the docking site for the Src homology-2 (SH2) domain of the Class IA regulatory subunits (3). PI3K enzymes are activated in B cells by recruitment of cluster of differentiation 19 (CD19) through the YXXM motif in its cytoplasmic tail (6, 7). As a result of this interaction, the p110 catalytic domain binds to the coiled-coil motif of the

SH2 domain and is recruited to the membrane where is able to phosphorylate phosphatidylinositol 4-phosphate (PI(4)P) to phosphatidylinositol 3,4-biphosphate (PI(3,4)P2) and phosphatidylinositol 4,5-biphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (5) (Table 1). These lipids are found to be highly concentrated in the plasma membrane of cells and are ligands for phosphoinositide-binding proteins (8).

Class IB PI3K enzyme contains a regulatory subunit p101 that facilitates the interaction between the single catalytic isoform p110γ and Gβγ subunits. This interaction leads to downstream activation of G protein coupled receptors (1, 2, 5). Catalytic subunit p110γ and Class IA subunit p110δ are restricted to leukocytes while both p110α and β are expressed by all cells types (5). However, little is known about Class II and III PI3K functions in lymphocytes. Class II PI3K enzymes contain 3 isoforms, C2α, C2β, C2γ (1, 2). These enzymes are mainly associated with membrane structures such as plasma and intracellular membranes and are able to phosphorylate PI and PI(4)P (2, 9) (Table 1). The N-terminus of the Class II enzymes lacks defined structural domains but contains proline-rich and coiled-coil motifs that play a role in protein interactions. The C-terminus has tandem PX and C2 domains, however it function remains undefined (1). Class III PI3K only contain one isoform, Vps34 that can only phosphorylate PI (1, 2, 9) (Table 1).

The PI3K enzymes are able to produce various 3-phosphorylated lipid products such as PI(3)P, PI(3,4)P2 and PIP3 that can recruit signaling proteins to the membrane through their PH domains leading to various downstream signaling events (3, 10-12). Throughout

this thesis, I will focus on the function of Class IA PI3K and the role they play in normal and malignant B cells.

Table 1: PI3K Classes and the Phosphoinositides they Phosphorylate.

PI3K Class	Substrates	Products
I	PI PI(4)P PI(4,5)P2	PI(3)P PI(3,4)P2 PI(3,4,5)P3
II	PI PI(4)P	PI(3)P PI(3,4)P2
III	PI	PI(3)P

b) Phosphoinositide Phosphatases

Activated PI3K phosphorylates PI(4,5)P2 on the D3 position to form PIP3, which is able to recruit various signaling proteins to the membrane through their PH domains leading to activation of many downstream signaling events. Lipid phosphatases such as, PTEN and SHIP antagonize PI3K signaling by dephosphorylating PIP3 leading to the activation of downstream signaling pathways resulting in cell death (Figure 1). Phosphatase and tensin homolog deleted from chromosome 10, PTEN, also known as MMAC1 is a 60kDa dual lipid/protein phosphatase that modulates many cellular processes such as proliferation, cell cycle and apoptosis (13-15). PTEN opposes PI3K activity by removing the 3-phosphate on PIP3 to form the original substrate of Class I PI3K, PI(4,5)P2 (13, 16, 17). This decreases the levels of PIP3 and its downstream signaling events (13, 18). PIP3 levels and its downstream signalling can become dramatically increased upon suppression of the lipid phosphatase PTEN (19, 20).

SH2-containing inositol phosphatase, SHIP is a 145 kDa lipid phosphatase that is known to be a negative regulator of PI3K signaling (21-23). The N-terminal of SHIP contains an

SH2 domain that forms sites for phosphotyrosine binding, while its C-terminal region contains proline-rich regions forming sites for SH3 domain interactions (Figure 2) (24).

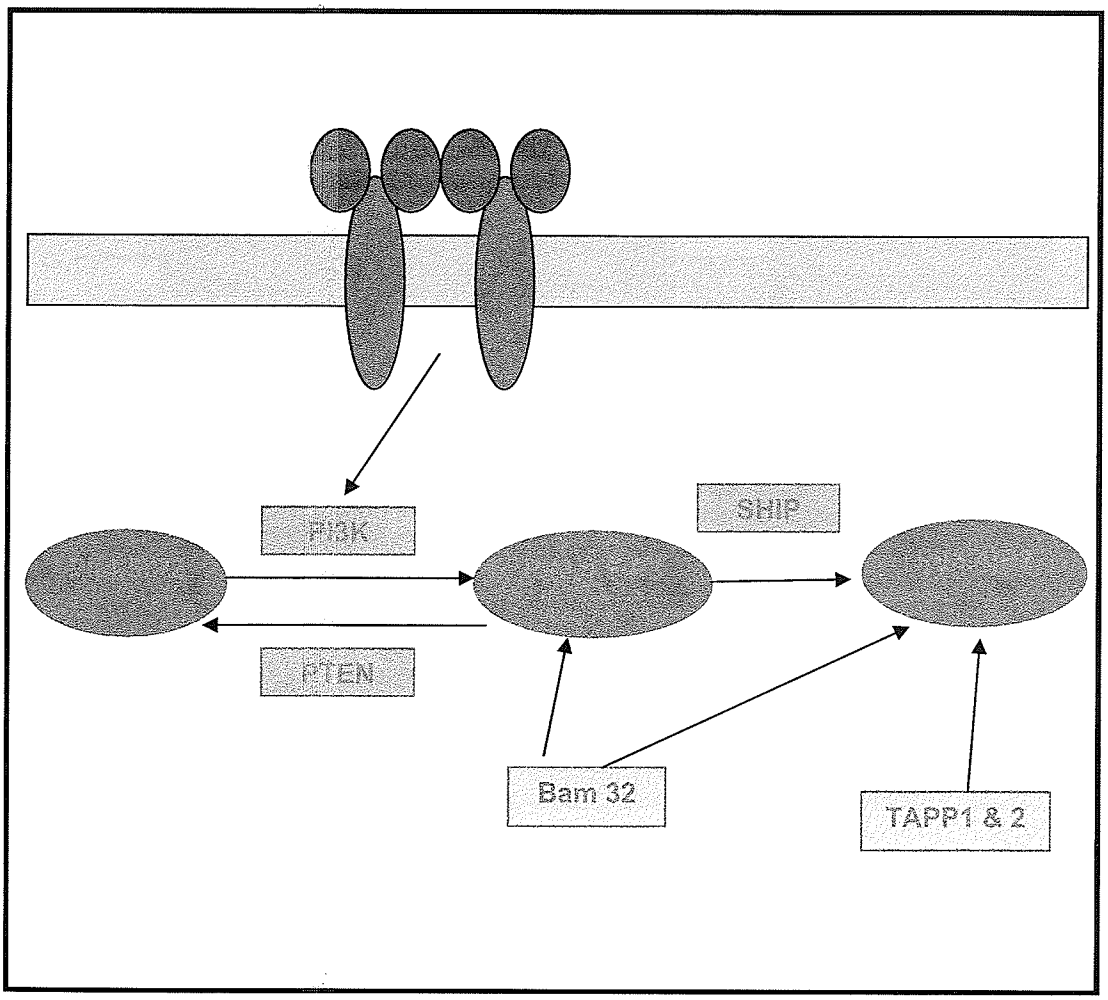


Figure 1: PI3K Pathway Components. This diagram shows PI3K activation. PI3K generates PIP3 and PI(3,4)P2. Both SHIP and PTEN antagonize the PI3K pathway. PTEN dephosphorylates PIP3 to PI(4,5)P2 and SHIP dephosphorylates PIP3 to PI(3,4)P2. Bam32 can bind to both PIP3 and PI(3,4)P2 at the membrane while TAPP1 and TAPP2 only bind to PI(3,4)P2.

SHIP is recruited to the membrane through its SH2 domain and negatively regulates BCR signaling. This negative regulation is increased by co-ligation of BCR and inhibitory $F_c\gamma$RIIB on B cells, which is a family of single chain low affinity IgG receptors (22, 24, 25). This coaggregation results in tyrosine phosphorylation of the FcγRII ITIM, a 13

amino acid sequence which is located in their intracytoplasmic domains (24, 26).

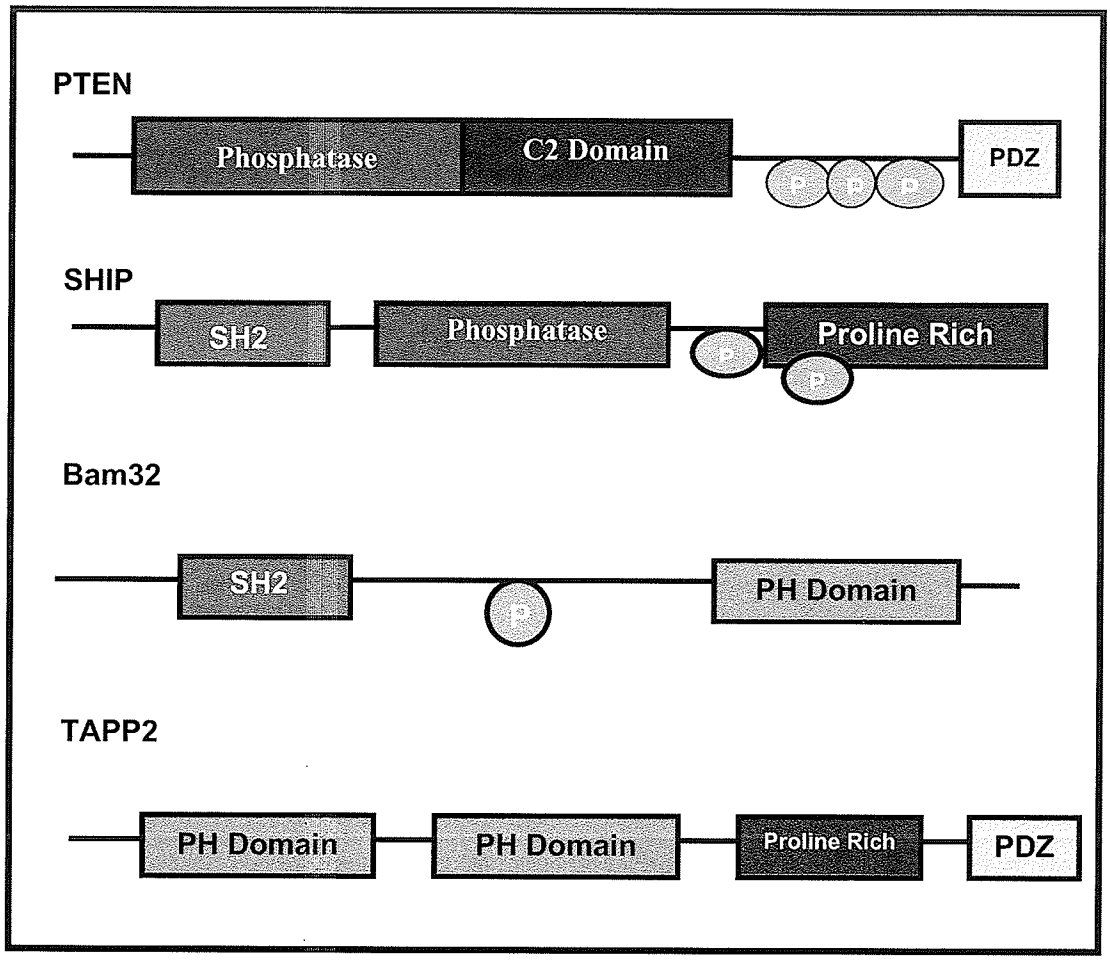


Figure 2: PI3K Pathway Components Structure.

This ITIM phosphorylation leads to recruitment and tyrosine phosphorylation of SHIP, ultimately generating a dominant negative signal in the pathway. Another way SHIP can be recruited to the membrane through its SH2 is by the formation of a ternary complex with Shc and Grb2 (21). Once at the membrane, SHIP removes the 5-phosphate of PIP3 to form PI(3,4)P2 therefore impairing some signaling downstream of PIP3, but also

increasing recruitment of some signaling proteins through their PI(3,4)P2-binding PH domain to activate other downstream signals (22, 23, 25, 26).

c) PH domains

Pleckstrin homology (PH) domains are protein modules of about 100-120 amino acids in length that are involved in intramolecular interaction in signal transduction, cytoskeletal organization and membrane dynamics (27-30). Some PH domains bind to inositol phospholipids located in the plasma membrane. The structure of the PH domain that allows for this binding consists of a core of seven stranded β-sheets that are capped at the c-terminal end with α-helix and at the other end by three inter-stranded loops (β1-β2, β3-β4 and β6-β7) which are involved in interaction of the phosphate groups from phosphoinositides that bind here (28, 31). PH domains discriminate between PIP3 and PI(3,4)P2 by the alignment of amino acids sequences of these loops. PIP3 binding requires 6 conserved residues on PH domains and mutation or substitution of any of these key amino acids was found to lead to PH domain not associating with the PIP3 (32).

Bam32 is known to bind to both PIP3 and PI(3,4)P2 since the 5-phosphate is able to be accommodated in the ligand binding pocket (31). However, the PH domain of TAPP cannot accommodate the 5-phosphate since there is no room for it in the binding pocket (33). This indicates that TAPP1 and TAPP2 PH domain are only able to bind to 3- and 4-phosphate groups therefore showing that they only bind to PI(3,4)P2 and not PIP3 (33). As well there are 3 conserved residues which are required in loops of the PH domain in order to allow for PI(3,4)P2 binding are as follows, Ala-Val-Met (31, 33). The presence

of the Ala in the β1- β2 loop sterically hinders binding of any 5-phosphate groups(33). One study showed that mutation of Arg to Leu prevented TAPP1 from binding to PI(3,4)P2. PH domains bind to specific phosphoinositides based on the structure of the PH domain and are recruited to the membrane. This recruitment facilitates downstream signaling (31). Certain PH domains such as Btk, Akt, Bam32 and the TAPPs translocate to the plasma membrane upon activation of PI3K and either preferentially bind to PIP3 or $PI(3,4)P_2$ (27, 29). Bam32, TAPP1 and TAPP2 PH domains that bind specifically to $PI(3,4)P_2$ (Figure3) will remain longer at the membrane than Btk that binds only PIP3 (27, 29, 30, 32).

d) TAPP1 and TAPP2 function in PI(3,4)P2 Pathway

TAPP1 and TAPP2 are adaptor proteins that are located in the cytosol of resting cells and upon receptor activation are physically recruited to the plasma membrane through their C-terminal PH domains. TAPP2 and TAPP1 preferentially bind to PI(3,4)P2 in the plasma membrane through their C-terminal PH domain where they are then involved in protein-protein interaction events (Figure 3) (34-37). The TAPPs also contain another PH domain in their N-terminus for which the function remains unknown, and a 3-residue PDZ binding domain that is known to be involved in protein-protein interactions (Figure 2) (35-37). TAPP1 and TAPP2 are both widely expressed in both human and mouse, however TAPP2 appears to be expressed at higher levels in lymphoid tissues (34, 37). Upon plasma membrane activation, recruitment of the TAPPs are relatively slow compared to Btk and this seems to correlate well with $PI(3,4)P_2$ production, not PIP3 production that occurs after BCR cross-linking (36, 37). PI(3,4)P2 production can be

enhanced with the overexpression of SHIP which also increases the recruitment of the TAPPs to the membrane (37). At the plasma membrane, TAPP1 and TAPP2 are found to preferentially accumulate with areas rich in ruffled F-actin (34, 37). This is an indication of a potential role for TAPP1 and TAPP2 in cytoskeletal reorganization.

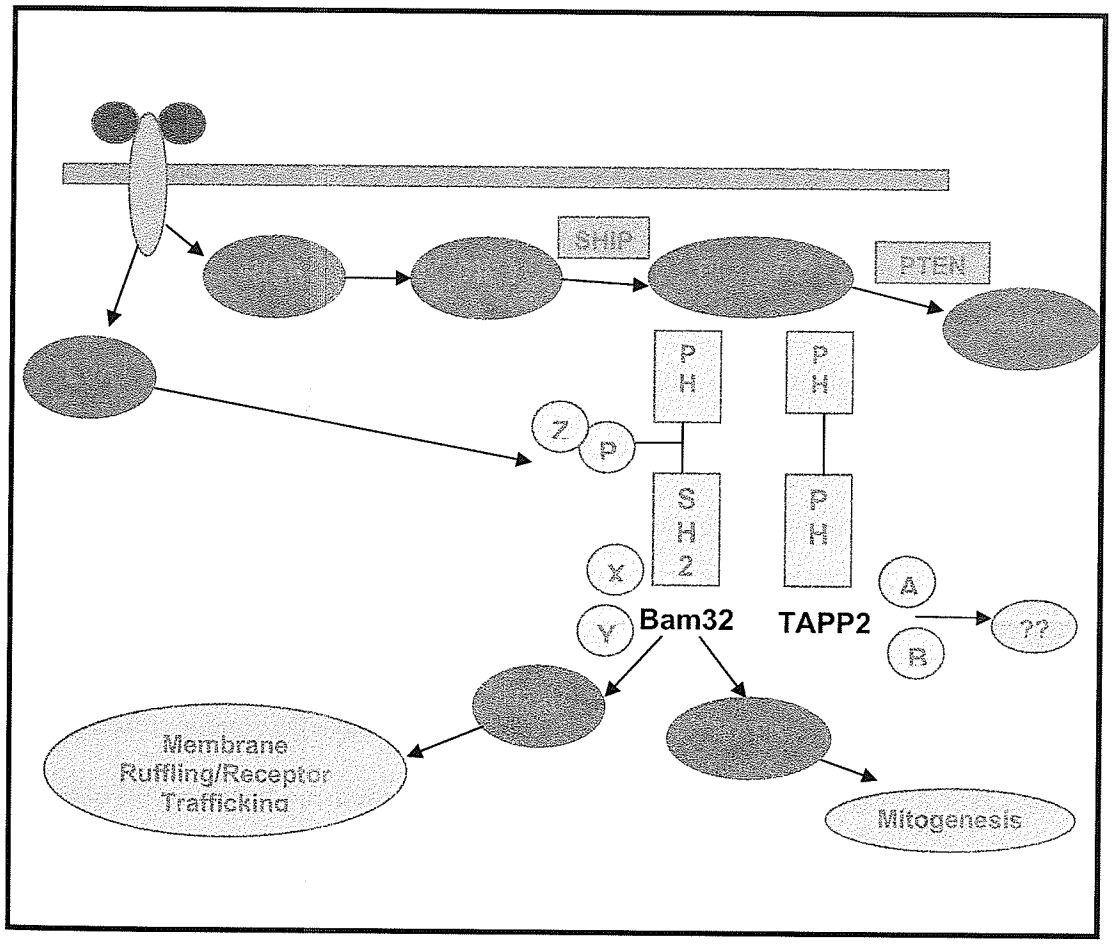


Figure 3: Basic Model for PH Adaptor Function. This diagram shows PH domain recruitment of adaptor proteins. PI3K generates PIP3. PIP3 is dephosphorylated by SHIP to PI(3,4)P2. As a result of activation, Bam32 and TAPP2 are recruited to the membrane where they bind to PI(3,4)P2 through their PH domains and activate various downstream signaling events.

2. PI3K Pathways Function in Cancer

a) p110α activating mutation

The p110 catalytic subunits of class IA PI3K have been known to be amplified or overexpressed in human cancers. Catalytic subunit, p110α has been found to be mutated in many different types of human cancers leading to the deregulation of the PI3K pathway (38). The gene that encodes p110α catalytic subunit of class IA PI3K is PIK3CA which is found to be mutated in multiple human cancers such as colorectal and breast cancer (38-41). PIK3CA gene is found to be amplified when PTEN, another gene that is often found to be deregulated in multiple human cancers (42). In many human cancers, genomic profiling revealed the presence of somatic point mutation, predominantly missense mutations, in PIK3CA which are nonrandomly distributed over the p110α structure (40). The mutations found in PIK3CA were found to cluster in 2 "hotspot" regions, exon 9 which encodes the beginning of the helical domain and then exon 20 which encodes the tail of the kinase domain (42, 43). The sites of these mutations that were most frequently affected are at residues 542, 545 and 1047. The glutamates that are found to be located at positions 542 and 545 are changed to lysines (E542K and E545K) within the helical domain and the histamine that is located at positions 1047 is often replaced by arginine (H1047L) within the kinase domain (40). The position of these point mutation result in increased lipid kinase activity (39, 40).

Mutations of PIK3CA are found to arise late in tumorigenesis and tend to coincide with invasion (39). A study by Samuels et al. screened the kinase domains of PI3K in colorectal cancer patients and found that PIK3CA was the only gene to have somatic

mutations (38, 43). Another study found PIK3CA to be the most mutated oncogene in breast cancer since 25% of their breast cancer patients contained mutations in this gene (44). This high mutation frequency also holds true for cancers of the colon, stomach and brain. In patients with colorectal cancer, another study found that PIK3CA mutations inhibit apoptosis, this leads to cellular survival and accumulation which then promotes tumor invasion (38, 42).

b) Function of PTEN and SHIP in cancer

PTEN and SHIP both regulate the levels of the PI3K lipid product PIP3, leading to decreased Akt downstream signaling and therefore leading to cellular apoptosis. PTEN is often found to be mutated or inactivated in multiple human cancers(13, 45, 46). This mutation leads to uncontrolled accumulation of both PIP3 and PI(3,4)P2 causing hyperactivation of Akt signaling pathway ultimately leading to malignant transformation (17, 18, 47-49). Cells that are defective in PTEN function seem to have alterations in cell cycle regulation, impaired apoptosis response and increase in motility and invasiveness of cells leading to formation of an aggressive tumor phenotype (16, 19, 50, 51). However, re-expression of this phosphatase leads to absence of tumors, normalization of these functions and decreased levels of PIP3 and Akt downstream signaling.

SHIP, a negative regulator of PI3K plays a major role in normal proliferative responses as a result of BCR signaling. B cells deficient in SHIP have impaired function and generation of new B cells (21). SHIP deficiency also leads to accumulation of PIP3, and

B cells tend to have hyperproliferative response but do not seem to become transformed, since no tumors of any kind are observed in SHIP-deficient mice (52).

PTEN-deficient mouse models, researchers were able to demonstrate the requirement for PTEN during embryogenesis (13, 14, 48, 53). Those mice that had complete knockout of PTEN were found to die before birth (14, 48, 50, 54). However, PTEN heterozyous mice developed normally but displayed a high incidence of tumors (14, 46, 48, 53). In contrast, SHIP-deficient mice do not seem to show signs of tumor formation, but display a lymphoproliferative disorder (46). These mice are viable and fertile but have a short lifespan characterized by autoimmune reactions such as, myeloid cell infiltration in kidney, lung and liver and progressive splenomegaly (23, 52, 55). $SHIP^{-/-}$ mice display an increase in myeloid cell proliferation as a result of PIP3 production and is therefore an indication of the role PI(3,4)P2 has in activating various mitogenic pathways(52).

PTEN and SHIP are both able to reduce the levels of PIP3 in cell, but PTEN has a more prominent role in controlling the transforming effects of the phosphoinositides, possibly because it reduces the levels of all lipids phosphorylated at the D3 position (15, 46). PTEN mutation lead to uncontrolled accumulation of both PIP3 and PI(3,4)P2, whereas SHIP mutation leads only to uncontrolled PIP3 levels (PI(3,4)P2 is normal or decreased) (15, 56, 57). This suggests that uncontrolled accumulation of PIP3 alone is not sufficient for tumor formation and it seems to require both arms of the PI3K pathway.

c) PI3K Effectors in Cancer

PI3K effectors are found downstream and carry out the function of PI3K activation. Akt family members contain 3 isoforms, Akt1, Akt2 and Akt3 (Figure 4) (41, 58-64). These isoforms all share similar domain structures and can be activated in a PI3K-dependent manner by various stimuli. Akt contains a PH domain in its amino-terminal end and serine/threonine kinase domain in its c-terminal end (41, 59, 61, 64, 65). Akt activation is PI3K-dependent. PIP3 is able to recruit Akt (which is also known as protein kinase B or PKB), a serine/threonine kinase, through its PH domain to the membrane from the cytosol. Akt binds to PIP3 and PI(3,4)P2 with high affinity and is known to be an important signaling molecule downstream of PI3K. Once at the membrane Akt can be activated by 3-phosphoinositide-dependent kinase 1 (PDK1) through phosphorylation of the Thr^{308} and Ser^{473} residues in the kinase domain (59, 61). This activation leads to downstream signals resulting in the accumulation of cells due to the suppression of apoptosis. Apoptosis is suppressed as a result of Akt phosphorylating BAD, a pro-apoptotic molecule. This phosphorylation results in BAD binding to 14-3-3 proteins which does not allow BAD to enter into the mitochondria and trigger the release of cytochrome c (41, 59, 66). As well, Akt targets pro-apoptotic tumor suppressor p53 by phosphorylating MDM2 which is the binding protein for p53 (59, 66). This phoshorylation results in targeting of p53 for degradation by proteasomes through ubiquitination (59, 66).

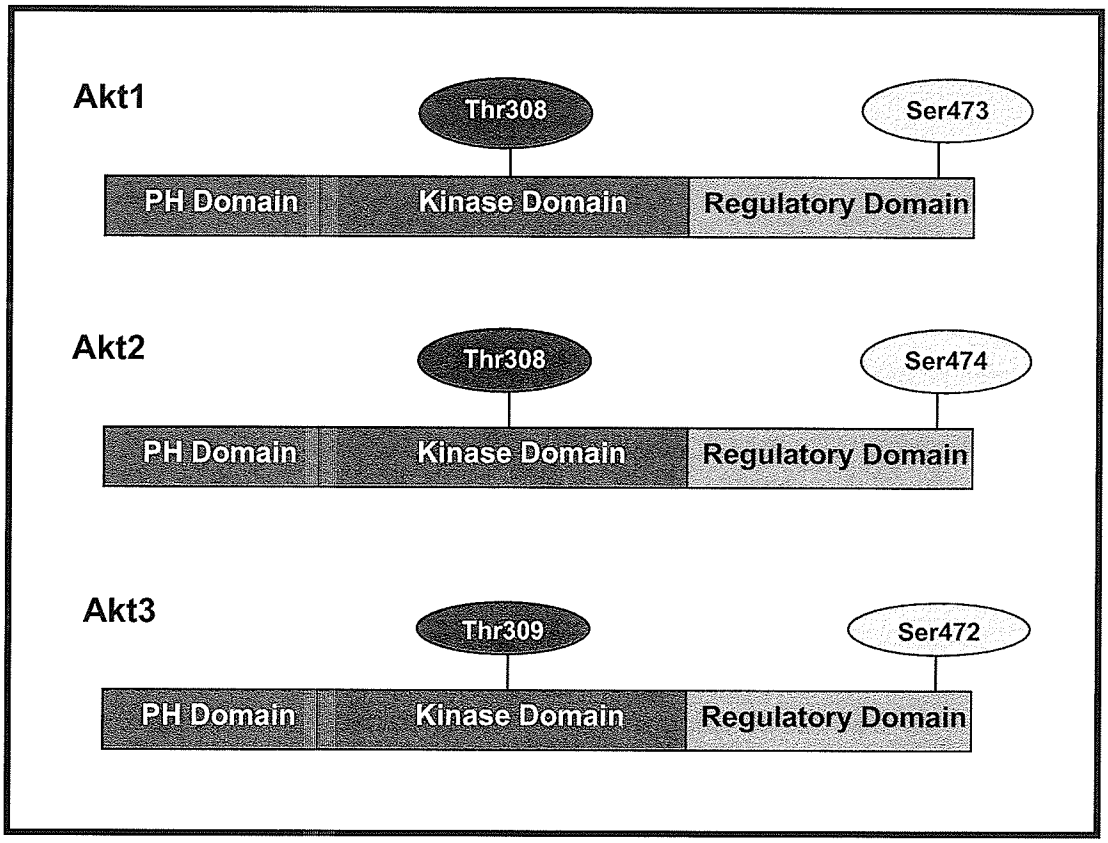


Figure 4: Akt Domain Structure. There are 3 different isoforms of Akt which consists of three functional domains.

In many human cancers, PI3K-Akt pathway has been disrupted. This disruption results in accumulation and invasiveness of malignant cells. Akt is found to be overexpressed in colorectal, pancreatic and breast cancers (66-68). Overexpression of Akt has been found to correlate with tumor cell resistance to treatments such as chemotherapy and signal molecule inhibitors (61, 69). A study showed that siRNA knockdown of Akt in cells overexpressing Akt resulted in significant tumor growth reduction and invasiveness (61, 70, 71). Inhibition of PI3K, which is upstream activator of Akt results in decreased Akt phosphorylation leaving these cells more susceptible to drug treatment and cell death. As

well, these tumors cells begin to undergo apoptosis and there was an arrest in tumor cell growth (61, 70, 71).

Another kinase that plays a role in cancer and is found downstream of PI3K pathway is mammalian target of rapamycin (mTOR) (72). mTOR is a serine/theronine kinase that is involved in regulating cell growth and proliferation (59, 73, 74). mTOR is colocalized with three peptides that aid in further downstream activation of mTOR pathway (75-77). The first protein is the regulatory-associated protein of mTOR (raptor), a scaffolding protein that presents various substrates to mTOR kinase domain (75-77). GbetaL is an other peptide that is able to stabilize raptor which ultimately increases mTOR interactions and activity (75-77). The third peptide is mLST8, however its role remains unclear (74-77). mTOR phosphorylates two downstream proteins, p70S6 (S6K) and eukaryotic translational initiation factor 4E (eIF4E) that are critical for the increase in translation of cap-dependent mRNAs (59, 73, 78). The increase in mTOR activity, overexpression and increase in translation via phosphorylation of eIF4E and S6K, as a result of PTEN loss in many human tumors leaves tumor cells dependent on mTOR for growth and sensitive to mTOR inhibitors. (75, 78-81).

mTOR pathway is found to be altered in many human cancers. eIF4E is often found to have low levels in cells, however when overexpressed it leads to deregulation in translation which ultimately leads to tumor formation (82-84). eIF4E is found to be the most commonly disrupted and overexpressed in solid tumors especially those of the breast, colon and neck (82, 85). This overexpression is found to be associated with

overexpression of PI3K/Akt kinases and/or increased Akt activation as a result of the loss of PTEN expression. High levels of eIF4E correlates with higher rates of relapse and death (75, 85-92). In several studies, eIF4E levels have been found to be elevated and lead to enhanced mRNA translation in cancers of the colon, breast, bladder and Hodgkin's lymphoma (75, 93). siRNA knockdown of eIF4E in one study showed that there was an inhibition of tumor cell growth, it was therefore suggested that this site could be a potential target for anticancer therapy (82, 94).

d) Targetting PI3K as Cancer Treatment

The PI3K pathway has become a promising target for drug development since in many cancers deregulation of this pathway results in increased cell proliferation, repression of apoptosis and resistance to treatments. PI3K inhibitors have been used widely as an experimental tool to determine the role of PI3K pathway in biological process. Wortmannin, an inhibitor of type I PI3K, it is a fungal metabolite that is able to inhibit the PI3K pathway at 2-4nM (41, 61, 63, 95, 96). The PI3K pathway is irreversibly inhibited by this potent inhibitor by binding covalently to a conserved lysine residue (Lys 802) which is located in the p110 catalytic subunit of the PI3K enzymes (59, 61, 63, 66). Wortmannin has been suggested as a valuable approach to cancer treatment as a result of its antitumor activity demonstrated both *in vitro* and *in vivo*. This activity is enhanced when combined with other treatments, such as radiation (41, 61, 63, 97-102). However, there are many disadvantages of this specific inhibitor that limits its use in clinical trial. It has been shown to be very toxic, has low stability in aqueous environments, and short half life in culture medium (41, 61). Although these problems make wortmannin an

unattractive agent for clinical trials, it has been shown in mouse models that restricted administration of this inhibitor had an effective antitumor activity with little toxicity (62, 103). A study in SCID mice that contained human mammary or pancreatic carcinoma cell xenografts and received a daily oral administration of wortmannin, showed a reduction in tumor size and inhibition of Akt phosphorylation compared to controls (62, 104).

Another PI3K inhibitor, LY294002 is a small flavonoid derivative that inhibits ATP binding sites competitively and reversibly on several different PI3K enzymes using a low micromolar range (41, 59, 61, 63, 66). LY294002 has been shown in several *in vitro* studies that when used alone it has both antiproliferative and proapoptotic activites. However when used in combination with cytotoxic drugs or radiation the effectiveness of the treatment is enhanced (41, 63, 102, 105-108). One study showed that activity of PIK3CA could be inhibited through the use of LY294002 (38). In studies with human gastric or pancreatic cancer cell lines, LY294002 was shown to inhibit cell cycle at G1 (63, 109, 110). Similar results were seen in non-small lung cancer cell lines with the addition of inhibition of Akt activity and induction of apoptosis (63, 106). In human studies, LY294002 has been found to be effective when used topically for melanoma or intraperitoneally for cancers of the pancreas, colon and ovaries (62, 103-105, 111, 112). As well when administered in human cancer xenografts, it has been shown to inhibit tumor growth and induces apoptosis (61, 112, 113). PI3K inhibitors used as cancer treatment may be possible in the future as a result of the next generation of isoform PI3K inhibitors that are being developed. There are reviews published promising to reduce toxicity of these inhibitors (114).

Rapamycin is an inhibitor that acts on mTOR activity which is found downstream of PI3K activation (41). Rapamycin is a macrolide antibiotic that was discovered originally as an anti-fungal agent (62). It was later Food and Drug Administration (FDA) approved to be used as an immunosuppressive agent for patients that had renal transplants (66). This inhibitor targets mTOR kinases which block 2 important downstream signaling proteins, S6K and eIF4E (75, 115, 116). This results in blocking the translation of mRNAs that are required for the progression through the cell cycle from G1 to S phase (75, 115, 116). High concentration of inhibitor rapamycin will have pro-apoptotic effects on several human tumors. There are currently 3 water soluble analogs of rapamycin that have been used in clinical trials, CCI-779, RAD001 and AP23573 and shown to be effective in inhibiting proliferation and growth in several *in vitro* human tumor cell lines and *in vivo* in human tumors (41, 62, 66, 117-121). CCL-779 is at the most advanced stage in clinical trails, Phase III for many cancers such as renal cell carcinoma and advanced breast cancer (62, 122). Phase I in clinical studies in these cancers using CCl-779 showed that it is tolerated at lower doses and Phase II indicated a response in 5% of renal cell carcinomas and 10% in cases of advanced breast cancer (62, 122). RAD001 showed similar results as CCL-779 and was also able to show an inhibition of tumor growth in phase I of clinical trails (75). It is currently being used in phase II of clinical trials in patients that have progressive or recurrent endometrial cancer (75). The last analog of rapamycin, AP23573 showed anti-proliferative activity *in vitro* in tumor cell lines of the prostate, breast, lung and colon that are deficient in PTEN (75, 115). This analog is currently in clinical trials as well. It has been tested on patients with refractory

or advanced solid tumors in two clinical trials (75). AP23573 is in a few preliminary phase II clinical studies of patients with advanced sarcoma and hematological malignanies (75). The overall outlook of these studies requires further testing and enrollment of more patients (75).

3. Chronic Lymphocytic Leukemia (CLL)

a) Basic Characteristics of the Disease

Chronic Lymphocytic Leukemia (CLL) is a common B cell leukemia that is predominately found in middle aged and elderly individuals (123, 124). This slow-progressing leukemia has the following phenotype: expression of CD19, CD20, CD5, CD31 and CD23 surface markers, but low or absent expression of CD22, CD79b and surface IgM and IgD (123, 125, 126). CLL is characterized by the accumulation of monoclonal $CD5^+$ and CD19 mature B cells in peripheral blood, bone marrow and lymph nodes where the majority of the leukemic cells are arrested in the G_O/G_1 phase of the cell cycle (126-130). The accumulation of B cells in these individuals is thought to result from defects in apoptosis including high levels of antiapoptotic protein Bcl-2 (123, 130, 131). The disease remains incurable. However, there are 2 commonly-used treatments, chlorambucil and fludarabine that are effective in decreasing the levels of accumulating cells by inducing apoptosis (123, 124). These treatments are enhanced when combined with such drugs like cyclophosphamide, mitoxantrone and topoiosomerase II inhibitors (123).

b) Subtype of CLL and Markers

CLL cases can be divided into 2 different subtypes. The first group contains leukemia cells that have undergone somatic hyper mutation (SHM), where the cells have mutated their IgV_H genes with 2% or more base-pair changes in the V region. This group is known as the mutated CLL patients (128). While the other group known as the unmutated CLL patients have not undergone SHM and their IgV_H genes contain less than 2% mutations in

the V region (123, 132). The malignant clones of those CLL cells that have mutated IgV_H are thought to have developed after stimulation with antigen in germinal centers (GC) and contain low levels of AID, the enzyme responsible for SHM. In contrast, unmutated CLL cells are thought to arise from naïve B cell that have encountered antigen but did not form a GC and did not express AID (133). These two groups have different clinical outcomes and diagnostic makers. Those patients that have a mutated phenotype tend to have a slowly progressing disease with a favourable prognosis, while unmutated CLL patients tend to have a more aggressive form of the disease with rapid progression and a less favourable prognosis (128).

Those CLL patients that contain unmutated IgV_H have higher expression of ZAP70 than the mutated CLL cells (125, 134-136). ZAP70 is a 70kDa protein tyrosine kinase that is normally expressed in T cells and not found in B cells. It is a signaling molecule that is involved in intracellular signaling that is initiated by T cell receptor ligation (133). There are other prognostic markers used to detect early CLL progression, such as surface molecule CD38 (124, 136, 137). Those CLL patients that have high levels of CD38 have a poor response to fludarabine and have a lower survival rate than those that do not contain CD38 (124). However, ZAP70 expression is currently the best marker correlated with clinical progression.

c) CLL and BCR Signaling

BCR signaling is initiated after receptor aggregation leading to the phosphorylation of Igα and Igβ ITAMs by Src and Syk family kinases (138). ITAM phosphorylation leads to

downstream BCR signaling and activation of many signaling pathways. Studies have shown that unmutated, but not mutated CLL patients are able to signal via their sIgM (136, 139, 140). High expression of Zap70 that is found in unmutated CLL patients is able to associate with various proteins through their sIgM following BCR engagement (141). This crosslinking in these patients that express ZAP70 further leads to greater phosphorylation of $p72^{Syk}$, BLNK and PLC-γ (136, 141).

BCR is composed of mIg that associate noncovalently to CD79a and b heterodimer which functions as the receptors primary signal transducer (131). In most CLL patients, the expression of CD79b is absent and these patients tend to have altered downstream signal transduction pathway (123, 131, 142). The absence of CD79b in these CLL patients leads to inhibition of apoptotic signal and ultimately leading survival of these cells (123, 131).

d) CLL survival

In contrast to normal B lymphocytes, CLL cells show an increase in survival as a result of their low levels of apoptosis. However, when cultured *in vitro* they die spontaneously and are difficult to keep alive for long periods. In vivo, CLL cells receive growth and survival signals from cells they associate with while migrating through lymph nodes and marrow. Signalling pathways that are responsible for CLL cell survival are not completely understood, however further studies on these pathways could lead to a better understanding of disease pathogenesis and development (143). One study showed that CD40 stimulation from lymphoid tissue, such as the bone marrow, resulted in rescuing

CLL cells from undergoing apoptosis (143). Apoptosis in these cells is inhibited as a result of NFκB activation through CD40 stimulation. NFκB is found to have high levels of activity in CLL cells (143, 144). As well PI3K activity has been shown to play a critical role in CLL cell survival. PI3K activity appears to be elevated and sustained in CLL cells (143). This sustained activation results in activation and upregulation of the Akt pathway which leads to cell survival as a result of phosphorylation and inactivation of proteins involved in apoptosis such as caspase 8 (64, 143, 145-147). PI3K/Akt constitutively activates CLL cell and maintains their survival by preventing the activation of procaspase 8, which is required for the initiation signal of apoptosis (148).

PTEN is another inhibitory phosphatase of BCR signaling. PTEN is found to be mutated in many different cancers. However, it was published that 1/5 of CLL patients have loss of heterozygosity at the gene locus where PTEN is expressed(149). This study showed that there was no mutation of PTEN found at the RNA level in CLL patients (149). But at the protein level, 11/41 patients has no expression of PTEN and 8 out of 41 had reduced expression (149).

Bcl2 is another important factor that is known to contribute to the survival of CLL cells and is often found to be upregulated in both treated and untreated patients (150-154). Bcl2 is a member of the Bcl2 family of proteins that is responsible for regulating apoptosis in many cellular systems. Bcl2 family proteins either promote apoptosis through expression of pro-apoptotic proteins, Bax, Bad and Bid or inhibit apoptosis through the expression of anti-apoptotic proteins Bcl2 and Mcl1 (150, 155-157). Cells

require a balance between these 2 sets of proteins in order to regulate apoptosis. Cells that contain a higher ratio of pro-apoptotic proteins vs anti-apoptotic proteins will result in apoptosis of the cells and the reverse is true for cellular survival. Bax, a pro-apoptotic protein, has an antagonistic role against Bcl2 and balanced expression of these proteins is required to regulate apoptosis (153, 154). In CLL patients, a high ratio of Bcl2/Bax correlated with their clinical resistance to treatment (151, 154). Another study found that *in vitro* downregulation of Bcl2 in CLL cells resulted in increase of cellular apoptosis (151, 153). Upregulation of Bax in CLL patients with high Bcl2 also caused these cells to undergo apoptosis (154).

Lysophosphatidic Acid (LPA) is another factor that plays a role in CLL cell survival. LPA is a naturally occurring soluble phospholipid that in B cells is known to act as a growth factor which will lead to increase in cellular proliferation, intracellular calcium and immunoglobulin formation (158-160). LPA acts through specific LPA receptors and is known to block death receptor-induced apoptosis. It activates Akt leading to the phosphorylation of Bad, resulting in inactivation of this pro-apoptotic protein, and activates NFκB which is responsible for the increase in expression of anti-apoptotic proteins such as Bcl2 (158, 161-163). It was shown that LPA is able to protect CLL cells from spontaneous apoptosis induced by fludarabine and chlorambucil (158). It was also found that compared to normal B cells, LPA_1 receptor is expressed at higher levels in CLL cells (158). LPA could be an important survival factor for CLL apoptosis since it is able to block both spontaneous and drug induced apoptosis.

e) CLL adhesion and migration

Adhesion and chemotaxis both play important roles in the survival and maintenance of CLL cells both *in vivo and in vitro.* Chemotaxis is the cells ability to detect and migrate in the direction of the extracellular chemical gradient. CLL cells migrate from the blood to the bone marrow (BM) and lymph nodes (LN) where they accumulate as a result of chemotactic gradients. CCR7 is a chemokine receptor that is found to be upregulated on peripheral blood (PB) CLL cells, and their migratory response to CCL12 is increased in CLL patients that have clinical lymphadenopathy (164, 165). This increase in CCR7 may be one of the many mechanisms involved in CLL cells ability to move from PB to BM and LNs (164).

Stromal cell-derived factor 1 (SDF-1) is another chemokine that has been shown to play multiple roles both in chemotaxis and survival. SDF-1 is known to signal through the chemokine receptor CXCR4 and plays a major role in B lymphopoiesis (141, 166, 167). This chemokine is produced in high levels by the stromal cells of the bone marrow which is the primary site for differentiation of B cells (141, 168, 169). B lymphopoiesis is regulated by SDF-1 by maintaining close contact of B cells with stromal cells and therefore preventing premature release of B cells into circulation (166, 170). SDF-1 has also been found in a few studies to have a direct effect on the growth and survival of CLL cells. CLL cells have been found to have mRNA level expression of CXCR4 and functional chemotaxis responses to SDF-1 (166, 167, 171).

As CLL cells migrate from the blood to the bone marrow, lymph nodes and other secondary lymphoid tissue, these cells adhere to endothelium, bone marrow stromal cells and ECM. Adhesion molecules which play an important role in interaction between individual cells or the cell and the cell matrix belong to families of the selectin, integrins, immunoglobulins and CD44 (141, 172). These molecules are not only involved in the adhesion of cells but play a role in regulating the signals required for cytoskeletal reorganization, cell cycle progression and survival (141, 173). Certain functional characteristics of CLL cells, such as the cells ability to adhere to substrates may be explained by the expression of various adhesion molecules. CLL cells have been found to express the following adhesion molecules: β1 and β2 integrins, variable amounts of α3, α4, α5 integrins, intracellular adhesion molecule-1 (ICAM-1), ICAM-2 and ICAM-3, L-selectins and CD44 (141, 166, 172). CLL cell interaction with stromal cells found in the bone marrow which requires β1 and β2 integrins (141, 166, 174). As well, CLL cells are also known to interact with stromal cells and nurse-like cells (NLC) as a result of SDF-1 which promotes CLL adhesion by inside-out activation of integrins (164). SDF-1 has been previously shown to have a prosurvival effect on CLL cells. CLL cells cultured *in vitro* undergo spontaneous apoptosis when they are cultured alone (164, 175). However, providing CLL cells with their favorable microenvironment of stromal cells or nurse-like cells, CLL cells adhere to stromal cells or NLC and are found to release high levels of SDF-1 leading to chemotaxis of more CLL cells from the PB to BM or LN (164, 176, 177).

CLL cells have also been found to express variable amount of immunoglobulin adhesion molecules such as CD44 and CD58 (172, 178). CLL patients that expressed CD58 were found to have higher incidence of spenomegaly (172, 178). One study found that patients with high levels of CD44 had more diffuse bone marrow infiltration of CLL B cells (172, 178). This is consistent with other findings stating that CD44 mediates adhesion of CLL cells to bone marrow stromal cells (172, 178). VLA4 is an integrin that is found to be widespread in normal B cells, while VLA3 is only present in a few germinal center cells (179, 180). However, this does not hold true in CLL cells. Many studies have shown that VLA3 is highly expressed in CLL cells while there is a reduction in VLA4 expression (179). Changes in the expression levels of adhesion molecules and chemokines could be one of the many reasons that CLL are able to migrate and adhere to secondary lymphoid tissue where they proliferate and survive.

There were only a few studies that showed PI3K signaling pathway played a critical role in CLL adhesion and chemotaxis. One study was able to show that CCR7 levels were increased on patients that were positive for ZAP70 compared to those that did not express ZAP70. As a result $ZAP70^{+}$ CLL patients, when compared to those patient negative for ZAP70, have increased responses to CCL19 and CCL21, leading to increases in cellular chemotaxis and F-actin polymerization (164). In another study, they were able to show that CLL cells undergo chemotaxis in response to SDF-1 and this chemotactic response could be inhibited by PI3K inhibitor wortmannin (166). This is an indication that stromal cells can attract CLL cells via CXCR4 signalling through PI3Ks, allowing CLL cells infiltrate into the bone marrow (166).

4.: Distinct Functional Role of PI(3,4)P2 and the TAPPs

a) PIP3 vs PI(3,4)P2

PIP3 and PI(3,4)P2 are both lipids formed after PI3K activation and are able to activate different downstream signal transduction pathways. The molecular basis for regulation of the responses of PIP3 vs PI(3,4)P2 are not completely understood. Although their regulation are not completely understood, it is known that there is a large variation in the ratio of PIP3 vs PI(3,4)P2 which is dependent on receptors engaged or cell types (57, 181). PIP3 is generated as a result of the phosphorylation of PI(4,5)P2 and is known to recruit many proteins that are involved in cancer. This arm of the PI3K pathway is known to be deregulated in many human cancers and lymphomas. Membrane targeting is known to be regulated by PIP3 which also leads to the activation of Tec family tyrosine kinases and Akt through binding to their PH domains (182, 183). Akt is known to bind to PIP3 and results in activation of downstream signals that lead to preventing apoptosis, leading to cell survival.

PTEN is known to equally dephosphorylate both PIP3 and PI(3,4)P2 (15, 46, 49, 184). This activity of PTEN leads to a decrease in cell survival and increase in cellular apoptosis (15, 18, 49). However, PTEN is often found to be mutated in multiple cancers, and as a result leads to the accumulation of PIP3 and PI(3,4)P2. This results in an increase in cell proliferation (15, 18, 19). The relative importance of PIP3 vs PI(3,4)P2 in cell transformation is not known and has not be studied in human cancers and leukemias. PI(3,4)P2 is known to function as a signaling lipid since it is found in low concentration in unstimulated cells and is only elevated upon activation by PI3K. After BCR activation,

PI(3,4)P2 production results from the dephosporylation of PIP3 by SHIP (185). PI(3,4)P2 can also be generated independently by phosphorylation of PI(4)P by PI3K (186). PIP3 production is rapid and transient and is known to correlate with Btk PH domain recruitment to the membrane (37). However, PI(3,4)P2 lipids are produced after PIP3 and in a manner that is delayed and sustained like the recruitment of the TAPPs to the membrane (37). Although Bam32 is known to be recruited to the plasma membrane by both PIP3 and PI(3,4)P2, its recruitment to the membrane correlates better with PI(3,4)P2 production than PIP3 (37). It was recently shown in our lab that cell treated with peroxide resulted in the recruitment of PI(3,4)P2-binding effectors Bam32 and the TAPPs (185).

b) Function of PI(3,4)P2 in Cell Activation and Transformation

The functional role of PI(3,4)P2 is not completely understood. PI(3,4)P2 is able to specifically recruit adaptor proteins TAPP1 and 2 through their PH domains and emerging evidence suggests a role in cellular adhesion and chemotaxis. A major goal this thesis is to determine some of the possible roles of PI(3,4)P2 in normal and leukemic B cells. A few studies have shown evidence that PI(3,4)P2 as plays an important role for Akt activation (56, 185, 187, 188). This suggests that PI(3,4)P2 may have a role in cancer through regulating Akt activation. There are other studies that suggest PI(3,4)P2 has possible function in adhesion and chemotaxis since cells that were overexpressing SHIP have enhanced cell spreading and scattering (189, 190). These findings suggests that the PI(3,4)P2 arm of the PI3K pathway could potentially have an important role in cancer and cell survival ability to adhere to tissue and metastasize to other areas of the body.

c) Function of TAPPs as PI(3,4)P2-Binders

TAPPs are known to specifically bind to PI(3,4)P2 and not PIP3, however their downstream signaling function are unknown. Mounting evidence (including results in this thesis) suggests that these adaptors may play a role in adhesion and chemotaxis. In order to determine the functional role of these proteins our lab has been looking at the role of the TAPPs in B lymphoma cell lines through overexpression and mutation of these adaptor proteins. Our studies have shown that TAPP1 and TAPP2 preferentially accumulate at the plasma membrane in ruffled F-actin rich areas. TAPP1 was shown by another group to associate with PDZ domains of multiple syntrophin isoforms, which are a known cytoskeletal regulator (191). In fibroblast cells, overexpression of TAPP1 inhibits formation of circular membrane ruffles, but this inhibition was reversed by coexpression of TAPP1 with syntrophin (191). These results provide evidence that TAPPs may have a role in cytoskeletal rearrangements.

TAPP1 was shown to bind to multi-PDZ domains in MUPP1 and protein tyrosine phosphatase like protein-1 (PTPL1) proteins (35, 36). PTPL1 binds to PI(3,4)P2 through formation of a complex with TAPP1 (35). In a breast cancer cell line, this group was able to show that overexpression of PTPL1 can limit the growth of these cancer cells (35). This overexpression was able to reduce the activity of Akt, which suggests that interaction of PTPL1 and the TAPPs provides a mechanism for negative feedback regulation of Akt in response of increased levels of PI(3,4)P2 (35). As a result of these findings it provides evidence that TAPPs could potentially play a role in regulating both cellular adhesion (through syntrophin/cytoskeletal regulation) and survival (through

PTPL1/Akt regulation). A goal of this thesis is to investigate the functions of TAPP adaptors in normal and leukemic B cells.

Rationale

Phosphatidylinositol 3-kinases (PI3K) are involved in regulating many processes important for B cell signaling, motility, adhesion and survival. PI3Ks generate several distinct lipid second messengers such as PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. Lipid phosphatase enzymes SHIP1, SHIP2 and PTEN are involved in regulating the lipids of PI3K signal transduction. The PH domains of many adaptor proteins and enzymes are recruited to cell membranes where they bind to these lipids. After binding of the lipids to these PH domains, the adaptor proteins are mobilized and enzymes are activated, to carry out various cellular functions. Here we will specifically focus on the adaptor proteins, Bam32, TAPP1 and TAPP2 since their PH domains preferentially bind to PI (3,4)P2 and mediate the function of this lipid. PTEN, discovered as a tumor suppressor gene, has been previously established to be involved in multiple types of cancers through regulating levels of PIP3 and PI(3,4)P2. However, the specific roles of PI(3,4)P2, which preferentially binds to TAPP1, TAPP2 and Bam32, has not been studied in B cell leukemias, although evidence is mounting that this part of the PI3K pathway may be important for B cell signaling.

Hypothesis

We hypothesize that the PI(3,4)P2 signaling pathway is important in normal B cell functions and is deregulated in B cell leukemias. Deregulation of PI(3,4)P2 signalling results in functional consequences that contribute to malignant transformation of B cells.

Material and Methods

Sample Collection

All cell lines (BJAB, Ramos, Jurkat, FDC-1) were cultured in RMPI 1640 medium (Invitrogen) containing 5% FBS (HyClone) and 5 mL of penicillin-streptomycin (Invitrogen) and incubated at 37 °C. A20 cell line, a mouse B cell lymphoma was cultured in RMPI 1640 medium containing 5% FBS, 5 mL of penicillin-streptomycin and 50 uM 2-mercaptoethanol (ME) (Fisher) and was incubated at 37 °C in a humidified CO_2 incubator (ThermoForma). These cells were either extracted using NP40 lysis buffer containing 1% NP40 (USB Corporation), 50 mM Tris (Invitrogen), 150 mM NaCl (Fisher) and 5 mM EDTA (Fisher) with Complete protease inhibitor cocktail tablet (Roche) for protein assays or Trizol (Gibco) for RNA assays. CLL cells were isolated from whole blood using standard processing used by the Manitoba CLL Bank. The Manitoba CLL Bank was established in 2003-2004 fiscal year. To date this bank contains over 120 patients enrolled and has collected over 2000 samples for storage in the bank. Research in this facility focuses on improving the understanding of and establishing better treatments for CLL. Patients with lymphocyte counts greater than $40x10^9$ cells/L were collected by standard ficoll. Patients with lymphocytes less than $40x10^9$ cells/L used the RosetteSep B cell enrichment Cocktail (StemCell Technology) to remove the contaminating T cells and monocytes from the buffy coat layer. Blood was collected from patients into heparin tubes (BD Bioscience) and was centrifuged at 1500 rpm for 10 minutes in order to separate out the plasma. RosetteSep B Cell Enrichment Cocktail (StemCell Technology) was added in order to remove all monocytes and T cells, and the mixture was incubated for 30 minutes. The sample was then diluted with equal volumes

of Hank's Buffered Saline Solution (HBSS) (Invitrogen), layered over ficoll and centrifuged at 1500 rpm for 30 minutes. The buffy coat containing isolated B cells was removed and washed with HBSS. ACK lysis buffer was added to lyse any remaining red blood cells. Cells were resuspended in an appropriate amount of HBSS and counted via Coulter Counter. Cells were frozen as pellets at $1x10^8$ cells/tube. Normal B cells were also isolated from whole blood using the same protocol except blood from normal patients was collected in EDTA tubes (BD Bioscience) and cells were counted using trypan blue (Invitrogen) and hemacytometer (American Optical). B cell purity was determined through FACS analysis. B cells were labeled with anti-CD20-FITC in order to determine B cell purity.

siRNA

Transfection with synthetic siRNA (Invitrogen) was carried out according to the Qiagen RNAiFect protocol. BJAB cells were resuspended at $3.0x10^6$/mL in serum-free, antibiotic-free RPMI 1640 medium and plated in 24 well plates (3 mL per well). Plates were placed at 37 °C in a humidified CO_2 incubator while siRNA mixture was prepared.

Specific siRNA sequences used were:

Control 2	CUCCUGUCCAAGUAGUAUUCUCUAC
TAPP1 238	UUGAAAUGUAGGUAAGCUUAAUGGC
TAPP2 416	CCCUGUCUCAGAGAUAUUUCCUUCA

The siRNA mixture was prepared to total 115ul per sample:

- 80 uL RPMI medium
- 20 uL siRNA product (2mg) (Invitrogen Stealth siRNA)
- 15 uL RNAiFect lipofection reagent (Qiagen)

This mixture was incubated at room temperature for 10 minutes and the added to the appropriate wells drop by drop. The 24 wells plates were then incubated at 37 °C in a

humidified CO_2 incubator for 24 hours. After 24 hours, 1.5 mL medium containing 5% serum were added to each well and cells were then incubated a further 24 hours prior to being harvested for RNA prep, protein prep or functional analysis.

Preparation of total RNA

Total RNA isolation was performed by lysising cells at 1 to 5 million cells per mL using Trizol reagent (Gibco). The cells were incubated in Trizol for 5 minutes to ensure complete dissociation of nucleoprotein complexes, then 200 uL of Chloroform (Fisher) was added, and the mixture was shaken vigorously for 15 seconds. This mixture was incubated at 25 °C for 3 minutes and then centrifuged at 12000 rpm for 15 min at 4 °C. After centrifugation, the upper aqueous phase was transferred into new eppendorf tube and 500 uL of isopropanol (Fisher) and 1 uL of Glycogen (Roche) was added in order to allow precipitation of RNA. This mixture was incubated for 10 minutes at 25 °C and then centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol (Fisher). This was mixed by vortexing and then centrifuged at 7500 rpm for 5 min at 4 °C. The ethanol was aspirated and the pellet was air dried for 5-10 minutes at 25 °C. The pellet was then dissolved in 50 uL of RNase free water (Gibco) and the OD reading from BioPhotometer (eppendorf) were taken in order to determine the amount total RNA in the sample. The prepared RNA was stored at -80 °C.

cDNA (Reverse Transcription)

The volume of RNA to add to cDNA synthesis is determined as follows:

X uL = 2 ug/RNA sample concentration (ug/uL)

This equation will determine the volume of each RNA sample (in uL) that needs to be added to the reverse transcription mixture and then can be diluted by the appropriate amount of RNase free water equaling 11 uL. Reverse transcription mixture was prepared to total 20 uL per sample on ice:

- 4 uL of 5x AMV RT Buffer (Roche)
- 2 uL of 10 mM dNTP (Roche)
- 1 uL of RNasin (Promega)
- 0.5 uL of Random Primer (Promega)
- 1.5 uL of AMV RT (Roche)
- RNase Free Water (Invitrogen) -depends on concentration of total RNA
- Total RNA-depends on concentration of total RNA

This mixture is then incubated in GeneAmp Thermocycler (Applied Bioscience) at 42 °C for 1 hour, then heated to 95 °C for 5 minutes and cooled to 4 °C. cDNA was used freshly for real-time RT-PCR and then stored at -80 °C.

Real Time RT-PCR

The primer sequences from Operon (arranged 5' to 3') used for real-time RT-PCR reactions are:

TAPP1 (T1F2): TTGGAGCCATTAAGCTTACCTAC
TAPP1 (T1R1): GTGGAAGCACGGCAGCCTGACG
TAPP2 (T2F1): TGGACACCCAGGCTAACTG
TAPP2 (T2R1): GTGGAAGCACGGCAGCCTGACG
SHIP1 (S1F1): TCCCAGTTTGTCTCCAAGAACG
SHIP1 (S1R1): TGCCCTGAGAGGTCTGCAGCTTG
SHIP2 (S2F2): GACCAGCTCAACCTGGAGCG
SHIP2 (S2R1): AGCCCTTTCTTGGAGATGAAC
Bam32 (BF1): TCACACAGCAATGCAGACAG
Bam32 (BR2): TCCACTCATCAGCTTCTACTC
PTEN (PTENF2): CTGGTGTAATGATATGTGCATA
PTEN (PTENR1): GTCATTATCTGCACGCTCTATACTGC
Zap70 (ZAPF1): CGCTGCACAAGTTCCTGGT
Zap70 (ZAPR1): GACACCTGGTGCAGCAGCT
G3PDH (G3PDH1): AGCAATGCCTCCTGCACCACCAAC
G3PDH (G3PDH2): CCGGAGGGGCCATCCACAGTCT

These primers were made into 100 uM stock by dissolving in 10 mM Tris, and then 20 uL of stock primer was added to 180 uL of 10 mM Tris to generate 10 uM working solution for use in RT-PCR. The RT-PCR reaction mixture was prepared on ice using the Roche LightCycler SYBR Green I Kit according to the Roche published protocol as follows:

- 13.7 uL of H_2O
- 0.8 uL of $MgCl_2$
- 1.25 uL of forward primer
- 1.25 uL of reverse primer
- 2.0 uL of SYBR Green Mix

In the LightCycler capillary tubes, 19 uL of reaction mixture was combined with 1 uL of cDNA per sample. The real-time RT-PCR reaction was carried out in the LightCycler (Roche) with the following cycle conditions:

For TAPP1, TAPP2 and Bam32:
Denature: 95° for 10 min
PCR: 95° for 15 sec
59° for 5 sec
72° for 32 sec
- this will cycle for 40 cycles

84° for 5 sec
- acquisition mode is single for this segment

Melt: 95° for 0 sec
57° for 15 sec
95° for 0 sec
- temperature transition 20/sec, continuous acquisition

Cool: 40° for 30 sec

As above for SHIP1, SHIP2 and PTEN, but using 57° annealing temperature during the cycling stage.

For G3PDH:
Denature: 95° for 10 min
PCR: 95° for 5 sec
59° for 10 sec
72° for 7 sec
- this will cycle for 40 cycles

84° for 5 sec

- acquisition mode is single for this segment

Melt: 95° for 0 sec
64° for 120 sec
95° for 0 sec

- temperature transition 20/sec, continuous acquisition

Cool: 40° for 30 sec

To ensure that the real product was being amplified, the resulting RT-PCR products were removed from the capillaries, mixed with 6x loading dye and loaded along with 100 kb ladder on 1.5% agarose gel that contained ethidium bromide. The gel was run for 1 hour and then detected under ultraviolet light. Gels were imaged using a BioRad gel documentation system.

Protein Isolation and Bradford

Cell lysates were prepared using an NP40 lysis buffer containing 1% NP40, 50 mM Tris, 150 mM NaCl and 5 mM EDTA with Complete protease inhibitor cocktail tablet (Roche). These cells were lysated at $1.0x10^6$ cells/mL, incubated in NP40 lysis for 15 minutes on ice and then centrifuged at 13000 rpm for 10 minutes at 4 °C. Supernatant was collected and protein concentration was determined using Bradford assay according to BioRad protocol (BioRad). Bradford is a method that quickly and accurately determines protein concentration in a solution by the binding of Coomassie Brillant Blue G-250 dye to proteins and standards. Depending on the protein concentration, the solution will turn different shades of blue and the concentration can be detected at 595 nm using an ELISA plate reader.

Western Blots

Protein samples were mixed with lamelli buffer (Sigma), then boiled for 5 minutes before being loaded into 10% SDS-PAGE gel. The SDS-PAGE gels were made as follows;

The 10% acrylamide lower gel was made as follows:

- 1.25 mL of 40% acrylamide-bisulfate (BioRad)
- 1.25 mL of 1.5M Tris pH 8.8 (Invitrogen)
- 50 uL of 10% SDS (Fisher)
- 2.43 mL of ddH_2O
- 2.5 uL of TEMED (Fisher)
- 25 uL of 10% Ammonium Persulfate (BioRad)

The lower gel was incubated at room temperature for 30 minutes to allow the gel to polymerize. The upper gel was made as follows:

- 250 ul of 40% acrylamide-bisulfate (BioRad)
- 630 uL of 0.5M Tris pH 6.8 (Invitrogen)
- 25 uL of 10%SDS (Fisher)
- 1.59 mL of ddH_2O
- 2.5 uL of TEMED (Fisher)
- 12.5 uL of Ammonium Persulfate (BioRad)

The SDS-PAGE gel was incubated at room temperature for 30 minutes and then loaded with the protein extracts along with PAGE ruler protein standards (Fermentas). The SDS-PAGE gel was run for 2 hours at room temperature and then transferred to an Immuno-Blot PVDF membrane (BioRad) for one hour at 20 V using semi-dry transfer method (Trans-blot SD Semi-dry Transfer Cell, BioRad). The membrane was washed 3 times with TBST and then incubated overnight at 4 °C in TBST and 1% skim milk (Santa Cruz). The next day the membranes were blotted with the following primary antibodies for 2 hours, washed 3x in TBST followed by incubation with secondary HRP-conjugated antibodies for 1 hour:

TAPP2-primary antibody-rabbit polyclonal IgG (Affinity BioReagents) and secondary anti-body-HRP anti-rabbit (Cell Signalling)

SHIP-primary antibody-mouse monoclonal IgG1 antibody (Cat # sc-8425 Santa Cruz Biotechnology) and secondary antibody-HRP anti-mouse (Jackson Laboratories)
PTEN-primary antibody-mouse monoclonal IgG1 antibody (Cat #sc7974 Santa Cruz Biotechnology) and secondary antibody-HRP anti-mouse (Jackson Laboratories)
Actin-primary antibody-anti-actin rabbit monoclonal antibody (Sigma) and secondary antibody-HRP anti-rabbit (Cell Signalling)
Zap70-primary antibody-anti-Zap70 mouse monoclonal IgG2a antibody (Cat # 05-253 Cedarlane) and secondary antibody-HRP anti-mouse (Jackson Laboratories)

Blots were then incubated with ECL chemiluminescence substrate (Amersham Bioscience) and signals were detected with Fluorchem 8800 chemiluminescence imager (Alpha Innotech).

Adhesion Assays

Adhesion assays were carried out in 96 well ELISA plates (Costar #3369) coated with the following extracellular matrix components overnight at 4 °C:

- uncoated
- coating buffer and fibronectin, 5 ug/ml (Calbiochem)
- coating buffer and laminin, 5 ug/ml (Sigma)

The next day, the plates were washed once with 1xPBS and blocked for 30 minutes at room temperature a blocking buffer containing HBSS solution + 2% FBS. After 30 minutes, PMA (50 ng/mL) and $F(ab')_2$ fragment goat anti-human IgM (10 ug/mL) (Jackson ImmunoResearch) stimuli were added to each well or left unstimulated. TAPP1, TAPP2 and control siRNA treated BJAB cells were then resuspended in blocking medium at a concentration of $1x10^6$ cells/mL and 100 uL were added to each well. CLL cells were used at a concentration of $3.0x10^6$ cells/mL. The plates were then incubated for 1-3 hour at 37 °C in a humidified CO_2 incubator. After incubation, plates were inverted to remove non-adherent cells and medium. After the addition of PBS, plates were covered with sealing tape, inverted and centrifuged at 100 rcf for 3min. After

careful removal of wash solution, adherent cells were fixed with addition of 1% glutataldehyde solution for 10 minutes at RT. Fixative was then removed and cells were stained with 0.1% crystal violet (Sigma) for 20 minutes. After complete removal of crystal violet solution, stained cells were dissolved 1% SDS solution. Crystal violet absorbance was read at 570 nm using an ELISA plate reader (Molecular Devices).

Chemotaxis Assays

Chemotaxis was determined using parental A20 cells, transfected A20 cells that overexpressed TAPP2 (ATF1), transfected A20 cells expressing a TAPP2 PH mutant (ATR3), or CLL cells. Migration buffer (RPMI + 0.5% BSA) was prewarmed and 600 uL was added to the bottom of transwell plates (Costar). Cells were washed and resuspended at a concentration of $1.0x10^6$/mL in migration medium. SDF-1 stimulus was added to bottom chamber of wells at the following concentrations, for A20 samples 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL and 100 ng/mL and for CLL samples 5 ng/mL, 10 ng/mL and 40 ng/mL. Cells were added to the upper chamber, and the upper chamber was then placed into the lower chamber that contained stimulus. Plates were then incubated at 37 °C in a humidified CO_2 incubator for 3 hours to allow for migration. After 3 hours, live cells in both upper and lower chamber were counted using a flow cytometer in order to determine migration of A20 and CLL cells. Every sample was counted for 30 seconds to determine the number of live cells and then graphed in excel using the following calculation in order to determine the percent migration:

% migration = lower chamber/ (lower chamber + upper chamber) x 100%

Confocal Microscopy

Live parental BJAB cells and transfected BJAB cells overexpressing TAPP2 or mutant TAPP2 were adhered to glass slides as follows. 8 well chamber slides (Nalge Nunc International) were pre-coated with 10 ug/mL fibronectin overnight at 4 °C. After 24 hours, fibronectin was aspirated from wells and wells were washed with 1xPBS. 200 uL of pre-warmed RPMI medium (Invitrogen) containing 2 ug/mL anti- human IgM and 200 uL of cells resuspended at $1x10^6$/ml were added to each well. Slides were incubated for 2 hours in a humidified CO_2 incubator to allow for cell adhesion. 400 uL of cold 4% paraformaldehyde (Fisher) was then added and slides were incubated at 4 °C for 2 hours to allow adherent cells to be fixed to the chamber slides. Cells were washed with 1xPBS and the upper plastic chamber was removed from the slide. Slides were allowed to air dry and then stored at -20 °C.

Cytospin slide preparations of CLL cells were done as follows. CLL cells were fixed with 2% paraformaldehyde for 1 hour on ice, washed twice with 1xPBS, resuspended at $1x10^6$/mL and 50 uL of cells were centrifuged onto glass slides using Cytospin 3 (ThermoShandon). Prior to immunofluorescence staining, cytospin slides had a 1 cm diameter area drawn around the cell spot with a hydrophobic marker (DakoCytomation) and cells were then permeabilized and blocked by 10 minute incubation with PBS containing 5% goat serum and 0.1% TritonX (LabChem Inc). After a brief rinse with PBS, primary antibodies were added at the following dilutions in 100 uL volume of PBS+5% goat serum+0.01% Triton:

TAPP2-rabbit polyclonal IgG antibody (Affinity BioReagents)-1/200 dilution

Biotin-conjugated F(ab')$_2$ Fragment goat anti-human IgM (Jackson ImmunoResearch)-1/200 dilution
Rhodamine-Phalloidin (Molecular Probes)-1/200 dilution
ZAP70- primary antibody-anti-Zap70 mouse monoclonal IgG2a antibody (Cat # 05-253 Cedarlane)-1/200 dilution

Slides were stained for 2 hours in a humidified chamber at room temperature. Slides were then rinsed with 1 mL PBS+0.01% Triton and then immersed in 1xPBS for several minutes. Secondary antibodies were added at the following dilutions in PBS+5% goat serum+0.1% Triton:

Streptavidin Alexa Fluor 647 (Molecular Probes)-1/500 dilution
Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes)-1/200 dilution
Alexa Fluor 568 goat anti-mouse IgG_1 (Molecular Probe)-1/200 dilution

Slides were incubated for 1 hour and rinsed as above. After staining, slides were air dried and mounted with ProLong Gold anti-fade reagent containing DAPI (Molecular Probes). For staining surface IgM, the cells were not permeabilized but blocked for 10 minutes prior to addition of anti-human IgM antibody for 15 minutes.

Microscopy analyses were performed using either Ultraview spinning disk confocal microscope (Perkin Elmer) or Olympus FV500 Confocal Microscope. The fluorescence intensity of TAPP2 and ZAP70 staining were determined using image analysis functions in Ultraview software.

Stastical Analysis

Normalized expression for Western Blots was analyzed using a non-parameteric t-test using Mann-Whiney with a 95% confidence interval. The median values were calculated to determine the significant difference between CLL and normal B cell expression. Correlation of ZAP70 with the other PI3K pathway components were analyzed using a

scatterplot and a line of best fit was drawn. The correlation was calculated using the correlation equation. Analysis of significance for adhesion and chemotaxis assay was done through the use of a t-test with a 95% confidence interval.

Results

Section 1: Expression Studies

Section Abstract

The PI3K pathway plays a critical role in normal B cell activation and differentiation and is often deregulated in multiple B cell derived cancers. PI3K generates several lipid second messengers, such as PIP3 and PI(3,4)P2 which are regulated through lipid phosphatases SHIP and PTEN. Adaptor proteins, TAPP1 and 2 are recruited to the membrane through their PH domains and bind to PI(3,4)P2 and mediate the function of this lipid (32, 34-36). PTEN is a commonly studied tumor suppressor that regulates the levels of PIP3 and PI(3,4)P2 and is often found to be deregulated in many cancers (17, 46, 50). One study showed that PTEN had no mutation in CLL patients, but half of their patients showed little or loss of PTEN expression at the protein level (149). We hypothesize that PI(3,4)P2, which preferentially binds the TAPPs, plays an important role in normal B cell function and is deregulated in B cell leukemias. We sought to determine the relative expression of regulators and targets of PI(3,4)P2 in CLL samples. I therefore developed quantitative RT-PCR assays to measure mRNA expression of PTEN, SHIP1, SHIP2, Bam32, TAPP1 and TAPP2; however, my results indicated low reproducibility in the expression patterns determined by this method. Through the use of Western blots, I was able to determine the expression of the TAPP2, ZAP70, PTEN and SHIP and determine expression relative to normal B cells. Collectively, this data indicated that a subset of CLL samples have an overexpression of TAPP2, and this expression has some correlation with ZA70, a prognostic marker of CLL clinical course.

a) Establishing Specific Real Time RT-PCR Assays for PI3K Signalling Components

RT-PCR primers were chosen from unique sequences from PI3K signalling components mRNA. For each mRNA sequence, I chose 2 forward and 2 reverse primers that spanned between exons and introns boarders therefore eliminating DNA contamination. To optimize primer conditions for real-time RT-PCR, I first tested various combinations of forward and reverse primers using the BJAB B lymphoma cell line to determine the best combination. The best primer combinations were chosen based on the sensitivity (low crossover points), the least amount of primer dimer seen on the melting curve and appearance of a single PCR product on agarose gels. Figure 5 shows quantification and melting curves for TAPP1 and TAPP2 primer combinations to illustrate primer sensitivity and different amounts of primer dimer vs full product. Another factor for chosing the primer was running an agarose gel using the products of the real-time RT-PCR to ensure that a single band was present at the predicted molecular weight. Figure 6a shows the results for various TAPP1 and TAPP2 primer combinations. This required in some cases a change in annealing temperature from 57° to 59° in order to eliminate non specific background bands (Figure 6b). I performed a similar primer selection process for SHIP1, SHIP2 and PTEN. G3PDH and ZAP70 primer sequences were obtained from the literature (192, 193).

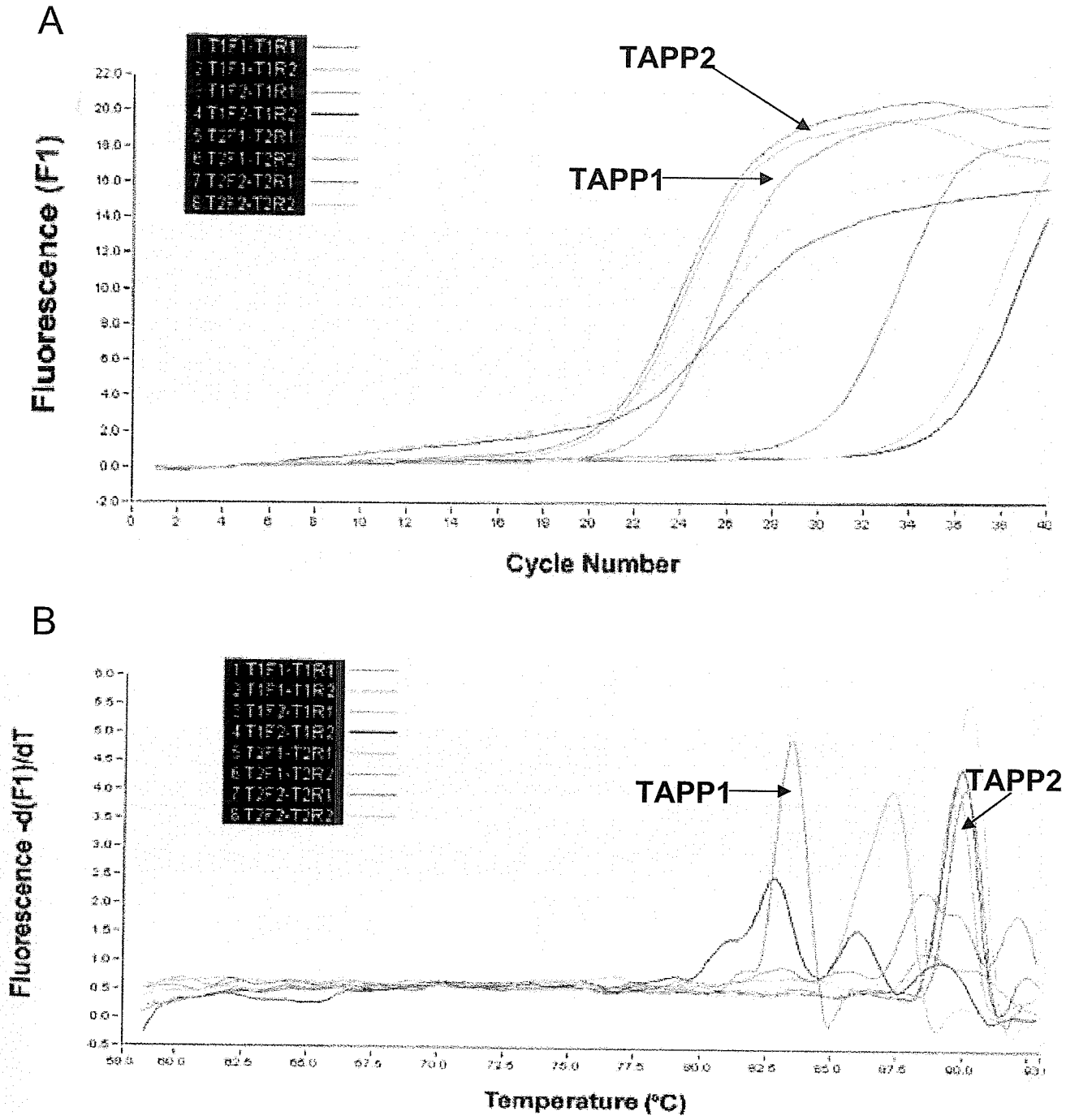


Figure 5: Real-time RT-PCR Quantification and Melting Curve Analysis for Screening Primer Combinations. BJAB cDNA was amplified in LightCycler assays with different primer combinations annealing to TAPP1 and TAPP2. Through the quantification graph in A I was able to determine the sensitivity of the primer through the crossover points. Those primers with lower crossover points were chosen. Through the melting curve I was able to determine that the product of the RT-PCR was real and not just primer dimers. The primers chosen for TAPP1 and TAPP2 were as follows: T1F2:T1R1 for TAPP1 (red) and T2F1:T2F2 for TAPP2 (light pink). These both displayed early crossover points and a single peak at their correct melting peak and there was no primer dimers present like that seen in the black curve which represents primer T1F2:T1R2.

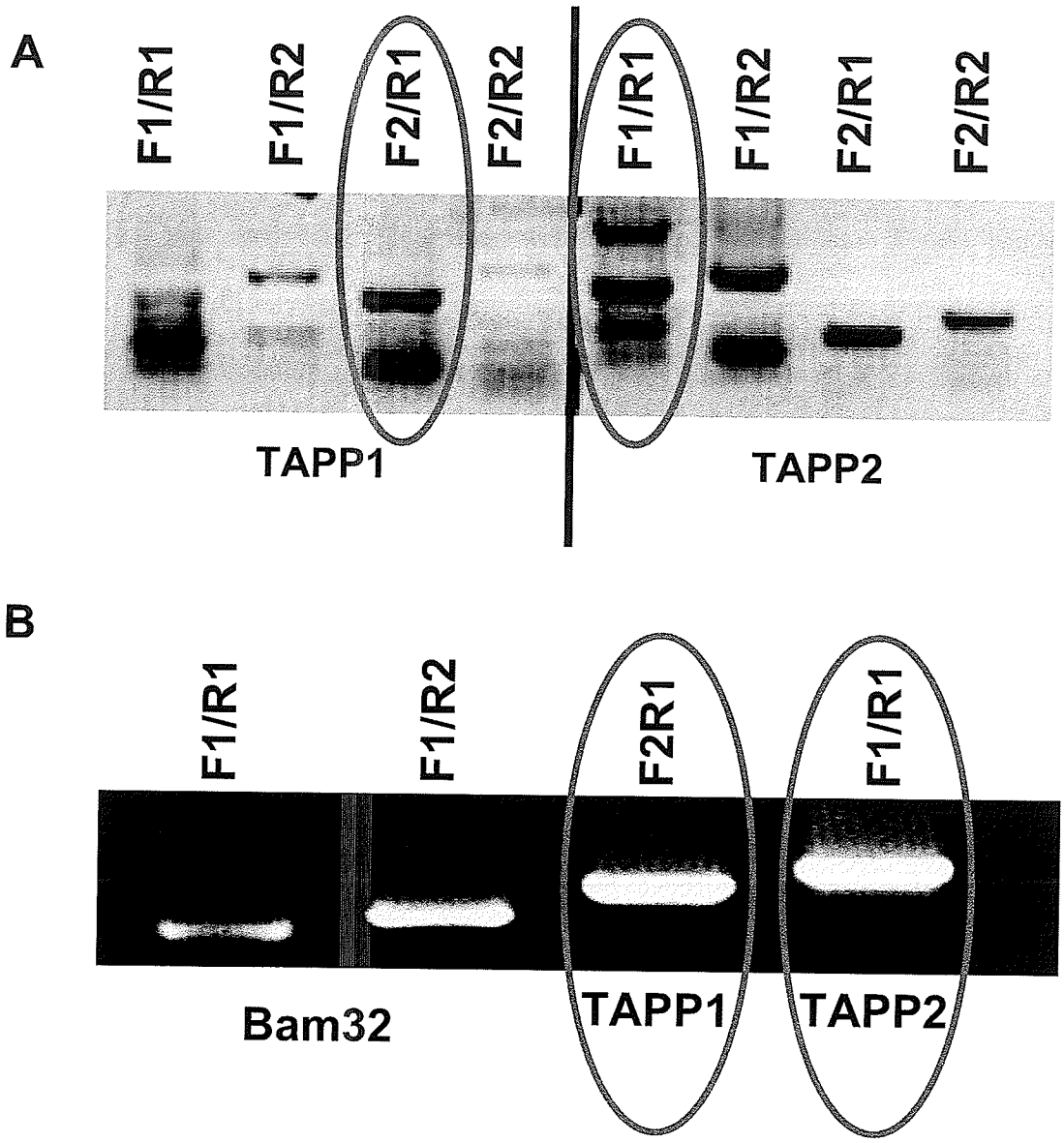


Figure 6: TAPP1 and TAPP2 Agarose Gel for Primer Optimization. The products from the real-time RT-PCR were run for 1 hour on a 1% agarose gel. A) Primers chosen for TAPP1 and TAPP2 are the following: TAPP1 F2/R1 and TAPP2 F1/R1. Using the agarose gel, it allowed us to determine if the primers had a single band at the correct molecular weight for that primer. B) Through increase of the annealing temperature from 57° to 59°, I was able to eliminate the background bands that appear in the upper gel photo.

b) Expression of PI3K signaling components in Various Cell Lines

The best primer combinations were then tested using various cell lines, such as Jurkat, a T cell line, Ramos and BJAB B cell lines and FDC-1, a non-hematopoietic adherent stromal cell line. These cell lines were used to confirm that primers can detect quantitative differences in gene expression. RNA was extracted from these cells using Trizol RNA isolation method, and real-time RT-PCR assays were performed. Bam32 expression is known to be restricted to B cells (194-196). I was able to find that Bam32 expression was significant in BJAB and Ramos, whereas Jurkat and FDC-1 cell lines had no expression (Figure 7a). This result correlated well with what is known about Bam32 expression and was a good indication that this primer combination can accurately detect Bam32 expression levels. Zap70 is known to be expressed in T cells but not B cells (136). This correlated with my findings since Jurkat, a T cell line, had a significantly higher expression than the other cell lines. The other cell lines showed little to no expression of Zap70 (Figure 7b). SHIP1 is known to have restricted expression to hematopoietic cells which was confirmed by real-time RT-PCR since there was no expression of in FDC-1 which is a non-hematopoietic cell line (Figure 7c) (21, 23). SHIP2 is known to be more widely expressed, and this was shown through its expression in all cell lines (Figure 7d) (23). My results indicated that TAPP1 is expressed in BJAB B cells, Jurkat T cells and non-hematopoietic FDC-1 cells (Figure 7e), which correlates with previously published work that TAPP1 is widely expressed and is found in most lymphoid tissues (34, 37). The low expression of TAPP1 in the Ramos B cell line is unexpected. B cells appear to have a greater expression of TAPP2 (Figure 7f), consistent with our previous study showing that it is highly expressed in spleen tissue (34, 37).

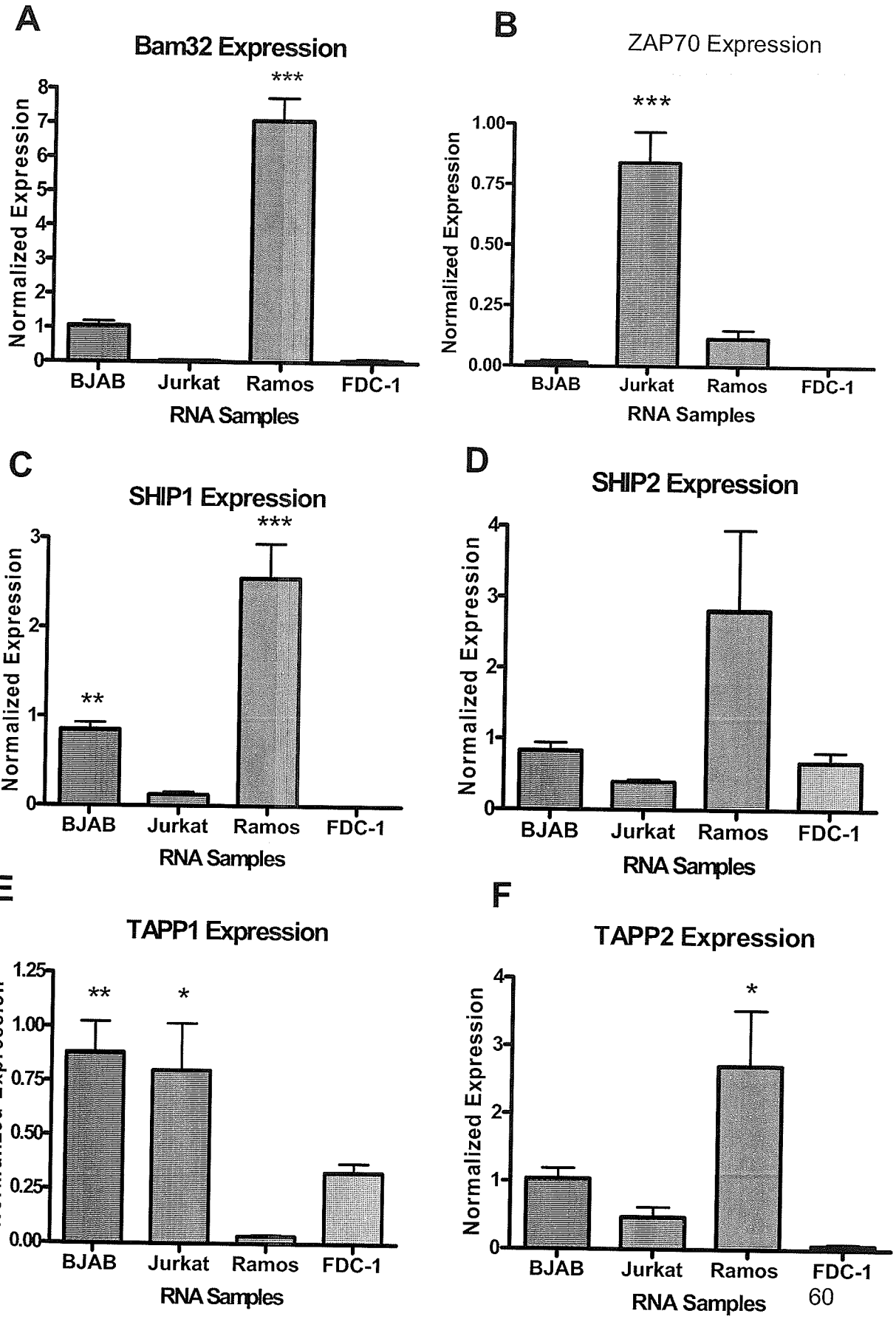

A
Bam32 Expression
Normalized Expression
***
BJAB
Jurkat
Ramos
FDC-1
RNA Samples
B
ZAP70 Expression
Normalized Expression
***
BJAB
Jurkat
Ramos
FDC-1
RNA Samples
C
SHIP1 Expression
Normalized Expression
**
***
BJAB
Jurkat
Ramos
FDC-1
RNA Samples
D
SHIP2 Expression
Normalized Expression
BJAB
Jurkat
Ramos
FDC-1
RNA Samples
E
TAPP1 Expression
Normalized Expression
**
*
BJAB
Jurkat
Ramos
FDC-1
RNA Samples
F
TAPP2 Expression
Normalized Expression
*
BJAB
Jurkat
Ramos
FDC-1
RNA Samples

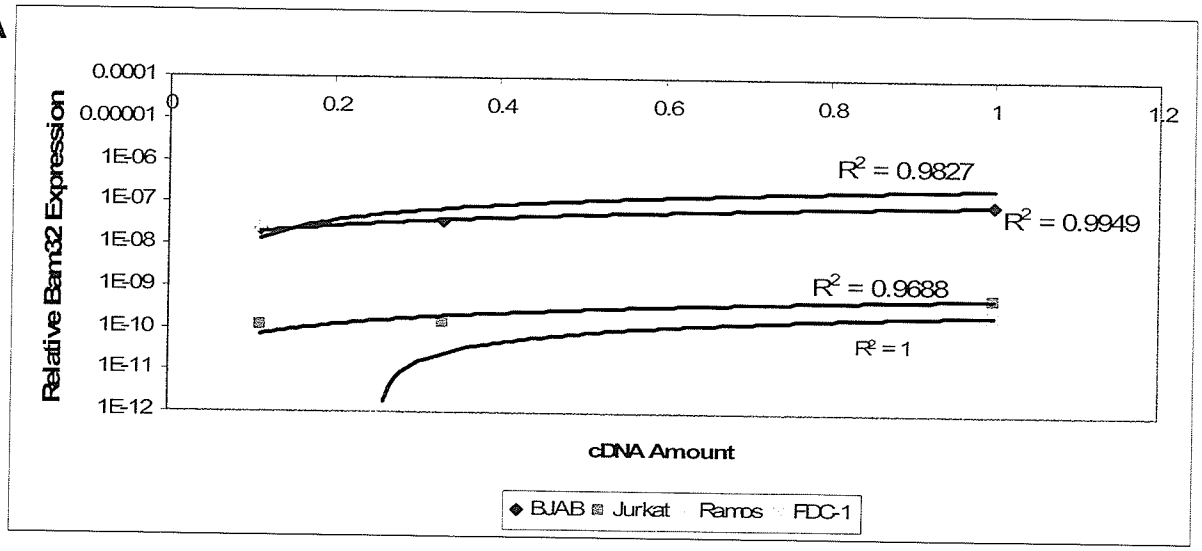
Figure 7: Validation of Real-Time RT-PCR Assays Using Cell Lines. Expression of PI3K pathway components was determined by using BJAB (B cell lymphoma cell line), Ramos (B cell line), Jurkat (T cell line) and FDC-1 (non-hematopoietic cell line) through the use of real-time RT-PCR. RNA from cell lines was extracted using Trizol method and RNA expression was determined using real-time RT-PCR. (A) Bam32 expression (B) ZAP70 Expression (C) Expression of SHIP1 (D) SHIP2 expression (E) TAPP1 Expression (F) TAPP2 Expression. These graphs are representative of 3 independent cDNA using the same RNA. T test revealed the following P-values *** $p< 0.0001$ ** $p<0.05$, * $p<0.01$

Repeat assays of cell line RNA samples gave similar results indicating good run to run reproducibility.

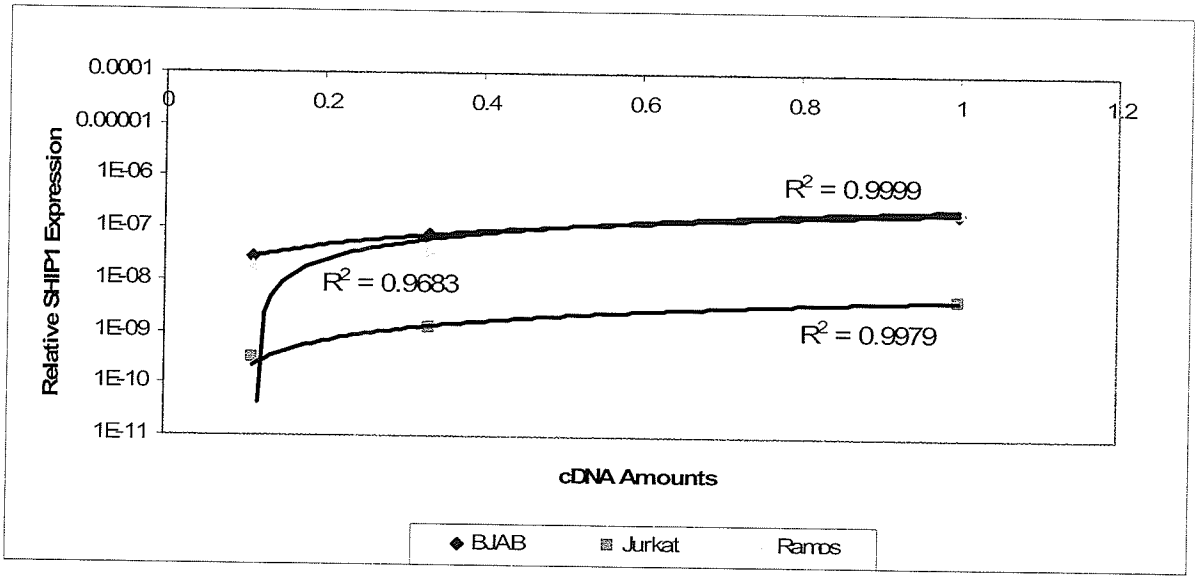
c) cDNA Dilution to Determine Quantitation of Primers

cDNA from BJAB, Ramos, Jurkat and FDC-1 cell lines was diluted with RNase free water in order to determine the ability of real-time RT-PCR assays to quantitate their specific target gene. It would be expected that with dilution of the cDNA, it would lead to a proportional decrease in the amplification of the cDNA in LightCycler assays. I was able to show that with increased dilution there was a decrease in amplification leading to later crossover points in the more diluted cDNA samples as compared to those that were undiluted. Through the use of a scatterplot, the R^2 value was greater than 0.9 in almost every sample. This is an indication of a linear relationship between the cDNA amount and the amplified product detected therefore showing that there was good quantitation (Figure 8).

A


Relative Bam32 Expression
0.0001
0.00001
1E-06
1E-07
1E-08
1E-09
1E-10
1E-11
1E-12
0
0.2
0.4
0.6
0.8
1
1.2
R2 = 0.9827
R2 = 0.9949
R2 = 0.9688
R2 = 1
cDNA Amount
BJAB
Jurkat
Ramos
FDC-1

B


Relative SHIP1 Expression
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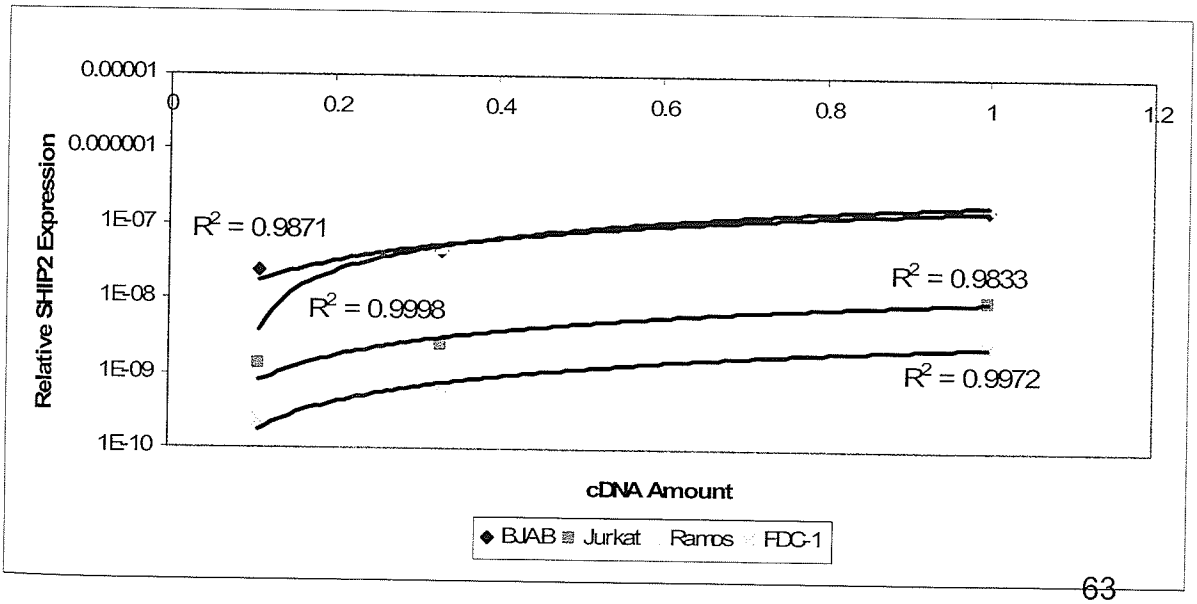
0.00001
1E-06
1E-07
1E-08
1E-09
1E-10
1E-11
0
0.2
0.4
0.6
0.8
1
1.2
R2 = 0.9999
R2 = 0.9683
R2 = 0.9979
cDNA Amounts
BJAB
Jurkat
Ramos

C


Relative SHIP2 Expression
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0.000001
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1E-08
1E-09
1E-10
0
0.2
0.4
0.6
0.8
1
1.2
R2 = 0.9871
R2 = 0.9998
R2 = 0.9833
R2 = 0.9972
cDNA Amount
BJAB
Jurkat
Ramos
FDC-1

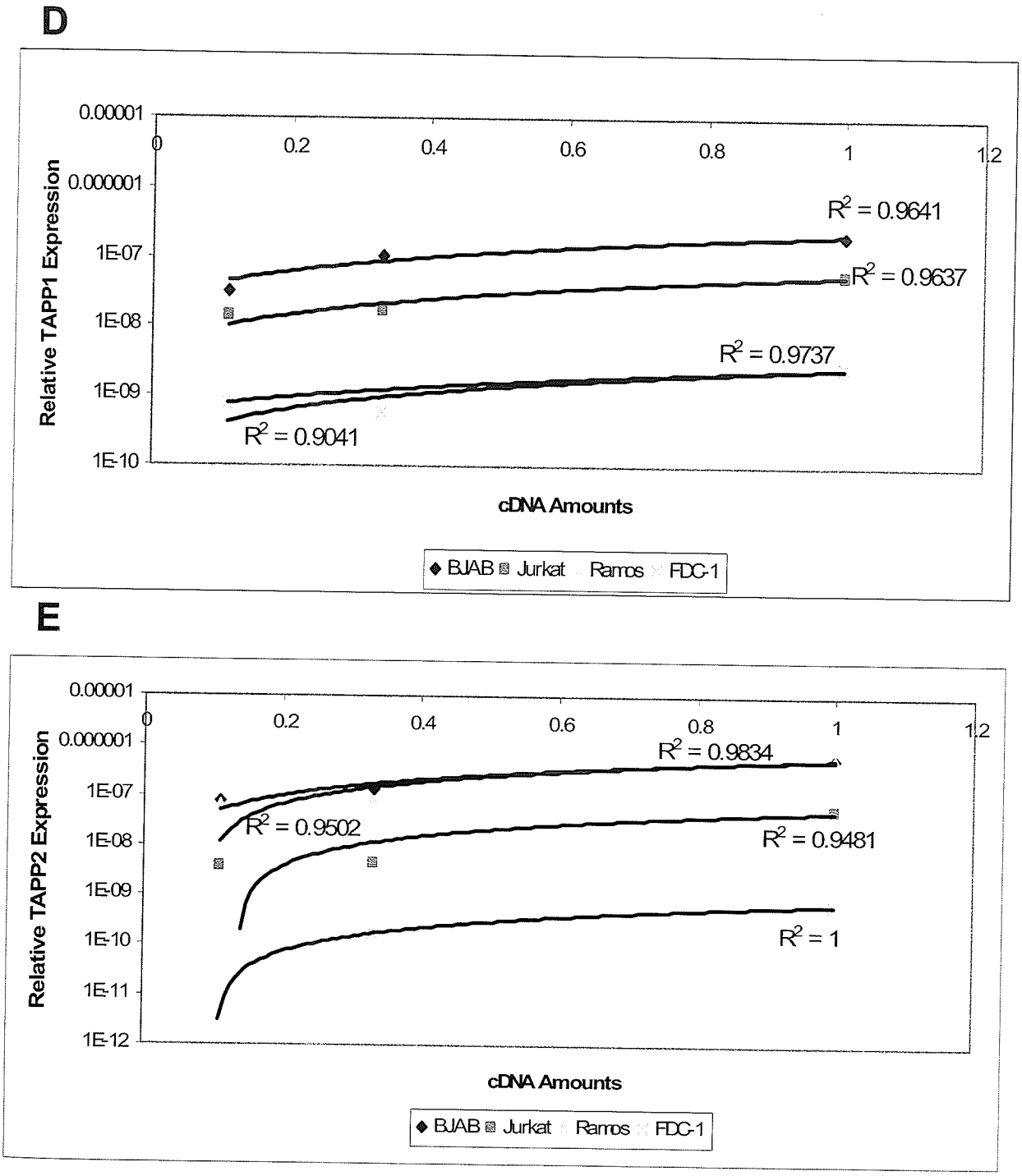


Figure 8: Serial Dilution to Determine Primer Sensitivity with Real-Time RT-PCR. cDNA was undiluted, 1/3 dilution or 1/9 dilution and PI3K components were detected using real-time RT-PCR. Those samples that were undiluted had lower crossover points compared to the more diluted cDNA samples. This was another way of indicating primer senstivity. (A) Bam32 expression, (B) SHIP 1 expression, (C) SHIP2 Expression, (D) TAPP1 Expression, (E) TAPP2 Expression. These graphs are representative of 3 independent cDNA from the same RNA.

d) RNA Expression of PI3K Components in Chronic Lymphocytic Leukemia and Normal B Cells

After real-time RT-PCR assays were validated using cell lines I went on to determine PI3K component expression in CLL and normal B cell samples. A set of patient samples were obtained from the Manitoba CLL Bank. The samples consisted of B cells isolated from whole blood using ficoll separation and a B cell enrichment kit if required (see Methods for detail). Cells were then stored frozen as cell pellets. Normal B cells were isolated from healthy donors using ficoll and B cell isolation cocktail, or were obtained as frozen samples in DMSO medium from the CLL bank. RNA was extracted using the Trizol method as done for the cell lines and the same procedures were followed. However, we found that reproducible results could not be obtained with these samples. Figure 9 shows TAPP2 expression as an example, illustrating that run to run reproducibility was poor. The reproducibility from run to run was different as seen in CLL 7 for example. The first and third runs showed high expression of TAPP2 (18 and 50 normalized expression values) but the second run then indicated that the expression in this sample was low (expression value of 1). Similar variability was seen for CLL 4, 8 and 9, where they are not showing the same relative expression levels from one run to the next. As a result of not being able to get convincing evidence of expression levels for any of the PI3K pathway components, I decided that western blot might be a more convincing approach.

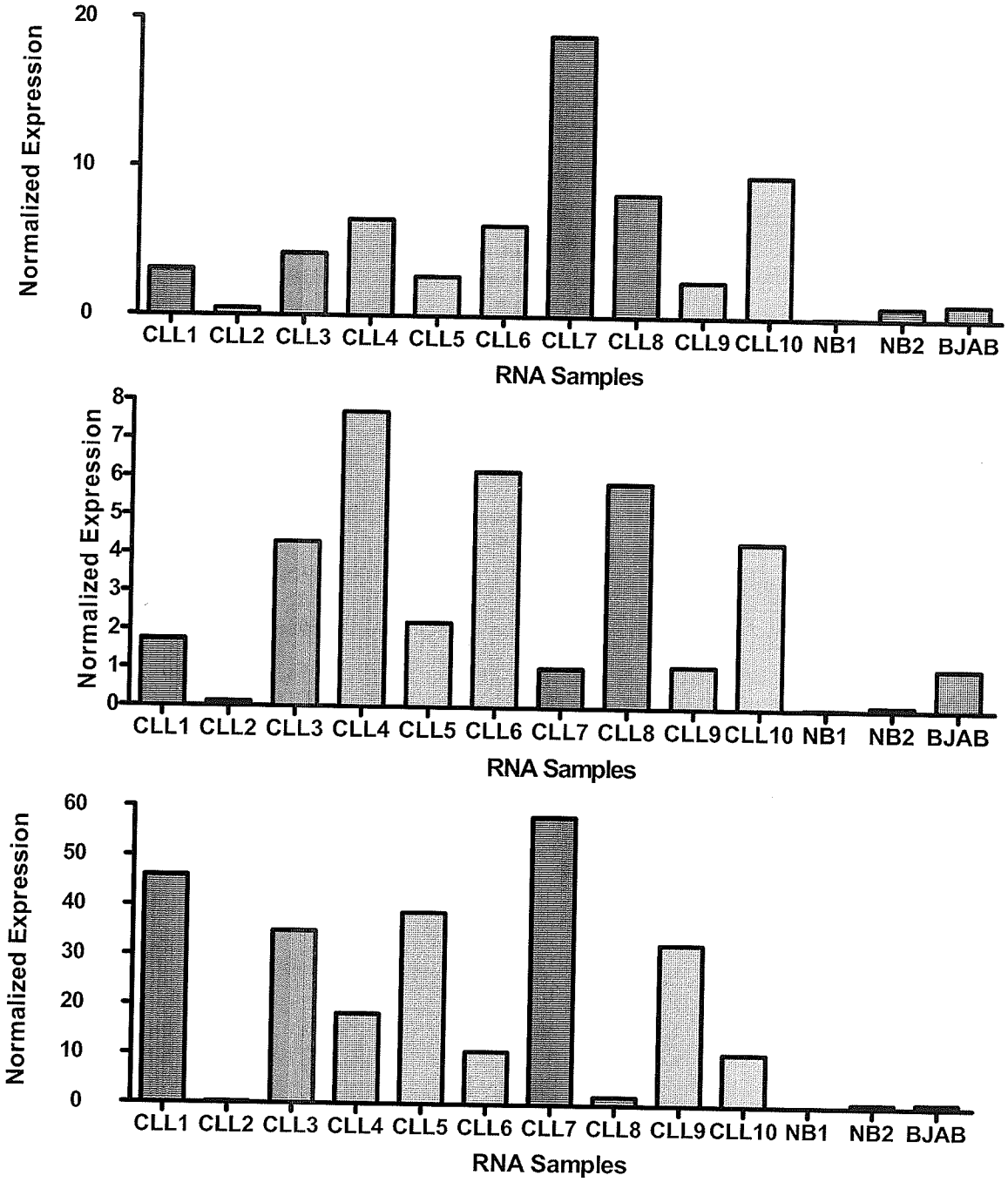


Figure 9: Real time RT-PCR reproducibility with CLL Samples. RNA was isolated from CLL pellets using Trizol RNA isolation method and real-time RT-PCR was run to determine PI3K component expression in CLL and normal B cell samples. All data was normalized to G3PDH which was our housekeeping gene. This data is representative of 3 independent cDNA from the same RNA. TAPP2 expression is shown as an example illustrating the lack of reproducibility in the measured expression. CLL1, 4, 6, 7 and 8 showed high expression in one run of TAPP2 expression and then low expression in another run.

e) Protein expression of PI3K pathway components

CLL samples were obtained as frozen pellets and cells were lysed in NP40 lysis buffer to extract proteins. Protein concentrations were determined through the use of Bradford assay, allowing even loading of samples. The amount of protein loaded ranged from 5-10 ug depending on which PI3K pathway component was being detected. The protein amounts used for each PI3K component were chosen based on sensitivity of being able to detect sample without saturating or using too little of the protein. CLL protein samples were loaded on SDS-PAGE gels and run, blotted and detected by appropriate antibodies. TAPP2 is used as an example here to show that reproducible differences in expression could be determined by Western blotting. Each independent blot shows the same pattern of samples being TAPP2 high or TAPP2 low (Figure 10). As a result of this reproducibility, PI3K components expression was determined through the use of Western Blots.

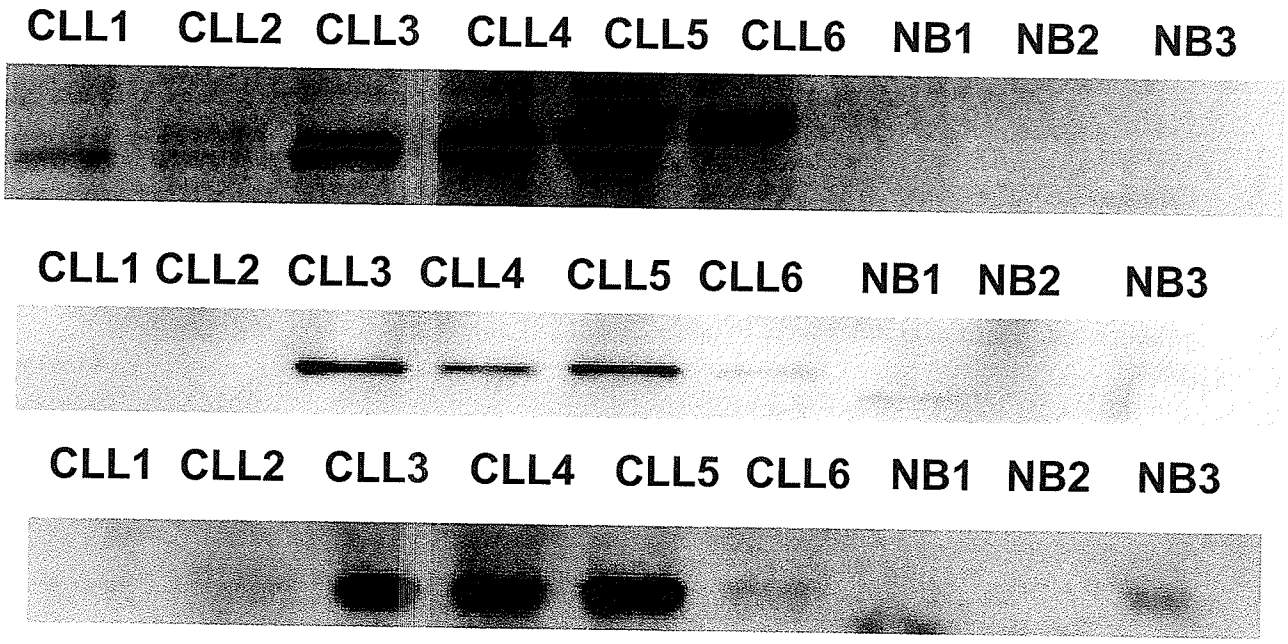


Figure 10: Western Blot Reproducibility with CLL Samples. CLL protein samples were isolated from cell pellets that were obtained from the CLL bank using NP40 lysis buffer. Protein concentrations were determined using Bradford assay and 5 ug of protein was loaded into SDS-PAGE gel in order to determine TAPP2 expression in CLL and normal B cells. These blots are representative of 3 runs of the same protein samples and reproducibility was obtained. In each blot, CLL3-5 have a higher concentration than normal B cells and CLL1,2 and 6 have little or no expression of TAPP2. As a result of this reproducibility expression of PI3K components in CLL and normal B cells was carried out using Western blots.

f) Chronic Lymphocytic Leukemia and Normal B Cell Protein Expression of Various PI3K Components

CLL samples were obtained from the Manitoba CLL bank in pellet form and protein was isolated using NP40 lysis buffer as discussed in the methods section. Actin expression was used as a housekeeping gene for normalization of protein loading (Figure 11). TAPP2 expression was determined and it appears that there is a subset of CLL samples that have robust expression of TAPP2 compared to normal B cell samples, which have relatively low TAPP2 expression (Figure 12). Zap70 expression is normally not found to be in B cell but is found in T cells. CLL samples that express ZAP70 are usually indicated as having a more aggressive form of the disease (134, 136, 164). My Western Blots confirm that there is a subset of our CLL patients that have a high expression of ZAP70, while the other samples have little or no expression (Figure 13).

PTEN is known to be mutated in many cancers. However, there was only one study looking at PTEN in CLL cells, which found no PTEN mutations at the DNA level but almost half of their patients showed no or little expression of PTEN at the protein level (149). Through Western blots, I was able to show that a few of our patients analyzed had little or no expression of PTEN, while a few of them had high expression of PTEN (Figure 14). Expression of another phosphoinositide phosphatase SHIP had not been analyzed in CLL, so I examined its expression in our patient samples. My results indicated that SHIP expression is more variable than PTEN, and seems to be overexpressed in a subset of CLL patients when compared to the normal B cell controls (Figure 15).

For quantitative analysis, chemiluminescence images were analyzed using Fluorchem software to determine the band intensity for each sample. I determined the band intensity by drawing a square around each band and then a square in the background. Through linking to my squares around the bands to the one made for the background I was able to eliminate the background and getting a reading of the band intensity. All samples were then normalized to Actin band intensities in order to indicate relative expression among CLL and normal B cell samples. A non parameter T test using Mann Whitney showed that there was no significant difference between TAPP2, Zap70, PTEN and SHIP expression in CLL versus Normal B Cell samples (Figure 16). The median expression of both CLL and normal B cell samples for TAPP2, ZAP70, PTEN and SHIP are around 1 arbitrary units (Figure16).

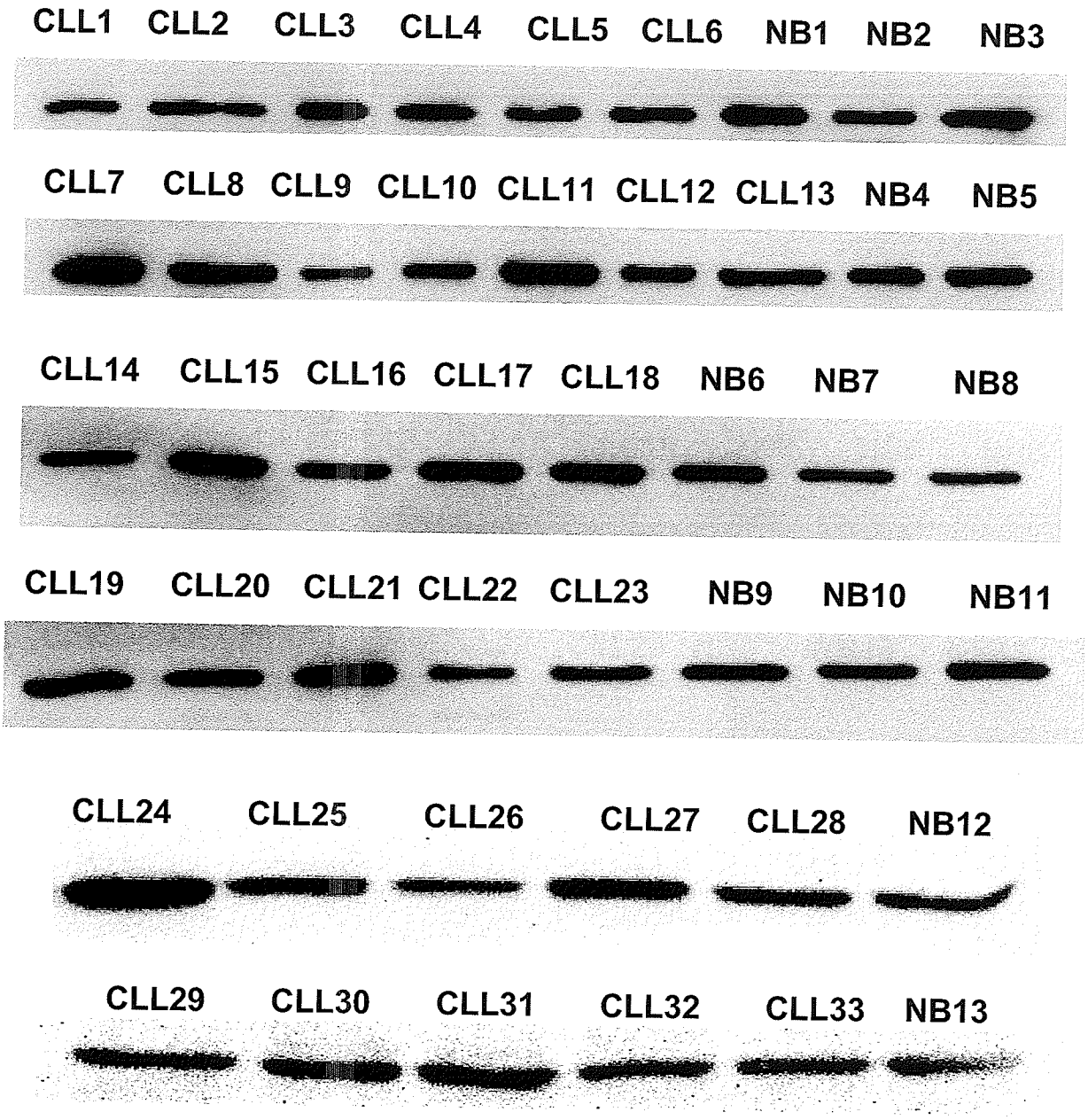


Figure 11: Actin Expression in Chronic Lymphocytic Leukemia and Normal B Cells. Actin expression in evenly distributed among all samples and was used to normalized housekeeping gene. These blots are representative of 3 independent experiments.

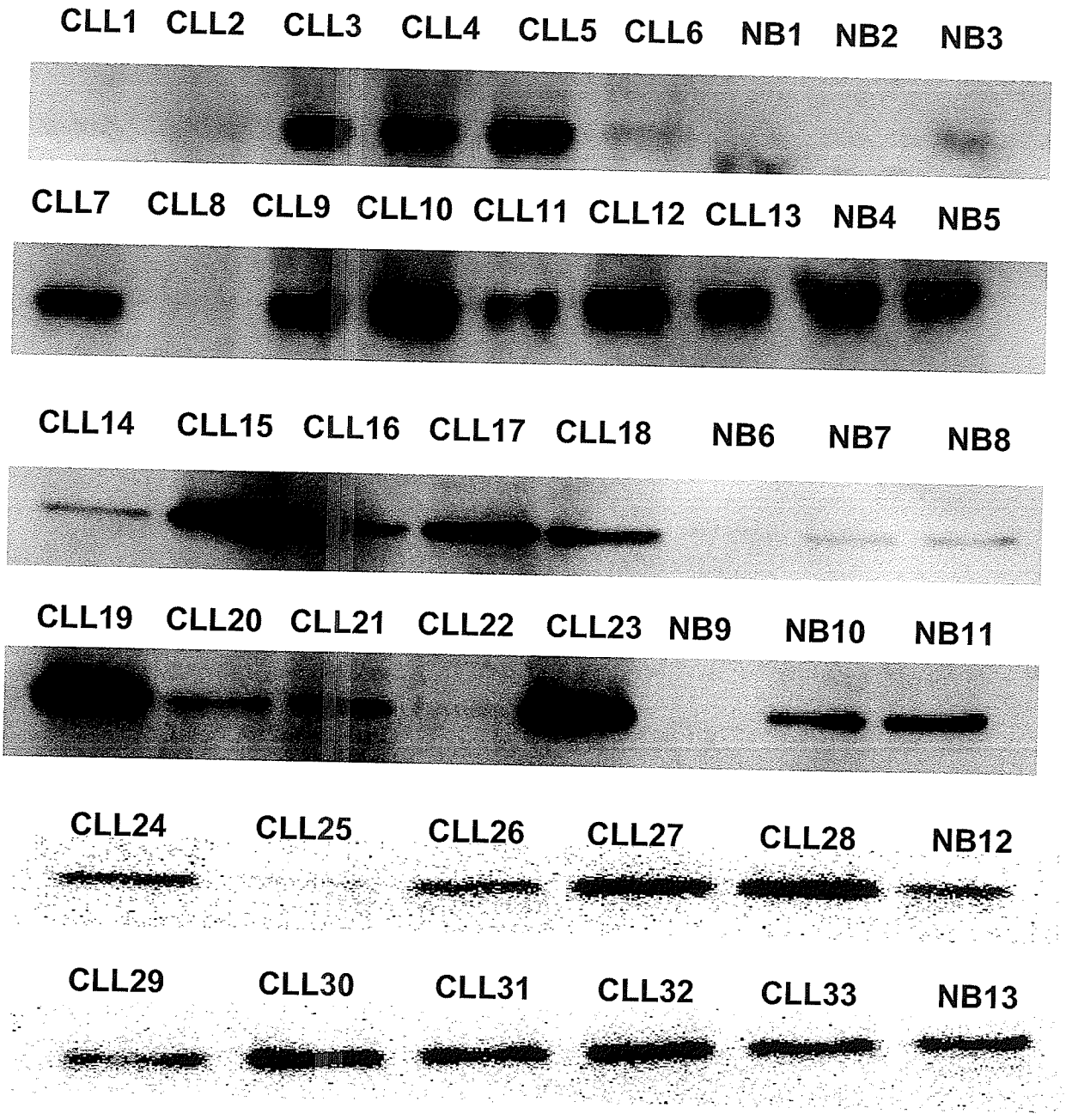


Figure 12. TAPP2 Expression in Chronic Lymphocytic Leukemia and Normal B Cells. CLL samples and normal B cells samples were isolated from whole blood using ficoll and B cell isolation cocktail. Cell lysates were made using NP-40 lysis buffer and then 5 ug protein were run on SDS-PAGE gel. TAPP2 expression was detected using α-TAPP2 antibody. These blots are representative of 3 independent experiments. Through quantification, there are a subset of CLL samples that have an overexpression of TAPP2 compared to normal B cell samples.

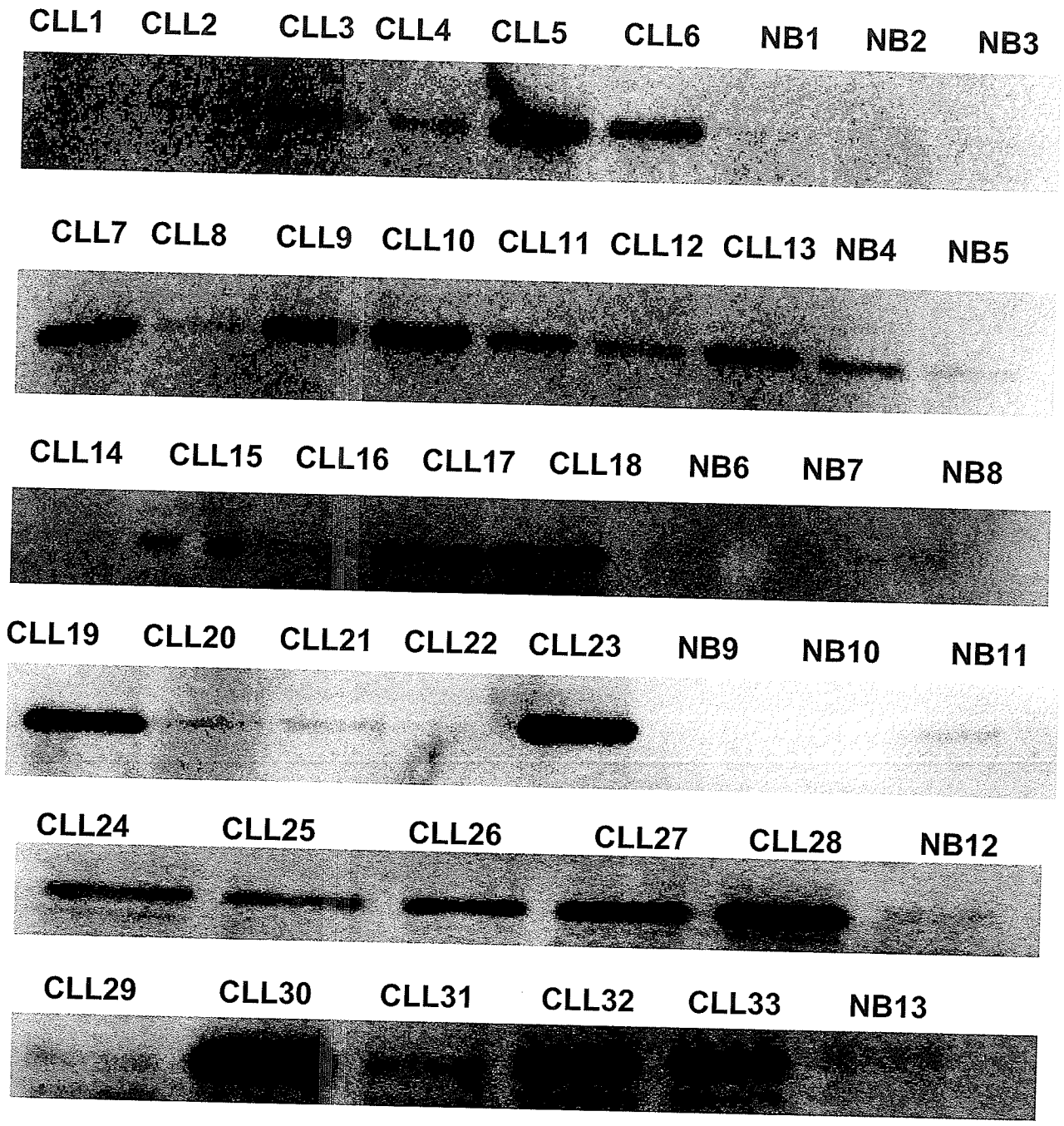


Figure 13: ZAP70 Expression in Chronic Lymphocytic Leukemia and Normal B Cells. CLL samples and normal B cells samples were isolated from whole blood using ficoll and B cell isolation cocktail. Cell lysates were made using NP-40 lysis buffer and then 5 ug protein were run on SDS-PAGE gel. ZAP70 expression was detected using α-ZAP70 antibody. These blots are representative of 3 experiments. Through quantification, there are a subset of CLL samples that have an overexpression of ZAP70 compared to normal B cell samples. Samples high in ZAP70 could be an indication of a more aggressive form of CLL.

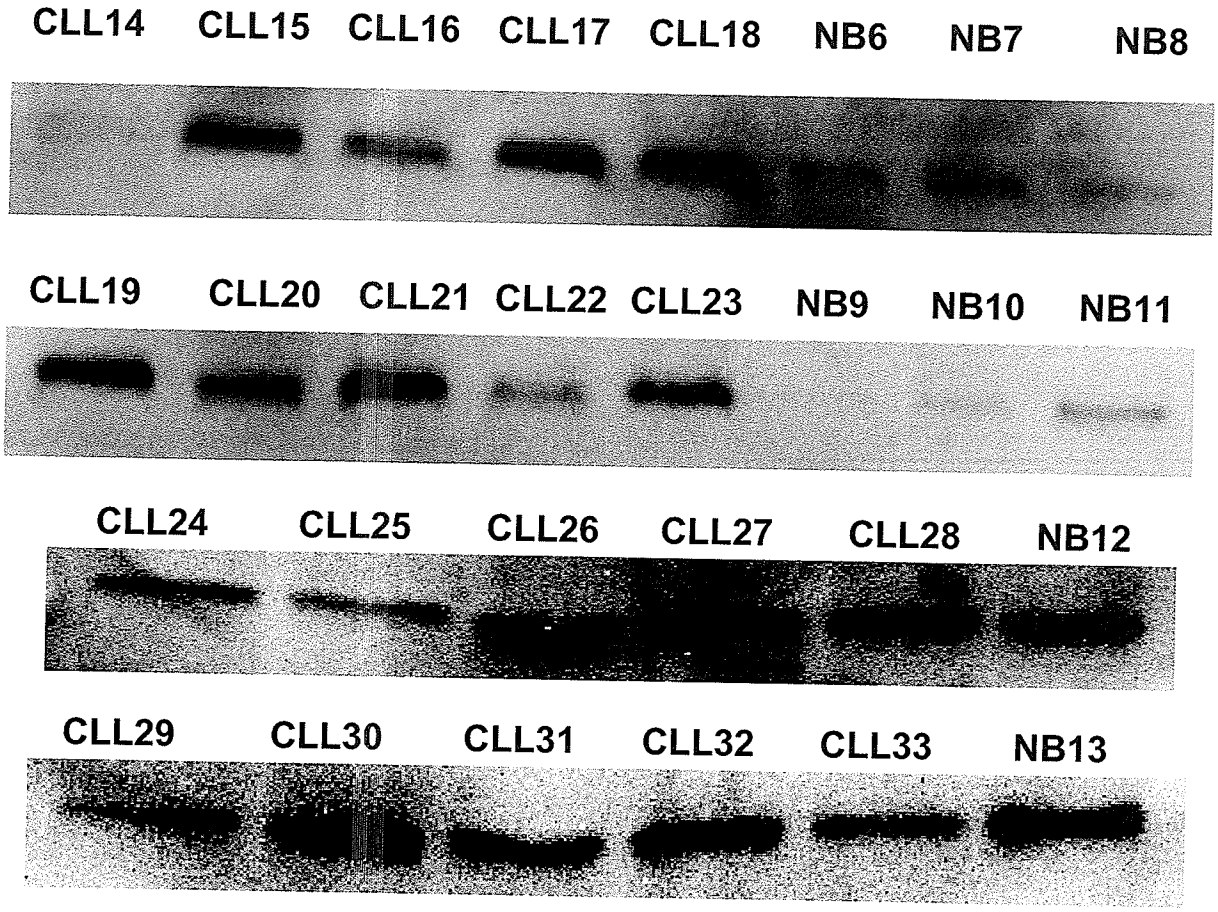


Figure 14: PTEN Expression in Chronic Lymphocytic Leukemia and Normal B Cells. CLL samples and normal B cells samples were isolated from whole blood using ficoll. Cell lysates were made using NP-40 lysis buffer and then 10 ug protein were run on SDS-PAGE gel. PTEN expression was detected using α-PTEN antibody. These blots are representative of 3 independent experiments. Through quantification, PTEN is strongly expressed among some of the CLL samples and it appears that PTEN has little or no expression in the other patients.

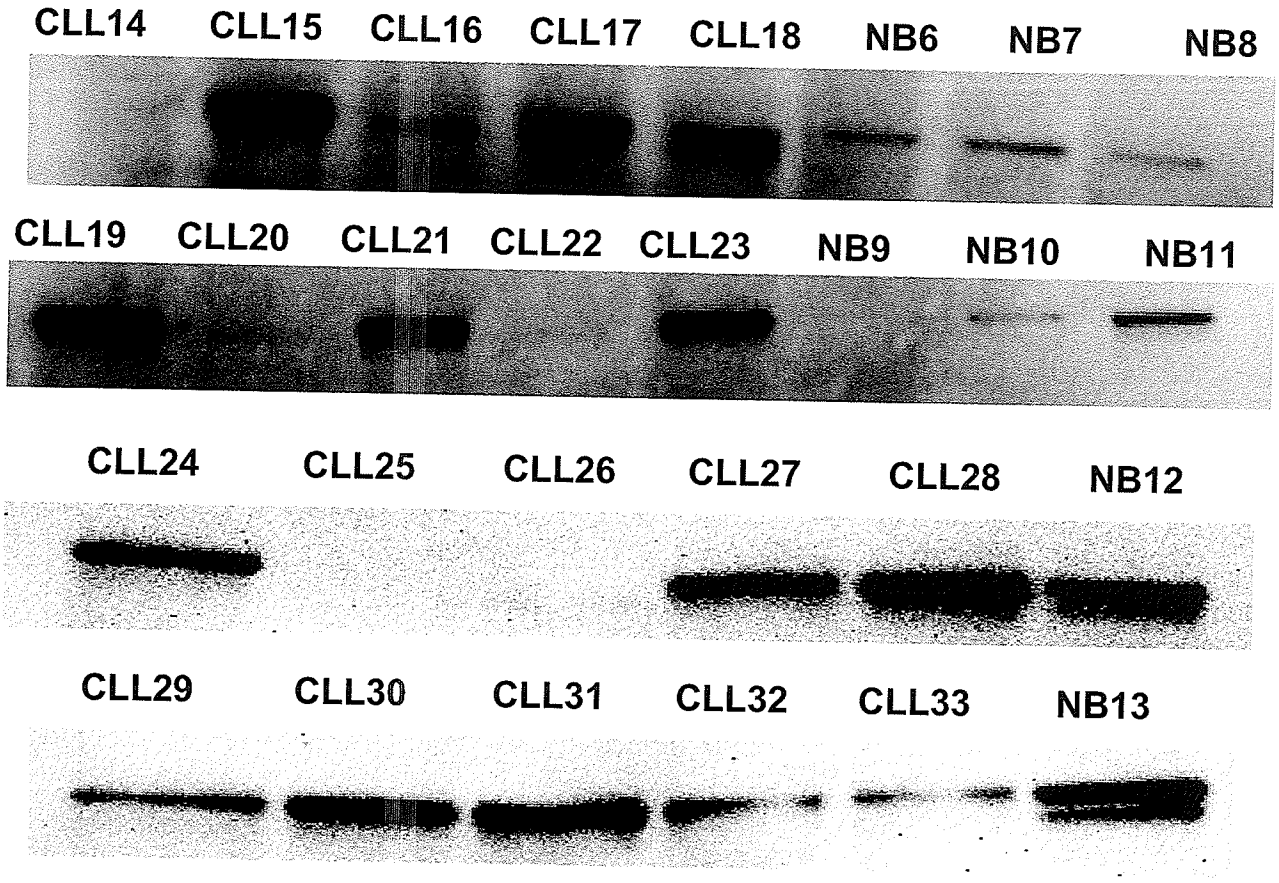


Figure 15: SHIP Expression in Chronic Lymphocytic Leukemia and Normal B Cells. CLL samples and normal B cells samples were isolated from whole blood using ficoll and B cell isolation cocktail. Cell lysates were made using NP-40 lysis buffer and then 10 ug protein were run on SDS-PAGE gel. SHIP expression was detected using α-SHIP antibody. These blots are representative of 3 independent experiments. Through quantification, there are a subset of CLL samples that have an overexpression of SHIP compared to normal B cell samples.

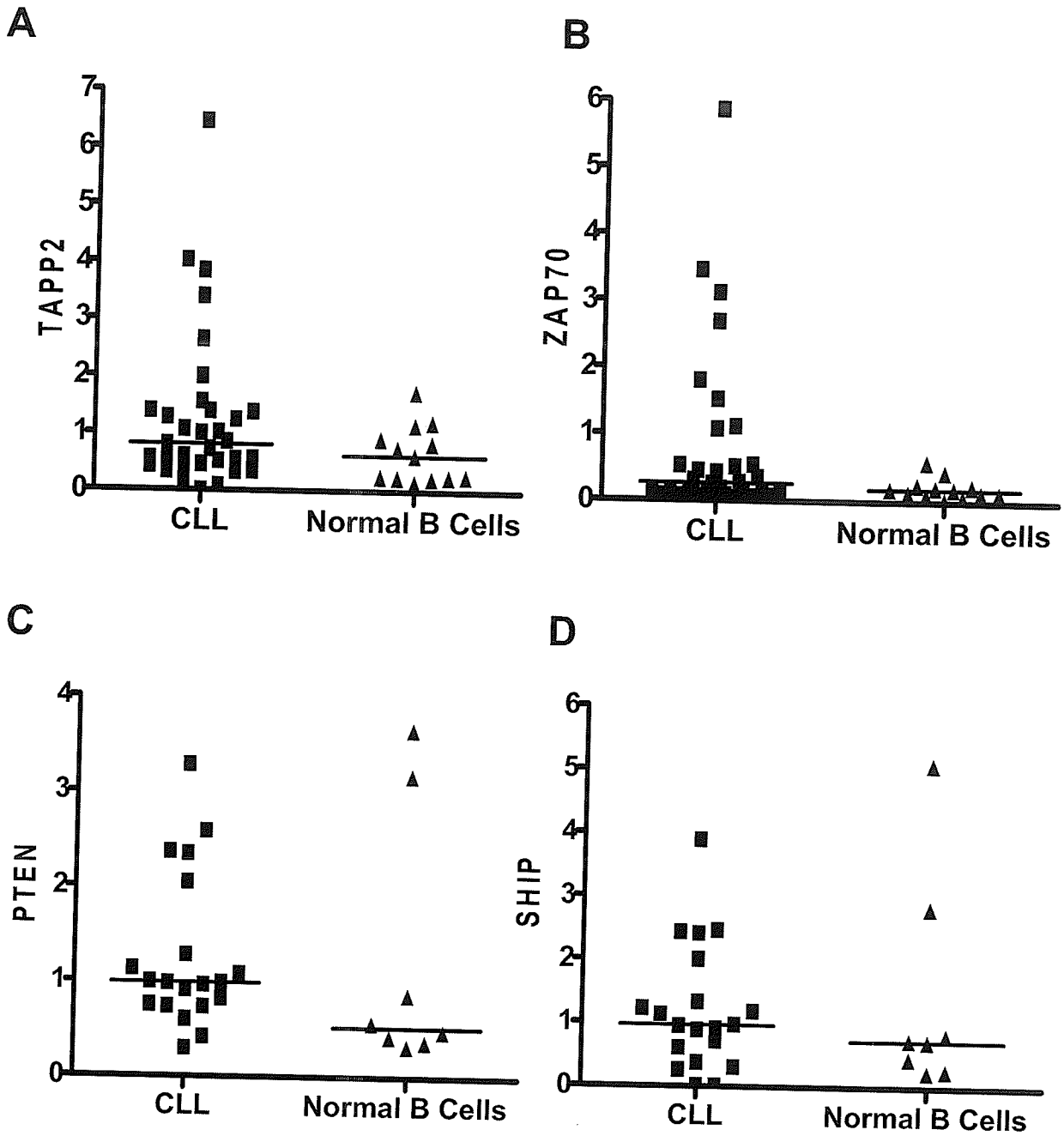


Figure 16: Normalized Expression of Normal B Cells and Chronic Lymphocytic Leukemia. Each sample was quantified using Fluorchem software and normalized to housekeeping gene β-Actin. TAPP2, ZAP70, PTEN and SHIP normalized expression was then determined in CLL samples vs normal B cell samples. TAPP2 and ZAP70 had significantly higher expression in CLL than in normal B cells (A,B). However, SHIP and PTEN seemed to have a evenly distributed expression in CLL than normal B cells but does not seem to be significant (C,D). These figures are pooled data from 4 independent experiments. A nonparameteric T test reveals no significant difference between CLL and Normal B Cell samples.

g) Correlation of ZAP70 and Various PI3K Components in Chronic Lymphocytic Leukemia

We examined whether the relative expression of PI3K components correlated with ZAP70 expression. As a result of the correlation graphs, ZAP70 and TAPP2 have the strongest correlation (66%). Therefore, TAPP2 high CLL samples correlate with ZAP70 high patients and the same is seen with the low expressing samples (Figure 17). Both PTEN and SHIP, although they usually correlate in Western blots, displayed a low r^2 value compared to TAPP2 (16-30%) (Figure 17). The Manitoba CLL Bank determines the IgV_H mutational status of each patient and we then went on to determine its correlation with ZAP70. It is known that ZAP70 is clinical prognostic marker for CLL patients correlating with unmutated IgV_H genes. Our results show that most patients that have mutated IgV_H and do not express Zap70 through Western blot, however two "outlier" patients with mutations of their IgV_H genes showed high expression of ZAP70. This may be due to T cell contamination in these samples. In contrast, most CLL samples with unmutated IgV_H did have detectable ZAP70. However, there is no significant difference between these categories in average Zap70 expression (Figure 18).

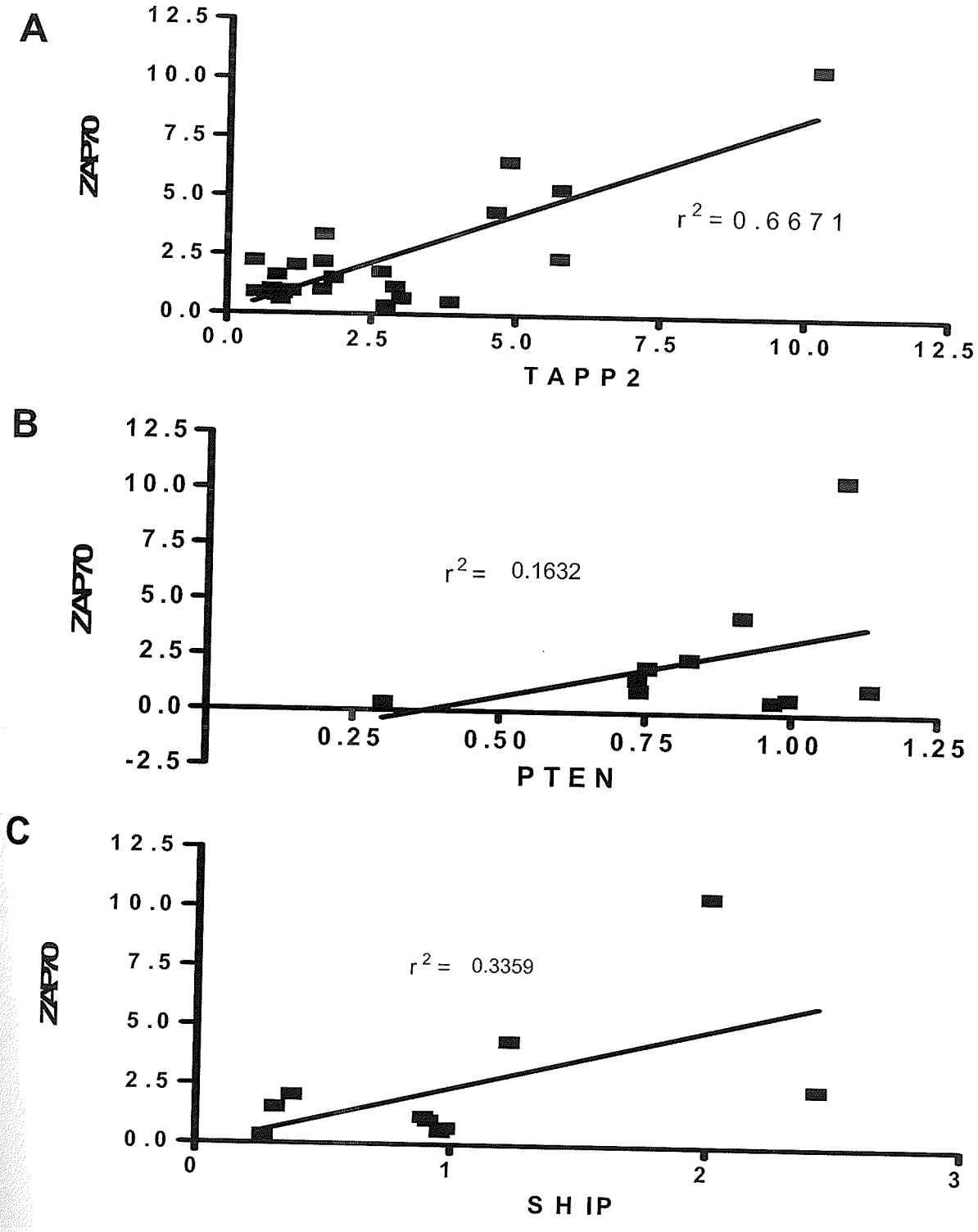


Figure 17: ZAP70 correlation with various PI3K components. These are the correlation graphs of ZAP70 with TAPP2, PTEN and SHIP. TAPP2 seems to have the highest correlation to ZAP70 compared to the other 2 PI3K components. In most cases, if a CLL patient was high for TAPP2 it also expressed ZAP70 and the same was seen for low TAPP2 and ZAP70.

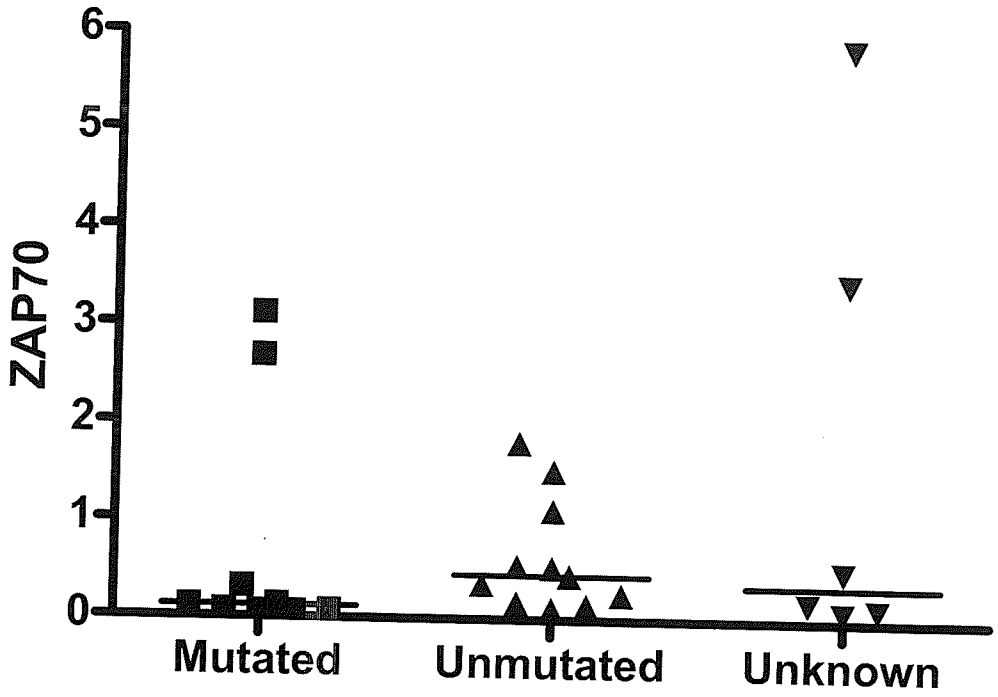
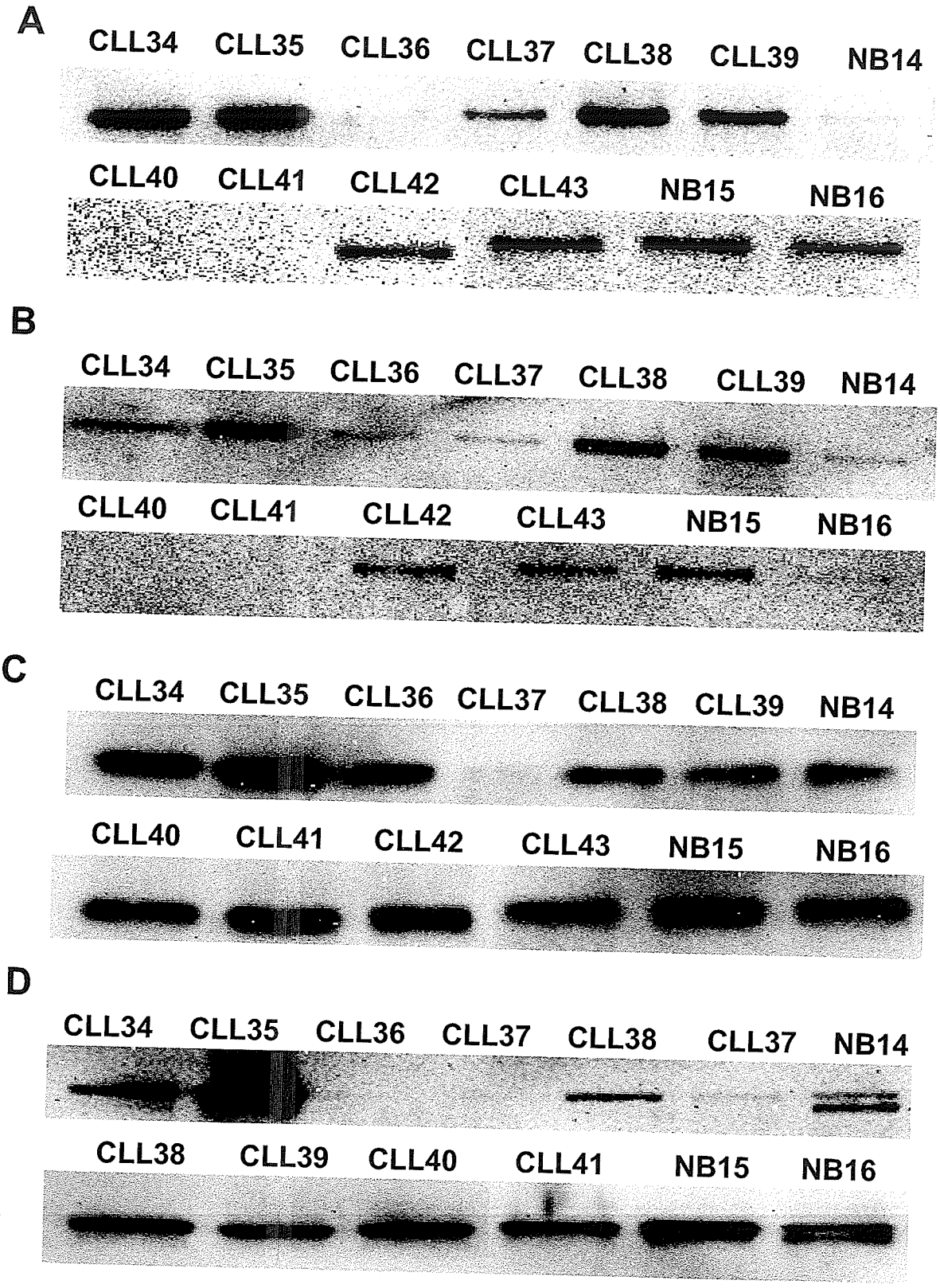


Figure 18: ZAP70 Expression vs Mutational Status. Patient mutational status was obtained from Manitoba CLL Bank and compared to ZAP70 expression through Western Blots. Figure shows groups divided into mutated, unmutated and unknown status. From the 33 patients that were examined for ZAP70 expression, only 26 of them expressed various levels of ZAP70. There were 7 patients that did not express ZAP70 and there mutational status was mutated. There is no significant difference between either groups. The few outlier in the mutated status could be a result of T cell contamination.

h) Expression of PI3K Components in Fresh CLL and Normal B Cell Samples

Fresh CLL samples were obtained from Manitoba CLL Bank and protein was extracted as mentioned in the above section. Actin expression was used as a housekeeping gene for normalization of protein loading. Actin expression is evenly expressed among all samples (Figure 19). As seen in the frozen cell samples, TAPP2 is overexpressed in a subset of CLL (ie. CLL 34/35/38/39) compared to normal B cell samples. TAPP2 expression was absent in CLL 36, CLL 40, CLL 41 and CLL 43 (Figure 19). I was able to show that a subset of CLL samples had an overexpression of Zap70 (ie. CLL 35/38/39) compared to normal control samples. Zap70 expression was absent in CLL 40, CLL 41 and CLL 43 and is also another indication that these samples have mutated IgV_H genes and will have less aggressive clinical progression. Lastly, NB15 in this blot contains Zap70, this is an indication of T cell contamination and therefore all T cells were not removed during B cell isolation cocktail step (Figure 19).

I was able to show little PTEN expression in some CLL samples (CLL 38 and 39) or even no expression (CLL 37 and CLL 43). However, many CLLs had strong PTEN expression, with some apparently exceeding the normal controls (CLL 34 and CLL 35 for example) (Figure 19). My analysis of SHIP expression indicated a subset of CLL that had an over-expression of SHIP and others that have little to no expression of SHIP (CLL 36/37/39/43) (Figure 19). These patterns of expression for each PI3K pathway components are similar to those seen in the frozen samples (Figure 11-15). There still appears to be a subset of CLL samples that overexpress TAPP2 and ZAP70 and there appears to be a correlation between the two (Figure 12 and 13). As well, some samples


A
CLL34 CLL35 CLL36 CLL37 CLL38 CLL39 NB14
CLL40 CLL41 CLL42 CLL43 NB15 NB16
B
CLL34 CLL35 CLL36 CLL37 CLL38 CLL39 NB14
CLL40 CLL41 CLL42 CLL43 NB15 NB16
C
CLL34 CLL35 CLL36 CLL37 CLL38 CLL39 NB14
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D
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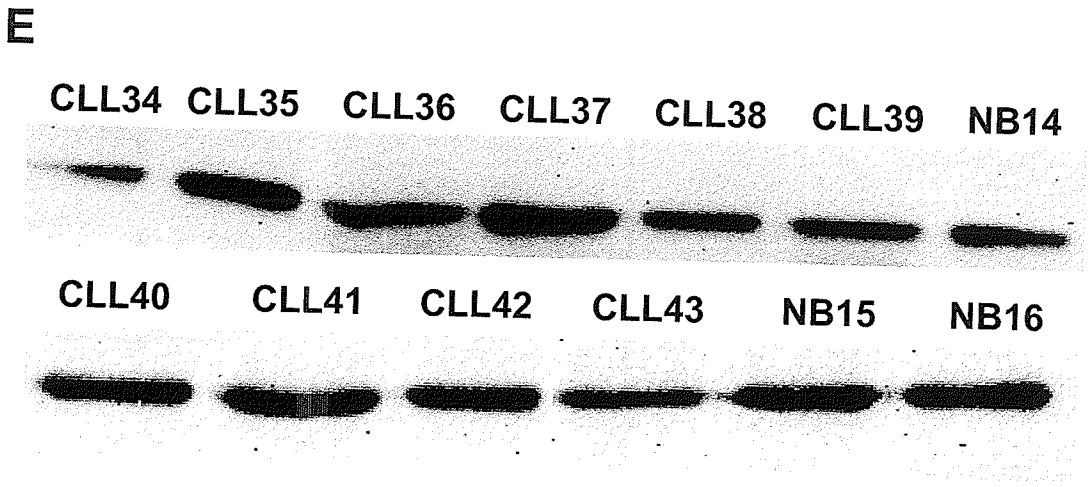


Figure 19: Expression of PI3K Pathway Components in Fresh CLL and Normal B Cells. Fresh CLL samples were obtained from the Manitoba CLL Bank. Cells were lysed in NP40 lysis buffer and run on SDS-PAGE gels. A)TAPP2 Expression B)ZAP70 Expression C) PTEN Expression D) SHIP Expression E) Actin Expression. The blots are representative of 3 independent runs.

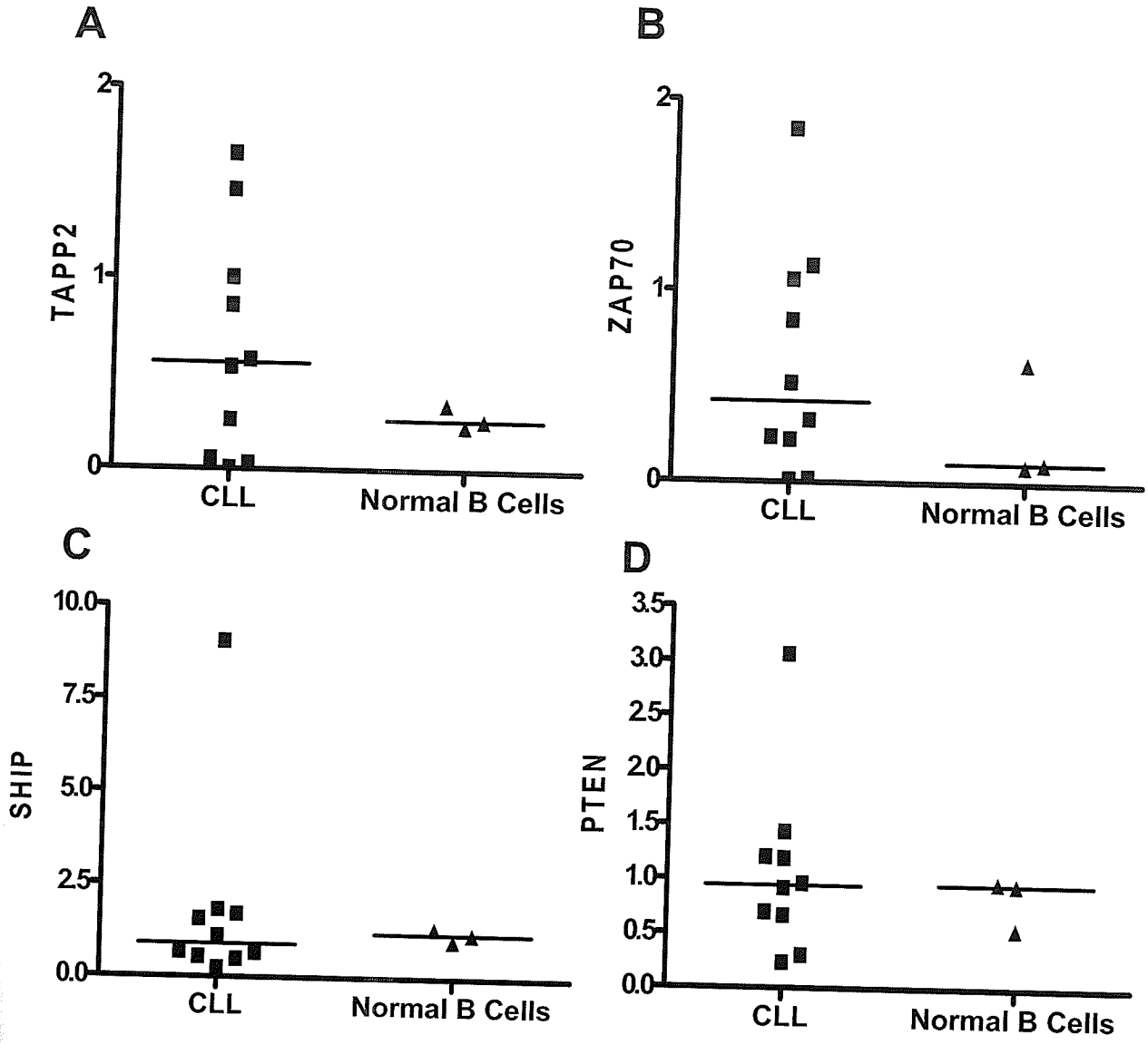


Figure 20: Normalized Expression of Fresh CLL and Normal B Cells. Each sample was quantified using Fluorchem software and normalized to housekeeping gene β-Actin. TAPP2, ZAP70, PTEN, and SHIP normalized expression was then determined in CLL samples vs normal B cell samples. In Figure A-D there is no significant difference between the expression of the PI3K pathaway components in CLL vs normal B cells. These figures are pooled data from 4 independent experiments.

have reduced expression of both PTEN and SHIP like that seen in the frozen samples (Figure 14 and 15).

i) Confocal Imaging of TAPP2 and ZAP70 in CLL Cells

Fresh CLL cells were cultured overnight in medium containing 10% FBS. Cells were then fixed with paraformaldehyde and adhered to slides using cytospin. Slides were stained with anti-TAPP2, DAPI and surface anti-IgM in order to determine TAPP2 expression level and membrane localization using confocal microscopy. In all figures, the blue stain (DAPI) is representative of the nucleus. These cells have a fairly large nucleus and little cytoplasm. Some CLL cells have lost expression of surface IgM. In figure 22, it shows that this CLL sample has high expression of surface IgM, while in figure 21 there is little surface IgM expression. TAPP2 expression is strong and seems to be localized mainly to the cytoplasm.

CLL cells were also stained with TAPP2 only or ZAP70 only in order to determine the expression of TAPP2 and ZAP70 in CLL cells. There was a subset of CLL cells that were brightly stained with anti-TAPP2 while others had dimly stained TAPP2. Examples of four CLL cell samples are shown in Figure 23. Most cells showed predominantly cytoplasmic localization of TAPP2, however some cells did show more concentrated staining at the cell periphery and/or at cell:cell contacts. ZAP70 staining on the other hand was not as bright, but samples did show clear ZAP70 staining, which was also redominantly cytoplasmic (Figure 24). A few samples showed a low frequency of

brightly stained ZAP70+ cells among dimly expressing CLL cells. This could be a possible indication of T cell contamination in those CLL samples (Figure 24b).

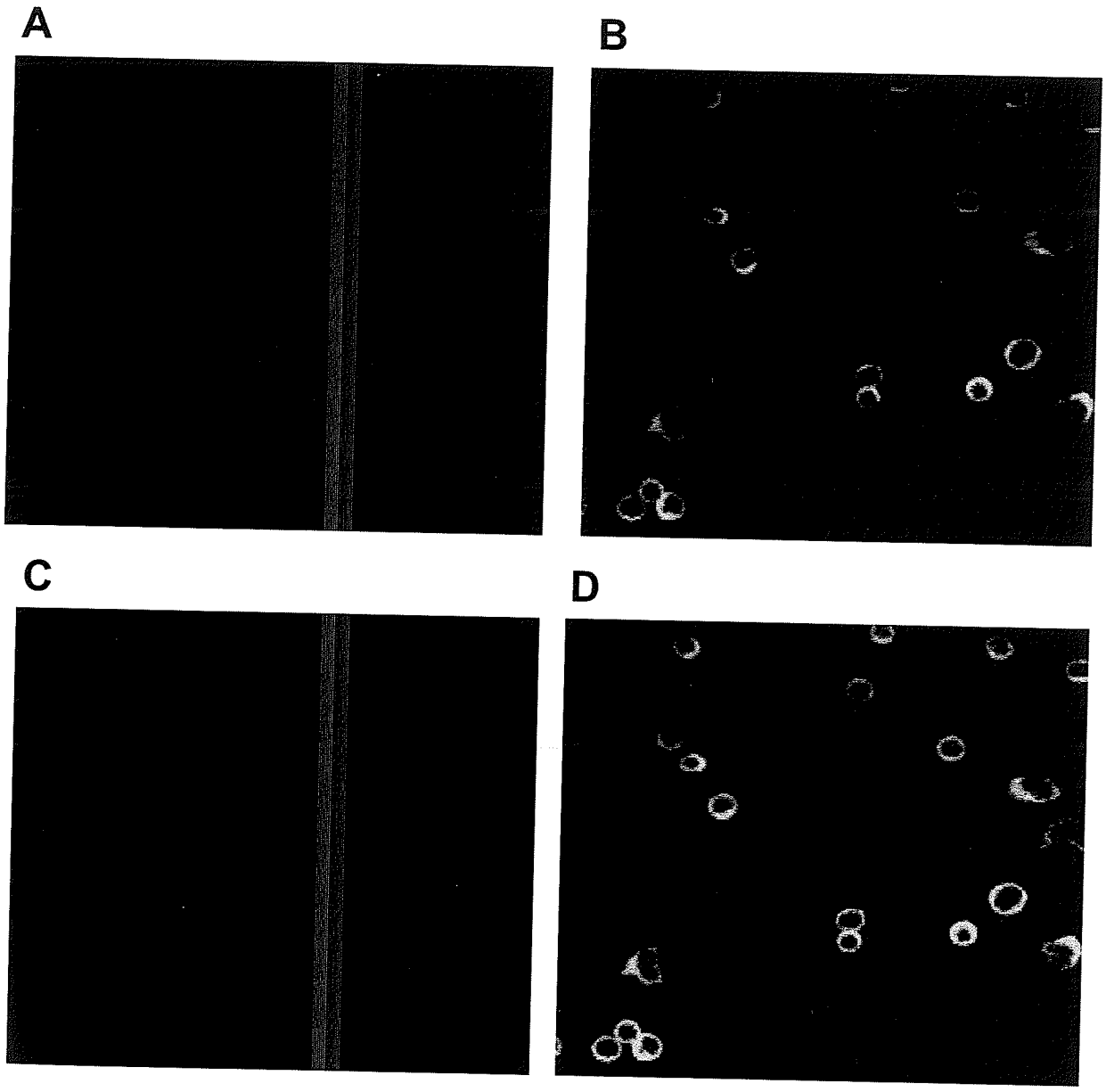


Figure 21: TAPP2 and Surface IgM Expression Among CLL Samples. CLL samples were fixed with paraformaldehyde and adhered to slides using cytospin. Cells were stained with TAPP2, surface IgM and DAPI and detected using confocal microscopy. A) DAP1 staining, B) TAPP2 staining, C) surface IgM, D) Merge of all staining. This picture is representative of 6 photos for CLL4.

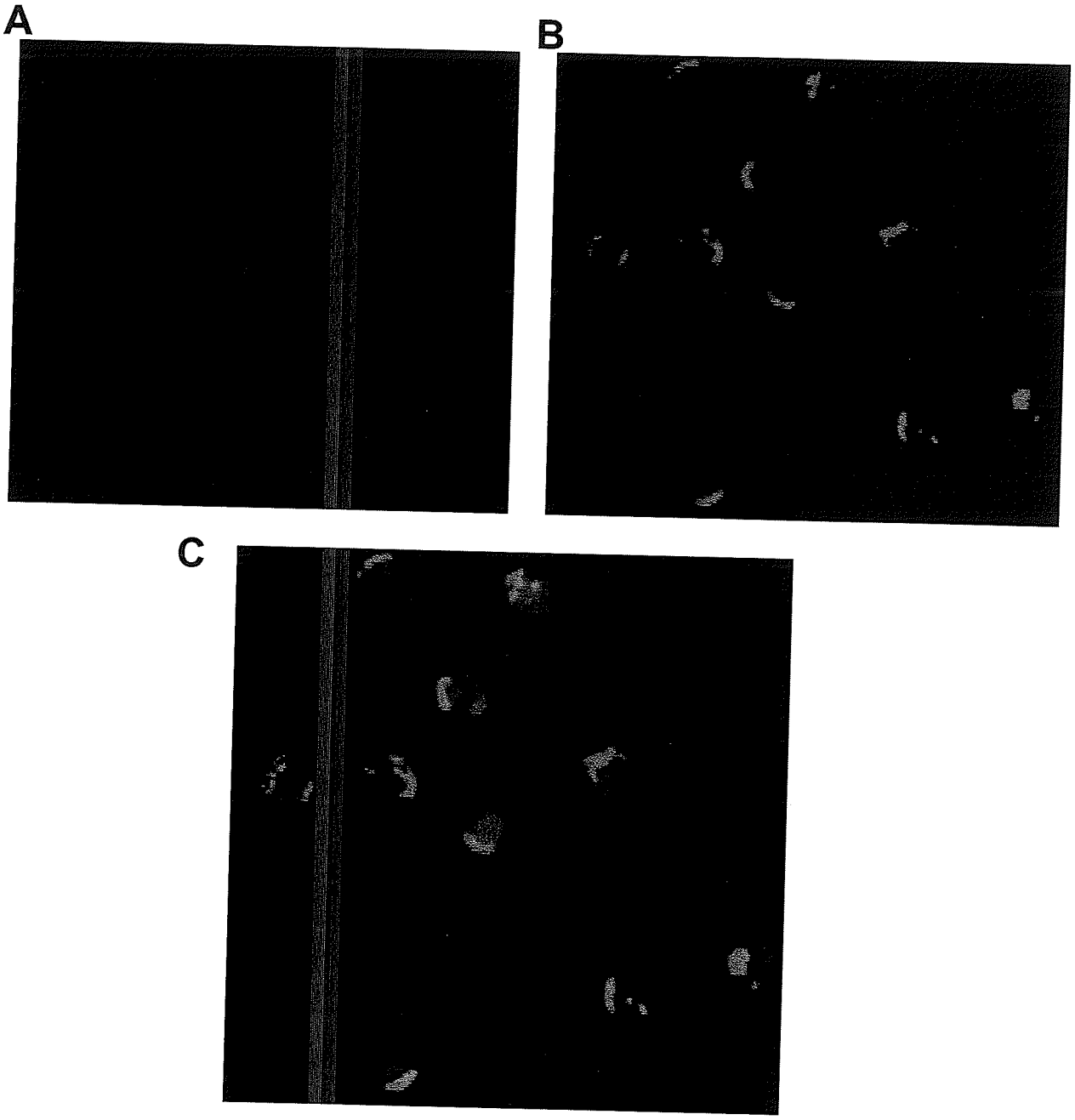


Figure 22: DAPI and Surface IgM Staining of CLL Cells. CLL samples were fixed with paraformaldehyde and adhered to slides using cytospin. Cells were stained with surface IgM and DAPI and detected using confocal microscopy. A) DAP1 staining, B) surface IgM, C) Merge of all staining.

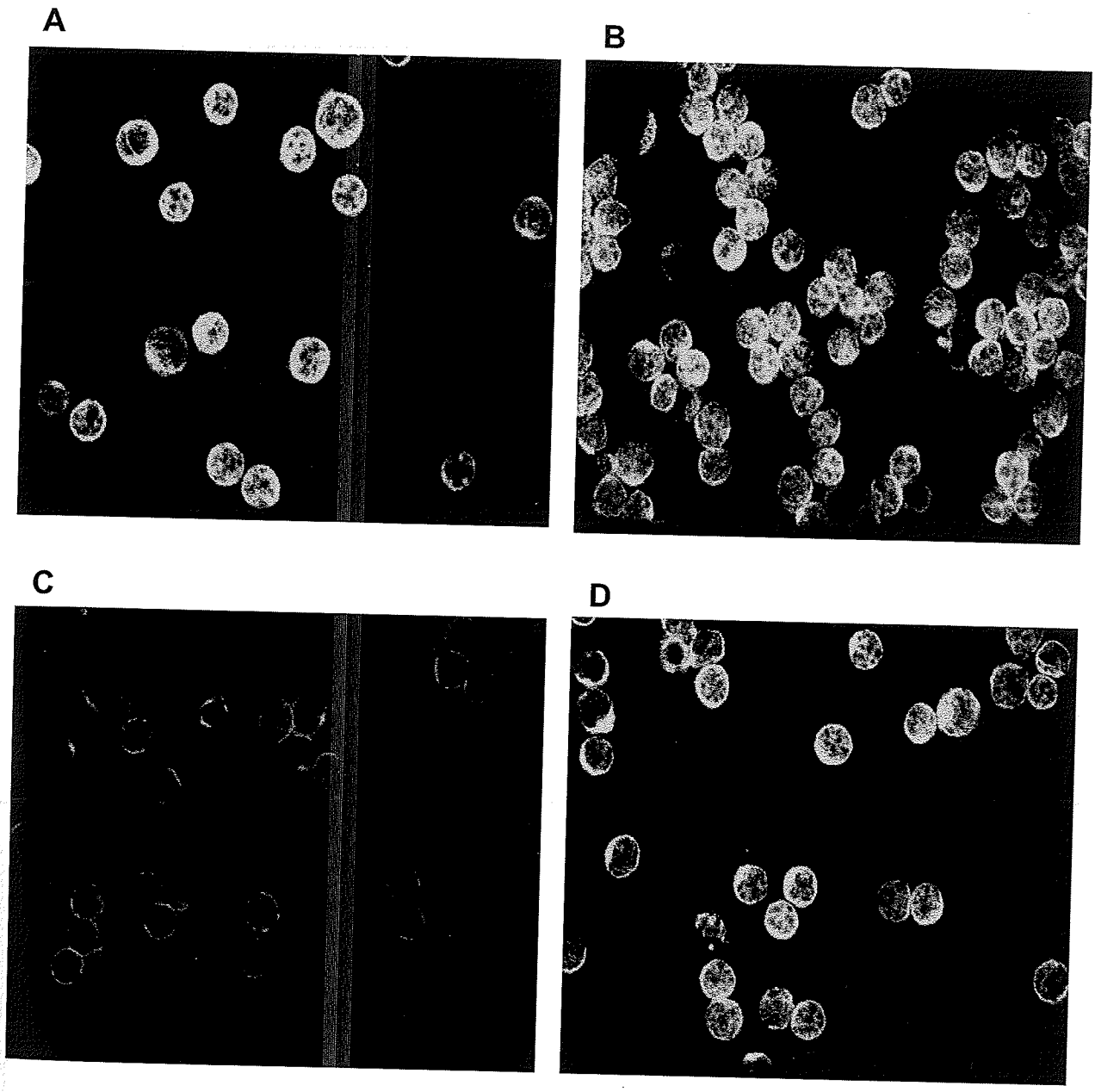


Figure 23: TAPP2 Staining of CLL Cells. CLL cells were fixed with paraformaldehyde and adhere to slides using cytospin. Slides were then stained with TAPP2 and detected using confocal microscopy. A),B), D) Bright TAPP2 Expression. C) Dim TAPP2 Expression.

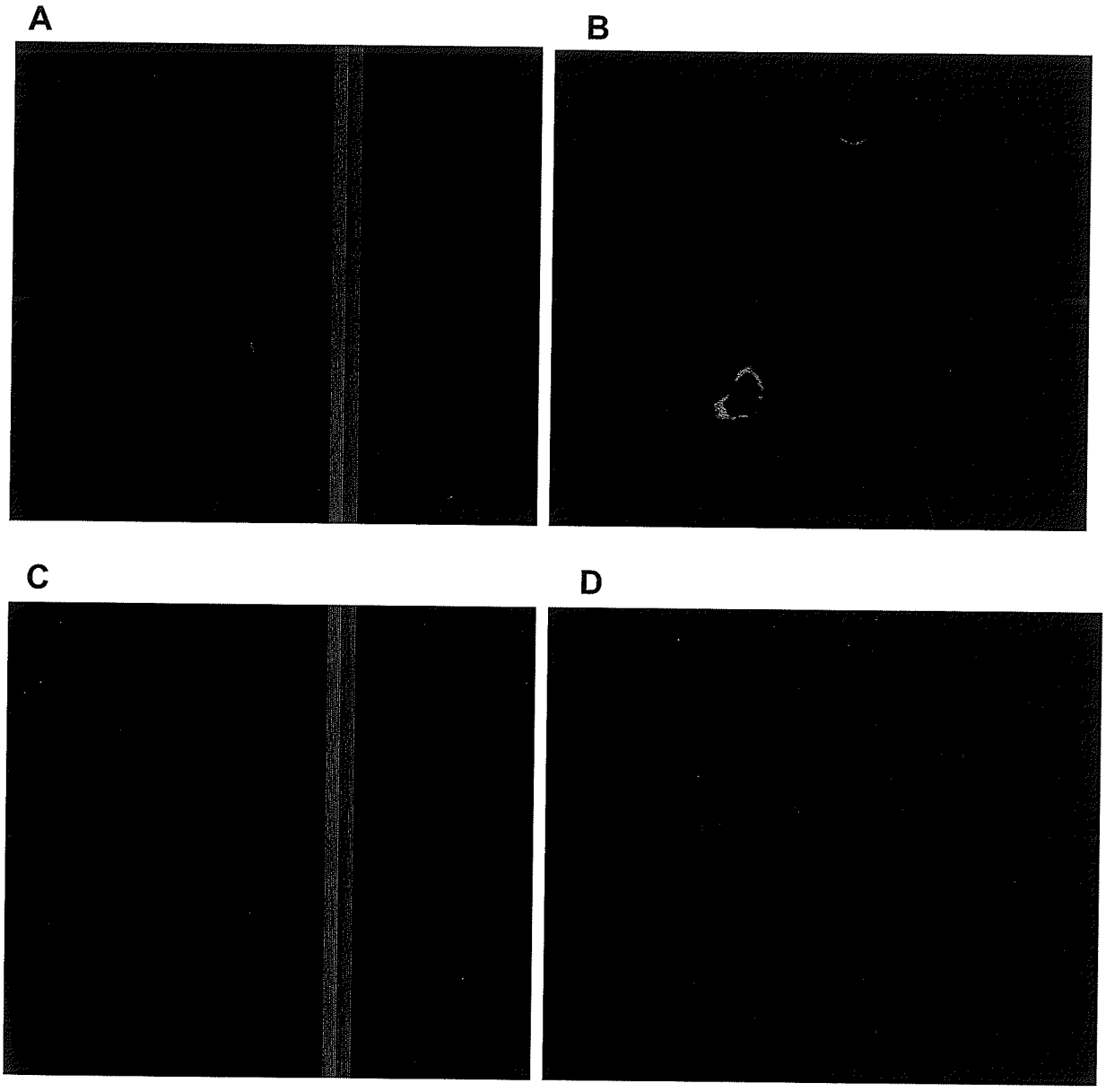


Figure 24: ZAP70 Staining of CLL Cells. CLL cells were fixed with paraformaldehyde and then adhered to slides using cytospin. Slides were stained with ZAP70 and then detected using confocal microscopy. A) Semi-Bright ZAP70 expression. B) Dim ZAP70 expression with 2 bright cells indicating T cell contamination. C) Dim ZAP70 expression. D) Bright ZAP70 Expression.

Section 2: Functional Studies

Section Abstract

The previous section examined the expression of the PI(3,4)P2 target protein TAPP2 in normal and leukemia B cells. This section will determine the functional role of TAPP2 in B lymphoma cells using overexpression, wild-type and dominant negative stable transfectants or by knocking down TAPP2 through the use of siRNA. Previous studies from our lab showed that TAPP2 co-localizes with filamentous actin and associates with several additional cytoskeletal proteins, suggesting this molecule may have a role in linking PI(3,4)P2 signals to cytoskeletal rearrangement processes. We therefore tested whether TAPP2 regulates B cell adhesion to matrix proteins or migration in response to chemotactic factors. TAPP2 over-expression resulted in an increase in adhesion, whereas expression of a PH domain mutant form of TAPP2 led to reduced adhesion. I was able to show that TAPP2 knockdown by siRNA decreases the cells ability to adhere, confirming the importance of this molecule in B cell adhesion. Previous cell line studies showed that overexpression of TAPP2 lead to enhanced migration and this migration was drastically decreased in those cells expressing the PH domain mutant. I examined in CLL cells whether variation in expression of TAPP2 correlates with ability of cells to adhere or undergo chemotaxis. My results did not indicate any correlation of TAPP2 expression with CLL cell adhesion or migration function. Those cells that have high TAPP2 expression did seem to have variable levels of migration, while low TAPP2 expression seemed to have a more consistent chemotaxis. Collectively, this data indicated that TAPP2 is involved in cellular adhesion to extracellular matrix and migration; however,

further studies will be required to determine whether there is a specific role in CLL or other other types of transformed lymphocytes.

a) Adhesion in over-expression of TAPP2 WT and PH mutant cells

Experiments performed by Sen Hou in our lab examined whether TAPP2 can regulate cell adhesion to matrix proteins. Prior to adhesion assay, plates were coated with laminin or fibronectin. Adhesion assay conditions were established in order to determine the ability of BJAB cells to adhere to laminin or fibronectin-coated plates. Cells adhered in BCR-stimulation dependent manner, and adhesion increased by 50% with stimulation dose. Those cells that were incubated with PI3K inhibitors, LY294002 and wortmannin, had reduced BCR-induced adhesion to fibronectin and laminin coated plates (Figure 25a). This is an indication that BCR-induced PI3K activation is required for increased adhesion. In Figure 25b, we compared parental BJAB cells with BJAB transfectant cells stably expressing wild-type TAPP2, membrane targeted TAPP2 (myristoylated) or PH mutant TAPP2 (R218L). Those transfectant cells expressing wild-type TAPP2 or membrane targeted TAPP2 had increased adhesion to fibronectin and laminin coated plates. However, cells expressing PH mutant TAPP2 had an inhibited ability to adhere. These results indicate that TAPP2 plays a role in BCR-induced cell adhesion.

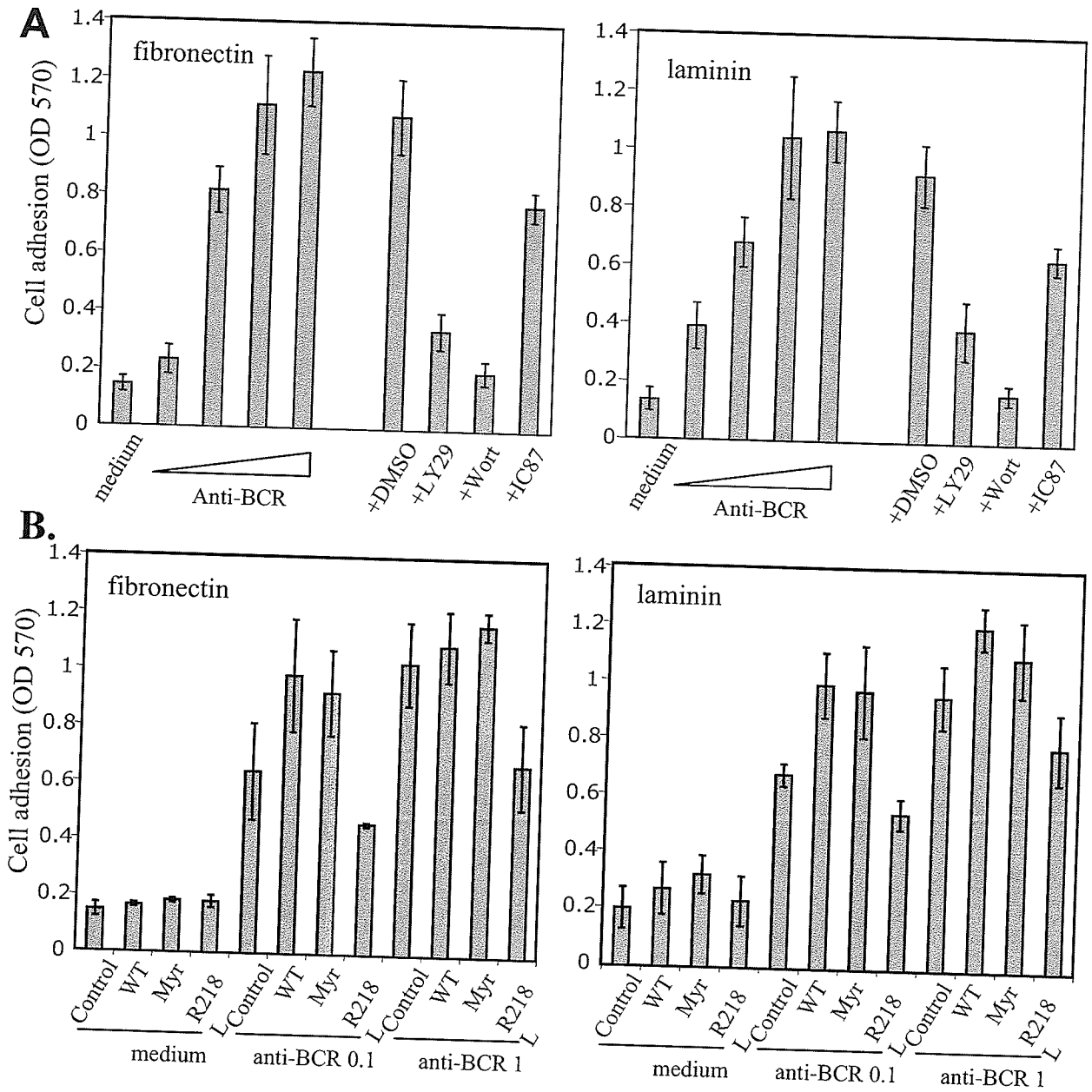


Figure 25: TAPP2 Regulates Cell Adhesion. Plates were coated with fibronectin and laminin prior to adhesion assays. Cells were stimulated with anti-BCR, unstimulated or inhibited with PI3K inhibitors, wortmannin and LY294002. After 1 hour non-adherent cells were removed, cells were fixed with glutataldehyde and stained with crystal violet. Stained cells were dissolved in SDS and the OD570 read on an absorbance plate reader. (A) BCR stimulated cells induced PI3K-dependent increase in BJAB adhesion to laminin and fibronectin. BJAB adhesion increased with anti-BCR doses (medium=no stimulus or 0.01, 0.1, 1 or 10 ug/mL anti-BCR). All inhibitor treated cells were stimulated with 10ug/mL anti-BCR (DMSO=0.5% DMSO with no inhibitor; LY29= 50 uM LY294002; Wort= 50 uM wortmannin; IC87= 50 uM IC87114) (B) BCR-induced adhesion in BJAB transfectant cells (Control=parental BJAB, WT=wild-type TAPP2, Myr= myristoylated TAPP2, R218L=PH mutant TAPP2). Graphs represent 3-5 independent experiments

b) siRNA Knockdown Optimization

The above over-expression experiments showed that TAPP2 can regulate adhesion to matrix proteins (Figure 25). To confirm over-expression results it is important to knockdown TAPP2 in order to determine its function, since overexpression of a protein may lead to functions that are not typical of that protein. Through siRNA I wanted to try to knockdown TAPP1 and TAPP2 in order to determine if they will have reduced adhesion and/or other functional changes. siRNA knockdown was done using synthethic double-stranded RNA sequences that were chosen using online RNAi design program that picks unique sequences for your chosen gene. These were then incubated with RNAiFect lipofection reagent that allows for the siRNA sequence to cross the membrane into the BJAB cells. After incubation in medium for 48 hours, cells were harvested for protein extraction. Through Western blot, I was unable to detect significant TAPP2 knockdown when compared to controls (Figure 26). After further research about siRNA it was brought to my attention that the assay needs to be done in a serum free environment for at least 24 hours to preserve siRNA integrity and allow entry into cells. FBS apparently nonspecifically binds to siRNA and can degrade it before it is able to get into cells. The same protocol was then followed in a serum free environment for 24 hours and through Western blot I was able to reproducibly show a knockdown of TAPP2 (Figure 27 and 28). However, we have not been able to verify that TAPP1 has been knocked down because we haven't been able to find a good antibody to detect TAPP1 expression.

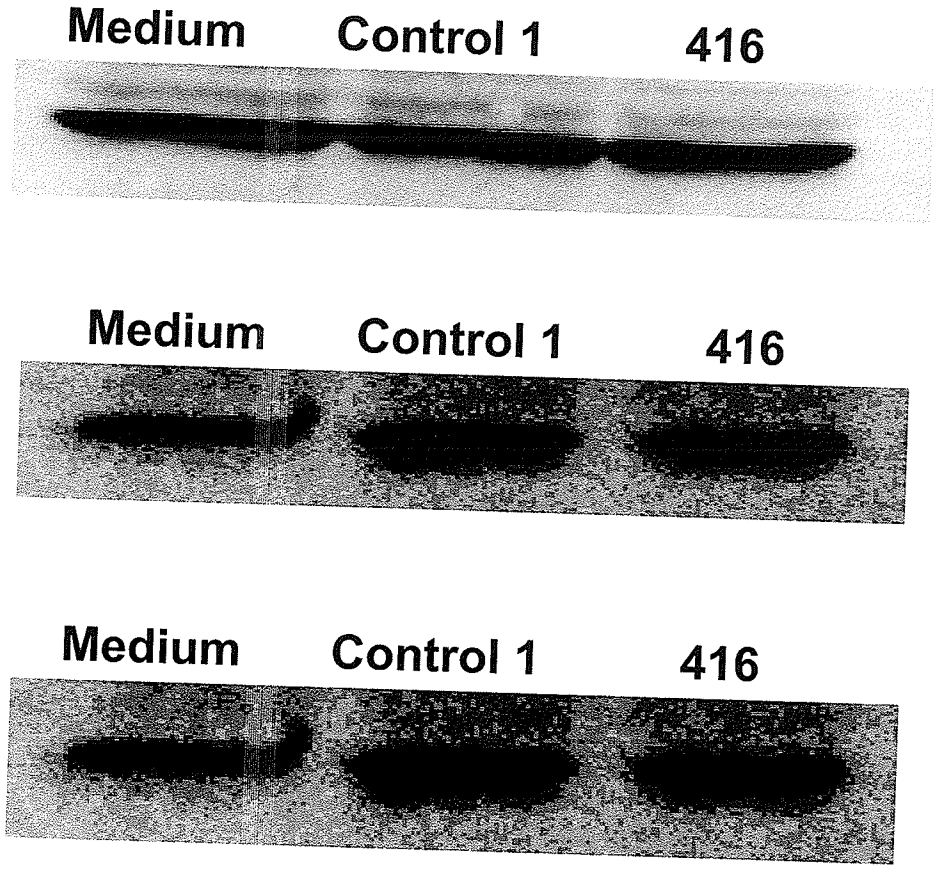


Figure 26: TAPP2 knockdown not obtained with the use of serum. TAPP2 knockdown was preformed in BJAB using synthetic RNA sequences and RNAiFect lipofaction. Cells were incubated for 48 hours and then harvested using NP40 lysis buffer and protein concentration was determined using Bradford protein assay. 5 ug of protein was run on SDS-PAGE gel and TAPP2 knockdown was determined. Through the use of Western Blots we were unable to see a knockdown of TAPP2 compared to medium and control 1. These blots are representative of 6 independent experiments.

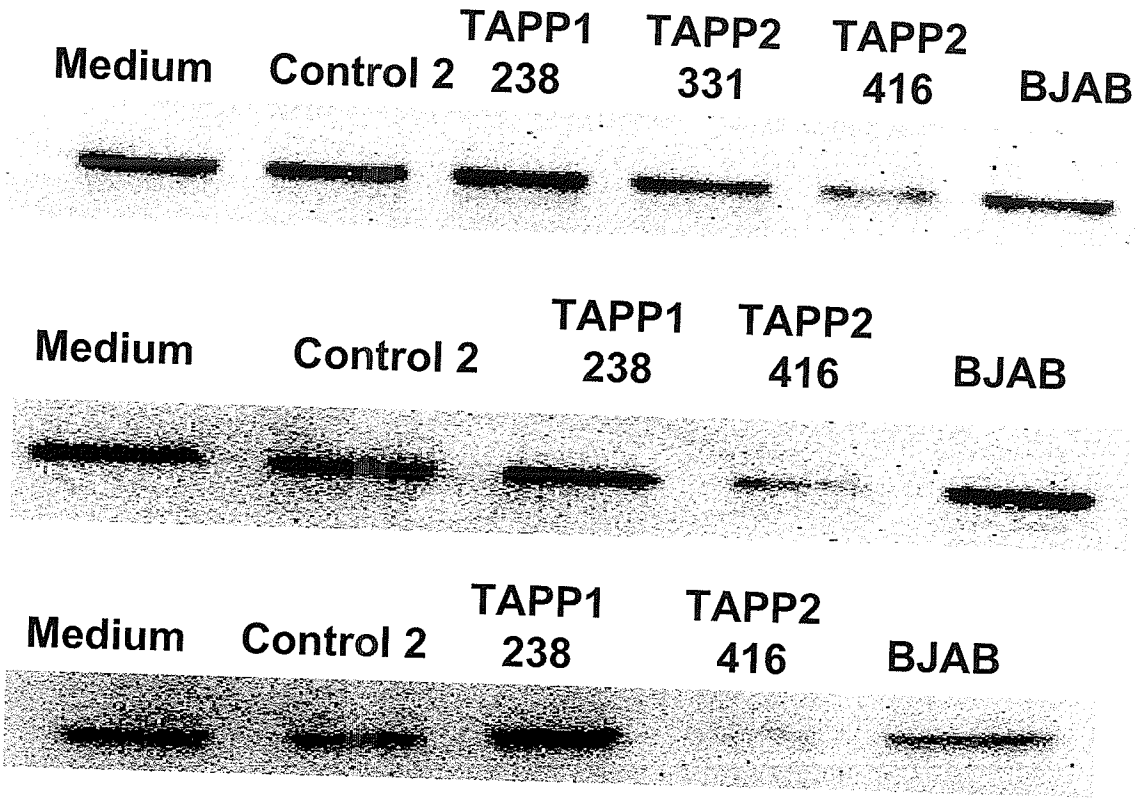


Figure 27: TAPP2 knockdown established with the removal of serum. TAPP2 knockdown was preformed in BJAB using synthetic RNA sequences and RNAiFect lipofaction. Cells were incubated for 48 hours in serum free medium for 24 hours and 10% FBS was than added. Cell were harvested after 48 hours using NP40 lysis buffer and protein concentration was determined using Bradford protein assay. 5 ug of protein was run on SDS-PAGE gel and TAPP2 knockdown was determined. Through Western Blot, we were able to see knockdown of TAPP2 compared to medium and control 2. These blots are representative of 5 independent experiments.

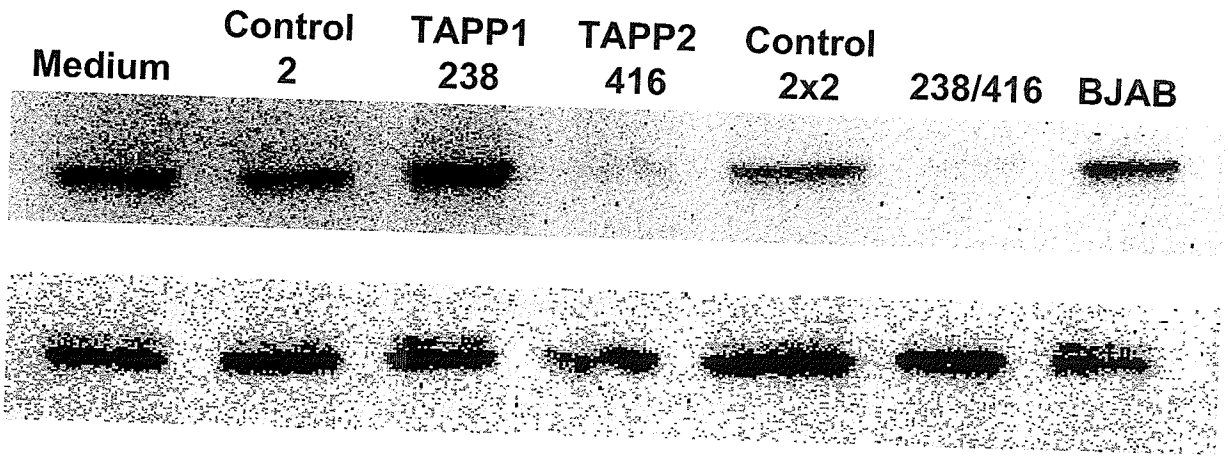


Figure 28: TAPP2 knockdown through the use of siRNA. TAPP2 knockdown was preformed in BJAB using synthetic RNA sequences and RNAiFect lipofaction. Cells were incubated for 48 hours in serum free medium for 24 hours and 10% FBS was than added. Cell were harvested after 48 hours using NP40 lysis buffer and protein concentration was determined using Bradford protein assay. 5 ug of protein was run on SDS-PAGE gel and TAPP2 knockdown was determined. Through Western Blot, I was able to show a knockdown of TAPP2 and both TAPP1/TAPP2 (top blot). The bottom blot is expression of Actin and it seems to be evenly distributed among all samples. These blots are representative of 3 independent experiments.

c) Reduced Adhesion in TAPP2 siRNA Knockdown Cells

After successful knockdown of TAPP2 in BJAB cells I wanted to determine their ability to adhere to plates coated with matrix proteins. Plates were coated with fibronectin or laminin prior to the experiment and cells were plated on these wells and stimulated with PMA, IgM or left unstimulated for 1 hour. Cell adhesion was detected using crystal violet stain and ELISA plate reader. Cellular adhesion in TAPP knockdowns was reduced by 50% when compared to controls (Figure 29). This demonstrated that TAPPs have an important role in cells ability to adhere.

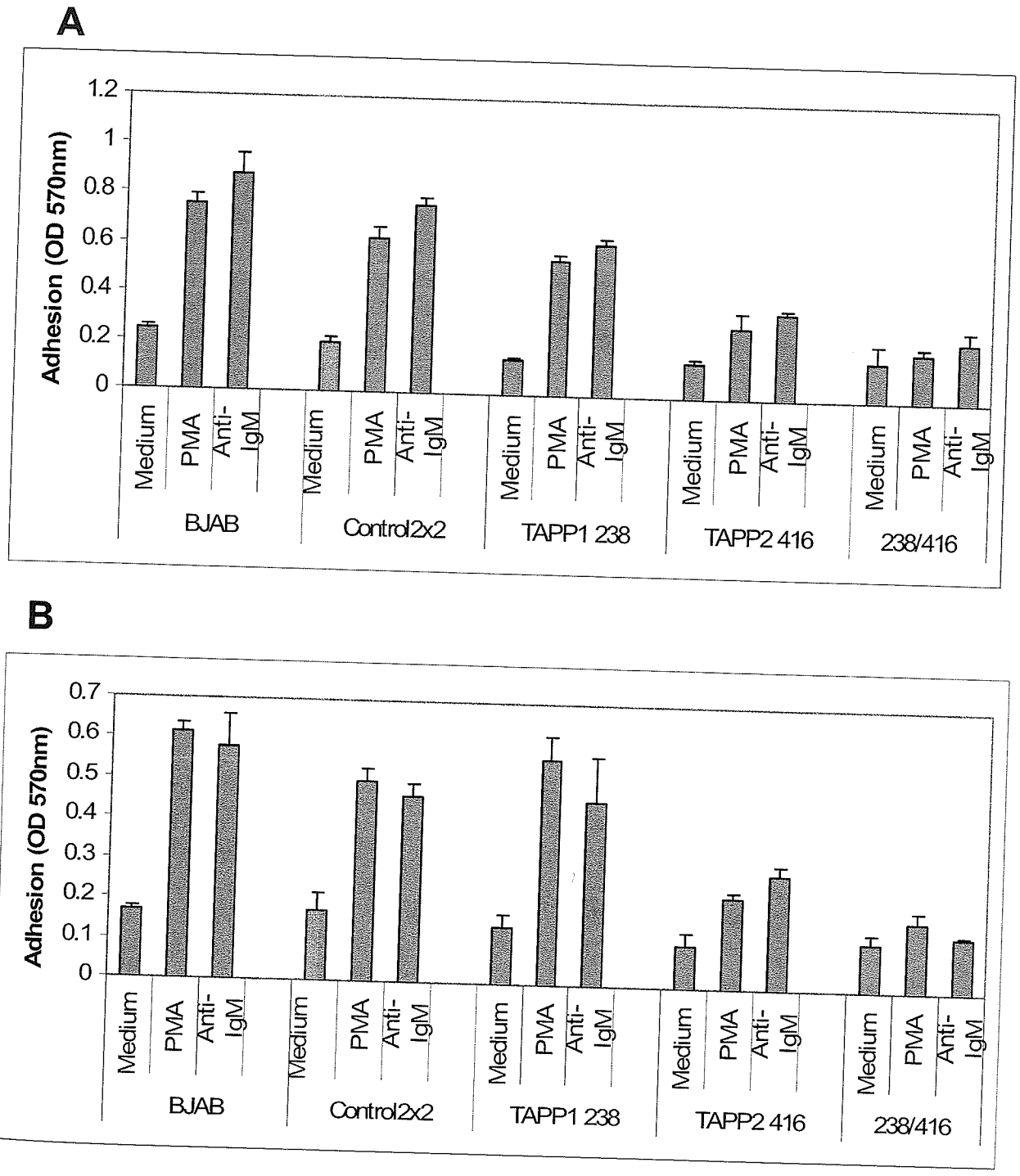


Figure 29: Reduced Adhesion in TAPP1 and TAPP2 knockdown cells. Plates were coated with laminin and fibronectin, cell matrix protein, prior to experiment. Cells were stimulated for 1 hour with PMA, IgM or left unstimulated. Adhesion was determined using crystal violet stain and an ELISA plate reader. TAPP2 and TAPP1/TAPP2 have a significantly reduced adhesion in fibronectin coated plates compared to medium and control 2. As well, similar results were seems with plates coated in laminin. This data is representative of 5 independent experiments.

d) Confocal Imaging of BJAB Adhered Cells

To determine TAPP2 subcellular localization during adhesion and whether TAPP2 affect cell morphology or F-actin during adhesion, we imaged cells that over-expressed TAPP2 or expressed PH mutant TAPP2. BJAB transfected cells were stimulated with anti-IgM and allowed to adhere to fibronectin-coated slides. Cells were fixed to slides with paraformaldehyde and stained with TAPP2 in order to determine membrane localization of TAPP2. TAPP2 was stained with Alexa 488-conjugated secondary antibody (green fluorescence dye) and F-actin was stained with rhodamine-phalloidin (red fluorescence dye). Slides contain the following cells: parental BJAB, BJAB stably over-expressing TAPP2 (BTF4), PH mutant TAPP2 (BTR3), or myristoylated, constitutively membrane-targetted TAPP2 (BTL1). Parental BJAB cells show cytoplasmic TAPP2 expression with some accumulation at the cell periphery and co-localization with F-actin at cell:cell junctions (Figure 30). BTF4 cells adhering to fibronectin show TAPP2 accumulation at the cell periphery and colocalization with F-actin (Figure 31). BTF4 cells also show an increase in the number of adhered cells, increased cell spreading and increased F-actin localization at the cell periphery (Figure 31). BTL1 cell show show an even stronger phenotype of more cell spreading and F-actin accumulation at the cell periphery (Figure 33). In contrast, BTR3 shows fewer cell adhered and less F-actin (Figure 32). These pictures confirm that, during B cell adhesion, TAPP2 is localized at the cell membrane via its PH domain and seems to promote cell spreading and accumulation of F-actin.

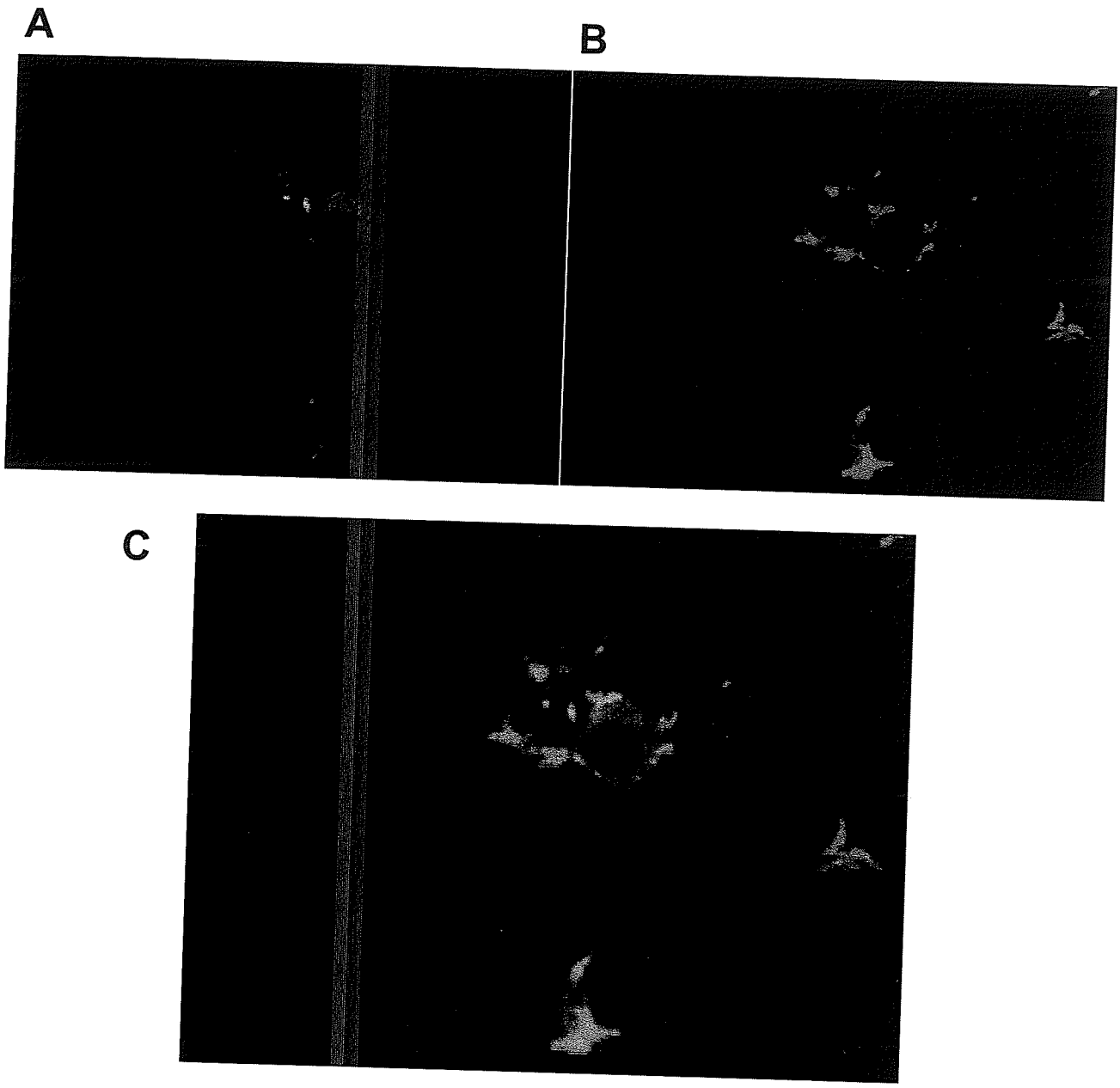


Figure 30: BJAB Cells stained with TAPP2 and Rhodamine-phalloidin. Slides were coated with fibronectin prior to cell adhesion. BJAB cells were stimulated with anti-IgM and allowed to adhere for 2 hours and then were fixed with paraformaldehyde. Cells were stained with TAPP2 and Rhodamine-Phalloidin. A) TAPP2 staining B) Rhodamine-Phalloidin C) Combined

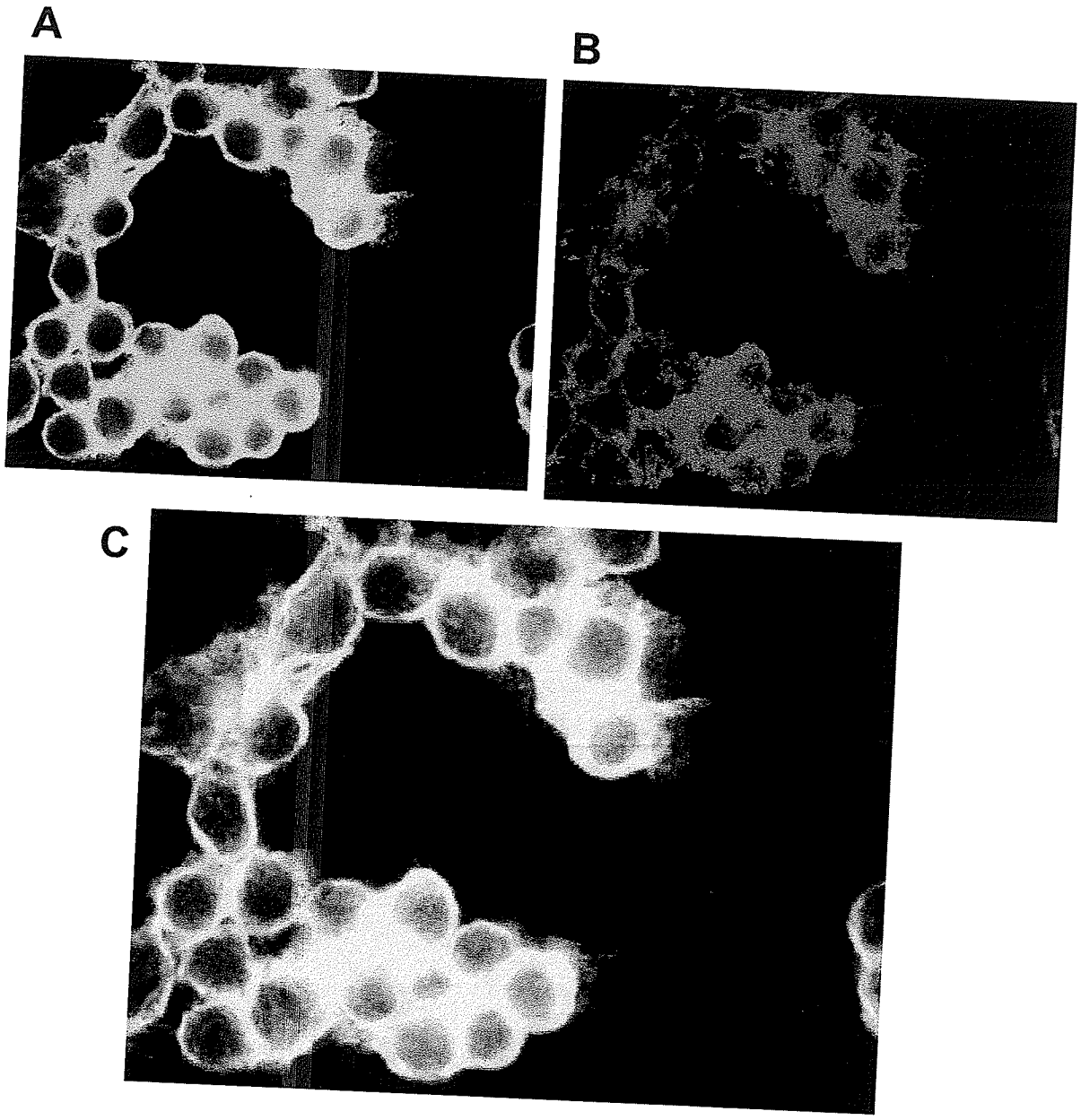


Figure 31: BTF4 Cells stained with TAPP2 and Rhodamine-phalloidin. Slides were coated with fibronectin prior to cell adhesion. BTF4 cells were stimulated with anti-IgM and allowed to adhere for 2 hours and then were fixed with paraformaldehyde. Cells were stained with TAPP2 and Rhodamine-Phalloidin. A) TAPP2 staining B) Rhodamine-Phalloidin C) Combined

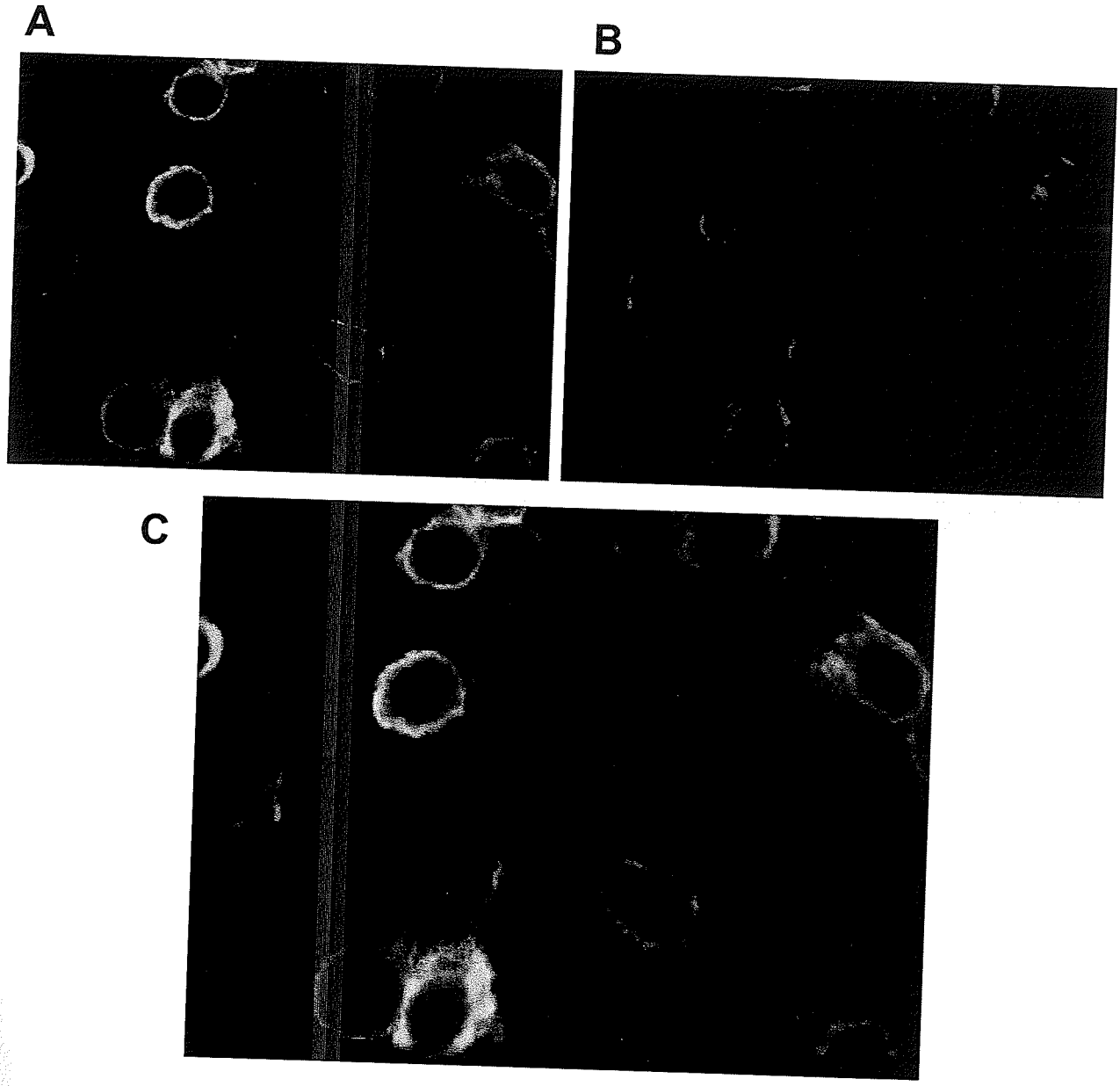


Figure 32: BTR3 Cells stained with TAPP2 and Rhodamine-phalloidin. Slides were coated with fibronectin prior to cell adhesion. BTR3 cells were stimulated with anti-IgM and allowed to adhere for 2 hours and then were fixed with paraformaldehyde. Cells were stained with TAPP2 and Rhodamine-Phalloidin. A) TAPP2 staining B) Rhodamine-Phalloidin C) Combined

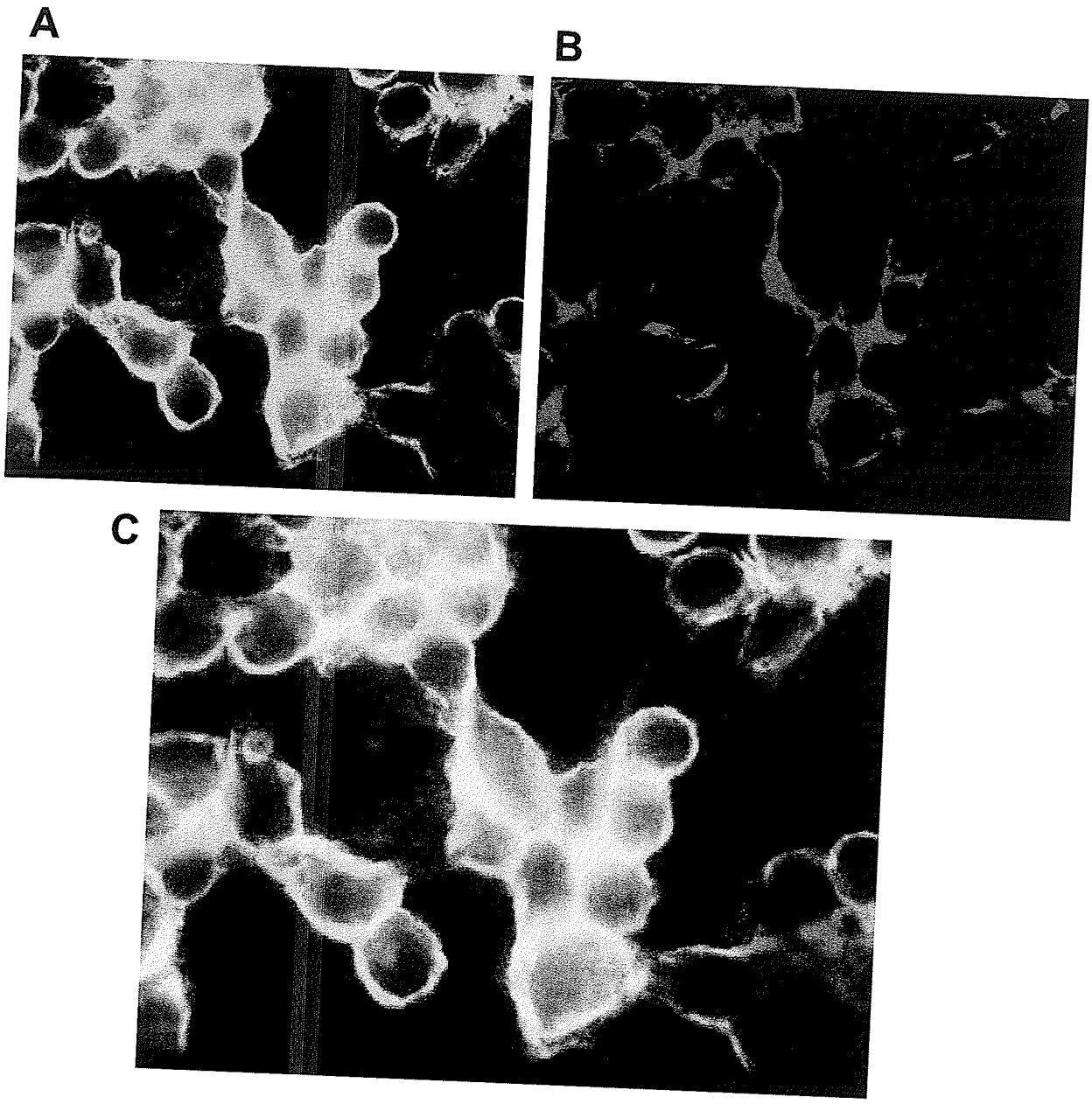
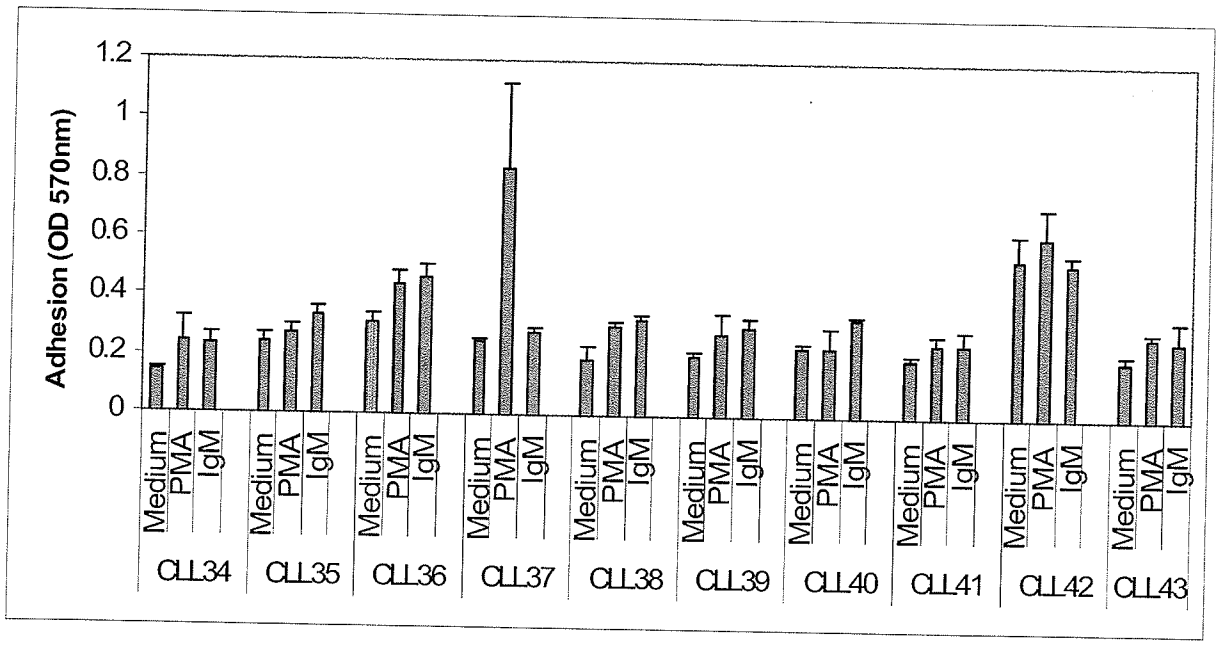


Figure 33: BTL1 Cells stained with TAPP2 and Rhodamine-phalloidin. Slides were coated with fibronectin prior to cell adhesion. BTL1 cells were stimulated with anti-IgM and allowed to adhere for 2 hours and then were fixed with paraformaldehyde. Cells were stained with TAPP2 and Rhodamine-Phalloidin. A) TAPP2 staining B) Rhodamine-Phalloidin C) Combined

e) CLL Cells Ability to Adhere

To determine whether our observed variation in TAPP2 expression in CLL patients correlate with their adhesion function, I carried out adhesion assays using the fresh CLL samples that were blotted for expression in the previous section. Cells were stimulated with PMA (50 ng/mL) or anti-IgM (10 ug/mL), plates were incubated for 3 hours in 37°C incubator and adhered cells were measured as previously. Cells plated in wells coated with laminin did not show increased adhesion after stimulation, and generally showed less adhesion than in fibronectin-coated wells (Figure 34). In plates coated with fibronectin, a few CLL samples, such as CLL 34, 36, 38 and 43 had an increase in adhesion when stimulated with PMA, while most CLL cells stimulated with IgM showed a slight increase in adhesion (10-30%) over the unstimulated control (Figure 34). Overall, we could not detect substantial activation-induced adhesion among CLL samples, and the small adhesion responses observed not correlate with TAPP2 expression as seen with BJAB cells (Figure 35).

A



B

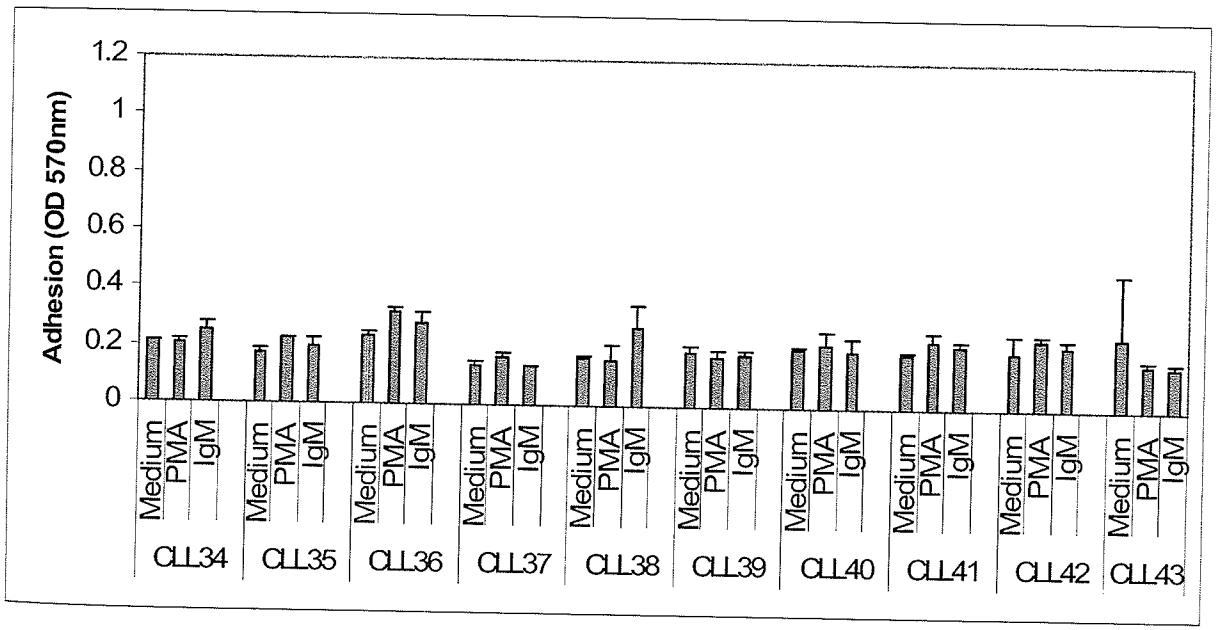


Figure 34: CLL Adhesion to Fibronectin and Laminin Coated Plates. Fresh CLL samples were obtained from Manitoba CLL Bank and cultured overnight in medium containing 10% FBS. Plates were coated prior to experiment with laminin and fibronectin. CLL cells were stimulated with PMA, anti-IgM or left unstimulated for 3 hours. Adhesion was detected through crystal violet stain and an ELISA reader. A)Fibronectin adhesion B)Laminin adhesion. Each condition contained triplicate wells and the average was obtained.

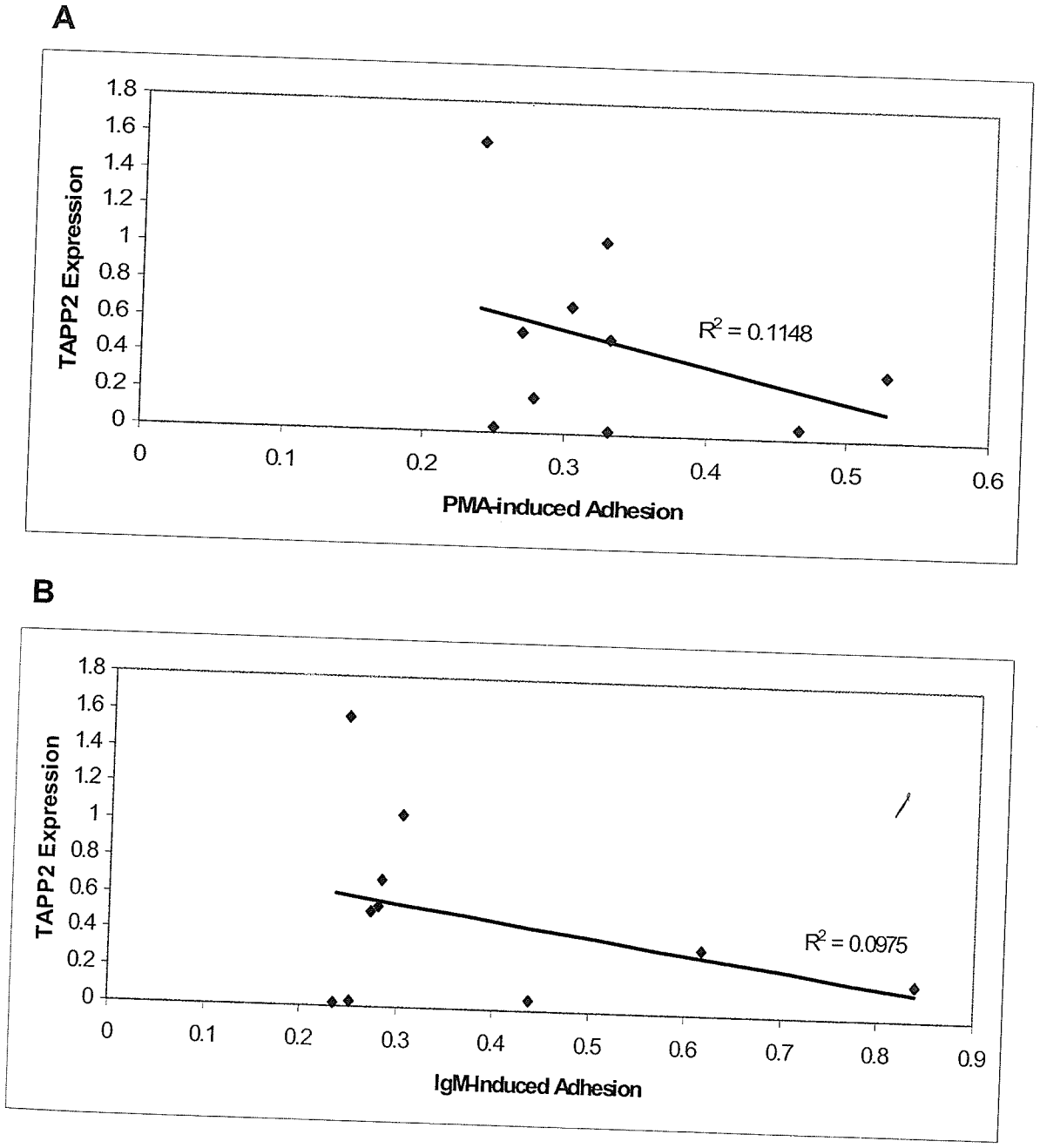


Figure 35: TAPP2 Corelation with Adhesion Assay. TAPP2 expression was determined using Western blots. Corelation was determined using R^2 value. There is no evident correlation between TAPP2 expression and PMA-induced or IgM-induced adhesion.

f) Role of TAPP2 in Chemotaxis

Previous experiments in our lab performed by Sen Hou examined whether TAPP2 can regulate cell ability to migrate. A20 murine B lymphoma cells were transfected with wild-type or PH mutant TAPP2. ATF1 (A20 TAPP2 overexpressing cells) and ATR3 (A20 TAPP2 PH mutant cells) were stimulated with different doses of SDF-1 in transwell chamber plates. After 2 hours, migration of cells towards SDF1 in the lower chamber was determined by counting cells in the upper and lower chambers. Parental A20 cells show a bell-shaped dose-response curve with maximal migration at 20 ng/mL which showed a 1% migration (Figure 36). ATF1 cells over-expressing TAPP2 showed a 50% increase in cell migration (4% migration) compared to parental A20 cells (Figure 36). In contrast, ATR3 cells expressing TAPP2 PH mutant have drastically reduced migration (less than 1% migration) when compared to both parental and TAPP2 overexpressing A20 cells (Figure 36).

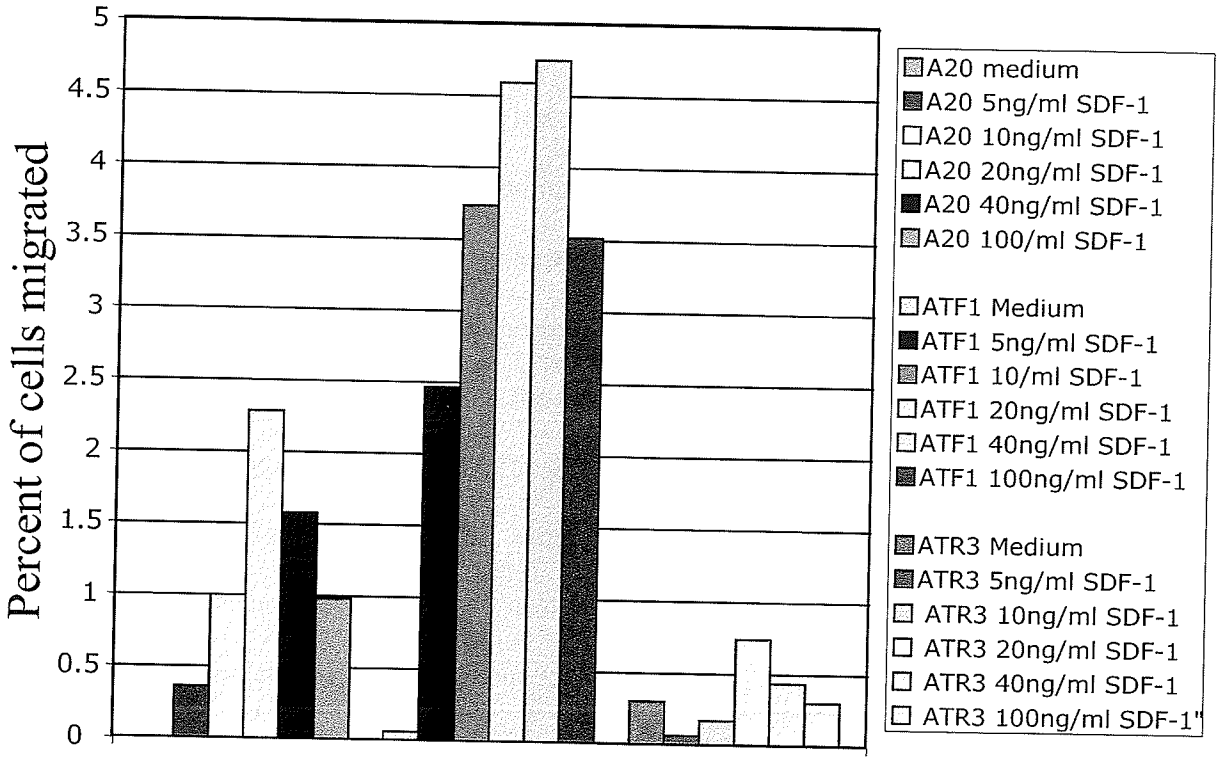


Figure 36: TAPP2 transfectants have altered chemotactic responses to the chemokine SDF1. A20 cells, TAPP2 transfectant (ATF1) or R218L TAPP2 transfectant (ATR3) cells were placed in transwell chambers with the indicated concentrations of SDF1 in the lower well. After incubating 2 hours, the number of cells in the upper and lower chambers were counted using flow cytometry to calculate the percent migration. This graph is represent of 4 independent experiments.

g) Chemotaxis with CLL Cells

To determine whether our observed variation in TAPP2 expression in CLL patients correlates with their chemotaxis function, we carried out migration assays with fresh CLL cells. CLL cells were stimulated with different doses of SDF-1 in transwell chamber plates that contain an upper and lower chamber to allow for migration. After 3 hours migration was determined by counting cells in the upper and lower chambers using a flow cytometer. Most CLL cells show dose dependent migration towards SDF1 (Figure 37). There is a marked variability in responses, with CLL 47, 48 and 52 shows close to a 50% increase in migration, while the others have migration close to 35% increase. The degree of migration has no significant correlation with TAPP2 or ZAP70 expression (Figure 39). However, those CLL samples that are TAPP2 high tended to have variable levels of chemotaxis (ie. CLL 45/46/47/48/50/52), while TAPP2 low patients tended to have a consistent migration at about 35% increase (ie. CLL 44/49/51) (Figure 39). Both ZAP70 high and ZAP70 low expressing patients have variable levels of chemotaxis (Figure 39). These variations are an indication that there are other factors that play a role in migration and TAPP2 may be a contributing factor.

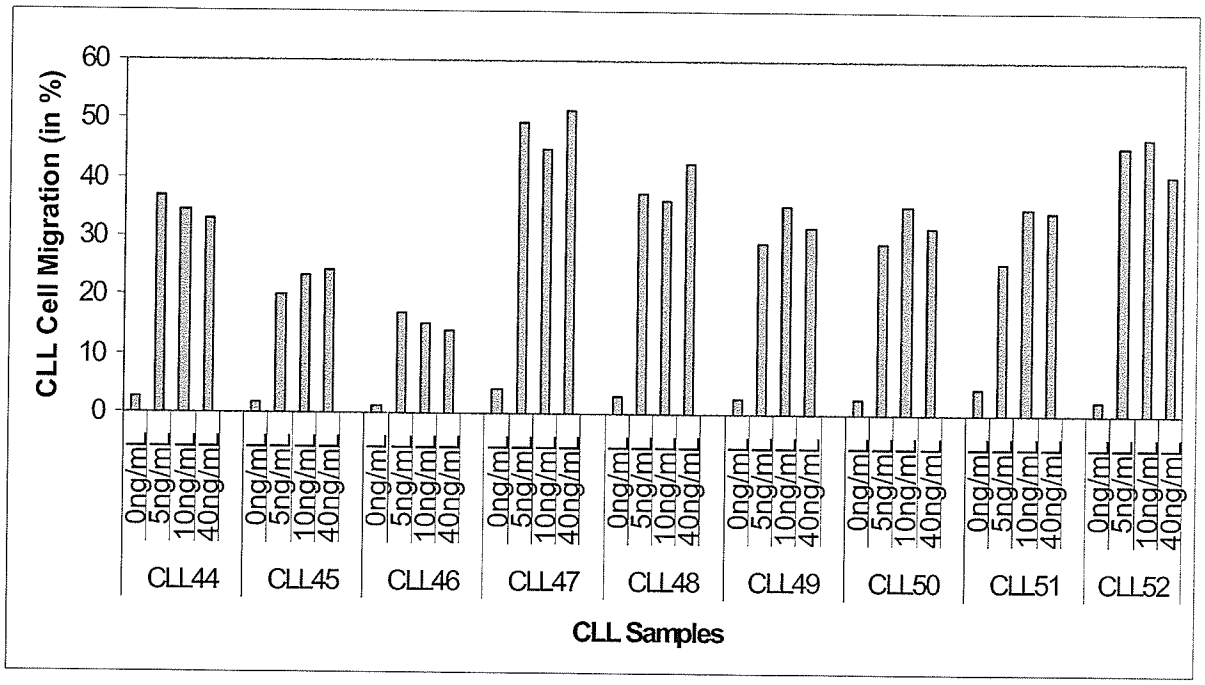


Figure 37: CLL cells ability to migrate. Fresh CLL cells were stimulated with 5, 10, 40 ng/mL of SDF-1 or left unstimulated for 3 hours. Cell migration was then determined by count cells in the upper and lower chambers using a flow cytometer. Cell migration seems to be increased in a subset of CLL samples.

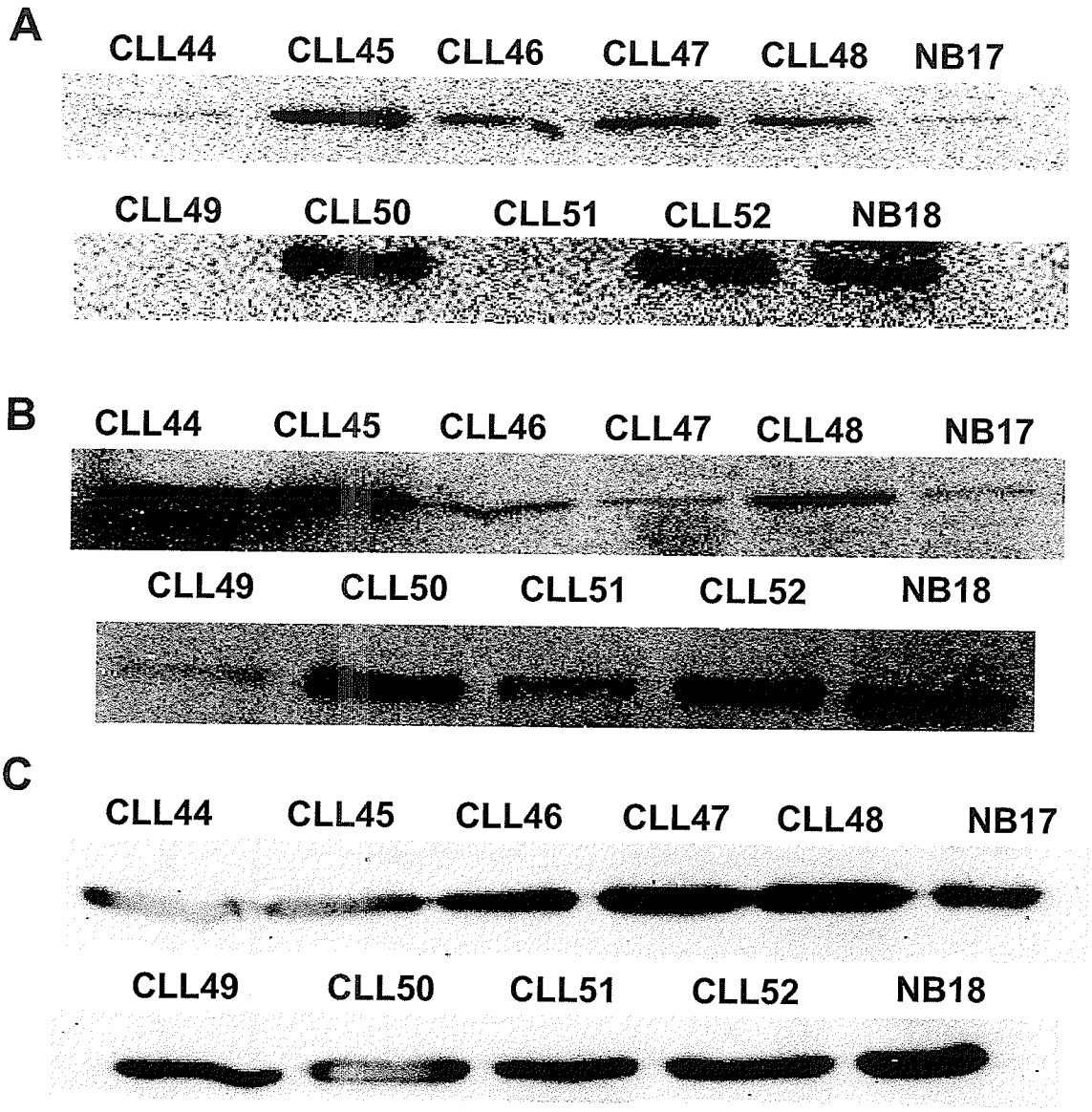


Figure 38: TAPP2 Correlation with Chemotaxis. TAPP2 expression was determined through Western blots. A) TAPP2 Expression. B) ZAP70 Expression C) Actin Expression. TAPP2 is overexpressed in a subset of CLL samples. Actin is evenly distributed among all samples. These blots are representative of 3 independent runs.

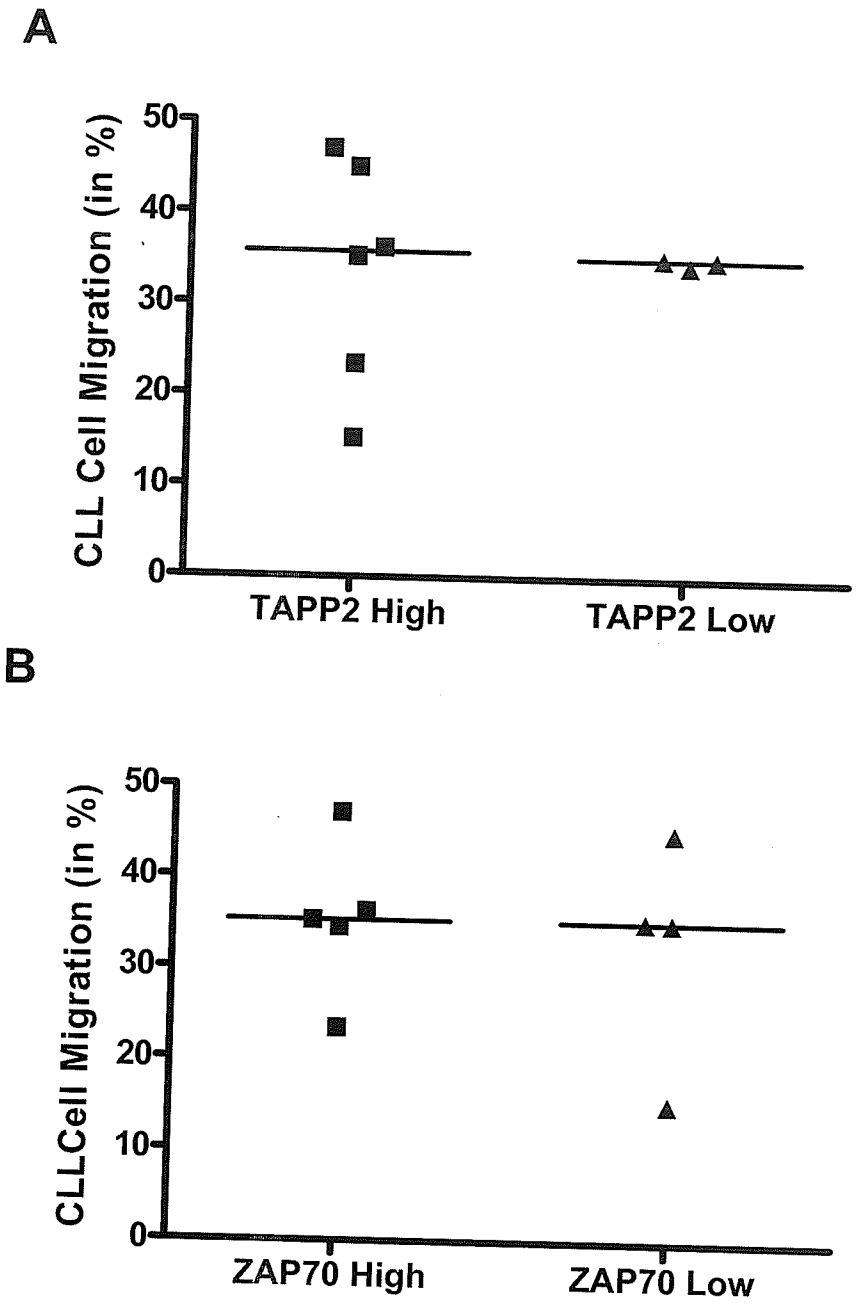


Figure 39: TAPP2 and ZAP70 Correlation with Chemotaxis.

Discussion

In section one of the results, I examined expression of PI3K pathway components are expressed in CLL and normal B cell samples. Through the use of real time RT-PCR I was able to choose primers for each of the PI3K components. The best primer combinations were chosen based on primer sensitivity and melting curves. Primer specificity was confirmed using RNA extracted from cell lines of different types. Bam32 expression is known to be restricted to B cells in humans (194-196). Consistent with this, I found that Bam32 is highly expressed in Ramos and BJAB (both B cell lines), and had little to no expression in T cells and non-hematopoietic cells. ZAP70 is known to be expressed mainly in T cells and not B cells (136). My real-time RT-PCR data confirmed ZAP70 expression in Jurkat, a T cell line, but little or no expression in B cell and non-hematopoietic cell lines. Lipid phosphatase SHIP1 has been shown to have restricted expression to hematopoietic cells, while SHIP2 is more widely expression (21, 23). I was able to confirm these previous finds through real-time RT-PCR where SHIP2 expression was shown among all cell lines, while SHIP1 was expressed in B cell and T cell lines but not expressed at all in non-hematopoietic cells. Adaptor proteins TAPP1 and TAPP2 are both found to be widely expressed; however TAPP2 was previously shown to have higher expression in lymphocytes (34, 37). These findings were consistent with my results and were a good indication that the primer selections were accurate and will be able to provide some promising results.

I attempted to determine expression of these PI3K components in CLL and normal B cells using the same primer combinations. I was unable to get run to run reproducibility

using these samples. This could be a result of many variables. Samples were obtained from the Manitoba CLL Bank and therefore were not isolated first hand. Factors that could variably affect RNA quality could be different amount of time individual samples were left at room temperature before freezings or variable exposure to RNases therefore leading to degradation of the RNA. These were variables that I was unable to control in my experiments but may have played a role in the inability to achieve reproducible results. Potential solutions could include immediate RNA preparation on the day of sample collection or isolation of CLL cells from blood using our own hands.

Using Western blots, I was able to show that there is a subset of CLL samples that overexpress certain PI3K components at the protein level. Both TAPP2 and Zap70 were significantly over-expressed in CLL compared to normal B cells and the expression of these two molecules positively correlate. Those CLL samples that were TAPP2 high were also high for ZAP70 and vice versa. ZAP70 expression in CLL is known to be associated with having unmutated IgV_H genes and having a more aggressive form of the disease (134, 136). I was able to confirm the correlation between ZAP70 and patient mutational status. Over 50% of the patients with unmutated IgH genes had clear ZAP70 expression. In contrast, the majority of patients with mutated IgH genes had undetectable ZAP70. Two "outliers" with mutated status, but very high ZAP70 expression may reflect T cell contamination in patients whose peripherial blood is less dominated by leukemia cells. Alternately these patients may represent a less frequent $ZAP70^+$ mutated leukemia, consistent with previous studies showing that patients with mutational status can also occasionally be ZAP70 positive (136).

SHIP expression was shown in one recently published study to correlate with prognostic marker ZAP70. SHIP is an inhibitory phosphatase that functions to counterbalance BCR signaling by limiting the duration and intensity of the signal (197). CLL cells that do not express ZAP70 seem to have impaired BCR signaling (197). It was recently published that SHIP is highly expressed in CLL cells that did not express ZAP70 (197). As well, these cells seem to have a greater tyrosine phosphorylation of SHIP than those CLL samples that express ZAP70, leading to the conclusion that ZAP70-negative CLL are more likely to be inhibited by SHIP than ZAP70+ CLL (197). My data do not fit well with this study, since the majority of my CLL samples that were high in ZAP70 were also high in SHIP. Thus, while my data confirms a marked variable SHIP expression among CLL, they did not show significant correlation of SHIP expression with ZAP70 status. The reasons for this discrepancy are not clear; however, the published results were based on a relatively small sample size.

A published analysis of PTEN expression in CLL cells showed little or no expression in about 50% of the patient samples (149). I found that there is a subset of CLL samples that had little or no expression when compared to normal B cells. However I also observed a subset of CLL samples that appear to over-express PTEN compared to normal B cells. Altered lipid phosphatase expression could be a reason for the accumulation of these B cells. This altered expression leads to deregulation of PI3K pathway ultimately leading to overexpression of PIP3 and PI(3,4)P2 which leads to enhanced downstream signals that results in accumulation of cells due to defect in apoptosis. This defect of apoptosis is one of the characteristics of CLL. This part of the pathway may be a potential target for

treatment in CLL patients. Addition or depletion of these altered lipid phosphatase may aid in induction of apoptosis and decrease in cellular apoptosis.

In the second section of the results, I was able to show that TAPP2 plays a role in B cell adhesion. TAPP2 is likely to play a role in adhesion and chemotaxis by linking BCR signaling to cytoskeletal rearrangement. Through previous experiments done in my lab by Sen Hou, she was able to show that when TAPP2 was overexpressed in BJAB transfected cells they had an increase in adhesion. In contrast, when these BJAB cells were transfected with TAPP2 PH mutants or subjected to PI3K inhibitors there was a decrease in their ability to adhere. These results showed that there may be a possible role for TAPP2 in cells ability to adhere, since those cells that had TAPP2 PH mutations were unable to migrate when stimulated. Sen was also able to show that when TAPP2 was overexpressed in A20 lymphoma cells they had an enhanced ability to migrate, while migration was drastically reduced when there was a mutation in PH domain of TAPP2 expressing A20 cells. These experiments led to my work trying to determine the role TAPP2 may play in adhesion through siRNA knockdown of TAPP2. I was able to show that when TAPP2 was knocked down through siRNA there was a decrease in their ability to adhere. This shows that not only is the PH domain of TAPP2 important for cells to adhere but the presence of the TAPP2 gene in cells plays an important role in adhesion. TAPPs ability to regulate adhesion was an indication could be a potential area of the pathway to target in cancer treatments. TAPP expression levels could be altered as a potential cancer therapy and possible lead to altered activity of these cancer cells. Alternatively, it may be possible to design inhibitors blocking interaction of TAPP PH

domains with PI(3,4)P2, therefore decreasing the adhesion and chemotaxis ability of cancer cell, limiting their invasiveness into tissues.

The same adhesion assay was then tried in CLL cells, since, through our expression data, we were able to show that some of the CLL have an overexpression of TAPP2 while others have little or no expression. I wanted to determine if those CLL samples that overexpressed TAPP2 would have an increase in adhesion like that seen in BJAB transfected cells. However, this was not the case in CLL samples. There was not a drastic increase in adhesion in any one sample. It is possible that the adhesion assay conditions used were not optimal for distinguishing functional differences in CLL adhesion capacity and differences might be observed using different matrix proteins and/or stimuli. Alternatively, this result may indicate that altered TAPP2 expression does not substantially affect CLL adhesion function. Clearly, there are other factors that might be playing a role in adhesion of human cancers. It is not as simple as the BJAB model where we are only changing one factor and keeping the rest the sample. In human leukemia like CLL there are many other changes occurring at the same time as overexpression of TAPP2 and they may also play a role in the cells ability to adhere.

We went on to determine CLL cells ability to migrate to various doses of SDF-1 since TAPP2 overexpression enhanced migration of A20 cells, and it was previously published that CLL cells have enhanced migration (164, 167, 176). I was able to show strong but variable SDF1-induced migration of CLL cells in our transwell chamber assay. We also examined TAPP2 expression in these CLL samples. Several of the TAPP2-high

expressing samples showed robust chemotaxis; however, the correlation is not 100% indicating that there are other factors that may play a role in migration of human cells, like that seen in the adhesion assays with CLL samples. Further studies with a larger sample size are required before any definite conclusions can be drawn. In addition, it may be important to look at lower "sub-optimal" doses of SDF1 to better detect the variations in chemotactic responses.

Through confocal microscopy analysis I was able to examine both TAPP2 expression levels and subcellular localization. I first looked at stimulated BJAB cells that were adhered to fibronectin and stained for TAPP2. I was able to show that transfected cells overexpressing TAPP2 had TAPP2 membrane localization and more cells that adhered to the slides when compared to TAPP2 PH mutant transfected cells. The transfectants expressing PH domain mutant also showed abnormal morphology and spreading on fibronectin. This is an indication that the PH domain is important for membrane localization of TAPP2 upon stimulation and seems to be required for cells to undergo cytoskeletal rearrangement required for adhesion to extracellular matrix proteins like fibronectin.

I also used confocal microscopy to examine TAPP2 expression and localization in freshly isolated CLL samples. CLL have small cytoplasm relative to their nucleus and this makes it hard to determine membrane localization of adaptor molecules. Using a DAPI staining I was able to determine through confocal microscopy the location of the nucleus in CLL cells. TAPP2 was showed highly variable expression levels among CLL patients,

consistent with Western blot analysis; however TAPP2 did not seem to be localized to the membrane in any of the CLL samples that I looked at. This may be an indication that CLL cells are not activated enough to lead to constitutive membrane localization of TAPP2 and that other stimulus may be required in order for this to occur. As well I stained for surface IgM and found that some CLL samples have surface IgM while others do not express it which is consistent with previous findings (123, 131, 198).

Summary

In this thesis, I was able to determine the expression of PI3K pathway components in CLL and normal B cell samples. I have showed that there are a subset of CLL samples that overexpress TAPP2 and ZAP70 and is a positive correlation. As well, I was able to show that PTEN and SHIP expression is highly variable, but not significantly different compared to normal B cells. Lastly, I was able to show that TAPP2 may have a possible role in cells ability to adhere and migrate. Those cells that had mutation or knockdown of TAPP2 seemed to have decrease ability to adhere or migrate. These findings indicate that the altered expression of PI3K pathway components in CLL patients could be a possible reason for their accumulation and altered cellular function. CLL cells accumulate as a result of their inability to undergo apoptosis which may be due in part to their over or underexpression of SHIP and PTEN. TAPPs possible role in migration and adhesion shows the importance of these adaptor proteins in linking the PI3K pathway to other important cellular functions. Altered TAPP expression could be a potential target for cancer treatments and may lead to altered downstream signals that may aid in restoration of normal function of cells.

Future Directions

It was recently shown in our lab that utrophin and syntrophin are able to associate with TAPP2. Utrophin and syntrophin are both part of the dystrophin family of cytoskeletal proteins and they have not been looked at as to what their role is in lymphocytes. I think it would be interesting to look at their expression in CLL and their functional role in adhesion and chemotaxis as a nice addition to the TAPP2 story.

Expression of Associated Proteins: It would be important to look at the expression of syntrophin and utrophin in CLL and normal B cell samples in order to determine if there is a correlation with TAPP2. It would also be interesting to look at the other TAPP2-associated protein, utrophin in order to determine its expression in CLL and normal B cells and if there is a correlation with TAPP2 expression. Interestingly, the utrophin-related protein dystrophin was identified in microarray experiments to be over-expressed in unmutated verus mutated CLL (199). Thus, it would also be interesting to determine whether dystrophin protein is expressed in our CLL patients and whether TAPP2 might also be associated with this protein.

Adhesion Assays: It would be interesting to determine whether utrophin and sytrophin have functional roles in adhesion to integrins. While these proteins are known to be important for adhesion of muscle cells through the dystrophin glycoprotein complex receptors, it is not known if they might be involved in lymphocyte adhesion to integrin ligands. This could be tested through siRNA knockdown of sytrophin and utrophin and to determine if there is a correlation with TAPP2 and its role in adhesion. As well it would

be interesting to look at siRNA knockdown of TAPP2 and associated proteins in CLL samples that are known to have high expression of these molecules. Although we did not see a significant increase in adhesion after stimulation of CLL cells, it would be interesting to determine if loss of multiple proteins plays a role in changes basal adhesion. Also, it may be important to try other adhesion conditions such as different integrin ligand coating (VCAM) and other stimuli to activate adhesion.

Confocal Imaging: We accessed plasma membrane localization, however no marker was used to determine that TAPP2 was localized to the membrane in CLL cells. Through the use of membrane fractionation, membrane localization would be more easily distinguished in CLL samples.

Chemotaxis: It would also be interesting to look at utrophin and syntrophin role in chemotaxis. This could be done in both siRNA knockdown and CLL cells to determine if TAPP2-associated proteins play a role in a cells ability to migrate.

Co-Immunoprecipitation: I think it would be important to look at utrophin and syntrophin association with TAPP2 in BJAB cells and the CLL cells. This would complete the story about TAPP2 associated proteins, since we know that TAPP2 associates with utrophin as a result of findings done previously in the lab. It would be interesting to show how well utrophin associates with syntrophin and syntrophins association with TAPP2.

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