Independent and Associative Functions of IKKs and c-Myc in Cancer

By

Mamoona Noreen

(2010-NUST-TfrPhD-V&I-33)

Atta-ur-Rahman School of Applied Biosciences

National University of Sciences and Technology

Islamabad, Pakistan

2015

Independent and Associative Functions of IKKs and c-Myc in Cancer

By

Mamoona Noreen

(2010-NUST-TfrPhD-V&I-33)

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

In

Virology and Immunology

Atta-ur-Rahman School of Applied Biosciences

National University of Sciences and Technology

Islamabad, Pakistan

2015

DEDICATED TO

HUMANITY

ACKNOWLEDGEMENTS

All praises to Almighty Allah, the Creator and Sustainer of the universe, Who is the supreme authority, knowing the ultimate realities of universe and source of all knowledge and wisdom. Without His will nothing could be happened. It has been deemed a great favor of Allah that I was bestowed upon the vision, initiative, potential and hope to complete my research project successfully.

All regards to the Holy Prophet Hazrat Muhammad (PBUH) who enabled me to recognize my Creator and His creations to understand the philosophy of life. Who is forever a torch of guidance and light of knowledge for mankind, for our conscience with the essence of faith in God, converging all His kindness and mercy upon us.

First of all the predecessor of my all work is my decorous Father (late) and whom I owe all that I've ever had in my life is my Mother (late). Limitless thankfulness to the Almighty Creator of love for blessing me the tenderness and carefulness of my great parents, whose encouragement has always been my energy, whose inspiration was my guideline, whose expectations were always my target, whose gratification was my aim, whose advice has always been my weapons, whose prayers were my treasure and whose cares were my fortification. Without their support this study would have been impossible.

I feel great pleasure to take this opportunity to express my regards and appreciation to respected, proficient and learned supervisor, Dr. Sheeba Murad; Assistant Professor ASAB, NUST and Dr. Johannes A. Schmid; Associate Professor, Medical University of Vienna, Austria, for their kind supervision and stimulating discussions. I am in debt for their guidance and sympathetic attitude. I consider myself to be fortunate to work under such an illustrious personality of science.

I promulgate scrupulous respect to the Principal ASAB; Dr. Peter John for his untiring effort in providing congenial environment for education and research.

Being Higher Education Commission, Pakistan scholar, I am thankful to HEC for giving me scholarship for completion of this study.

I would also like to express my regards and sincere thanks to my teachers Dr. Aneesa Sultan; Assistant Professor, QAU, Islamabad, Dr. Aneela Javed;

Assistant Professor ASAB, NUST and Dr. Touqeer Ahmed; Assistant Professor ASAB, NUST, Dr. Mohammad Arshad; Chairman Department of Zoology, University of Sargodha, for their supporting behavior, guidance and invaluable suggestions.

While convening my work I was emanated by the generous help and high fidelities switched in the counsels by my fellows Naila Malkani, Naureen Ehsan Elahi, Kalsoom Sugra, Zia-Ur- Rehman Farooqi, Muhammad Imran, Hayat Khan, Sadia Salahuddin, Sana Gul, Altaf Ahmad, Syed Fazal Jalil, Muhammad Ali, Malik Nawaz Shuja, Sajib Mansoor, Ayaz Anwar and Imran Raza. Their knack of perceiving things early were the key factors behind the successful completion of my research project. I found myself reliant on their support and almost every step of my project. I am also thankful to them for providing me refreshing society, cheerful company, sincere suggestions and cooperation. I would admit that they are among the precious asset that I have earned in my Ph.D.

I owe a great debt of appreciation and gratitude to staff members of ASAB, NUST for their generous help, gracious cooperation and skilful advices through my research work which ultimately impinged upon the fruition of this research.

It will be injustice not to acknowledge the cooperation of staff members of oncology department of Sheikh Zayed Hospital, Rahim Yar Khan and Histopathology department of Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan in providing me the tissue samples for my project.

Man has the power to speak and write to express his feelings but there are some relationships where words always fall short. I feel no way to convey my gratitude to my sisters Shagufta, Nadia and Sobia, niece Maria and Mawra, nephews Furqan and Abdullah, brother in law Muhammad Sadiq and Dr. Raheel Atif Hameed for their support, blessing and love. I would like to pay special thanks to very respectable Muhammad Usman, Nasir Ali, Mehboob Alam, Zia Kiyani and Julius Victorius for their encouragement and moral support which gave me confidence and courage to fight problems. It is due to their prayers that I have completed my research work.

I do appreciate all of those who remembered me in their prayers and encouraged me through out my life and educational career.

Mamoona Noreen

TABLE OF CONTENTS

Chapter -3

Materials and Methods 26

Chapter -4

Results 56

LIST OF ABBREVIATIONS

AFIP	Armed forces institute of pathology
ANK	Ankyrin
APS	Ammonia persulphate
BAFF	B-cell activation factor
BC	Breast Cancer
BCL-2	B-cell lymphoma 2
bHLHLZ	Basic helix-loop-helix leucine zipper
BLC	B lymphocyte chemoattractant
BSA	Bovine serum albumin
BSE	Breast self examination
Btk	Bruton's tyrosine kinase
CBE	Clinical breast examination
CC	Coiled-coil domain
CD	Cluster of differentiation
CD14	Cluster of differentiation 14
CDK1	Cyclin-dependent kinase 1
CFLAR	FADD like apoptosis regulator chemokine
cIAPs	Cellular inhibitor of apoptosis proteins
CMV	Cytomegalovirus
COX-2	Cyclooxygenase 2
CPRG	Chlorophenol red-β-D-galactopyranoside
CSC	Cancer stem cell
Ct	Crossing threshold
DAB	Diaminobenzidine
DD	Death domain
DMEM	Dulbecco's modified Eagle's medium
ELC	Epstein-Barr virus–induced molecule 1 ligand
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
FADD	Associated protein with death domain

FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
FRET	Fluorescence resonance energy transfer
GFP	Green florescent protein
GMCSF	Granulocyte-macrophage colony-stimulating factor
GSK3	Glycogen synthase kinase 3
HBS	Hepes Buffered Saline
HER2	Human epidermal growth factor receptor 2
HH	Hedgehog
HLH	Helix-loop-helix
HRP	Horseradish peroxidase
ICAM-1	Intercellular cell adhesion molecule 1
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRAKs	IL-IR associated kinases
IRF3	Interferon regulatory factor 3
IκB	Inhibitor of nuclear factor kappa-B
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LFS	Li-Fraumeni syndrome
LPS	Lipopolysaccharide
LTβ	Lymphotoxin β
LZ	Leucine zipper
MALP 2	Macrophage activating lipopeptide 2
MAP	Mitogen activated protein
MAX	Myc associated factor X
MB	Myc box
MCP-1	Monocyte chemoattractant protein-1
MCS	Multiple cloning site
MD-2	Myeloid differentiation protein-2

MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloproteinase
MSCS	Median sequence conservation score
Myc	Myelocytomatosis
NBD	NEMO-binding domain
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NIK	NF-κB inducing kinase
nsSNP	Nonsynonymous SNP
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDB	Protein data bank
PI3K	Phosphatidylinositol 3' –kinase
PIAS1	Protein inhibitor of activated STAT1
PLA2	Phospholipase A2
PolyPhen-2	Polymorphism phenotyping version 2
PR	Progesterone receptor
PSIC	Position-specific independent counts
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RFP	Red fluorescent protein
RHD	Rel homology domain
RIP1	Receptor interacting protein-1
RIPA	Radio Immuno precipitation assay
SCF	Skp1-Cul1-F-box
SDF-1	Stromal cell–derived factor-1
SDS	Sodium dodecyl sulfat
SIFT	Sorting Intolerant from Tolerant
SLC	Secondary lymphoid tissue chemokine
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase

TA	Trans-activation domain
TAB-1	TAK-1-binding protein 1
TAK1	Transforming growth factor -B-activated kinase 1
TANK	TRAF associated NF-kappa-B activator
TB	Transcription blocker
TEMED	Tetra-ethylethylene-diamine
TI	Tolerance index
TIR	Toll/interleukin-1 receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain containing adaptor inducing interferon
VCAM-1	Vascular cell adhesion molecule 1
VEGFR	Vascular endothelial growth factor receptor
ZF	Zinc-finger domain

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Cancer related mortality rate is exceptionally high in developing countries including Pakistan. Lack of awareness regarding early warning signs and potential risk factors often contributes to high mortality rate. Complex molecular mechanisms are involved in the pathogenesis of various malignancies. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is one of the key pathways involved in cancer progression by cellular and immune regulation. This pathway is usually kept inactive in cytoplasm of normal cells by another group of proteins known as inhibitor of NF-κB (IκB). Degradation of IκB is regulated by a set of proteins called IκB kinases (IKKs) resulting in deregulated activation of NF-κB pathway in malignant cells. IKKs have been reported to interact with cellular Myelocytomatosis (c-Myc), thereby suggesting NF-κB-independent roles of IKKs in tumors. Fluorescence Resonance Energy Transfer (FRET) microscopy was employed to analyze the association of IKKs and c-Myc in human embryonic kidney (HEK 293) cell line. The current study revealed for the first time that the helix loop helix (HLH) and leucine zipper (LZ) domains of IKKα play an indispensable role in the interaction of IKKα with c-Myc. Further the effect of the IKKs and c-Myc interaction was tested at transcriptional and translational levels. IKKs were not found to affect the c-Myc expression at transcriptional and translational level, but IKKs were found to reduce transcriptional activity of c-Myc, thereby suggesting a possible regulatory role of IKKs on c-Myc and cancer. The interaction of IKKα with c-Myc was found to be comparatively stronger as compared to IKKβ. Therefore, in order to explore the independent role of IKKα in Breast cancer, the paraffin embedded tissue sections were immunohistochemically stained with IKKα. The immunohistochemical analysis of IKKα protein showed its decreased expression in Progesterone receptor (PR) positive BC biopsies suggesting an inverse correlation between the two proteins. An association between IKKα expression with tumor grade was observed but was found to be statistically insignificant.

Proto-oncogenes such as c-Myc are often found to be up-regulated in most of the human neoplasmas often in association with genetic changes including single nucleotide polymorphism (SNPs). Therefore, the effect of 27 non synonymous SNPs (nsSNPs) on the structure and function of c-Myc protein was found through an in silico analysis. Among the various nsSNPs studied; rs114570780 (Tyr47His), rs150308400 (Cys148Tyr), rs137906262 (Leu159Ile) and rs200431478 (Ser362Phe) were found to have noteworthy biological worth and may be considered while developing personalized therapeutic approaches. Taken together, these findings have potential implications in the development of diagnostics and therapeutic approaches against malignancies.

Chapter 1

INTRODUCTION

Cancer is a group of diseases resulting from an uncontrolled division of abnormal cells with the propensity to invade or spread to other parts of the body. Presently, there are more than 100 types of cancers that can affect humans (Smyth *et al.*, 2006). Cancer is found to be the most frequent cause of death (accounting for 13 % of total deaths) across the globe. In last few decades tremendous work has been done to investigate the pathogenic mechanisms involved in cancer progression but still a lot is to be done (Hoesel and Schmid, 2013).

Cancer progression and sustenance is directly controlled by proper regulation of molecular activities as a signaling cascade. Abnormal activation of these pathways leads to excessive inflammation, escape from immune system and ultimate development of cancer (Hayden and Ghosh, 2012). A significant hallmark of cancer pathogenesis involves regulation of well known nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling pathway. Aberrant activation of NF-κB in tumor microenvironment leads to excessive inflammation and cancer progression. (Hanahan and Weinberg, 2011).

Activity of NF-κB transcription factors is controlled by a group of inhibitory molecules called inhibitor of NF-κB (IκB). These inhibitors make complexes with NF-κB through non-covalent association thus inhibiting its translocation to the nucleus (Whiteside and Israel, 1997). IκB is phosphorylated by the catalytic subunits of IKK complex, called IκB kinase alpha (IKKα) and IκB kinase beta (IKKβ), allowing the ubiquitinylation of IκB and its degradation by the proteasome. Degradation of IκB leads to the liberation of NF-κB subunits to translocate to the nucleus where they bind to specific DNA targets as transcription factors to promote their activation (Bonizzi and Karin, 2004; Hayden and Ghosh, 2012).

Accumulating evidence has indicated involvement of IKKα in endurance and development of cancers independent of NF-κB (Israel, 2010; Jiang *et al.*, 2010). IKKα potentially regulates cyclin D1 expression which is a well known oncogene involved in the pathogenesis of the breast cancer (BC) (Arnold and Papanikolaou, 2005). Moreover, the modulation of E2F1 transcription factor and consequent regulation of oestrogen induced cell-cycle progression reveal a considerable role of IKKα in BC (Tu *et al.*, 2006). In an *in vitro* study the invasion and propagation of BC cells is reported to be mediated by IKKα downstream of the membrane bound epidermal growth factor receptor Her2 (Merkhofer *et al.*, 2010). More detailed molecular characterization and exploration of new targets of IKKs can help to clarify their potential role in cancer biology.

IKKα regulated transcription factors such as NF-kB and E2F are directly involved in expressional regulation of a pleiotropic oncogenic Myc family of proteins. (Wierstra and Alves, 2008; Levens, 2010). c-Myc belongs to the basic helix-loop-helix leucine zipper (bHLHLZ) containing family of transcription factors (TFs) and is among the most well characterized and influential transcription factors as it is evidently involved in the regulation of almost 15% of human genes (Xu *et al.*, 2010). Extensive research during last three decades manifested the crucial role of Myc protein in cell growth, proliferation, apoptosis, gene expression and cancer metabolism (Henriksson and Luscher, 1996; Thompson, 1998; Lee and Dang, 2006; Miller *et al.*, 2012).

c-Myc is actively involved in the regulation of a variety of genes related to the cancer development and persistence (Gustafson and Weiss, 2010). The c-Myc plays a role in the cancer development either by translocation or extrinsically deactivating the critical regulatory mechanisms (Dave *et al.*, 2006). c-Myc plays a crucial role in angiogenesis (Baudino *et al.*, 2002) along with the regulation of metabolism, cell growth and metastasis to promote oncogenesis (Adhikary and Eilers, 2005; Dang *et al.*, 2006). Moreover, c-Myc activates cyclin D1, CDC25A, CDK4, E2F1 and E2F2 genes to endorse cell cycle progression (Meyer and Penn, 2008) and transcriptional repression of the p27 CDK inhibitor gene by c-Myc assists in cell proliferation (Yang *et al.*, 2001).

BC is one of the most commonly diagnosed malignancies and the second leading cause of cancer-related deaths among women worldwide (Jemal *et al.*, 2011). According to the World Health Organization (WHO) more than 1.4 million people are victimized globally by BC and 458,000 die of this disease every year (Asif *et al.*, 2014). BC associated mortality rate is on rise in developing countries (Forouzanfar *et al.*, 2011). Developing countries are predicted to face rapid death rate due to BC as compared to the developed countries (Donnelly *et al.*, 2013). Pakistan has highest incidence and mortality rate of BC among Asian countries (Bhurgri *et al.*, 2000; Asif *et al.*, 2014).

One of the leading causes of BC related mortality is the late diagnosis in Pakistan. Late stage diagnosis usually involves metastasis and is hard to treat thus, reduces the survival rate. Likelihood of up to 5 years survival is about 85% among the cases diagnosed at an early stage while it reduces to just 10 % among cases diagnosed at BC stage IV (Gilani *et al.*, 2003). Lack of public awareness about preventable risk factors and early warning signs is main cause of late diagnosis. Increase in survival rate of patients can be achieved by a significant approach of persuading awareness in population, better screening and in time detection of breast cancer at an initial stage.

Going through all reported data this study was aimed to explore:

- *In vitro* study of molecular role and cross talk between IKKs and c-Myc onco-protein.
- *In silico* analysis of genetic variations in c-Myc onco-protein and their structural and functional role.
- *In vivo* expression of IKKα in breast cancer patients and its correlation with clinicopathological features of Pakistani population.

Chapter 2

REVIEW OF LITERATURE

Cancer is a group of diseases involving abnormal cell growth with the propensity to invade or spread to other parts of the body. There are more than 100 different types of known cancers that can affect humans (Smyth *et al.*, 2006). In last few decades tremendous steps have been taken to explore the mechanisms involved in cancer progression. So far, numerous discoveries are made in this concern, but slew of still unanswered questions cued that cancer is actually an infinitely complex disease (Hayden and Ghosh, 2012). Knowledge to date indicates that fortunately, one third of all cancers can be prevented and further one third can also be cured if diagnosed at an early stage (Parkin, 1994).

2.1 CANCER AND SIGNALING PATHWAYS

Cell functions are carried out by controlled activities of molecules working together in form of a signaling cascade. Abnormal activation of these pathways leads to development of cancer. Considerable role of several signaling pathways in development of cancers has recently been made evident. Identification of extracellular environment and consequential cellular response involves cross talk of various signaling pathways (Eroles *et al.*, 2012). Any disruption in these integrated pathways can lead to initiation and progression of cancer. The epidermal growth factor receptor (EGFR) of ErbB family is abnormally activated in several cancers including BC (Carpenter, 1987). Four structurally related receptors of ErbB family include HER1/ErbB-1/ EGFR, HER2/ ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 (Martin, 2006). The ligand binding to cysteine-rich extracellular domain activates EGFR which undergoes autophosphorylation at the tyrosine kinase domain consequently activating EGFR regulated downstream signaling pathways including signal transducer and activator of transcription (STAT), mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK), phosphatidylinositol 3' -kinase (PI3K/AKT), and protein kinase C pathways (Atalay *et al.*, 2003).

The STATs family of transcription factors mediates changes in gene expression after interaction of cytokines and growth factors with their receptors on the cell surface. In general the STATs remain transiently activated but tyrosine-phosphorylated STATs especially STAT3 and STAT5 are reported to be constitutively active in a wide range of human cancers (Hynes and Gullick, 2006). STAT3 is implicated to promote BC development. Elevated levels of activated STAT3 is reported to be associated with increased breast cancer cell proliferation, endurance and metastasis while suppression of STAT3 expression in breast cancer leads to apoptosis, inhibition of cell growth and reduction of invasive potential. (Sato *et al.*, 2011).

Highly conserved Notch signaling pathway involves four Notch receptors and five ligands in mammals. It plays crucial role in cell growth, apoptosis, differentiation and angiogenesis through transcriptional regulation of cyclin D1, p21, c-Myc, Nuclear factor kappa light chain enhancer of activated **B** cells (NF-κB), vascular endothelial growth factor receptors (VEGFR) and HER2 (Al-Hussaini *et al.*, 2011). Disruption of the Notch pathway has been implicated in development of BC. It interacts with HER2 signaling pathway, which is active in approximately 20% BC cases and results in more aggressive outcome of the disease. Expression of HER2 is down regulated by inhibition of Notch signaling pathway (Osipo *et al.*, 2008). This pathway significantly promotes tumor growth and proliferation by renewal of treatment resistant tumor-initiating cells (TIC) and endorsement of tumor angiogenesis through MAPK activation (Zeng *et al.*, 2005). Aberrant activation of the Hedgehog (HH) signaling pathway has also been implicated in development of skin, digestive tract, breast, prostrate, brain and lung cancer (Ali, 2012). HH signaling is reported to be involved in invasiveness and metastasis of cancer (Feldmann *et al.*, 2007).

2.2 CANCER AND NF-κB SIGNALING PATHWAY

A significant hallmark of cancer pathogenesis involves regulation of well known NF-κB signaling pathway which is conserved in multi-cellular animals (Hanahan and Weinberg, 2011). The tumor microenvironment deregulates the pathway, leading to inflammation and cancer development. Aberrant activation of

NF-κB provides mechanistic basis for a number of cancers. Physiological, physical, or/and oxidative stress results in inflammation that can activate NF-κB signaling pathway (Ben-Neriah and Karin, 2011). Inflammation generally and NF-κB pathway particularly exhibit a double-edged role in cancer development. Constitutive activation of NF-κB leads to pro-tumorigenic role in a number of cancers. While on the other hand, activation of the NF-κB signaling pathway by activity of cytotoxic immune cells results in elimination of transformed cancer cells (Disis, 2010). This anti-tumorigenic role of the immune system involving NF-κB pathway has been designated as cancer immune surveillance (Smyth *et al.*, 2006). As this immune defense system of the body against cancer, is not strong enough to eliminate all the cancer cells thus a successive shift to an equilibrium phase and then an escape phase makes the cancer cells able to surpass the immune system (Dunn *et al.*, 2004).

NF-κB fuels inflammation through production of Cyclooxygenase 2 (COX-2) enzymes and inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), IL-6 and IL-8. Moreover, it causes stimulation of cell proliferation, promotion of inflammation and evasion of apoptosis by activation of cancer stem cells (CSCs) (Chefetz *et al.*, 2011) and up-regulation of several anti-apoptotic genes such as B-cell lymphoma 2 (BCL-2), BCL-XL, caspase8 and Fas-Associated protein with Death Domain (FADD) like apoptosis regulator (CFLAR) and cellular inhibitor of apoptosis proteins (cIAPs) (Basseres and Baldwin, 2006) (Figure 2.1).

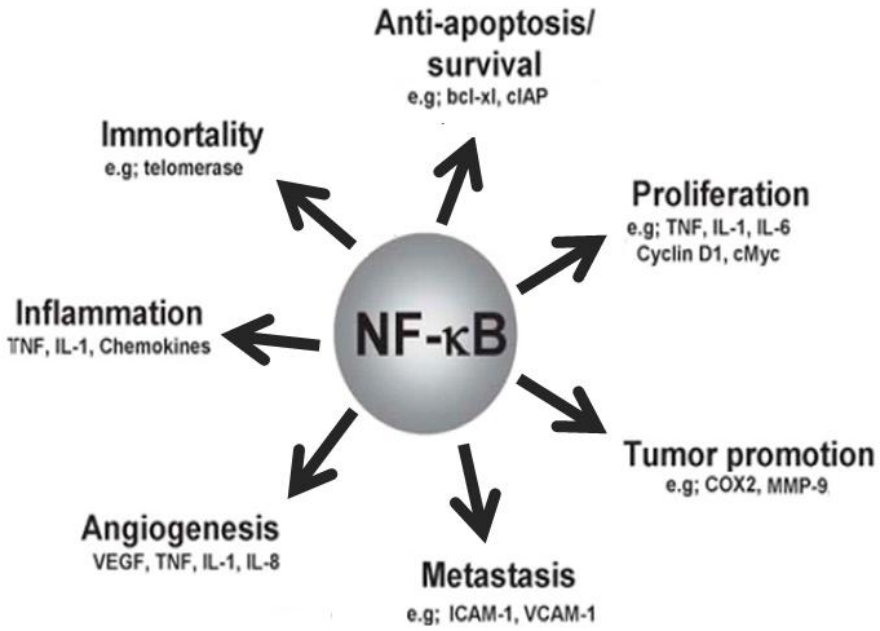

Figure 2.1: Role of NF-κB signaling pathway in cancer cell progression.

NF-κB signaling is reported to play a vital role in cancer progression by controlling up-regulation of VEGF and VEGFR responsible for enhanced blood supply to tumors through vascularization and angiogenesis. NF-κB is also involved in epithelial to mesenchymal transition and metastasis by targeting the adhesion factors including the vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1) and matrix metalloproteinases (MMPs) which help in loosening the extracellular matrix for evasion of the cancer cells (Huber *et al.*, 2004; Hoesel and Schmid, 2013) (Figure 2.1). Enhanced release of cytokine, presence of genetic variations in NF-κB genes and/or oncogenes involved in activation of NF-κB signaling pathway can directly elevate NF-κB activity in tumor microenvironment (Ben-Neriah and Karin, 2011). The steroid hormone receptors PR and ER also play significant roles in inflammation and cancer development. PR and NF-*κ*B are reported to suppress each other's activity (Van Der Burg and Van Der Saag, 1996).

2.2.1 Members of NF-κB Signaling Pathway

Initially the proteins of NF-κB family were characterized due to their interaction with the immunoglobulin light chain enhancer of the B cells (Sen and Baltimore, 1986). The family of NF-κB transcription factors comprises mainly five proteins i.e. RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Nabel and Verma, 1993) (Figure 2.2). A conserved Rel homology domain (RHD) of approximately 300 amino acids is common among these proteins. RHD is responsible for homo and heterodimerization along mediation of DNA contact. C-terminal trans-activation domains (TAs) are found among subset of RelA, RelB and c-Rel proteins which are needed for transcriptional activity (Shih *et al.*, 2011). Initially the NF-κB1 (p105) and NF-κB2 (p100) are produced as large inactive precursors that are ultimately degraded through ubiquitin proteasomal pathway to yield smaller active proteins; p50 and p52 respectively (Ghosh *et al.*, 1998). These active smaller proteins then form homo and heterodimers and are translocated to the nucleus where they bind to specific DNA targets as transcription factors to promote their activation (Bonizzi and Karin, 2004; Hayden and Ghosh, 2012).

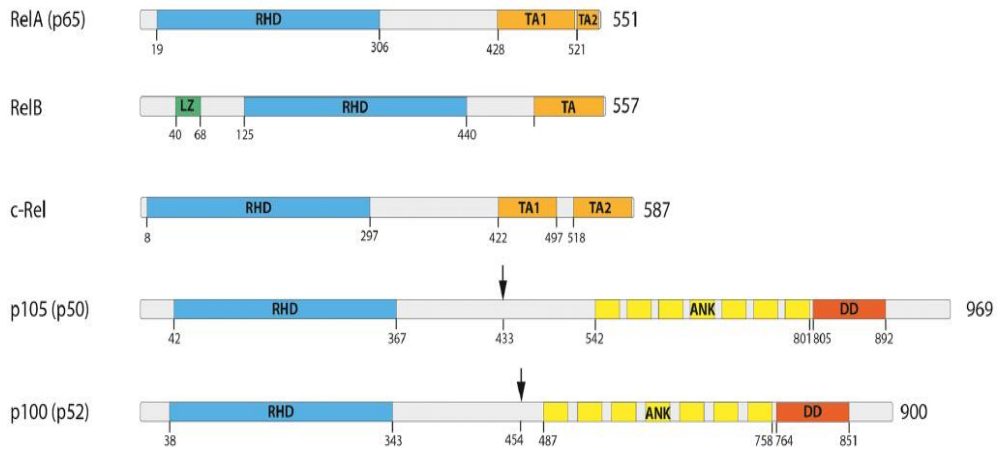

Figure 2.2: Members of the NF-κB family of proteins. NF-κB family of proteins contains five members; RelA (p65), RelB, c-Rel, p105 and p100. p105 and p100 yield their shorter forms p50 and p52, respectively. All members contain a N-terminal Rel homology domain (RHD) while three members (RelA, RelB and c-Rel) harbor C-terminal transactivation domain (TA). Leucine zipper (LZ) is present in RelB while Ankyrin (ANK) repeats and death domain (DD) are found in p105 and p100 (Hoesel and Schmid, 2013).

2.2.2 Inhibitor of NF-κB (IκB)

Activity of NF-κB transcription factors is controlled by a group of inhibitory molecules called inhibitor of NF-κB (IκB). The IκB family of proteins includes three members; IκBα, IκBβ and IκBε (Figure 2.3). Ankyrin (ANK) repeats are present among these proteins which are responsible for making complexes with NF-κB through non covalent association thus inhibiting its translocation to the nucleus (Hoesel and Schmid, 2013). In addition to the ANK repeats IκBα and IκBβ are characterized by the presence of PEST domains that are rich in proline (P), glutamate (E), serine (S) and threonine (T) and are necessary for constitutive turnover (Figure 2.5). Phosphorylation of IκB on two nearby serine residues (Ser32/Ser36; found in the N-terminal signal-response domain) is followed by polyubiquitination by specific Skp1-Cul1-F-box (SCF) type E3 ubiquitin-protein ligase and ultimate degradation by 26S proteasome (Karin and Ben-Neriah, 2000; Chen, 2005; Schmid and Birbach, 2008).

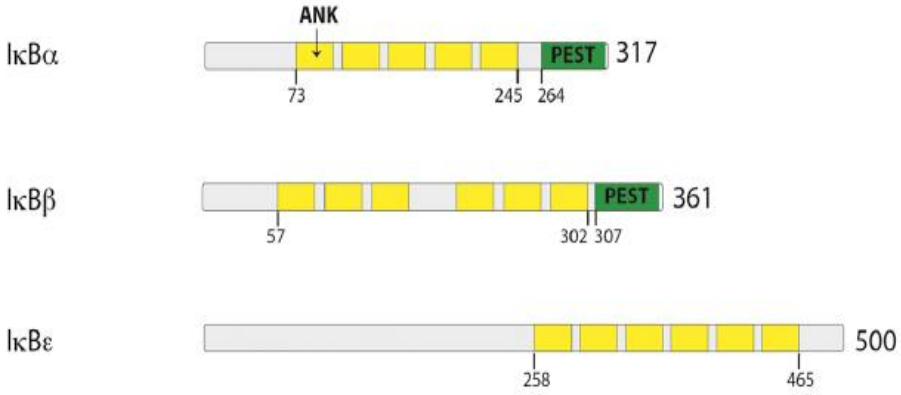

Figure 2.3: Members of the IκB family of proteins. Inhibitor of NF-κB (IκB) family of proteins includes three members; IκBα, IκBβ, IκBε. In addition to the ANK repeats IκBα and IκBβ have PEST domains (Hoesel and Schmid, 2013).

2.2.3 IKK Complex

Cytoplasmic IκB kinase (IKK) complex of about 700–900 kD was discovered to be responsible for phosphorylation of IκB. The IKK complex/IKK signalosome consists of two catalytic subunits: IκB kinase alpha (IKKα/IKBKA/IKK1), IκB kinase beta (IKKβ/IKBKB/IKK2) (Zandi *et al.*, 1997) and a non-enzymatic accessory NF-κB essential modulator (NEMO) (Yamaoka *et al.*, 1998) named IKK gamma (IKKγ) (Rothwarf *et al.*, 1998) (Figure 2.4). IKKα and IKKβ exhibit 50% similarity in sequence. IKKs contain an amino terminal kinase domain, helix-loop-helix (HLH) domain and a leucine zipper (LZ). IKKα contains a nuclear localization signal (Sil *et al.*, 2004) and is reported to shuttle between nucleus and cytosol. Despite the structural similarity of two kinase subunits and their presence in the same complex, they involve distinct proteins to perform different functions in the cell. It leads to two signaling pathways; canonical and non-canonical pathways (Hoesel and Schmid, 2013).

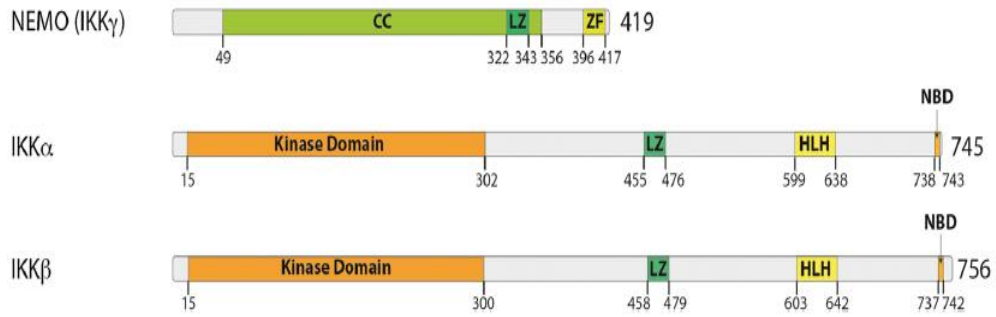

Figure 2.4: Members of IκB kinase (IKK) complex. IKK complex includes essential modulator (NEMO or IKKγ), IκB kinase α, (IKKα) and IκB kinase β (IKKβ). Essential domains of IKKs are Coiled-coil domain (CC), Leucin-zipper (LZ), death domain (DD), zinc-finger domain (ZF), helix-loop-helix domain (HLH), NEMO-binding domain (NBD) (Hoesel and Schmid, 2013).

2.2.4 Canonical and Non-canonical NF-κB Signaling Pathway

Canonical pathway is initiated by proinflammatory stimuli, such as lipopolysaccharides (LPS), TNFα and IL-1β and involves phosphorylation of IκBα principally by IKKβ (Perkins and Gilmore, 2006; Schmid and Birbach, 2008). Non-canonical pathway is initiated by different stimuli such as B-cell activation factor (BAFF), CD40 ligand, and lymphotoxin β (LTβ) and predominantly involves NF-κB inducing kinase (NIK) which phosphorylates IKKα (Park *et al.*, 2005) (Figure 2.5).

In the canonical NF-κB signaling pathway the tumor necrosis factor α (TNFα), lipopolysaccharides (LPS) or interleukin-1 (IL-1) activate their respective receptors. Involvement of a number of proteins results in activation of IKKβ, which phosphorylates IκBα. This phosphorylation is followed by polyubiquitination and proteasomal degradation of IκBα liberating NF-κB to translocate into the nucleus to start transcription activation of target genes. The canonical NF-κB signaling pathway results in transcriptional activation of different cytokines like TNF-α, IL-1β, IL-6 and Granulocyte-macrophage colony-stimulating factor (GMCSF), chemokines e.g. IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), adhesion

molecules (VCAM-1, ICAM-1, E-selectin) and enzymes like COX-2 and phospholipase A2 (PLA2) (Luo *et al.*, 2005).

In the non-canonical NF-κB signaling pathway, attachment of CD40 ligand, B-cell activation factor (BAFF) or lymphtoxin β (LTβ) to their respective receptors induces NF-κB-inducing kinase (NIK) which activates IKKα. The p100 after being phosphorylated by IKKα undergoes polyubiquitination and subsequent proteasomal processing to p52. Heterodimers of p52 and RelB are then translocated into the nucleus to initiate transcriptional activation of target genes (Figure 2.5). The non-canonical NF-κB signaling pathway results in transcriptional activation of different cytokines (BAFF), chemokines like B lymphocyte chemoattractant (BLC), secondary lymphoid tissue chemokine (SLC), stromal cell–derived factor-1α (SDF-1), Epstein-Barr virus–induced molecule 1 ligand chemokine (ELC) and lymphoid organogenesis genes like GlyCAM-1 (Luo *et al.*, 2005).

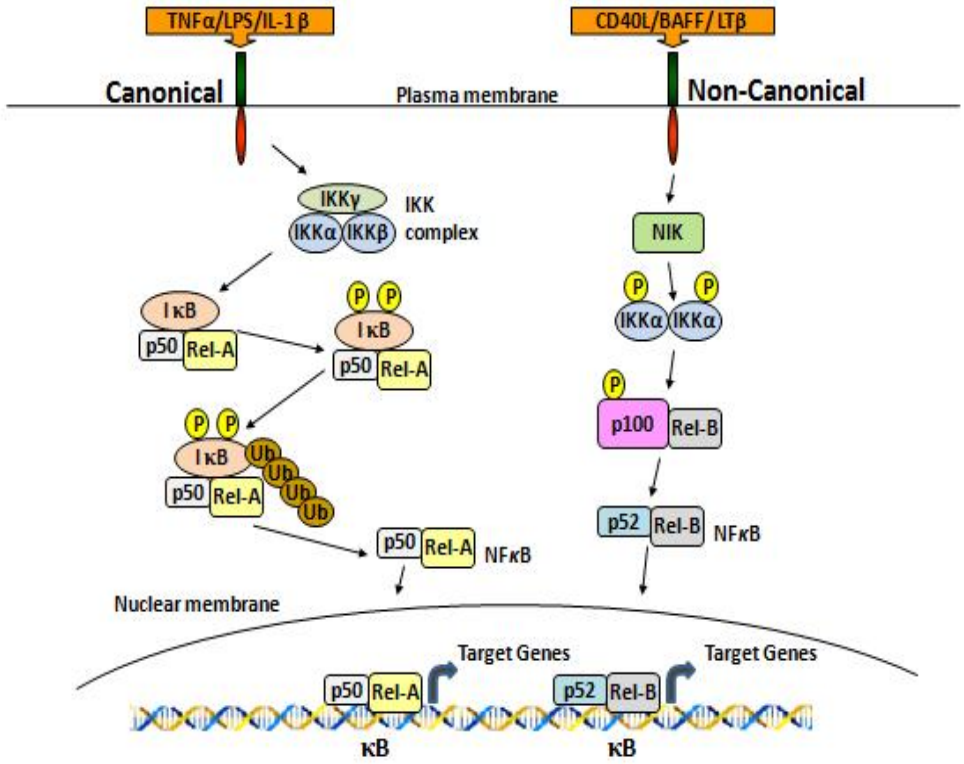

Figure 2.5: The canonical and non-canonical NF-κB signaling pathway. Canonical NF-κB signaling pathway is triggered by attachment of tumor necrosis factor α (TNFα), lipopolysaccharides (LPS), or interleukin-1 (IL-1) to their respective receptors. IKKβ is activated through involvement of a variety of adapter proteins and signaling kinases and phosphorylates IκB. Phosphorylated IκB is subjected to polyubiquitination and subsequent degradation allowing NF-κB homo or heterodimers to translocate to the nucleus and activate transcription of target genes (TNF, IL-1, IL-6 etc.). Non-canonical NF-κB signaling pathway is activated by attachment of CD40, B-cell activation factor (BAFF) or lymphtoxin β (LTβ) to their respective receptors. This activation leads to activation of IKKα through NF-κB-inducing kinase (NIK). Activated IKKα then phosphorylates p100 which is further subjected to polyubiquitination and proteasomal processing to p52. Heterodimers of p52-RelB is then translocated to the nucleus for transcriptional activation of target genes (BCL, BAFF etc.).

2.2.5 Role of IKKs in Cancer

Accumulating evidences have indicated that IKK family of proteins regulates many physiological and pathological processes by targeting a variety of substrates, which significantly widens their crucial role in development of cancers where their activity is enhanced and constitutive (Israel, 2010; Jiang *et al.*, 2010). IKKs can also affect a number of proteins independent of NF-κB involvement (Lee and Hung, 2008; Chariot, 2009). IKKα affects gene expression and function of cyclin D1 which is a major driver in the pathogenesis of multiple types of human cancers (Albanese *et al.*, 2003). Involvement of cyclin D1 in growth factor signaling pathway and cell cycle regulation designate it to be a potent oncogene. Activation and dysregulation of the cyclin D1, often by amplification or rearrangement result in loss of normal cell cycle control during tumorigenesis. More than 50% of human breast cancers are characterized by Cyclin D1 overexpression. (Arnold and Papanikolaou, 2005; Velasco-Velazquez *et al.*, 2011). IKKα regulates cyclin D1 through transcriptional activation, phosphorylation of cyclin D1 (Kwak *et al.*, 2005), estrogen receptor-α (ERα) (Park *et al.*, 2005) and steroid receptor coactivator (SRC3) (Wu *et al.*, 2002). IKKα is also reported to induce the promoter of cyclin D1 through activation of p52 (Rocha *et al.*, 2003) and stabilization as well as phosphorylation of β-catenin oncoprotein. IKKα inhibits polyubiquitylation of β-catenin thus increases its protein level and transcriptional activation (Carayol and Wang, 2006).

Activation of IKKβ is reported to stimulate pro-inflammatory, proliferative and anti-apoptotic pathways (Luo *et al.*, 2005) (Kucharczak *et al.*, 2003) however, its pro-apoptotic role can also not be ignored (Herrmann *et al.*, 2005; Baxter *et al.*, 2006). IKKβ can promote tumor endurance by phosphorylating FOXO3a which is a well known tumor suppressor that can induce apoptosis and cell-cycle arrest. This phosphorylation leads FOXO3a to nuclear exclusion and ultimate degradation (Hu *et al.*, 2004). Conversely, IKKβ is also found to induce apoptosis by activating FOXO3a in regressing mammary glands (Baxter *et al.*, 2006). IKKβ helps in cell proliferation by phosphorylating 14-3-3β protein (Gringhuis *et al.*, 2005). IKKβ regulates pro-proliferative MAPK signaling pathway by inducing proteolysis of

p105 subunit of NF-κB (Beinke *et al.*, 2004; Waterfield *et al.*, 2004) and phosphoralyting an adaptor docking protein 1 (DOK1) which down regulates MAPK and promotes cell motility (Lee *et al.*, 2004).

Some transcriptions factors are also among potential substrates of IKKs. IKKα directly phosphorylates protein inhibitor of activated STAT1 (PIAS1) upon TNFα stimulation. Phosphorylated PIAS1 is then recruited to the promoter of NF-κB target genes and inhibits the DNA-binding of p65 consequently repressing the transcription of genes responsible for production of pro-inflammatory cytokines and chemokines (Liu *et al.*, 2007). Moreover, modulation of E2F1 transcription factor and consequent regulation of estrogen induced cell-cycle progression reveal a considerable role of IKKα in breast cancer (Tu *et al.*, 2006). IKKs are also reported to prolong the stability of Myc oncoprotein (Yeh *et al.*, 2011). More detailed molecular characterization and exploration of new targets of IKKs can help to clarify their potential role in cancer biology.

2.3 MYC PROTEIN

The pleiotropic oncogenic c-Myc protein was first identified as a homolog of v-Myc avian myelocytomatosis viral oncogene about 3 decades back (Vennstrom *et al.*, 1982). Myc family of oncoproteins arises from three distinct gene family members; c-Myc, N-Myc, and L-Myc. Although these proteins differ in potency (Nesbit *et al.*, 1998) and patterns of expression (Zimmerman *et al.*, 1990), they are functionally related. Myc belongs to the basic helix-loop-helix leucine zipper (bHLHLZ) containing family of transcription factors and is among the most well characterized and influential transcription factors as it is evident by its involvement in the regulation of almost 15% of human genes (Xu *et al.*, 2010). N-terminal region contains four virtually identical and highly conserved domains, identified as Myc Box I (MBI), MBII, MBIII and MBIV (Cowling *et al.*, 2006). N-terminal region contains several well characterized phosphorylation and ubiquitination sites (Figure 2.6). The basic region (BR) of c-Myc mediates the DNA while the helix-loop-helix (HLH) and leucine zipper (LZ) domains mediate heterodimerization with other proteins (Hann, 2006). Extensive research during

last three decades manifested the crucial role of Myc protein in cell growth (Henriksson and Luscher, 1996), proliferation (Lemaitre *et al.*, 1996), apoptosis (Thompson, 1998), gene expression (Lee and Dang, 2006) and cancer metabolism (Miller *et al.*, 2012).

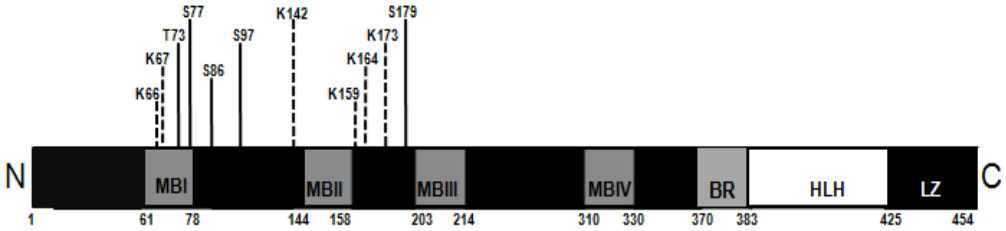

Figure 2.6: Schematic diagram of the significant domains and position of phosphorylation and ubiquitination in c-Myc protein. It details the location of highly conserved Myc homology boxes (MBI, MBII, MBIII and MBIV), basic region (BR), helix-loop-helix (HLH) and leucine zipper (LZ) domains. The numbers below represent the amino acid that borders each significant domain of protein. Specific N-terminal features, including the location of phosphorylation and ubiquitination (lysine residues) are indicated on upper side.

2.3.1 c-Myc Isoforms

Translational initiation of c-Myc gene at different start sites results in the production of three isoformes i.e. c-Myc1, c-Myc2/c-Myc (Hann *et al.*, 1988) and c-MycS (Hann, 1995) which have similar carboxy terminus while different N-terminal region. Translational start at AUG start site in second exon encodes a 64 kDa isoform i.e. c-Myc2 comprising 439 amino acids. Another translational start at a non canonical CUG located 15 codons upstream of the c-Myc2 initiation start results in addition of 15 amino acids at the N-terminal region and production of c-Myc1 (p67) , a 67 kDa longer isoform of 454 amino acids (Hann *et al.*, 1988). Translational start at AUG site positioned 100 codons downstream of c-Myc2 start site yields N-terminally-truncated 339 amino acid protein called c-MycS. This isoform lacks well conserved MBI motif found in 100 amino acids region of the trans-activation domain at N-terminus in major isoform (Spotts *et al.*, 1997).

2.3.2 c-Myc Regulation

The Myc protein makes a heteromeric complex with Myc associated factor X (MAX), which binds to E-boxes to activate transcription (Blackwood and Eisenman, 1991). Many coactivators and protein complexes also recruit to the E-box elements for this transcriptional initiation (Adhikary and Eilers, 2005). Myc expression in turn is regulated by other transcription factors such as NF-κB, E2F, STAT, and β-catenin (Wierstra and Alves, 2008; Levens, 2010). Myc mediated biological activities are mainly regulated by an intricate series of phosphorylations and dephosphorylations. In asynchronously growing cells , Thr58 (73 in c-Myc2) , Ser62 (77 in c-Myc2), Ser71(86 in c-Myc2) (Gupta *et al.*, 1993; Henriksson *et al.*, 1993; Pulverer *et al.*, 1994), Ser82 (97 in c-Myc2) and Ser164 (179 in c-Myc2) (Lutterbach and Hann, 1997) are identified to be the potential phosphorylation sites so far (Figure 2.6).

The conserved region MBI of c-Myc1 harbors crucial Thr58 and Ser62 at N-terminus. Selection of proline-directed kinase (s) to phosphorylate c-Myc is dependent on stimuli and cellular conditions. Phosphorylation of Myc at Ser62 by the c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) or cyclin-dependent kinase 1 (CDK1) results in stability of protein (Hann, 2006). Then Thr58 is phosphorylated by glycogen synthase kinase 3 (GSK3) (Gregory *et al.*, 2003) followed by dephosphorylation at Ser62 by protein phosphatase 2A (PP2A). At this stage monophosphorylated protein is subjected to ubiquitin/proteasome system for degradation. Although the advancement in research has revealed noteworthy effects of this kinase/phosphatase system on biological and molecular activities of c-Myc, however, the knowledge about exact mechanism and involved cofactors is still deficient (Hann, 2006; Wierstra and Alves, 2008). Some unidentified kinases can be involved in this outcome.

2.3.3 c-Myc and Cancer

c-Myc is actively involved in regulation of a variety of genes related to cancer development and persistence (Gustafson and Weiss, 2010). Lower

expression of c-Myc in normal resting cell becomes enormously high in the transformed cell stimulated via a broad range of signaling pathways, designating it to be an early response gene. It is not clear whether c-Myc over expression is the cause or the consequence of cell transformation (Miller *et al.*, 2012). The c-Myc plays a role in cancer development either by translocation or extrinsically deactivating the critical regulatory mechanisms (Dave *et al.*, 2006). Induction of c-Myc in most of the common cancers is typically brought about by gene amplification (Vita and Henriksson, 2006). Not only the number of copies of c-Myc (Escot *et al.*, 1986; Mariani-Costantini *et al.*, 1988) but the frequency of gene amplification is noteworthy in various human malignancies (Singhi *et al.*, 2012).

c-Myc plays a crucial role in angiogenesis (Baudino *et al.*, 2002) along with regulation of metabolism, cell growth and metastasis to promote oncogenesis (Adhikary and Eilers, 2005; Dang *et al.*, 2006). c-Myc brings about remarkable changes in cell morphology and promotes human mammary epithelial to mesenchymal transition (EMT) (Cowling and Cole, 2007). In breast cancer cells c-Myc regulates G1-S transition of cell cycle by activation of cyclin E/CDK2 and repression of CDK inhibitor p21 (Prall *et al.*, 1998; Mukherjee and Conrad, 2005). Moreover, c-Myc activates cyclin D1, CDC25A, CDK4, E2F1 and E2F2 genes to endorse cell cycle progression (Meyer and Penn, 2008). Transcriptional repression of the p27 CDK inhibitor gene by c-Myc assists in cell proliferation (Yang *et al.*, 2001).

2.3.4 c-Myc Polymorphisms and Cancer

Cancer risk is also influenced by the existing polymorphisms in the genome. One of the most frequent types of polymorphisms found in human genome is single nucleotide polymorphisms (SNP) (Noreen *et al.*, 2012). Generally the SNPs associated with disease risk are missense or nonsynonymous SNPs (nsSNPs) involving substitution of amino acids. These SNPs are likely to bring structural and functional changes in the protein. Genetic variations in cis-regulators of transcription at 8q24 can significantly change the germline expression levels of c-Myc thus contribute to cancer susceptibility (Sole *et al.*, 2008). Many studies are conducted to find association of a well documented SNP; rs13281615 harboring

the non-coding chromosomal region 8q24 near c-Myc and susceptibility of breast cancer, prostate cancer and colorectal cancer (Tomlinson *et al.*, 2007; Meyer *et al.*, 2009; Pei *et al.*, 2013). However, the results were inconsistent.

A meta-analysis including more than 100,000 subjects was performed to evaluate the role of this SNP in risk of breast cancer development. Increased risk of breast cancer development was found to be significantly associated with presence genotype "GG" (OR: 1.20, 95% CI: 1.12–1.29, p value: 0.001), and allele "G" (Odds Ratio (OR): 1.10, 95% Confidence interval (CI): 1.06–1.14, p value: 0.001) while the genotype "AA" was found to be a protective factor against risk of breast cancer development (OR: 0.89, 95% CI: 0.84–0.93, p value: 0.001) (Gong *et al.*, 2013).

Chromosomal region 8q24.21 harbors another discussable cancer risk SNP; rs6983267. Presence of allele "G" is variably frequent among all the populations ranging from 31% in native Hawaiians to 85% in African American population. In Europeans the allele frequency is around 50% (Wang *et al.*, 2014). The rs6983267 is reported to up regulate the c-Myc transcription (Takatsuno *et al.*, 2013) and alter the physical interactions of c-Myc by affecting binding of transcription factor 7-like 2 (TCF7L2) and TCF4 (Pomerantz *et al.*, 2009). Presence of rs6983267 is associated with susceptibility to gastric cancer, inflammatory breast cancer, prostate cancer, colorectal cancer and colorectal adenoma (Guo *et al.*, 2011; Bertucci *et al.*, 2012; Os'kina *et al.*, 2012; Wang *et al.*, 2014).

A cluster of four pathogenetically valuable nsSNPs; VAR_063384 (Glu39Asp), VAR_063385 (Pro57Ser), VAR_063386 (Pro59Ala) and VAR_063387 (Asn86Thr) was reported to harbor 2nd and 3rd exon of c-Myc2 trans-activation domain in Burkitt lymphoma samples (Bhatia *et al.*, 1993). A rare nsSNP (rs4645959) lying in N-terminal trans-activation domain of c-Myc2 was reported to be putatively functional. Heterozygous carriers of this variant among Polish and German subjects were observed to have an increased risk of familial breast cancer (Wirtenberger *et al.*, 2005). Later on a large population based centralized pathology review was conducted revealing no biological relevance of rs4645959 with risk, tumor characteristics or endurance of breast cancer (Figueiredo *et al.*, 2007). Molecular epidemiological association studies mainly

focused on the nsSNPs lying in coding region of the gene (Ramensky *et al.*, 2002; Savas *et al.*, 2004; Zhu *et al.*, 2004). Studying the structural and the functional impact of the nsSNPs on the protein can assist in the selection of functionally important nsSNPs.

2.4 BREAST CANCER

A group of uncontrollably proliferating cancer cells which originates from cells of the breast is known as BC. This malignancy can spread to surrounding and distant parts of the body. Breast cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among women across the globe (Jemal *et al.*, 2011). According to World Health Organization (WHO) more than 1.4 million people are victimized globally by breast cancer 458,000 die of this disease every year (Asif *et al.*, 2014). Male breast cancer is rare and is not thoroughly investigated (Johansson *et al.*, 2014).

BC diagnosis has been regularized in developed countries in 1980, however the BC incidences and the associated mortality rate is on rise in developing countries (Forouzanfar *et al.*, 2011). Developing countries are predicted to face rapid death rate due to BC from 2002 to 2020 as compared to the developed countries (Donnelly *et al.*, 2013). Information regarding morbidity and mortality of BC in Pakistan has been inadequate due to lack of cancer registration system at national level. One in every nine Pakistani women is diagnosed to be a breast cancer patient thus making the Pakistan a country with highest incidence rate of BC among Asian countries (Bhurgri *et al.*, 2000; Asif *et al.*, 2014). Despite of same socio-cultural environment in Pakistan and India the occurrence rate of breast cancer among Pakistani women is significantly higher (50/100,000) than that of Indian women (19/100,000) (Rasheed, 2013).

The female breast tissue is complex overlying the chest (pectoral) muscles. Breast has a number of lobules (milk producing glands) which have small sacs called alveoli. The lobules are linked together by a number of small ducts which ultimately open into larger ducts at areola. This network of ducts is responsible for

supply of milk to the nipple during breast feeding. Spaces around the lobules, ducts, blood and lymphatic vessel are filled with ligament, fat and connective tissue (cancer.org, 2014) (Figure 2.7).

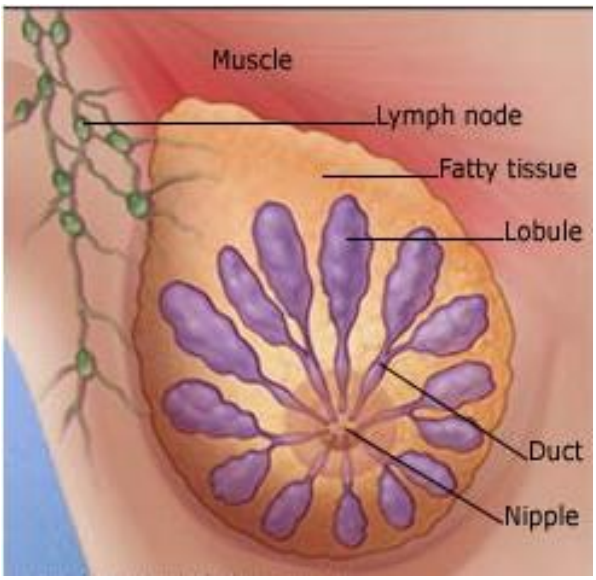

Figure 2.7: Anatomy of breast (www.webmd.com)

In most of the cases BC originates from the cells covering the ducts of breast and is called ductal carcinoma. Cancer starting in the cells that surround the lobules is known as lobular cancer of breast. Rarely the cancer can start from rest of the breast tissue (cancer.org). Non-invasive cancers stay benign and do not spread beyond their site of initiation while the invasive cancers not only invade surrounding healthy but can also metastasize to distant organs of the (Figure 2.8) (www.iamstillawoman.com). Unfortunately, invasive BC occur more commonly. (breastcancer.org, 2014). BC can be classified into different types depending on the area from where the tumor starts primarily. The most common (80%) type of BC is the ductal carcinoma. About 12% cases are of lobular carcinoma while others like Mucinous carcinoma, tubular carcinoma, Paget‘s carcinoma and lymphoma occur rarely (8%) (Bocker, 2002).

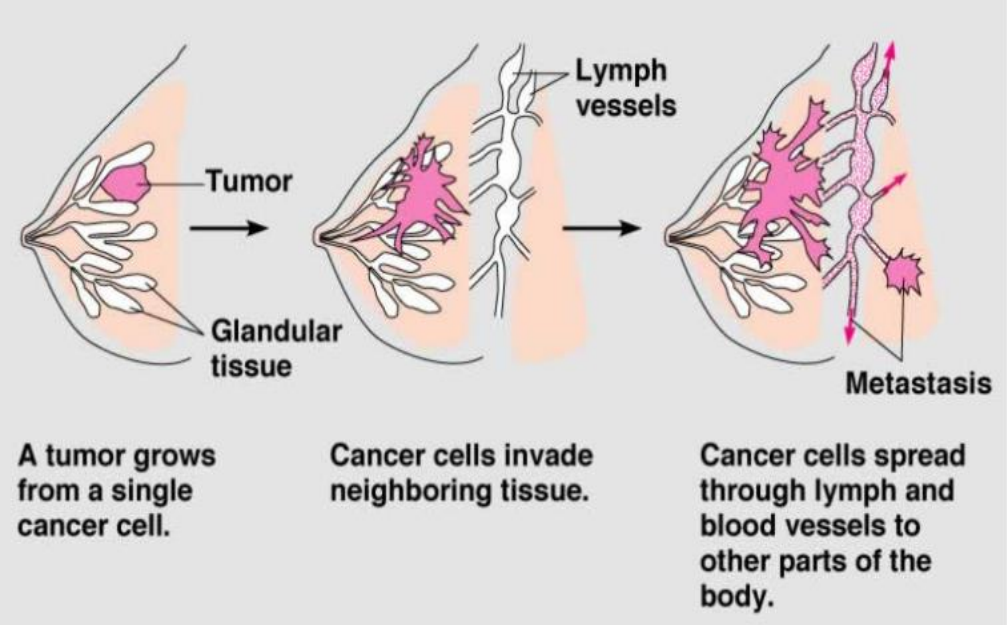

Figure 2.8: Types of breast cancer (adapted from www.iamstillawoman.com)

2.5 RISK FACTORS FOR BREAST CANCER

Anything that affects a person's chance of getting BC would be narrated as risk factor for BC. But risk factors do not elaborate all possible causes of the disease. Having one or more than one risk factors does not necessarily mean that a person will develop disease. A woman can develop BC in absence of any apparent risk factor or can remain healthy throughout her life even in the presence of one or more risk factors. Exact contribution of a specific risk factor is hard to know. What exactly is the cause of breast cancer is not completely known nevertheless, some factors are known to increase a person's chance of getting BC. These factors are usually of genetic, socio-biological, environmental and physiological nature (Hunter, 2000).

2.5.1 Non-Genetic Risk Factors

Risk of breast carcinoma development increases with increase in age but the trend varies among ER-positive and ER-negative cases. Mostly women at postmenopausal stage tend to have ER-positive tumors (Yasui and Potter, 1999).

Both; early menarche (<12 years) and late menopause (>55 years) are BC risk factors. Probable reason of this effect corresponds to long term exposure of body to endogenous hormones (Kelsey *et al.*, 1993). Delay of 2 years in regular menses leads to 10% risk reduction (Hsieh *et al.*, 1990). Risk of BC is increased up to 3% by each year's delay in the start of menopause (Trichopoulos *et al.*, 1972). Women who bear first full-term pregnancy at younger age have reduced BC risk in future life (Macmahon *et al.*, 1970). Parous women have lower risk of BC than nulli-parous women (Lambe *et al.*, 1994).

Breast feeding is reported to reduce the risk of breast cancer. A large scale study done by Collaborative Group on Hormonal Factors in BC (2002) analyzed data of 47 epidemiological studies conducted in 30 countries, revealed risk reduction of 4.3% for every addition of 12 months of breast feeding. This reduction in risk corresponds to the reduction of a woman's lifetime estrogen exposure due to ovulation suppression during breast feeding (Byers *et al.*, 1985). Breast feeding also causes terminal differentiation of epithelial cells of breast tissue. Differentiated cells have longer cell cycle, thus have more time available for DNA repair and are indifferent to carcinogenic effects (Russo and Russo, 1994). Presence of more dense breast tissue (Kerlikowske *et al.*, 2010), higher body mass index (BMI) (Zhu *et al.*, 2005), diabetes mellitus (Noto *et al.*, 2013), use of oral contraceptives (Hunter *et al.*, 2010) and postmenopausal hormone replacement (HRT) (Kerlikowske *et al.*, 2010) have also been reported to have an association with an increased risk of breast carcinoma. Socio-economic status, smoking and consanguineous marriage conferred to increase the risk of breast cancer (Asif *et al.*, 2014).

Lack of public awareness results in late diagnosis of BC; metastasized to other organs. Survival rate depends markedly on the stage of diagnosis. Detection at an early stage increases chances of treatment and the likelihood of up to 5 years survival is about 85% while five years survival rate at stage IV is just 10% (Gilani *et al.*, 2003). Increase in survival rate of patients can be achieved by a significant approach of persuading awareness in population, better screening and early detection of breast cancer.

The breast self examination (BSE), clinical breast examination (CBE) and mammography are the recommended screening methods (Okobia *et al.*, 2006). During BSE a woman can simply look and feel each of the breasts for presence of lump, swelling, distortion or any other significant change in the area of the breast. Physical examination performed by a health professional to identify changes in breast comes under the fold of CBE, while use of low energy X-ray with a higher sensitivity to detect presence of distinct masses and/or microcalcifications in human breast tissue is called mammography. BSE is a simple, inexpensive, easy and effective practice which can be carried out by the women themselves. It does neither require a professional expertise nor a visit to the hospital involving particular equipment. Regular performance of BSE can help in the early detection of Breast cancer and thus increasing chances of early treatment and survival rate in women (Al-Naggar *et al.*, 2011).

2.5.2 Genetic Risk Factors

Several genes are related to high risk of BC. Family history of breast cancer is one among well-documented risk factors. Presence of first degree relative with BC doubles the risk. Repair of breaks in DNA double-strand needs involvement of BRCA1 and BRCA2 (Chen *et al.*, 1999). Mutations in these genes lead to incompletion of this role leading to tumor development (Murphy and Moynahan, 2010). Presence of mutated BRCA1 and BRCA2 genes ascertain about 5% to 10% of all breast cancer cases. Their pattern of inheritance is autosomal-dominant (Evans *et al.*, 2005). Deletion mutation in either gene can result in 10-fold enhanced risk of breast cancer development (Mavaddat *et al.*, 2013). Carriers of Mutated BRCA1 and BRCA2 have about 65-85% and 40-85% risk of breast cancer respectively (Chen and Parmigiani, 2007; Evans *et al.*, 2008). Mostly patients having mutated BRCA1 are triple negative, i.e. the tumor cells do not have estrogen receptors (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Papelard *et al.*, 2000). Presence of BRCA2 mutations is associated with sporadic cancers mostly expressing ER and PR involving just 16% triple negative cases (Mavaddat *et al.*, 2012).

BC has a well known link with another high-penetrance gene; P53. Mutated P53 is related to Li-Fraumeni syndrome (LFS) which makes women more susceptible to breast cancer development. Mutation in another tumor suppressor gene; PTEN of MAPK/mTOR pathway results in PTEN hamartoma tumor syndrome/Cowden syndrome which significantly increases risk of breast cancer (Walsh and King, 2007). Mutation of PTEN is less prevalent but there is 85% life time BC risk for those who have germline PTEN mutations (Tan *et al.*, 2012). Numerous low-penetrance genes are also reported to increase risk of BC. Mutated ATM, BRIP1, CHEK2, NBS1, PALB2, and RAD50 can lead to an increase of 2-4 folds in breast cancer risk (Walsh and King, 2007). Further studies are being done to explicate more genes associated with breast cancer tumorigenesis.

Keeping all the literature in view, this study was aimed to assess expressional and interactional analysis of IKKs in oncogenic cellular transformation and *In Silico* evaluation of potential nsSNPs in c-Myc protein. Furthermore, knowledge of early warning signs and potential risk factors of breast cancer in population was also among aims of study.

Chapter 3

MATERIALS AND METHODS

3.1 PREPRATION OF BUFFERS

3.1.1 Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8 g NaCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml distilled water. The pH was adjusted to 7.4 using hydrochloric acid (HCl). Total volume was made up to 1 liter (1 L) by adding additional distilled water and buffer was sterilized by autoclaving.

3.1.2 Radio Immunoprecipitation Assay (RIPA) Buffer

RIPA buffer was prepared by mixing 150 mM Sodium chloride, 50 mM Tris (pH 8), 1.0% triton X100 and 0.5% Sodium dodecyl sulfat (SDS).

3.1.3 Resolving Gel Solution

10% resolving gel solution was prepared by mixing 4.8 ml water, 2.5 ml acrylamide/bis-acrylamide (37:1) solution (40%), 2.5 ml of 1.5M Tris base (pH 8.8) and 100 µl SDS (10%). After that 100 µl freshly prepared 10% ammonia persulphate (APS) solution and 10 µl Tetra-ethylethylene-diamine (TEMED) was added to start polymerization (Table 3.1).

Table 3.1: Recipe of 10% resolving gel

Water (ml)	3.6	4.8	7.2
40% Gel solution (ml)	1.9	2.5	3.75
Tris 1.5M (pH 8.8)	1.9	2.5	3.75
10% SDS (µl)	75	100	150
10% APS (µl)	75	100	150
TEMED (µl)	7.5	10	15
Total Volume	7.5 ml	10 ml	15 ml

3.1.4 Stacking Gel Solution

5ml stacking gel solution was prepared by mixing 3.645 ml water, 625 µl acrylamide/bis-acrylamide (37:1) mixture (40%), 630 µl of 1 M Tris base (pH 6.8) and 50 µl SDS (10%). After that 50 µl freshly prepared APS (10%) and 5 µl TEMED was added to start polymerization.

Table 3.2: Recipe of stacking gel

Water (ml)	2.185	3.645	5.84
40% Acrylamide mixture (ml)	0.375	0.625	1
Tris 1.5M (pH 6.8)	0.38	0.63	1
10% SDS (µl)	30	50	80
10% APS (µl)	30	50	80
TEMED (µl)	3	5	8
Total Volume	3 ml	5 ml	8 ml

3.1.5 SDS Transfer Buffer

SDS transfer buffer was prepared by adding 2.9 g glycine (25 mM), 200 ml methanol, 5.8 g Tris base (50 mM), 0.4% SDS in water and pH was maintained at 8.0.

3.1.6 Assay Buffer

Assay buffer was prepared by mixing 20 µl $MgSO_4$ (20 mM), 200 µl adenosine triphosphate (ATP) (4 mM) and 780 µl Glacylglicine buffer (25 mM). The pH was adjusted to 7.8 using HCl.

3.2 PLASMID CONSTRUCTS

Plasmids containing DNA; GFP (pEGFP-C1, Clontech) (Figure 3.1) , RFP (pDsRed2-C1, Clontech) (Figure 3.2), GFP tagged IKKα, GFP tagged IKKβ, GFP tagged truncated IKKα (HLH and LZ of IKKα was cut out using EcoRV and Sal I restriction enzymes (Fermentas, Leon-Rot, Germany), DsRed tagged c-My, c-Myc luciferase expression construct pMyc-TA-Luc (Clontech) (Figure 3.3) and constitutively expressing β-Galactosidase normalization construct pub6/V5-His/lacZ (Invitrogen) (Figure 3.4) were used in this study.

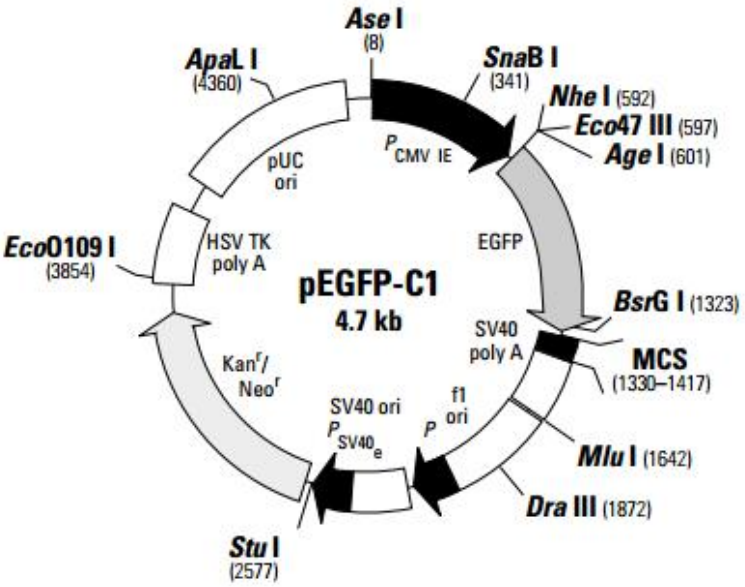

Figure 3.1: Restriction Map of pEGFP-C1 Vector. The IKKα and IKKβ cDNA, with signal sequence were separately cloned downstream of the cytomegalovirus (CMV) promoter in the expression vector pEGFP-C1. The origin of replication along with kanamycin/neomycin resistant genes, multiple cloning site (MCS) and unique restriction sites (bold) for some crucial restriction enzymes in the plasmid are shown.

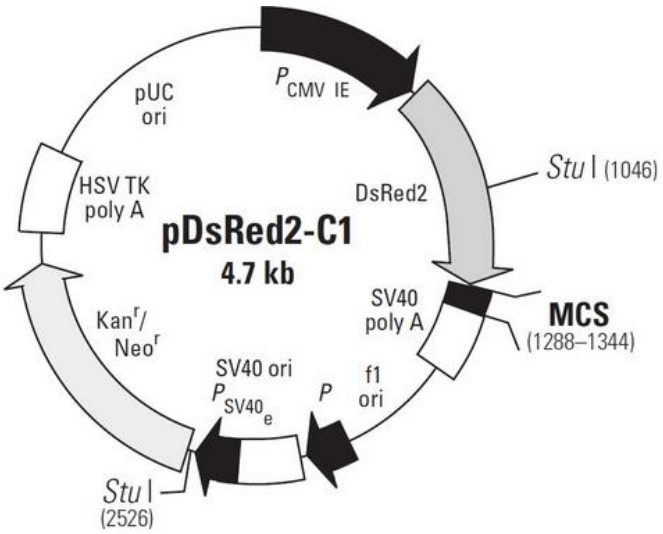

Figure 3.2: Map of pDsRed2-C1 Vector. cDNA of the c-Myc gene, with signal sequence was cloned downstream of the cytomegalovirus (CMV) promoter in the expression vector pDsRed2-C1. The origin of replication along with kanamycin/neomycin resistant genes, multiple cloning site (MCS) and unique restriction sites for some crucial restriction enzymes in the plasmid are shown.

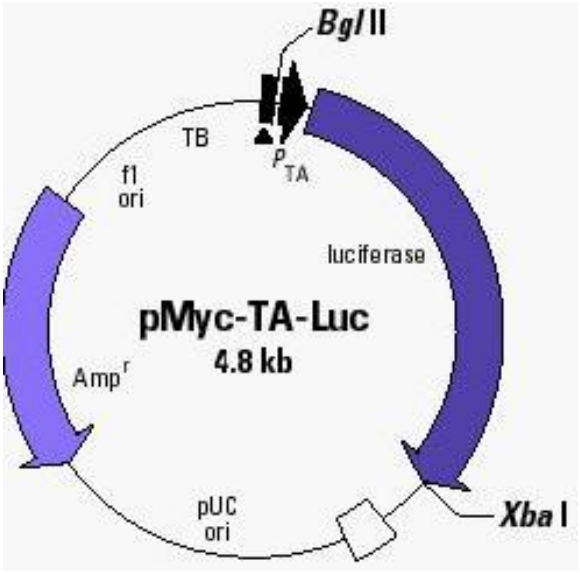

Figure 3.3: Map of pMyc-TA-Luc Vector. The origin of replication along with ampicillin resistant gene, transcription blocker (TB), luciferase gene and unique restriction sites (bold) for some crucial restriction enzymes in the plasmid are shown.

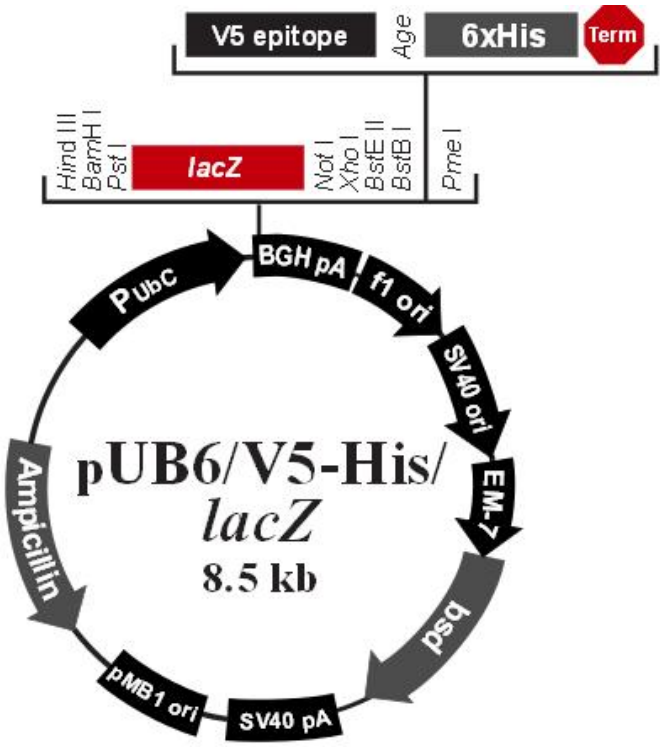

Figure 3.4: Map of pub6/V5-His/lacZ Vector. The origin of replication along with ampicillin resistant gene, SV40 promoter and unique restriction sites for some crucial restriction enzymes in β-Galactosidase normalization construct pub6/V5-His/lacZ are shown.

3.3 PRIMERS USED FOR qPCR

The coding sequences of IKKα, IKKβ, c-Myc and GAPDH were taken from GenBank and one set of forward and reverse primers for each gene was developed using the Primer3 (http://frodo.wi.mit.edu/primer3/) software for qPCR (Table 3.3).

Table 3.3: Primers for qPCR

Gene	Primers	Annealing temperature	Amplicon size (bp)
IKKα	Forward: 5'-GAAGGTGCAGTAACCCCTCA-3'	58°C	169
	Reverse: 5'-ATTGCCCTGTTCCTCATTTG-3'	57°C	
IKKβ	Forward: 5'-GCTGCAACTGATGCTGATGT-3'	58°C	157
	Reverse: 5'-TGTCACAGGGTAGGTGTGGA-3'	59°C	
c-Myc	Forward: 5'-TCAAGAGGCGAACACACAAC-3'	58°C	110
	Reverse: 5'-GGCCTTTTCATTGTTTTCCA-3'	57°C	
GAPDH	Forward: 5'-CCTGTTCGACAGTCAGCCG-3'	59°C	193
	Reverse: 5'-CGACCAAATCCGTTGACTCC-3'	58°C	

3.4 PLASMID DNA PREPARATION

For plasmid containing DNA preparations the GeneJET Plasmid Midiprep Kit (Cat No: K0481, Thermoscientific, Lithuania) was used. 250 ml Erlenmeyer flasks containing 50 ml LB broth and appropriate selective antibiotics (ampicillin 100 µg/ml or kanamycin 25 µg/ml) were inoculated with the desired transformed single colony of E. coli strain and incubated at 37°C with vigorous shaking at 200 rpm in orbital shaker.

The cells were harvested by centrifugation at 5000 g for 10 minutes in centrifuge (Biofuge Primo R, Thermo Scientific, Germany). The supernatant was discarded. The pelleted cells were resuspended in 2 ml of resuspension solution by vortexing with vortex mixer (Heidolph, Schwabach, Germany) and pipetting up and down until no cell clumps remained. 2 ml of lysis solution was added and mixed gently by inverting the tube 4-6 times until the solution became viscous and slightly clear. Then the solution was incubated for 3 minutes at room temperature. 2 ml of the neutralization solution was added and mixed immediately by inverting the tubes 5-8 times. After it, 0.5 ml of the endotoxin binding reagent was added and mixed immediately by inverting the tubes 5-8 times and was incubated for 5 min at room temperature.

The mixture was centrifuged for 20 minutes at 20,000 rpm (48,000 g) to pellet cell debris and chromosomal DNA. After transferring the supernatant into a 15 ml tube, 1 volume of 96% ethanol was added and mixed immediately by inverting the tube 5-6 times. Part of the sample (5.5 ml) was transferred to the supplied column pre-assembled with a collection tube (15 ml) and was centrifuged for 3 min at 2,000 g in a swinging bucket rotor. The flow-through was discarded and the column was placed back into the same collection tube. Remaining lysate was processed in the same way through the purification column. After that, 4 ml of Wash Solution I (diluted with isopropanol) was added to the purification column and was centrifuged for 2 minutes at 3,000 g in a swinging bucket rotor. The flow-through was discarded and the column was placed back into the same collection tube.

Then 4 ml of Wash Solution II (diluted with ethanol) was added to the purification column and centrifuged for 2 minutes at 3,000 g in a swinging bucket rotor. The flow-through was discarded and the column was placed back into the same collection tube. Column wash was repeated with Wash Solution II. Then it was centrifuged for 5 min at 3,000 g in a swinging bucket rotor to remove residual wash solution. The collection tube containing the flow-through was discarded and the column was transferred into a fresh 15 ml collection tube (provided). Then 0.35 ml elution buffer was added to the center of the purification column membrane and was incubated for 2 minutes at room temperature. Then it was centrifuged for 5 min at 3,000 x g in a swinging bucket rotor to elute plasmid DNA.

The purification column was discarded and the purified plasmid DNA was stored at -20°C. DNA was taken out by adding 250 µl elution buffer. Then 500 µl DNA precipitation solution was added in it and the mixture was kept on -20°C overnight. Next day, mixture was centrifuged at 1100 rpm for 15 mints at 4°C. Visible pellet of DNA was separated by throwing off the supernatant. 70% ethanol was added to the tube and was incubated at room temperature for 10 minutes. Tube was again centrifuged at 4°C for 7 minutes. Ethanol was thrown off and pellet was resuspended in 200 µl distilled water.

3.5 FLUORESCENT MICROSCOPY

Fluorescence resonance energy transfer (FRET) analysis was carried out in order to find the interaction of IKKα and IKKβ with c-Myc protein using laser scanning microscope containing a photometrics coolsnap camera and suitable fluorescence filters controlled by Zeiss LSM 510 software. PixFRET plugin of ImageJ software was used to examine and analyze the interaction. The graph was plotted to find mean FRET efficiency ± SEM (standard error of mean) of ten different cells for negative control and the cells indicating interaction.

3.5.1 Cell Culture

Immortalized, adherent human embryonic kidney (HEK 293) cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 µg/ml), streptomycin (100 µg/ml), glutamine 2 mM. Cells were cultivated in 75 cm^2 tissue culture flasks at 37°C, in a humidified (90%) and CO_2 (5%) containing atmosphere. Cells were trypsinized and passaged every second or third day in proportion of 1:4 or 1:6, respectively. Passaging was done in laminar flow hood. For passaging the cell culture medium was removed, about 13 ml of PBS was used to wash the cells. PBS was removed completely and about 3 ml trypsin solution (37°C) was pipetted into the flask and spread evenly all over the bottom.

Excessive trypsin was removed and the flask was placed in the incubator for 3 minutes. With an inverted microscope (Nikon TMS F 4X or 10X objective) it was checked if the cells were already detached and round in shape. Trypsinized cells were gently resuspended by several times up and down pipetting in 10 ml complete DMEM and 2 ml were transferred into a new tissue culture flask containing 13 ml of DMEM complete medium. Cells in tissue culture flasks were placed at 37°C in a humidified (90%) and CO_2 (5%) containing atmosphere.

3.5.2 Cell Seeding on Cover Slip

Cells were checked under inverted microscope (Nikon TMS F 4X or 10X objective) for confluency. Healthy, live, intact and about 90% confluent cells were subjected to passaging. Trypsinized cells were gently resuspended by several times up and down pipetting in 24 ml complete DMEM. Sterile glass cover slip (15 mm) were again sterilized by washing with 70% ethanol and were kept to dry on tissue paper. One completely dry glass cover slip was placed in each well of 12 wells cell culture plate. Cells were resuspended gently by up and down pipetting and 2 ml medium with cells was dropped on each glass cover slip. Plate was rocked back and forth to assure equal distribution of cells over the glass cover slip. Plate was placed in humidified (90%) and CO_2 (5%) containing incubator at 37°C.

3.5.3 Transfection

After 24 hours, at 70% confluence the cells were transfected. Transfection of GFP alone, RFP alone, GFP and RFP in combination as negative control, GFP-IKKα, GFP-IKKβ, GFP-Trun. IKKα and DsRed-c-Myc was carried out using $CaCl_2$ method. For each well, 36 µl of 1X Hepes Buffered Saline (HBS) was placed in a sterile tube. 2.1 µg respective DNA was added followed by addition of 2.1 µl $CaCl_2$ (2 M) to each tube and mixed immediately. After incubation for 20 minutes at room temperature the transfection mixture was added in a drop wise fashion on surface of the cells in cell culture dish. Plate was rocked back and forth and was put in 5% CO_2 incubator at 37°C for 24 hours.

3.5.4 Live Cell Microscopy and Analysis

Transfection of the cells was analyzed through detection of fluorescently labeled transfected cells under fluorescent microscope. An appropriate metallic chamber having a central hole of 13 mm was taken. Edges of the chamber hole were greased to hold the glass cover slips. Glass cover slip was fixed in metallic chamber taking care of the cells facing upwards. Cells were covered with 80 µl DMEM medium and a new plain glass cover slip was placed over the medium in metallic chamber. Glass cover slips were fixed carefully avoiding any bubble or leakage of the

medium. Chamber was fixed on stage and focus was adjusted for live cell fluorescent microscopy. FRET microscopy was carried out using laser scanning Zeiss Axiovert 135 microscope containing a photometrics coolsnap camera and suitable fluorescence filters controlled by Zeiss LSM 510 software. Images were acquired with 40X oil immersion objective using the 3-filter method. Fluorescence of GFP was imaged by exposing the cells to 10% intensity of the 488 nm argon laser beam for using bandpass emission filter (505–550 nm) while that of DsRed was acquired with 100% intensity of the 543 nm argon laser beam and the spectral detector set to longpass emission starting at 574 nm. All the images were acquired under exactly the same conditions. Calculation of spectral bleed through for the cells expressing only GFP or DsRed and determination of corrected FRET efficiency indicating physical interaction of candidate proteins was brought about using PixFRET plugin of ImageJ software.

3.6 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

3.6.1 Cell Culture and Transfection

For qRT-PCR HEK 293 cells were cultured in 12 wells tissue culture plate. After one day transfection of 80% confluent cells was carried out. Plasmid DNA of IKKα, IKKβ, si-Myc, si-Myc control and c-Myc were transfected in triplicates using ExGen 500 (Cat: R0521, Roche, Germany). For each well, 2 µg of DNA was diluted in 100 µl of 150 mM NaCl. The mixture was vortexed gently and centrifuged briefly. Then 7.7 µl of ExGen 500 was added to the diluted DNA (not the reverse order) and the solution was vortexd immediately for 10 seconds. Mixture was then incubated for 10 minutes at room temperature. ExGen 500/DNA mixture was added slowly as drops to the cells in each well. The plate was gently rocked back and forth and from side to side to achieve even distribution of the complexes. After centrifugation at 280 g for 5 minutes the cell culture plate was placed in a CO_2 incubator at 37°C for 24 hours.

3.6.2 RNA Isolation

After 48 hours transfection of the cells was confirmed by observing the cells under fluorescent microscope. Media was sucked out and the cell layer was rinsed with 1 ml ice cold PBS. To each well, 500 µl TRIZOL reagent was added to lyse the cells in cell culture plate. Cell lysate from each well was transferred to properly labeled 1.5 ml eppedorf tube. Tubes were centrifuged at 300 X g for 5 minutes. Supernatant was removed and the pellet of cell lysate was resuspended in 0.5 ml ice cold PBS. Tubes were incubated for 5 minutes at room temperature and centrifuged to remove the cell debris. The supernatant was transferred to new 1.5 ml eppedorf tube.

After addition of 150 µl chloroform to each tube, the tubes were vortexed vigorously for 15 seconds and were incubated at room temperature for 5 minutes. Tubes were centrifuged at 12,000 g for 15 minutes at 8°C. This centrifugation

resulted in distinguishable separation of the mixture into lower phase, middle interphase, and an upper colorless aqueous phase. The RNA was intact in the upper most aqueous phase. Therefore, upper phase was taken out very carefully avoiding the middle interphase and was transferred to the fresh eppedorf tube. To each tube, 250 µl Isopropanol was added and incubated at 25^{o}C for 10 minutes. Tubes were centrifuged at 11,000 g for 10 minutes at 4^{o}C and the RNA pellet became visible at bottom of the eppedorf tube. The RNA pellet was washed with 70% ethanol and centrifuged at 7,000 g for 5 minutes at 4^{o}C, three times. Tubes were left open to get air dry for 8 minutes and 100 µl diethylpyrocarbonate (DEPC) treated water was added to each pellet and RNA was mixed in it gently.

3.6.3 cDNA Synthesis

cDNA synthesis kit (Thermoscientific, Rockford, USA) was used to synthesize cDNA from template RNA. Kit components were mixed briefly after thawing. Template RNA was measured on nanodrop and 500 ng RNA from each sample was diluted in nuclease free water into a sterile PCR tube placed on ice. Then 1 µl random hexamer primer was added to the mixture and volume was made up to 12 µl. After that, 4 µl 5X reaction buffer, 1 µl RNase inhibitor (20 u/µl), 2 µl dNTP mixture (10 mM) and 1 µl reverse transcriptase (200 u/µl) was added to the mixture to make total volume of 20 µl.

PCR mixture was gently mixed, centrifuged and placed in thermocycler (Applied Biosystem, Foster City, USA). The reaction mixture was incubated at 42°C for 60 minutes. Reverse transciption reaction was further processed for 25 cycles through thermocycling conditions of 5 minutes at 25°C for primers annealing and 80 seconds at 45°C for extension. Reaction was terminated by heating at 70°C for 10 minutes.

3.6.4 qRT-PCR

SYBR green PCR master mix (Applied biosystems, USA) was used for qRT-PCR. 1 µl of template cDNA, 1 µl (500nM) forward and 1 µl (500nM) reverse primer was added to each reaction mixture. Reaction was run on a StepOne

Plus machine (Applied Biosystems, Germany). Crossing threshold (Ct) points were calculated using StepOne Plus software while delta/delta-Ct method was used to calculate the relative expression of target genes.

3.7 WESTERN BLOT ANALYSIS

3.7.1 Cell Culture and Transfection

HEK 293 cells were cultured in 6 well cell culture plate. After one day two wells of 70% confluent cells were left untreated, while each two wells were transfected with IKKα , mutant IKKα (inactive), c-Myc and IKKα+c-Myc together using X-tremeGENE 9 transfection reagent (Cat: 06365787001, Roche, Germany). Vial of X-tremeGENE 9 was brought to 25°C. After vortexing and mixing the transfection reagent was diluted with serum-free DMEM to a concentration of 3 µl reagent/100 µl medium for ratio of 3:1 in a sterile 1.5 ml eppendorf tube. Then 1 µg DNA was added and gently mixed to 100 µl of diluted transfection reagent. Transfection reagent:DNA complex was incubated for 15 minutes at room temperature. Transfection complex was added to the cells in form of drops. Plate was gently swirled to ensure even distribution over the entire plate surface and was incubated in 5% CO_2 incubator at 37^oC for 48 hours. Then one well each of untransfected cells, transfected with IKKα , mutant IKKα (inactive) and IKKα+c-Myc were treated with Tumor necrosis factor α (TNFα) for half an hour prior to cell extract formation to stimulate expression and activity of IKKα.

3.7.2 Cell Extract Preparation

In order to prepare cytosolic cell extracts, 100 µl RIPA buffer and 1X protein inhibitor was added to each well. Plates were incubated for 1 minute to ensure effective lysis of the cells. Effective lysis of the plasma membrane was monitored with the help of inverse microscope under 10 X objective. After lysis the whole cell lysate from each well was taken in 1.5 ml eppendrof tube and centrifuged at 14000 g at -20°C for 20 minutes to eliminate the nuclei and parts of plasma membrane. The supernatant was shifted to fresh properly labeled 1.5 ml eppendrof tube.

3.7.3 Protein Quantification

Cell extracts of each sample were diluted as 1:10 in distilled water. In a 96 wells plate 200 µl of diluted Bradford reagent was pipetted out in each well. 1 µg/ µl bovine serum albumin (BSA) was dissolved in PBS. For the generation of BSA standard curve, 0 to 5 µg of BSA was pipetted into first 5 wells of 96 well plate. Then 1µl of diluted cell extract of each sample was added for quantification of protein to stay in the linear range. After 5 minutes incubation at room temperature the absorbance was measured at 595 nm with an Elisa reader (SLT Lab instruments 340 ATTC). The standard curve of BSA was evaluated in MS Excel sheet. After analysis of linear regression the protein amount of each sample was quantified.

3.7.4 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also performed. 10 ml resolving gel mixture (Table 3.1) and 5 ml stacking gel mixture (Table 3.2) was prepared for preparation of 1 gel (6 cm x 9 cm x 0.015 cm). The gel cast system (BioRad Mini Gel system, Germany) was filled with water to check its proper fitting. Then water was poured off and the separating gel solution was filled up to 5 cm height in gel cast. A layer of isopropanol was pipetted on top to cover surface completely to prevent oxygen contact with gel solution. As the oxygen contact disables polymerization. Polymerization of resolving gel completed in 30 minutes, thus the layer of isopropanol was poured off and the stacking gel solution was immediately layered on top of the resolving gel. Comb (1 mm) was inserted carefully between glass plates avoiding bubbles and the gel was kept undisturbed for 30 minutes.

Protein lysate of each sample containing 30 µg of protein was mixed with water to make the total volume up to 20 µl. Then 5 µl of 5X SDS sample buffer was added to each sample and cooked on 95 °C for 5 minutes and centrifuged at full speed for 2 minutes in a table top centrifuge machine. The gel sandwich was taken out of the gel cast and placed carefully in the electrophoresis tank. 1x SDS running buffer was filled in the inner and outer chamber of the tank and comb was

removed. Slots of the gel were cleansed with running buffer pipetting up and down few times to remove half-polymerized pieces of gel. 7 µl prestained protein marker and 25 µl of all the samples were loaded in the gel. Lid of gel tank was closed and the gel was allowed to run immediately to avoid diffusion of the loaded samples to surrounding area of the gel. Electrophoresis parameter of power supply was firstly adjusted on 90 volts for 10 minutes so that proteins stage on border of the stacking gel and then on 140 volts for 1 hour.

3.7.5 Wet Electrophoretic Gel Transfer

Transfer of polyacrylamide gel to polyvinylidene fluoride (PVDF) membrane was carried out by wet electrophoretic transfer method. Equal sized piece of PVDF membrane, blotting sponge and 3MM whatman filter paper were cut. The blotting sponge and 3MM whatman filter paper were soaked in 1X SDS transfer buffer. The PVDF membrane was charged with 100% methanol for 3 seconds. The blotting sandwich was built by placing one soaked blotting sponge lying immediately on blotting cassette then two soaked 3MM whatman filterpapers then the activated PVDF membrane, then gel then again two soaked 3MM whatman filterpapers and then again soaked blotting sponge. The blotting cassette (BioRad) was closed and positioned in the transfer chamber. Transfer was allowed to happen by adjusting the blotting parameters of power supply on 150 mA for 45 minutes.

3.7.6 Western Blot

After 45 minutes the power supply was disconnected and PVDF membrane was taken out of the gel transfer sandwich. In order to block the unspecific binding sites, the PVDF membrane was incubated for 1 hour at 37 °C in blocking solution continuing gentle shaking. Blocking solution was prepared by mixing 5% fat free dried milk in PBST (PBS containing 0.1% Tween-20). The primary antibodies used in this study were, mouse monocolonal antibody against IKKα (B-8, Santa Cruz Biotechnology, Europe), rabbit polycolonal IgG against p.H3 (Sc-8 Santa Cruz Biotechnology, Europe) and rabbit polyclonal antibody against Tubulin (H-

235 Santa Cruz Biotechnology, Europe). A 50 ml falcon tube was taken to dilute concerning primary antibodies (1:500) in 5 ml antibody solution. After blocking step the membrane was shifted to the falcon tube containing primary antibody. Membrane was carefully placed to assure the protein side of membrane oriented towards the inner side to come in direct contact with antibody. Falcon tube was placed in rotor at 4°C for 24 hours.

Next day, membrane was taken out of falcon tube and was rinsed 3 times with 1X PBST for 10 minutes. Secondary antibodies used in this study were anti-mouse (Amersham biosciences, England) and secondary anti-rabbit (GE Healthcare UK). Concerning secondary antibody was diluted (1:1000) and membrane was shifted to the falcon. The falcon containing the membrane was placed in the rotor for 1 hour at room temperature. Chemiluminescence method was used to detect the immunoblotted proteins. Substrate solution and pierce super signal west pico solution was mixed in 1:1. Mixture was dropped on membrane and left undisturbed for 5 minutes.

After incubation the membrane was placed in a plastic wrap carefully avoiding wrinkles of the plastic wrap. Excessive substrate solution was removed by with a tissue paper. For chemiluminescence detection the blot was exposed to BioMax film detection system (LumiImager, Roche, Germany). Images were captured and analyzed using ImageJ software.

3.8 LUCIFERASE REPORTER GENE ASSAY

3.8.1 Cell Culture and Transfection

Cells of human prostate adenocarcinoma cell line (LNCaps) were cultured in 24 wells cell culture plate. Next day 70% confluent cells were transfected with respective DNA using X-tremeGENE 9 transfection reagent (Cat: 06365787001, Roche, Germany). A c-Myc Luciferase expression construct (pMyc-TA-Luc from Clontech) along with constitutively expressing β-Galactosidase normalization construct and various c-Myc, IKKα and IKKβ expression constructs were transfected to the cells in triplicates in 24 wells plate. Three wells of the cells were treated with 10 μM Bay11-7082 (Selleckchem, Germany) and three wells were treated with IKKβ inhibitor; 4 μM 2-[(aminocarbonyl) amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1) (Selleckchem, Germany) for 12 hours prior to cell lysate formation.

3.8.2 Cell Lysate formation

After 48 hours of transfection media was completely sucked out and 50 μl lysis buffer was added to each well of 24 wells cell culture plate. Lysis buffer (pH 7.8) contained 0.1M KH_2PO_4 buffer, 25 X protease inhibitor in ratio 25:1 and Triton-X 100 (0.1%). Plate was kept for incubation at 4°C for 20 minutes. Then the cell lysate was transferred from the wells to the 1.5 ml eppendorf tube. Samples were centrifuged on 14,000 rpm at 4°C for 20 minutes. The supernatant was carefully transferred to new properly labeled 1.5 ml eppendorf tube letting the pellet undisturbed.

3.8.3 Luminometry

Luminometry was performed to measure the luciferase activity. In each well of 96 well white plate 50 μl assay buffer was dropped. Then 20 μl of lysate was added to each well. Injection buffer was prepared by adding 750 μl luciferin (1 mM) and 2250 μl Glacylglicine buffer (25 mM). 2.5 μl injection buffer was injected to each well in order to measure luciferase activity. Wallac 1420 VICTOR2-Luminometer (Perkin Elmer, Vienna, Austria) was used to measure the light emission for 5 seconds.

3.8.4 Photometry for Beta Gal

All the content from white 96 wells plate was transferred to the transparent 96 well plate. 50 µl chlorophenol red-β-D-galactopyranoside (CPRG) was added to each well. Plate was incubated for half an hour at room temperature to develop color. To normalize b-galactosidase activity the samples were exposed to 590 nm colorimetric detection to measure the Beta GAL values after specific time interval. Readings were tabulated in MS word excel sheet and graphs were plotted using GraphPad Prism software.

3.9 IN SILICO EVALUATION OF EFFECTS OF CODING SNPS ON C-MYC

3.9.1 Identification of SNPs in c-Myc

Neucleotide and protein sequence in FASTA format were obtained from NCBI (http://www.ncbi.nlm.nih.gov). The longest isoform of c-Myc was selected for in silico analysis. In order to identify the SNPs related to longest isoform of human c-Myc proto onco-gene protein. Four public SNP databases named dbSNP (http://www.ncbi.nlm.nih.gov/SNP), UniProt (http://www.uniprot.org), SNP500cancer (http://snp500cancer.nci.nih.gov/home.cfm) and GeneCards (http://www.genecards.org/) were used. Being in gene region, 165 SNPs were found to be related to c-Myc (gene ID: 4609, NCBI Reference Sequence: NP_002458.2). Out of 43 SNPs found in coding region of c-Myc, 27 non synonymous (nsSNPs) were kept into analytical consideration.

3.9.2 Prediction of Tolerated and Deleterious SNPs and Functional Consequences of nsSNPs Using SIFT

Sorting Intolerant from Tolerant (SIFT version 2) algorithm was used to predict probable effect of an amino acid substitution on protein function. SIFT is known to make a distinction between tolerated and deleterious amino acid changes in proteins (http://blocks.fhcrc.org/sift/SIFT.html). It is based on basic principle that substitution of some fundamentally conserved amino acids in a protein can have a potential influence on protein function and can be deleterious.

Protein sequence in FASTA format was submitted as input to SIFT in order to predict possible effect of any probable amino acids substitution at every position of protein sequence. This investigation by FIST involved search for homologous sequences using TrEMBL 34.3 and SWISS-PORT version 51.3 databases. Then multiple alignments of available sequences were performed until a median sequence conservation score (MSCS) for the protein sequence approached 3.00. Tolerant and intolerant effect of nsSNPs of coding region was predicted as

tolerance index (TI) score which was normalized probability that the amino acid substitution is tolerated or not. It ranges from 0.0 to 1.0 while threshold for intolerance is ≤0.05. Deleterious predictions with median score of 3.25 or less were made with low confidence due to lack of sequence diversity for protein alignment.

3.9.3 Prediction of Functional Consequences of nsSNPs Using PolyPhen

Polymorphism Phenotyping version 2 (PolyPhen-2) (http://genetics.bwh.harvard.edu) was used in order to predict detrimental outcome of nsSNPs on structural and functional basis. For each of the 27 variations in coding region of c-Myc protein, the position of SNP in protein sequence, wild type amino acid and present in protein and its substitute along with protein sequence were provided as an input to the algorithm.

Homologs of the submitted sequence were searched using BLAST+ and aligned using MAFFT. Secator algorithm formerly executed in ClusPack software was used to gather reliably aligned sequences. Analyzed sequences and its homologes were taken into further consideration. PolyPhen also searched for 3D protein structures and contact information about amino acids in a number of structure based databases in order to firstly calculate the position-specific independent counts (PSIC) scores for allelic variants of SNP and then to evaluate its difference. On basis of all the information the effect of nsSNP on protein was predicted. Highly reliable score was predicted as ‘‘possibly damaging’’, an option of “may or may not”, meant that the nsSNP was ‘‘probably damaging’’ while being unlikely to phenotypic alteration in protein designated a nsSNP as ‘‘benign’’.

3.9.4 Prediction of Phenotypic Effects of nsSNPs Using SNPeffect

SNPeffect was used as a platform to forecast the molecular phenotypic influence of nsSNPs found in coding region of c-Myc protein. It worked beyond the scores got on conservational basis and mainly emphasizes to map the effect of SNPs on the capability of cells to uphold suitable concentration of the properly folded proteins in appropriate cellular region. For this evaluation, wild type protein

sequence in FASTA format and its variants were provided to the SNPeffect server (http://snpeffect.switchlab.org) as input. Homology threshold was adjusted to 90%. SNPeffect used TANGO which predicts the regions of protein sequence that are more prone to aggregation and measures the TANGO score with wild and variant amino acids. Effect of these variants on protein aggregation was evaluated on the basis of difference of the TANGO score (dTANGO).

WALTZ server determined the aggregate morphology. dWALTZ score predicted amyloid-forming regions in protein sequence. Chaperone binding propensity was predicted by LIMBO for the Hsp70 chaperones and effect of variant was determined by dLIMBO score. SNPeffect did also use high resolution crystal structure of proteins from Protein Data Bank (PDB) and modeled the variants using the empirical force field FoldX (version 2.5) to evaluate possible effects on stability and binding properties of the protein.

3.10 IMMUNOHISTOCHEMISTRY

Protein expression of IKKα was evaluated in the tissue sections of IDC by Immunohistochemisrty (IHC). Scoring was categorized as low and high based on successive increase in percentage of positively stained cells for IKKα.

3.10.1 Collection of Data and Samples

Formalin fixed paraffin embedded (FFPE) tissue sections of 83 IDC patients were taken from oncology department of Sheikh Zayed Hospital, Rahim Yar Khan and Histopathology department of Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. The hospitals kindly provided us with tissue sections of 4 μm in thickness along with the patient's history about age, sex, metastasis, lymph node status and grade etc. from their registered record (Table 3.4).

Table 3.4: Clinicopathological features of Breast cancer patients

Clinicopathological Features		Number of patients
Age (n=74)	≤ 50	37
	> 50	37
Histological Grade (n=44)	II	35
	III	9
Lymph node status (n=36)	No metastasis	16
	Metastasis	20
Progesterone Receptor (n=52)	Positive	26
	Negative	26

3.10.2 Fixation

Slices of 4 μm thickness were cut from each FFPE tissue sample using a microtome. Thin slices were put onto glass slide and were passed through cold and hot water (70ºC) gradually. After the attachment of tissue to the glass slide, the slides were incubated on 55ºC for half an hour to get them heat fixed.

3.10.3 Deparaffinization

The glass slides containing samples were mounted on a plastic slide holder and were dipped in 100% Xylene for 15 minutes. Tissue samples were completely deparaffinized after removal of residual paraffin in this step. This step was repeated twice for complete deparaffinization.

3.10.4 Rehydration

For rehydration purpose the samples were subjected to ethanol solutions with gradually decreasing concentration. Samples were dipped firstly in 100% ethanol for 10 minutes, then 90% ethanol for 5 minutes, 70% ethanol for 5 minutes, 50% ethanol for 5 minutes, 30% ethanol for 5 minutes and finally in water for 2 minutes. The tissue was clearly visible as white patch on the glass slides after this step. The tissue was precautiously kept wet to be safe from non specific staining in the background.

3.10.5 Blocking

In order to block the non specific binding of antibodies to the tissue, the samples were treated with mixture of 30% Hydrogen peroxide (H_2O_2) in water. The slides were placed on a water soaked tissue paper in covered tray to provide them moist environment. After 10 minutes incubation at room temperature the samples were washed with water to remove the blocking solution.

3.10.6 Primary Antibody Incubation

The primary antibodies anti- IKKα (ab54628, abcam UK) was diluted to a 1/50 concentration in 1% BSA. 1% BSA was prepared by dissolving 1 g of BSA in 100 ml of 1X PBS. Diluted primary antibody was applied to the tissue sample and was incubated at room temperature for 1 hour.

3.10.7 Secondary Antibody Incubation

In order to remove the primary antibody, the samples were subjected to wash by dipping the slides in autoclaved 1X PBS for 5 minutes. Horseradish peroxidase (HRP) labeled secondary antibody (ab47827, abcam, UK) was diluted to 1/500 in 1% BSA dissolved in 1X PBS. After entire removal of primary antibody, the diluted secondary antibody was applied to the tissue samples. The Samples were cautiously placed on wet tissue papers kept in a covered dark box for 1 hour at room temperature.

3.10.8 Detection

Diaminobenzidine (DAB) is characterized to impart an intense brown color to the HRP labeled proteins. A mixture of DAB substrate and chromogen was prepared using DAB Stainning Kit (Abcam Cat: ab64238, UK). This mixture was applied to the tissue samples and the samples were incubated for 10 minutes at room temperature. The stain was washed with tap water. Hemotoxylin was used to counterstain the nuclei of cells. The samples were then subjected to thorough washing in order to remove the hemotoxylin. Samples were air dried at room temperature and mounted with mounting media. Then cover slips were placed on the tissue.

3.10.9 Visualization

The 40 X and 100 X lens of Labomed TCM400 inverted microscope (Labo America Inc., USA) was used to visualize the staining of the slides. Images of stained sections of tissue samples were captured using ProgRes Capture Pro 2.6

(JENOPTIK Laser, Optik, Germany) and the images were ranked as low and high based on intensity of protein expression.

3.10.10 Evaluation of Immunohistochemical Findings

Slides were given to two histopathologist for their blind assessment of the stained tissue samples. Brown color staining in cytosol and dark blue in nuclear region was designated as positive staining. Positively stained cells were counted using 200X lens in 3 different fields for each sample.

3.10.11 Statistical Analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS), version 16.0 (Chicago, IL, USA). Spearman's Rank Order correlation was run to evalute the relationship between expression of the IKKα and different clinicopathological features of the patient. Correlation with $p<0.05$ was taken to be statistically significant.

3.11 EVALUATION OF BREAST CANCER AWARENESS

3.11.1 Study design

A community based cross sectional descriptive analysis was carried out with the objective to assess the knowledge that young female students of the University possess regarding early warning signs and the risk factors involved in breast cancer.

3.11.2 Study Area and Target Population

The study was conducted in two major cities; Rahim Yar Khan and Bahawlpur of Southern Punjab, Pakistan. We involved female non medical students of Post Graduate Colleges in Rahim Yar Khan and Islamia University, Bahawalpur. While Medical students of Sheikh Zayed Medical College, Rahim Yar Khan and Quaid-e-Azam Medical College, Bahawalpur participated in this study.

3.11.3 Sampling

Sample selection was based on probability systematic random sampling method. An ample sample size was calculated using the Epi Info Program. Hypothesized proportion of outcome factors of the target population was 50%, absolute precision was 5%, confidence limit was 95%, and design effect was kept 2. Appropriate sample size was calculated to be 316 non-medical and 235 medical students. After adding chance of 5% non-respondents the final sample size was of 332 non-medical and 247 medical female students. Students with mild mental incapability or learning disability were not included in study population. Those students, who were not interested to answer, were not provided with questionnaire. Six questionnaires returned by non-medical students and seven from medical students presented insufficient information thus were excluded from the study. Final results were based on information provided by 326 non-medical and 240 medical participants.

3.11.4 Instrument

The questionnaire was developed to assess basic awareness of early warning signs and risk factors of breast cancer among the female students of University. Experts were involved to ascertain the content validity of the questionnaire which consisted mainly of four sections. The first section included five questions about demographic characteristics of the students such as age, religion, ethnicity, marital status and residency of rural or urban area. The source of concerning knowledge was also asked from the participants.

The second part of questionnaire comprised of five questions about basic knowledge of breast cancer. Third part included seven questions about the early warning signs and the fourth section consisted of twenty questions about the knowledge of breast cancer risk factors. Therefore 32 questions in total were included in the last three sections of the questionnaire. A score of 1 was awarded to the correct answer while 0 was given to the wrong answer. Thus, 32 was the maximum score one could achieve. The response of participants was grouped into "poor", "insufficient", "satisfactory" and "good" on the basis of obtained score of ≤ 8, ≤ 16, ≤ 24 and ≤ 32 respectively.

3.11.5 Statistical Analysis

Students were classified as non-medical and medical group. Percentage and frequency distribution were calculated to demonstrate the categorical variables. Data was statistically analyzed, the results were tabulated and graphs were plotted using Graph Pad Prism Version 5 for Windows (San Diego, California USA). The Chi-Square test (x^2) was performed to evaluate association between variables with a significance level set at $p < 0.05$.

Chapter 4

RESULTS

4.1 INTERACTION OF IKKs WITH c-MYC

IKKs have been reported to enhance the stability of c-Myc protein suggesting a possible tumor promoting role. Although, as a known proto oncogene, c-Myc has been an intense area of research especially in relation to oncogenesis, but not much is known about the interaction between IKKs and c-Myc protein. Therefore, the first part of the study aims to investigate the physical interaction between IKKs and c-Myc protein.

4.1.1 IKKα and IKKβ Interact with c-Myc

In order to gain insight into the physical interactions between IKKs and c-Myc protein, a live cell imaging technique- fluorescence resonance energy transfer (FRET) was employed in cell culture based system. It is the most effective way of analyzing the molecular interactions as it allows to measure small distances of around 1-10 nm in live cells.

HEK-293 cells were transfected with fluorescently labeled IKKα (pEGFP-C1), IKKβ (pEGFP-C1) and c-Myc (pDsRed2-C1). FRET analysis revealed the interaction of DsRed tagged c-Myc, GFP tagged IKKα and IKKβ in nucleus and cytosol respectively (Figure 4.1). The graph was plotted to find mean FRET efficiency ± SEM (standard error of mean) of ten different cells for negative control and the cells indicating interaction (Figure 4.2).

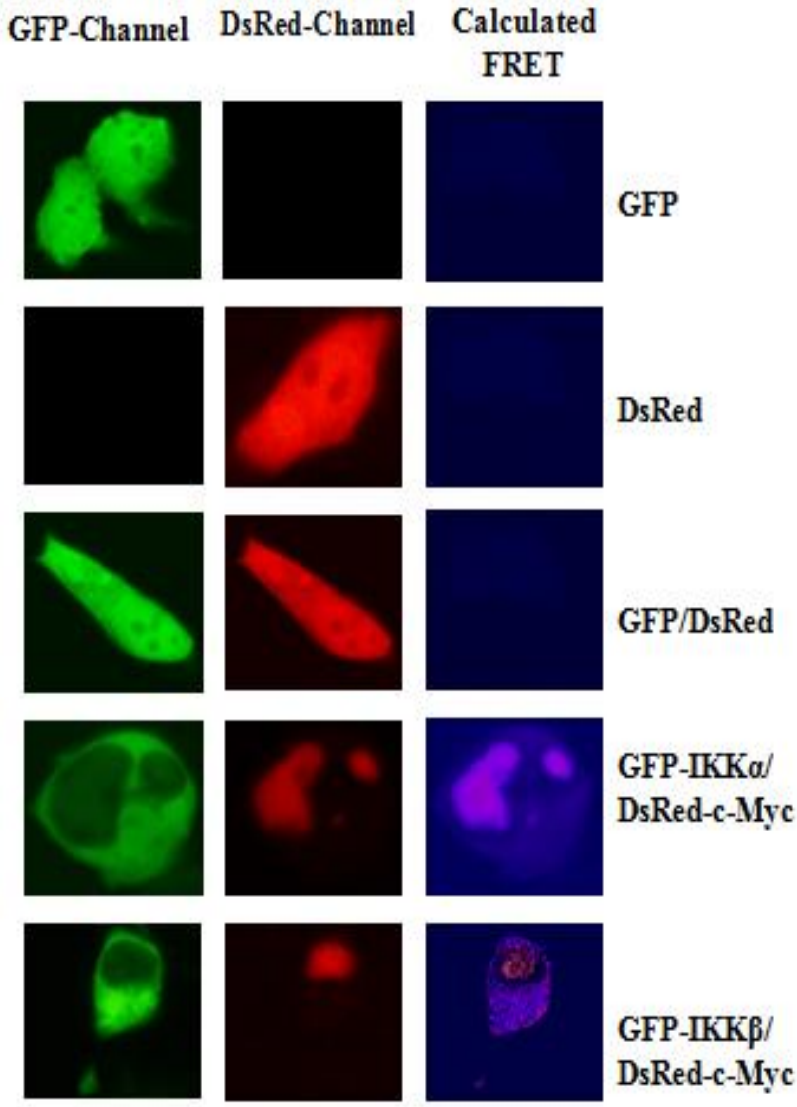

Figure 4.1: Live cell imaging showing molecular interaction between IKKs and c-Myc. Fluorescence resonance energy transfer (FRET) analysis was performed to visualize the interaction between fluorescently labeled IKKα, IKKβ and c-Myc in HEK-293 cells. From top to bottom, Lane 1-5; left to right 1) GFP mock transfected positive control cells, 2) DsRed mock transfected positive control cells, 3) both GFP and DsRed mock transfected negative control cells, 4) GFP-IKKα and DsRed-c-Myc double transfected cells showing colocalization in nucleus of the cells, 5) GFP-IKKβ and DsRed-c-Myc double transfected cells showing colocalization in cytosol of the cells. Experiment was repeated three times and data was analyzed using ImageJ to calculate FRET efficiency.

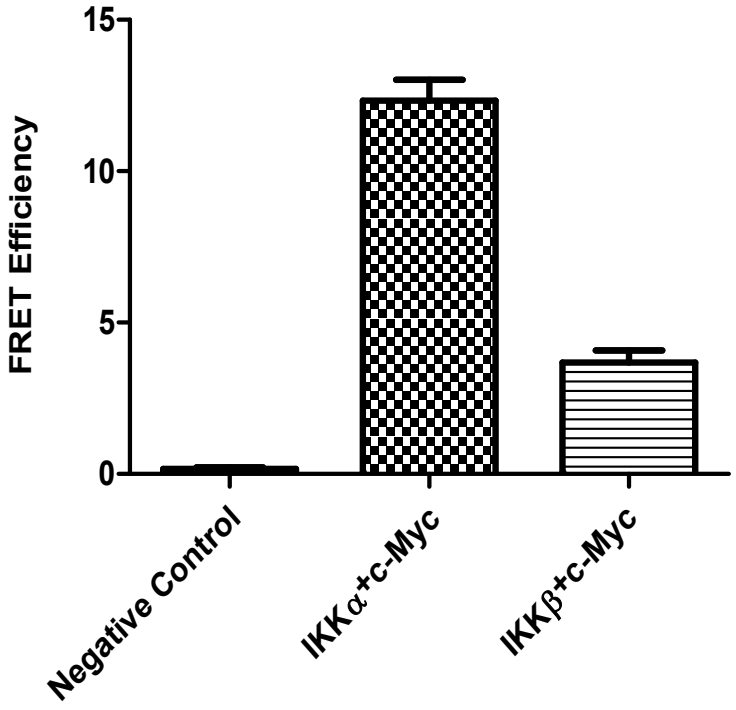

Figure 4.2: Graphical representation depicting molecular interactions between IKKs and c-Myc in HEK 293 cells. The calculations were based on the FRET efficiencies measured through strength of fluorescent signals using PixFRET plugin of ImageJ software. Fluorescence intensities were normalized to control cells expressing only GFP and DsRed. Mean FRET efficiency ± SEM of ten representative cells of each category was calculated using GraphPad prism software. From Left to right; Negative control is the GFP and DsRed transfected cells showing no FRET signal, calculated FRET efficiency shows efficient interaction between IKKα and c-Myc and moderate association between IKKβ and c-Myc.

4.1.2 Interaction Domain of IKKα with c-Myc

In order to find the interacting domain of IKKα with c-Myc, IKKα was deprived of helix loop helix (HLH) and leucine zipper (LZ) domains and was tagged with GFP. FRET analysis was carried out to find interaction of truncated IKKα with c-Myc using laser scanning microscope containing a photometrics coolsnap camera and suitable fluorescence filters controlled by Zeiss LSM 510 software.

Interaction of DsRed tagged c-Myc (pDsRed2-cMyc) with GFP tagged IKKα (pEGFP-IKKα), as well as GFP tagged truncated IKKα (pEGFP- IKKα ΔHLH-LZ) was clearly observed in nucleus. GFP-IKKα interaction was observed again as positive control while the interaction of GFP alone with c-Myc was used as negative control. No interaction of GFP tagged truncated IKKα with DsRed tagged c-Myc was observed, indicating the importance of HLH and LZ domains in the c-Myc and IKKα interaction (Figure 4.3). PixFRET plugin of ImageJ software was used to examine and analyze the interaction. Based on the results the graph was plotted to find mean FRET efficiency ± SEM of ten different cells for negative control and the cells indicating interaction (Figure 4.4).

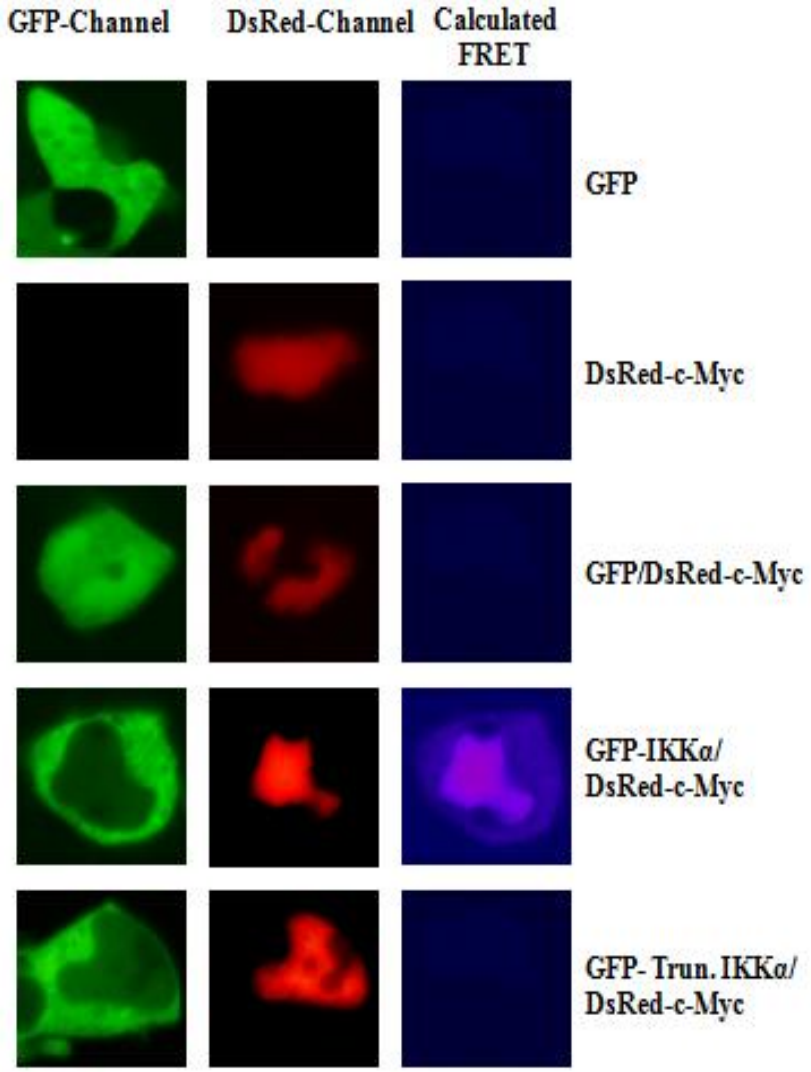

Figure 4.3: Live cell imaging showing molecular interaction between IKKα and c-Myc. Fluorescence resonance energy transfer (FRET) analysis was performed to visualize the interaction between fluorescently labeled IKKα, truncated IKKα IKKαΔHLH-LZ(the HLH and LZ loop was removed) and c-Myc in HEK-293 cells. From top to bottom, Lane 1-5; 1) GFP mock trasfected positive control cells, 2) DsRed-c-Myc trasfected positive control cells, 3) both GFP and DsRed mock trasfected negative control cells, 4) GFP-IKKα and DsRed-c-Myc double transfected cells showing colocalization in nucleus of the cells, 5) GFP-Trun.IKKα and DsRed-c-Myc double transfected cells showing no colocalization in the cells. Experiment was repeated three times and data was analyzed using ImageJ to calculate FRET efficiency.

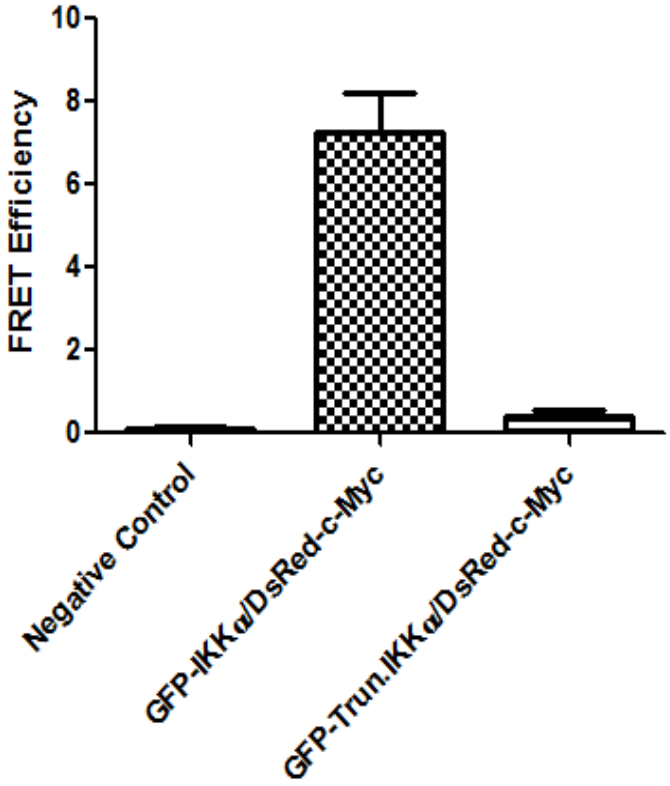

Figure 4.4: Graphical representation depicting molecular interactions between IKKα, Trun.IKKα and c-Myc in HEK 293 cells. The calculations were based on the FRET efficiencies measured through strength of fluorescent signals using PixFRET plugin of ImageJ software. Fluorescence intensities were normalized to control cells expressing only GFP and DsRed. Mean FRET efficiency ± SEM of ten representative cells of each category was calculated using GraphPad prism software. From Left to right; Negative control is the GFP and DsRed transfected cells showing no FRET signal, calculated FRET efficiency shows efficient interaction between IKKα and c-Myc and no association between Trun.IKKα and c-Myc.

4.2 EFFECT OF IKKs AND c-Myc INTERACTION ON TRANSCRIPTIONAL EXPRESSION

The FRET analysis was evident of the physical interaction between IKKs and c-Myc proteins in HEK 293 cells (Figure 4.1, 4.3), therefore it was intended to determine the functional relevance of IKKs and c-Myc interactions on each other at transcriptional level. In order to do so qRT-PCR was performed.

4.2.1 No Effect of IKKs on c-Myc Transcription

In order to determine the effect of IKKα or IKKβ overexpression on transcription of c-Myc, qRT-PCR was used. HEK 293 cells were transfected with fluorescent expressing plasmid constructs harbouring IKKα, IKKβ genes and gene silencing (si) constructs for c-Myc, si-Myc control and c-Myc in triplicates. Transfection efficiency was assessed by observing transfected cells under inverted fluorescent microscope. RNA was extracted manually and reverse transcribed using random hexamer primers to synthesize cDNAs. Housekeeping gene; GAPDH was used. SYBR green PCR master mix was used and crossing threshold (Ct) points were calculated using StepOne Plus software while delta/delta-Ct method was used to calculate the relative expression of target genes. No significant effect of IKK over expression on the transcriptional expression of c-Myc was observed (Figure 4.5).

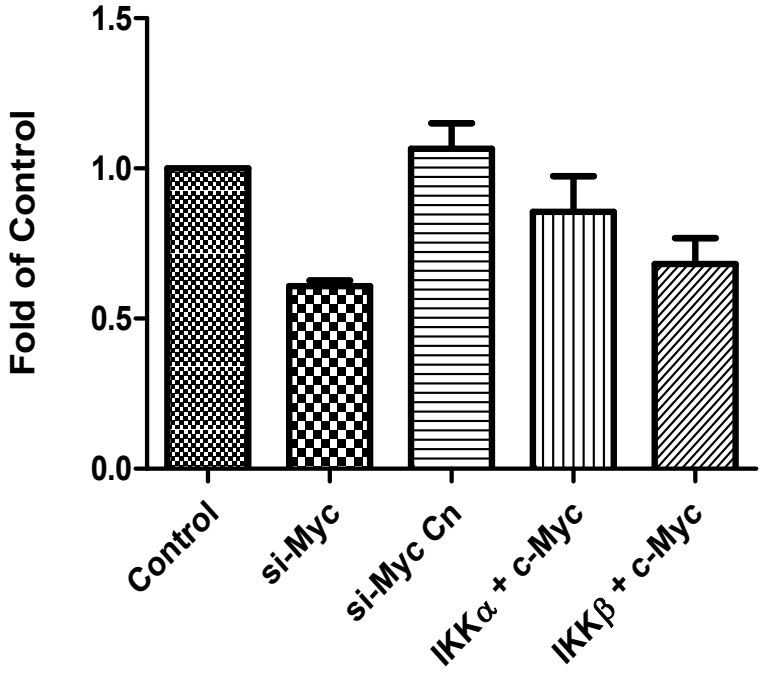

Figure 4.5: Histogram showing effect of IKKα or IKKβ on transcription of c-Myc. q-PCR was performed with SYBR green detection to find effect of IKKα and IKKβ over expression on transcription of c-Myc mRNA. Expression was normalized taking GAPDH as a control. Mean of transcriptional expression ± SD was calculated. No significant effect of IKKα and IKKβ on transcription of c-Myc was noted.

4.2.2 No Effect of c-Myc on Transcription of IKKα

HEK 293 cells were transfected with c-Myc plasmid DNA in triplicates. Transfection efficiency was assessed by observing transfected cells under inverted fluorescent microscope. qRT-PCR was carried out to determine the effect of c-Myc overexpression on transcription of IKKα mRNA. RNA was extracted manually and reverse transcribed using random hexamer primers to synthesize cDNAs. Housekeeping gene was GAPDH. SYBR green PCR master mix was used and crossing threshold (Ct) points were calculated using StepOne Plus software while delta/delta-Ct method was used to calculate the relative expression of target genes. No significant effect was observed in transcriptional level of IKKα (Figure 4.6).

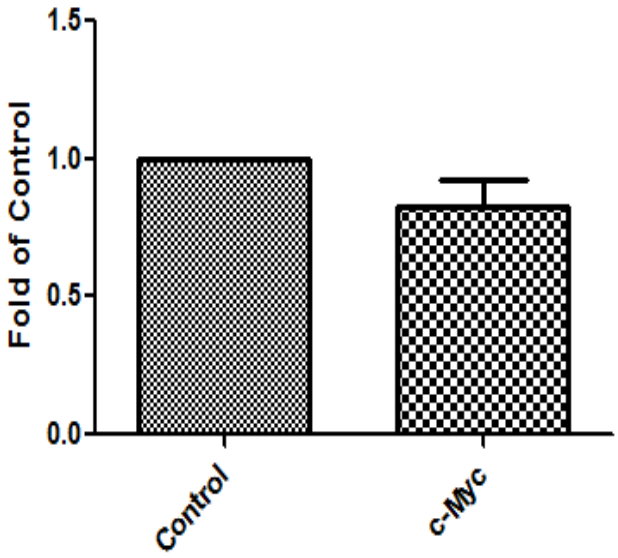

Figure 4.6: Histogram showing no effect of c-Myc overexpression on transcriptional expression of IKKα gene. q-PCR was performed with SYBR green detection to find effect of c-Myc overexpression on transcription of IKKα. Expression was normalized taking GAPDH as a control. Mean of transcriptional expression ± SD was calculated. No significant effect of c-Myc overexpression on transcriptional expression of IKKα gene was noted.

4.2.3 No Effect of c-Myc on transcription of IKKβ

qRT-PCR was carried out to determine effect of c-Myc over expression on transcription of IKKβ mRNA. HEK 293 cells were transfected with fluorescent expressing plasmid constructs harbouring DNA of c-Myc in triplicates. RNA was extracted by Trizol manual method and reverse transcribed using random hexamer primers to synthesize cDNAs. Housekeeping gene was GAPDH. SYBR green PCR master mix was used and crossing threshold (Ct) points were calculated using StepOne Plus software while delta/delta-Ct method was used to calculate the relative expression of target genes. No significant effect was observed in transcriptional level of IKKβ (Figure 4.7)

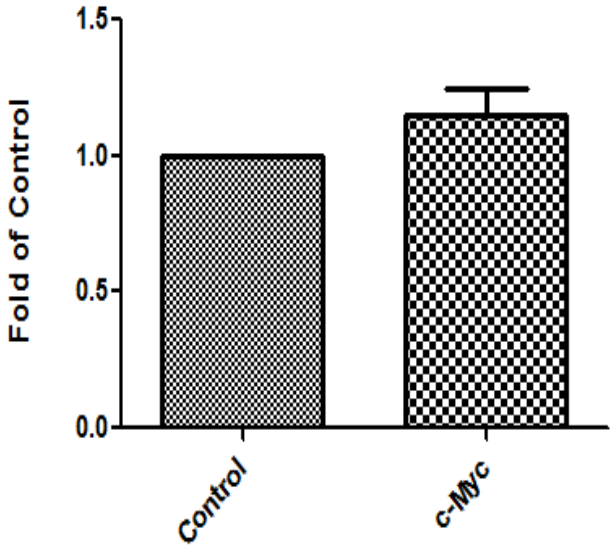

Figure 4.7: Histogram showing no effect of c-Myc over expression on transcriptional expression of IKKβ gene. q-PCR was performed with SYBR green detection to find effect of c-Myc over expression on transcription of IKKβ mRNA. Expression was normalized taking GAPDH as a control. Mean of transcriptional expression ± SD was calculated. No significant effect of c-Myc over expression on transcriptional expression of IKKβ gene was noted.

4.3 EFFECT OF C-MYC ON IKKα EXPERESSION AND ACTIVITY

c-Myc was found to have no effect on the transcriptional expression of IKKα but the effect could also be on IKKα protein. Therefore, in order to analyze the possible effect of c-Myc on protein expression of IKKα, western blot analysis was performed.

4.3.1 Effect of c-Myc on IKKα Protein Expression

HEK 293 cells were treated with TNFα for half an hour to stimulate expression and activity of IKKα, in HEK293 cells transfected with pEGFP- IKKα), mutant IKKα-KD (kinase dead) and (pDsRed2- c-Myc). Samples were run on 10 % SDS poly acrylamide gels and Western blot was performed using Mouse monocolonal primary antibody against IKKα (B-8, Santa Cruz Biotechnology, Europe) and secondary anti-mouse antibody (Amersham biosciences, England) was used to find protein expression of IKKα. c-Myc was not found to effect protein expression of IKKα (Figure 4.8).

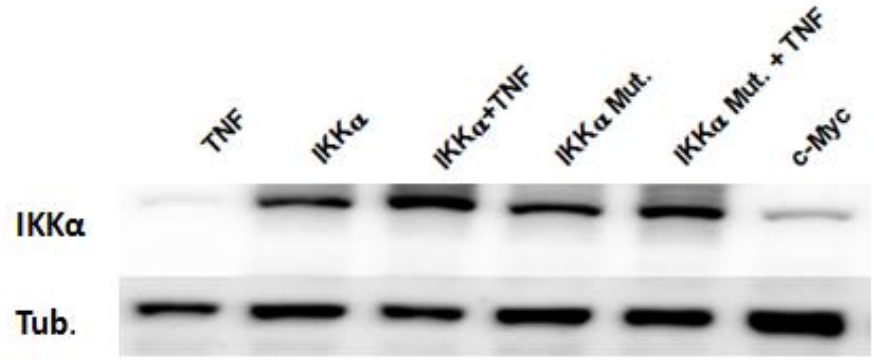

Figure 4.8: Western blot showing no effect of c-Myc on IKKα protein expression. SDS-PAGE and Western Blot was performed to evaluate effect of c-Myc over expression on expression of IKKα. HEK-293 cells were transfected with IKKα, Mutant IKKα (inactive) and c-Myc. Untransfected cells were treated with TNFα to stimulate expression and activity of IKKα. Blots were analyzed with ImageJ. c-Myc seems to have no effect on IKKα expression.

4.3.2 Effect of c-Myc on IKKα Activity

HEK 293 cells were treated with TNFα for half an hour to stimulate expression and activity of IKKα and were transfected with c-Myc (pDsRed2-cMyc) with GFP tagged IKKα (pEGFP-IKKα). All the protein samples were run on 10 % SDS poly acrylamide gels and then were transferred to the Polyvinylidene fluoride (PVDF) membrane using wet electrophoretic transfer method. As Histone H3 is nuclear target of of IKKα thus Rabbit polycolonal IgG antibody against phosphorylated histone (p.H3) (Sc-8, Santa Cruz Biotechnology, Europe) and secondary anti-rabbit (GE Healthcare UK) antibodies were used to find effect of c-Myc protein on nuclear activity of IKKα. c-Myc was not found to effect activity of IKKα (Figure 4.9).

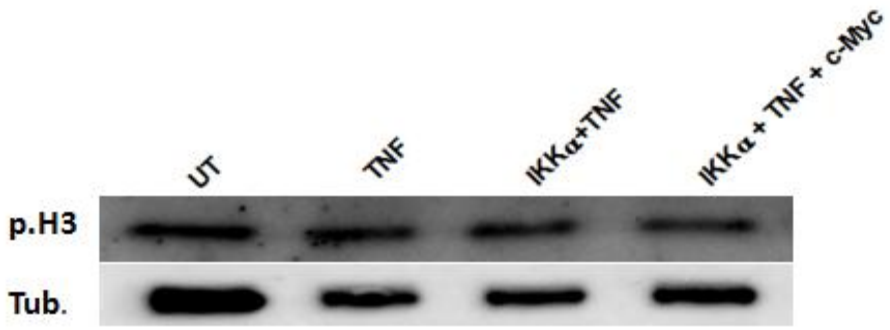

Figure 4.9: Western blot showing no effect of c-Myc over expression on IKKα activity. SDS-PAGE and Western Blot was performed after transfection of HEK-293 cells with IKKα and c-Myc. Untreated cells and the cells transfected with IKKα alone were treated with TNFα for half an hour to stimulate expression and activity of IKKα. Blots were analyzed with ImageJ. c-Myc seems to have no effect on nuclear activity of IKKα.

4.4 EFFECT OF IKKs ON TRANSCRIPTIONAL ACTIVITY OF c-MYC

c-Myc was found to have no effect on the translational expression and activity of IKKα. As c-Myc is a transcription factor thus IKKs may affect the transcriptional activity of c-Myc. Luciferase assay was performed in order to analyze the possible effect of IKKs on transcriptional activity of c-Myc.

4.4.1 IKKα Reduces Transcriptional Activity of c-Myc

A Myc luciferase expression construct Myc-luc (pMyc-TA-Luc) along with constitutively expressing β-Galactosidase normalization construct, IKKα (pEGFP-C1), and c-Myc (pDsRed2-C1) expression constructs were transfected to human prostate adenocarcinoma cell line (LNCaps) in triplicates. Basal expression of c-Myc is less in LNCaps as compared to HEK-293 cells thus LNCaps was used to perform this experiment. Luciferase activity was measured by use of luminometer. IKKα was found to reduce the transcriptional activity of c-Myc (Figure 4.10). Cells were also treated with inhibitor of IKK i.e. 10 μM Bay11-7082 for 12 hours (Yeh *et al.*, 2011) in order to confirm the results. Increase in the transcriptional activity of c-Myc in presence of inhibitors confirmed that IKKα is responsible for indicated reduction (Figure 4.11).

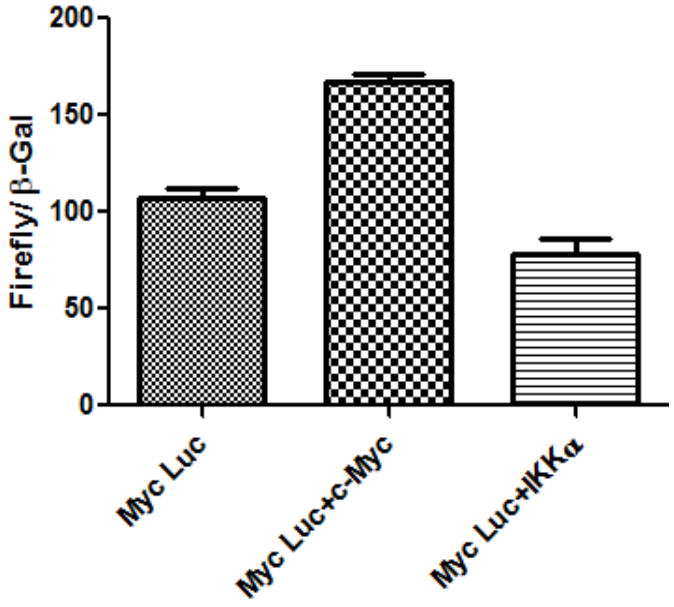

Figure 4.10: Histogram showing reduced c-Myc transcriptional activity due to IKKα. LNCap cells were transfected with c-Myc luciferase reporter, c-Myc and IKKα. Luciferase activity was normalized to constitutive β-Galactosidase expression. Standard deviation (SD) was used for descriptive error bars.

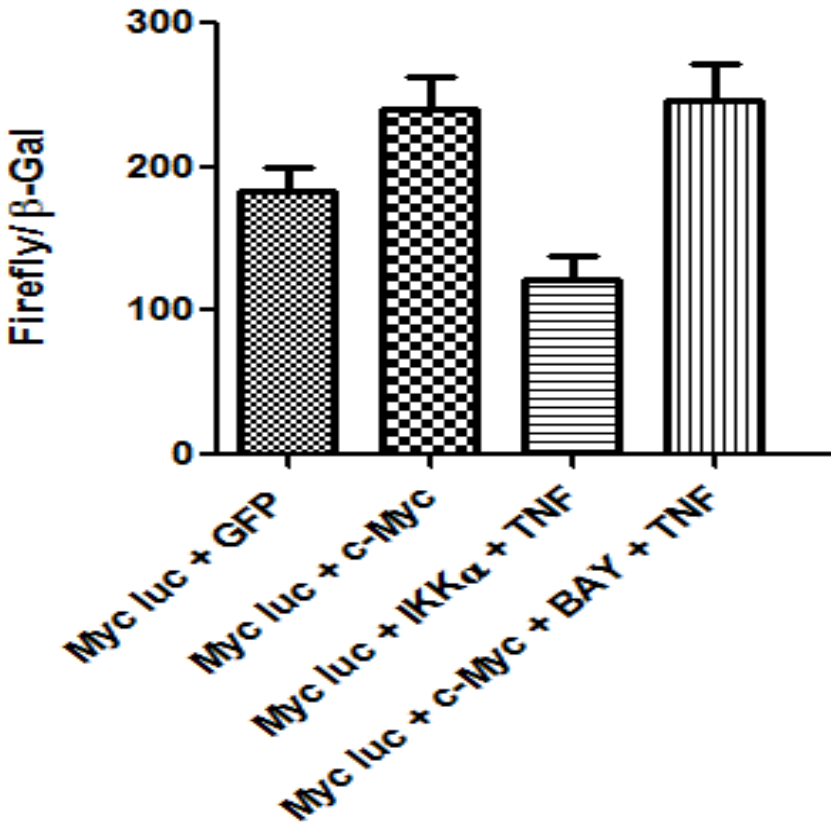

Figure 4.11: Histogram showing decrease in the IKKα caused reduction of c-Myc transcriptional activity by inhibitors of IKKα. LNCap cells were transfected with c-Myc luciferase reporter, c-Myc and IKKα. Cells were stimulated with TNF alpha. Luciferase activity was normalized to constitutive β-Galactosidase expression. Cells treated with 10 μM Bay11-7082 for 12 hours show significant increase in transcriptional activity of c-Myc. Standard deviation (SD) was used for descriptive error bars.

4.4.2 IKKβ Reduces Transcriptional Activity of c-Myc

A Myc luciferase expression construct Myc-luc (pMyc-TA-Luc) along with constitutively expressing β-Galactosidase normalization construct, c-Myc (pDsRed2-C1) and constitutively active IKKβ (Ca-IKKβ) (pEGFP-C1) expression constructs were transfected to LNCaps in triplicates. Luciferase activity was measured by use of luminometer. IKKβ was found to reduce the transcriptional activity of c-Myc (Figure 4.12). Cells were also treated with IKK inhibitor; 10 μM

Bay11-7082 (Yeh *et al.*, 2011) for 12 hours and potent IKKβ inhibitor; 4 μM TPCA-1 for 12 hours in order to confirm the results. Increase in the transcriptional activity of c-Myc in the presence of inhibitors confirmed that IKKβ to be responsible for indicated reduction (Figure 4.13).

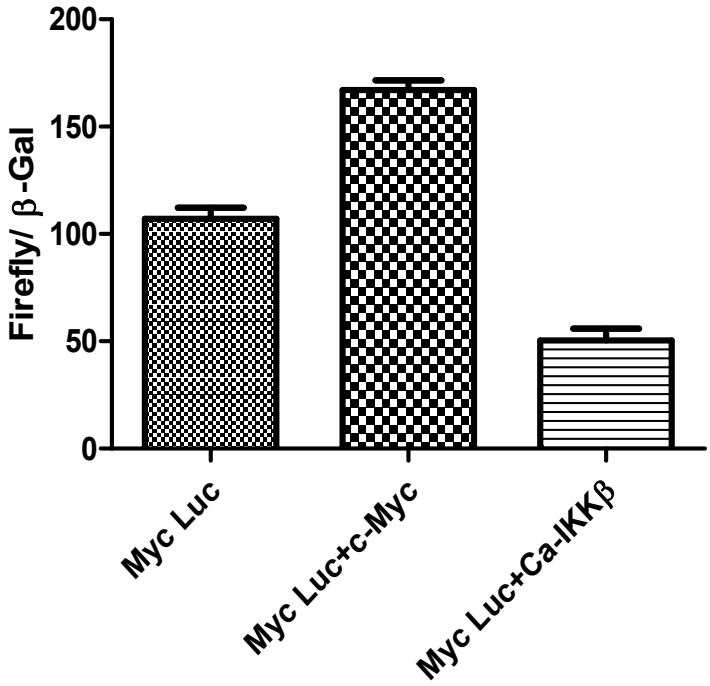

Figure 4.12: Histogram showing reduced c-Myc transcriptional activity due to IKKβ. LNCap cells were transfected with c-Myc luciferase reporter, c-Myc and constitutively active IKKβ (Ca-IKKβ). Luciferase activity was normalized to constitutive β-Galactosidase expression. Standard deviation (SD) was used for descriptive error bars.

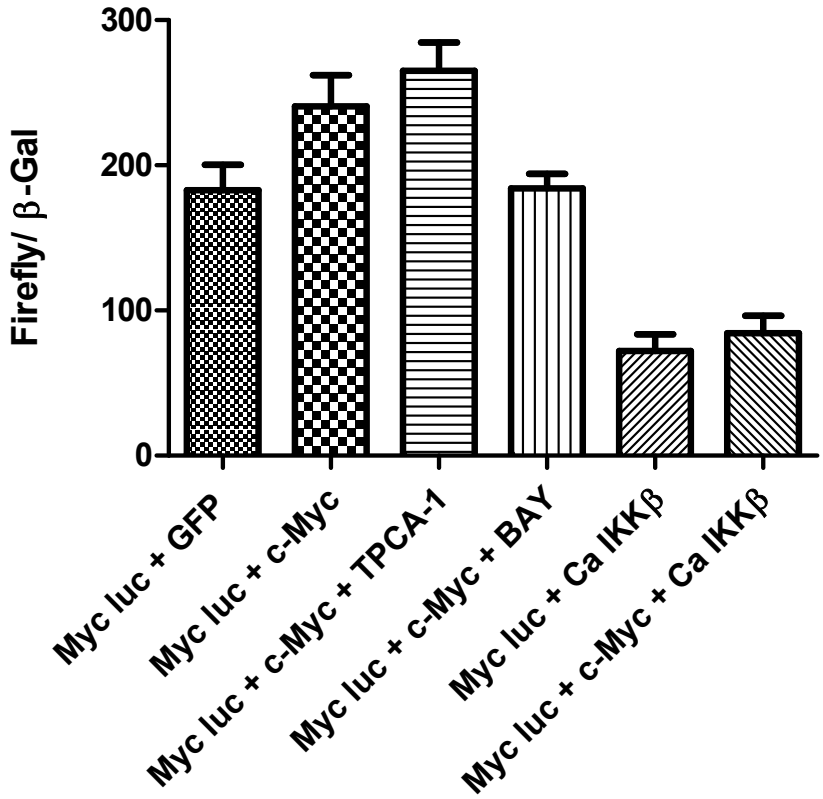

Figure 4.13: Histogram showing decrease in the IKKβ caused reduction of c-Myc transcriptional activity by inhibitors of IKKβ. LNCap cells were transfected with c-Myc luciferase reporter, c-Myc and constitutively active IKKβ. Luciferase activity was normalized to constitutive β-Galactosidase expression. Cells treated with 10 μM Bay11-7082 for 12 hours and potent IKKβ inhibitor; 4 μM TPCA-1 for 12 hours show significant increase in transcriptional activity of c-Myc. Standard deviation (SD) was used for descriptive error bars.

4.5 IN SILICO ANALYSIS OF CODING SNPs AND THEIR EFFECT ON C-MYC PROTEIN

c-Myc is actively involved in the regulation of a variety of genes related to cancer development and persistence (Gustafson and Weiss, 2010). Disease risk and progression is commonly influenced by the existing polymorphisms in the genome. The most frequent type of polymorphisms found in human genome is SNP (Noreen *et al.*, 2012). Studying the structural and functional effect of the nsSNPs on the protein can help in the selection of functionally important SNPs. Some sequence and structural homology based algorithms were used to find those nsSNPs which can play potential role in structural and functional alteration of c-Myc proto-oncoprotein.

4.5.1 Dataset compilation

Total 165 human c-Myc SNPs from the dbSNP database were further scrutinized. GeneCards database included four additional pathologically important SNPs of c-Myc2 proto oncogene reported in Burkitt lymphoma samples; VAR_063384 (Glu39Asp), VAR_063385 (Pro57Ser), VAR_063386 and VAR_063387 (Asn86Thr). The non-conding region of gene was found to contain 122 SNPs out of which 38 (23%) were in the region of 5' near gene, 13 (8%) were in 5' UTR, 51 (31%) were in intronic region, 11 (7%) were in 3' UTR and 9 (5%) were in region of 3' near gene. Coding region harbored 43 SNPs out of which 29 (17%) were non-synonymous and 14 (9%) were synonymous (Figure 4.14).

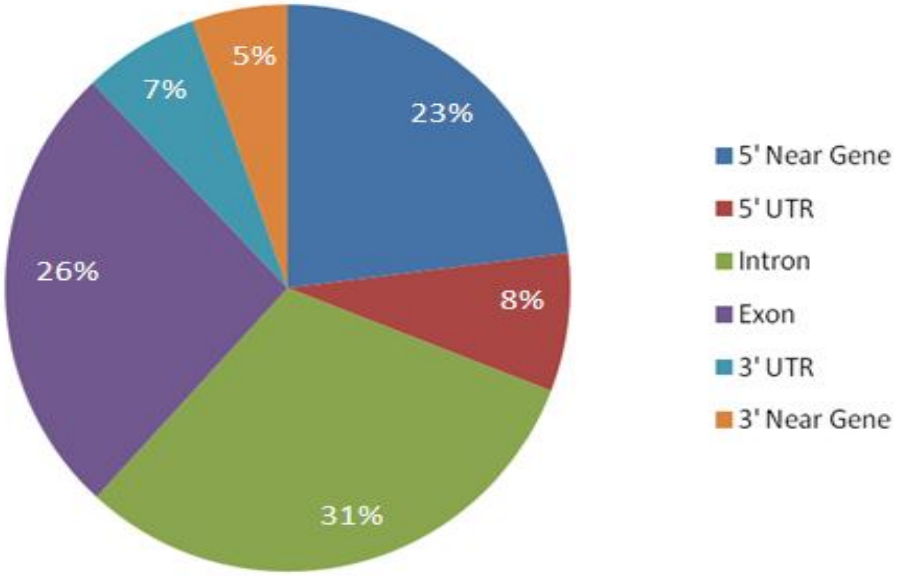

Figure 4.14: Pi chart showing distribution of SNPs in human c-Myc gene region

4.5.2 Non-synonymous SNPs of c-Myc Gene

Non-synonymous SNPs of c-Myc gene were supposed to be of more importance as their variants can directly affect protein structure and function. Detailed information about all nsSNPs of coding region included their dbSNPid, chromosomal position, mRNA position, dbSNP allele, codon position and amino acid position (Table 4.1). The significant domains and position of phosphorylation, ubiquitination and location of nsSNPs (predicted to be damaging by SIFT, PolyPhen or SNPeffect algorithms) in c-Myc1 protein are indicated in Figure 4.15.

Table 4.1: nsSNPs of c-Myc coding region

dbSNP id	Chromosome Position	mRNA Position	dbSNP Allele	Codon Position	Amino Acid Position
rs139294902	128750498	560	C/G	2	12
rs146505192	128750527	589	T/C	1	22
rs148915481	128750534	596	A/G	2	24
rs4645959	128750540	602	A/ G	2	26
rs114570780	128750602	664	T/C	1	47
rs148228388	128750735	797	C/A	2	91
rs199561469	128750773	835	G/C	1	104
rs150308400	128750906	968	G/A	2	148
rs137906262	128750938	1000	C/A	1	159
rs61755060	128750967	1029	G/T	3	168
rs147329312	128750977	1039	A/C	1	172
rs4645960	128750986	1048	G/T	1	175
rs4645960	128750986	1048	G/C	1	175
rs4645961	128751016	1078	G/A	1	185
rs112602073	128751121	1183	C/T	1	220
rs147506213	128751160	1222	C/A	1	233
rs186663828	128751185	1247	C/T	2	241
rs148544254	128751200	1262	C/T	2	246
rs150629172	128752729	1415	C/A	2	297
rs139697494	128752745	1431	C/A	3	302
rs146971340	128752799	1485	T/A	3	320
rs4645968	128752849	1535	C/T	2	337
rs200431478	128752924	1610	C/T	2	362
rs141095253	128753029	1715	C/T	2	397
rs145561065	128753141	1827	G/C	3	434
rs148863193	128753155	1841	G/T	2	439
rs201337668	128753196	1882	T/G	1	453
rs143501729	128753200	1886	C/A	2	454

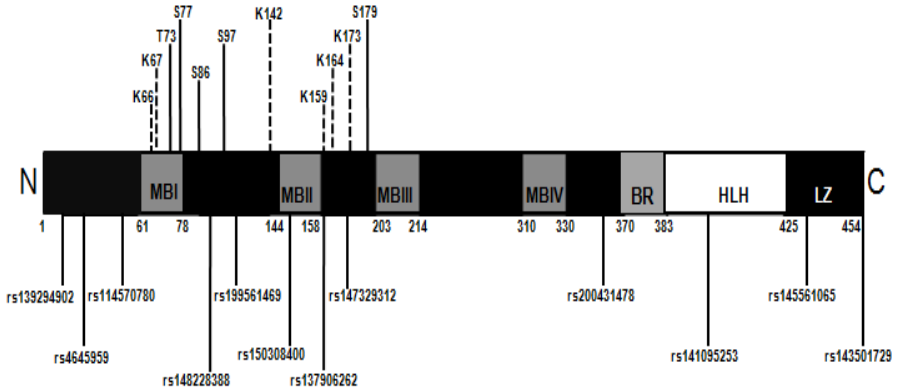

Figure 4.15: Schematic diagram of the significant domains and position of phosphorylation, ubiquitination and nsSNPs in c-Myc protein. It details the location of highly conserved Myc homology boxes (MBI, MBII, MBIII and MBIV), basic region (BR), helix-loop-helix (HLH) and leucine zipper (LZ) domains. The numbers below represent the amino acid that borders each significant domain of protein. Specific N-terminal features, including the location of phosphorylation and ubiquitination (lysine residues) are indicated on upper side while location of nsSNPs (predicted to be damaging by SIFT, PolyPhen or SNPeffect algorithms) are indicated below.

4.5.3 Prediction of Tolerated and Deleterious SNPs and Functional Consequences of nsSNPs Using SIFT

The SIFT program predicts the tolerant or deleterious effect of amino acid change on protein function. The analysis mainly considers the alignment of homologous sequences, physical properties of amino acids and the effect of natural nonsynonymous polymorphisms on phenotypic alterations by aligning paralogous and orthologous protein sequences. The prediction of possible substitutions for positions 1-454 amino acids was also done. Possible substitutions for those nsSNPs are shown in Figure 4.16 which were predicted to have some damaging effect by SIFT, PolyPhen or SNPeffect algorithms. 'Seq-Rep' is actually the fraction of sequences that has one of the fundamental amino acids. Low fraction shows that the position is unalienable or is severely gapped providing little information which results in poor prediction. Amino acids are shown in color codes i.e. basic

(red), acidic (blue) non polar (black); uncharged polar (green). Capital letters signifies the amino acids which come into view during the alignment while lower case letters are the consequence of prediction (Figure 4.16).

Out of these eight nsSNPs, rs139294902 (Pro12Arg), rs4645959 (Asn26Ser), rs114570780 (Tyr47His), rs150308400 (Cys148Tyr) and rs137906262 (Leu159Ile) are located within N-terminal trans-activation domain while rs145561065 (Leu434Phe) and rs143501729 (Ala454Glu) harbor the C-terminal domain of c-Myc1 protein (Figure 4.15). Sequence homology-based tool of SIFT was used to predict tolerant and deleterious effect of 27 nsSNPs present in coding region of MYC proto onco-gene protein. As a result 8 nsSNPs were found to be deleterious in total, out of which 2 had low confidence due to median score >3.25 (Table 4.2, Figure 4.17). Out of these eight nsSNPs, rs139294902 (Pro12Arg), rs4645959 (Asn26Ser), rs114570780 (Tyr47His), rs150308400 (Cys148Tyr) and rs137906262 (Leu159Ile) are located within N-terminal trans-activation domain while rs145561065 (Leu434Phe) and rs143501729 (Ala454Glu) harbor the C-terminal domain of c-Myc1 protein (Figure 4.15).

Predict Not Tolerated	Position	Seq Rep	Predict Tolerated
whyfmcrqedilkn	12P	0.10	vgPsAT
miwvfl cyrp qathe sgDK	26N	0.96	N
h qrn kdge pct smvi wl	47Y	0.98	AYF
whyfi mqnr d	91A	0.24	e l kcv TsGPA
whyfi m	104G	0.23	q r n de l kcvtPSGA
ywvtsr q pnml ki h gfeda	148C	0.99	C
ywvtsr q pnmki hg f edca	159L	0.99	L
cwdfmi yvh s nl t e	172K	0.99	AQGPRK
wf ymh c l i eqgvkpda	362S	0.95	RSNT
w h yf mqi r ncdekvt gaLS	397P	0.95	P
cwd f myi vgpsnat eH	434L	0.95	r LKQ
whyf m i q r ndecl kvt p g	454A	0.17	SA

Figure 4.16: Prediction of possible substitutions of amino acids in human c-Myc1 protein using SIFT algorithm. Amino acids are shown in color code: basic (red), acidic (blue) non polar (black); uncharged polar (green). Capital letters signify amino acids which come into view during the alignment while lower case letters are the consequence of prediction. 'Seq-Rep' is actually the fraction of sequences that have one of the fundamental amino acids. Low fraction shows that position is unalienable or is severely gapped providing little information which results in poor prediction.

Table 4.2: SIFT analysis of nsSNPs in coding region of human c-Myc proto oncogene protein

dbSNP id	Amino Acid Variant	SIFT Prediction	Tolerance Index
rs139294902	Pro [P]/Arg [R]	Intolerant *	0.00
rs146505192	Phe [F]/Leu [L]	Tolerant	1.00
rs148915481	Asn [N]/Ser [S]	Tolerant	0.99
rs4645959	Asn [N]/Ser [S]	Intolerant	0.01
rs114570780	Tyr [Y]/His [H]	Intolerant	0.00
rs148228388	Ala [A]/Glu [E]	Tolerant	0.06
rs199561469	Gly [G]/Arg [R]	Tolerant	0.36
rs150308400	Cys [C]/Tyr [Y]	Intolerant	0.00
rs137906262	Leu [L]/Ile [I]	Intolerant	0.02
rs61755060	Gln [Q]/His [H]	Tolerant	0.45
rs147329312	Lys [K]/Gln [Q]	Tolerant	0.11
rs4645960	Gly [G]/Cys [C]	Tolerant	0.12
rs4645960	Gly [G]/Arg [R]	Tolerant	0.13
rs4645961	Val [V]/Ile [I]	Tolerant	0.26
rs112602073	Pro [P]/Ser [S]	Tolerant	0.93
rs147506213	Pro [P]/Thr [T]	Tolerant	0.64
rs186663828	Ser [S]/Leu [L]	Tolerant	0.19
rs148544254	Pro [P]/Leu [L]	Tolerant	0.26
rs150629172	Pro [P]/His [H]	Tolerant	0.15
rs139697494	His [H]/Gln [Q]	Tolerant	0.15
rs146971340	His [H]/Gln [Q]	Tolerant	0.74
rs4645968	Ala [A]/Val [V]	Tolerant	1.00
rs200431478	Ser [S]/Phe [F]	Intolerant	0.01
rs141095253	Pro [P]/Leu [L]	Tolerant	0.05
rs145561065	Leu [L]/Phe [F]	Intolerant	0.01
rs148863193	Arg [R]/Leu [L]	Tolerant	0.12
rs201337668	Cys [C]/Gly [G]	Tolerant	0.64
rs143501729	Ala [A]/Glu [E]	Intolerant *	0.00

Note: Variants with tolerance index ≤0.05 score are considered as deleterious while others are taken to be tolerant.

* Predictions are made with low confidence because MSCS > 3.25, thus the interpretations should be made cautiously.

4.5.4 Prediction of Functional Consequences of nsSNPs Using PolyPhen

PolyPhen-2 server was used to predict structural and functional effects of 27 nsSNPs reported in the coding region of c-Myc proto oncogene protein. Using structure and sequence based predictive features PolyPhen searched for homologous protein sequences for multiple alignment and 3D protein structures to find probability score and predicted possible influence of variants on the protein. Out of 27 mutations, 7 were predicted to be probably damaging with probabilistic scores above 0.85, one variant was forecasted to be possibly damaging with probabilistic score above 0.15 while all others were considered to be benign (Table 4.3 Figure 4.17). Out of these eight nsSNPs, rs114570780 (Tyr47His), rs148228388 (Ala91Glu), rs199561469 (Gly104Arg), rs150308400 (Cys148Tyr), rs137906262 (Leu159Ile) are located within N-terminal trans-activation domain while rs200431478 (Ser362Phe), rs141095253 (Pro397Leu) harbor the C-terminal domain of c-Myc1 protein (Figure 4.15).

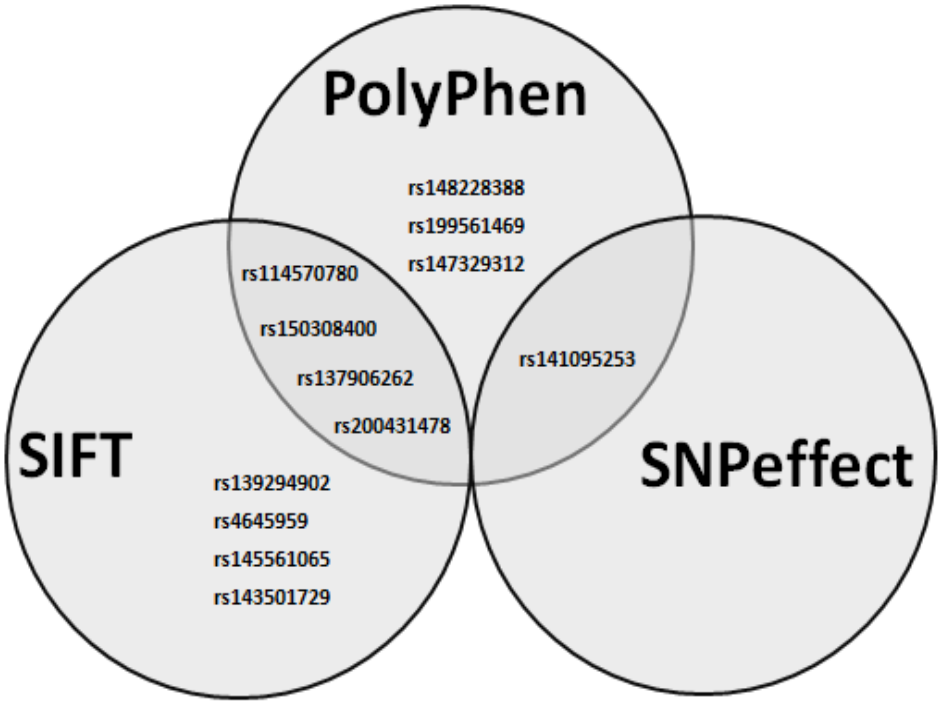

Figure 4.17: nsSNPs of human c-Myc1 protein predicted by SIFT, PolyPhen and SNPeffect algorithms to have some biological importance

Table 4.3: Polyphen analysis of nsSNPs in coding region of human c-Myc proto oncogene protein

dbSNP id	Amino Acid Variant	PolyPhen Prediction	Polyphen Score
rs139294902	Pro [P]/Arg [R]	Benign	0.027
rs146505192	Phe [F]/Leu [L]	Benign	0.008
rs148915481	Asn [N]/Ser [S]	Benign	0.007
rs4645959	Asn [N]/Ser [S]	Benign	0.160
rs114570780	Tyr [Y]/His [H]	Probably Damaging	0.955
rs148228388	Ala [A]/Glu [E]	Possibly damaging	0.452
rs199561469	Gly [G]/Arg [R]	Probably Damaging	0.915
rs150308400	Cys [C]/Tyr [Y]	Probably Damaging	0.999
rs137906262	Leu [L]/Ile [I]	Probably Damaging	0.997
rs61755060	Gln [Q]/His [H]	Benign	0.155
rs147329312	Lys [K]/Gln [Q]	Probably Damaging	0.971
rs4645960	Gly [G]/Cys [C]	Benign	0.048
rs4645960	Gly [G]/Arg [R]	Benign	0.269
rs4645961	Val [V]/Ile [I]	Benign	0.248
rs112602073	Pro [P]/Ser [S]	Benign	0.135
rs147506213	Pro [P]/Thr [T]	Benign	0.002
rs186663828	Ser [S]/Leu [L]	Benign	0.270
rs148544254	Pro [P]/Leu [L]	Benign	0.273
rs150629172	Pro [P]/His [H]	Benign	0.001
rs139697494	His [H]/Gln [Q]	Benign	0.041
rs146971340	His [H]/Gln [Q]	Benign	0.327
rs4645968	Ala [A]/Val [V]	Benign	0.009
rs200431478	Ser [S]/Phe [F]	Probably Damaging	0.914
rs141095253	Pro [P]/Leu [L]	Probably Damaging	0.999
rs145561065	Leu [L]/Phe [F]	Benign	0.100
rs148863193	Arg [R]/Leu [L]	Benign	0.193
rs201337668	Cys [C]/Gly [G]	Benign	0.00
rs143501729	Ala [A]/Glu [E]	Benign	0.014

Note: Variations with probabilistic score above 0.85 and 0.15 are considered to be "Probably damaging" and "possibly damaging" respectively while all the resting are categorized to be "Benign".

4.5.5 Prediction of Molecular Phenotypic Effects of nsSNPs Using SNPeffect

SNPeffect server was used to predict the effect of molecular phenotype of nsSNPs present in coding region of c-Myc protein. dTANGO, dWALTZ and dLIMBO scores were calculated to find effect of 27 nsSNPs on aggregation tendency, amyloid propensity and chaperone binding tendency respectively. Threshold for some significant effect was ranging from -50 to 50.

The dLIMBO score for Pro/Leu variation at position 397 (rs141095253) equals -277.70, which means that the mutation decreases the chaperone binding tendency of c-Myc protein. The scores for rest of the variants were not observed to be more than 50 or less than -50. The result shows that none of these nsSNPs other than rs141095253 is able to bring significant change in the aggregation tendency, amyloid propensity and chaperone binding tendency of c-Myc protein (Table 4.4). The difference in free energy of the mutation was calculated using empirical protein design force field (FoldX). The submitted sequence had 98.80 percent homology with 1nkp. Using FoldX a homology model was built starting from this PDB. The difference in free energy due to Pro/Leu variation at position 397 was of 2.68 Kcal/mol. This entails that the mutation reduces the protein stability (Figure 4.18). For rest of the variations SNPeffect could not predict possible changes in the stability of protein which could have been brought by variants due to the unavailability of adequate structural information of c-Myc in the form of PDB model.

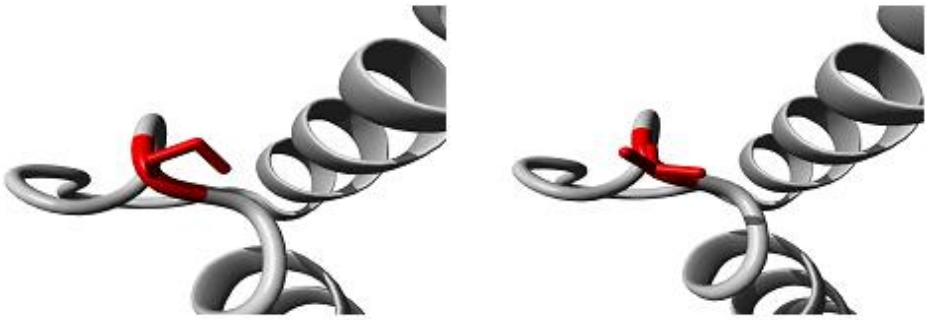

Figure 4.18: Molecular visualization of the wild type; Pro (left) and variant; Leu (right) residues colored in red at position 397 in c-Myc protein.

Table 4.4: SNPeffect analysis of nsSNPs in coding region of human c-Myc proto oncogene protein

dbSNP id	dTANGO	Agg. Tend.	dWALTZ	Amyl. Prop.	dLIMBO	Chap. Bind. Tend.
rs139294902	0.38	No effect	-0.05	No effect	0.00	No effect
rs146505192	-1.21	No effect	-0.93	No effect	0.00	No effect
rs148915481	-0.01	No effect	-0.36	No effect	0.00	No effect
rs4645959	0.00	No effect	-0.06	No effect	0.00	No effect
rs114570780	0.04	No effect	-10.16	No effect	0.00	No effect
rs148228388	-14.97	No effect	0.00	No effect	0.00	No effect
rs199561469	0.41	No effect	-0.05	No effect	0.00	No effect
rs150308400	9.73	No effect	0.24	No effect	0.00	No effect
rs137906262	0.00	No effect	8.63	No effect	0.00	No effect
rs61755060	0.04	No effect	-0.28	No effect	0.00	No effect
rs147329312	-0.41	No effect	0.05	No effect	0.00	No effect
rs4645960	0.00	No effect	0.00	No effect	0.00	No effect
rs4645960	0.41	No effect	-0.05	No effect	0.00	No effect
rs4645961	0.00	No effect	-0.00	No effect	0.00	No effect
rs112602073	0.00	No effect	0.00	No effect	0.00	No effect
rs147506213	0.00	No effect	0.07	No effect	0.00	No effect
rs186663828	0.00	No effect	0.04	No effect	0.00	No effect
rs148544254	0.00	No effect	0.00	No effect	0.00	No effect
rs150629172	0.04	No effect	-0.00	No effect	0.00	No effect
rs139697494	-0.05	No effect	0.01	No effect	0.00	No effect
rs146971340	-0.05	No effect	1.21	No effect	0.00	No effect
rs4645968	0.00	No effect	0.00	No effect	0.00	No effect
rs200431478	0.00	No effect	0.00	No effect	0.00	No effect
rs141095253	1.17	No effect	0.06	No effect	-277.70	Decrease
rs145561065	0.00	No effect	0.16	No effect	-0.05	No effect
rs148863193	-0.68	No effect	-0.02	No effect	0.02	No effect

rs201337668	0.00	No effect	-0.00	No effect	0.00	No effect
rs143501729	0.00	No effect	0.00	No effect	0.00	No effect

Note: Variations with dTANGO, dWALTZ and dLIMBO between -50 and 50 are supposed to have no affect on aggregation tendency, amyloid propensity and chaperone binding tendency respectively. Agg. Tend. = Aggregation Tendency, Amyl. Prop. = Amyloid Propensity and Chap. Bind. Tend. = Chaperone Binding Tendency.

4.6 ASSOCIATION OF IKKα EXPRESSION WITH CLINICOPATHOLOGICAL FEATURES OF BC PATIENTS

IKKα has been reported to play pro-tumorogenic as well as anti-tumorogenic role. To explore the molecular details and ultimate role of IKKα one of the objectives included the expressional analysis of IKKα in breast cancer tissue samples and its correlation to clinicopathological features of Pakistani BC patients. In 83 IDC tissue samples, IKKα expression was analyzed. Results of 9 samples were not predictable probably due to improper formalin fixation thus were excluded from the study. 74 remaining samples included 37 patients aged ≤50 years while 37 were of >50 years. 26 samples with PR positive status and 26 with PR negative status were stained and analyzed (Table 4.5).

IKKα expression was categorized as low or high depending on percentage of IKKα positive cells in IDC tissue samples (Figure 4.19) Analyses was performed using the SPSS software program (SPSS Standard version 16.0, Chicago, IL). Comparison of the IKKα expressions in breast cancer tissues was performed using paired t-test analysis for the clinicopathological feature having equal number of tissue samples in both groups to be compared, while for the unequal number of samples correlation was analyzed through spearman test. The p value <0.05 was considered significant.

Histological grade directly affects the propensity to invasion and metastasis of breast cancer. Low grade tumors morphologically resemble normal cells and are well differentiated while high grade tumors are highly undifferentiated. Highly undifferentiated cells are more likely to invade surrounding organs of the body causing metastasis and poor prognosis (cancer.gov, 2014). Total 35 samples were of grade II and 9 of grade III. Low expressional level of IKKα was observed in 14 of grade II and 4 of grade III tissue samples. However, no significant correlation (p=0.814) between IKKα expression and histological grade was observed (Table 4.5).

Metastasis is the hall mark of breast cancer. A cancer escapes the immune surveillance by invading the lymph and blood vessels gaining ability to metastasize at distant organs of the body. 20 samples were positive for lymph node metastasis while 16 were non metastatic samples. Low expression of IKKα was observed in 5 samples with no lymph node metastasis and 10 metastatic samples. While high expression was observed in 10 metastatic and 11 non metastatic samples. Although no significant correlation (p=0.270) between IKKα expression and lymph node metastasis was observed but a trend of low IKKα expression in non metastatic samples compared to metastatic ones was observed (Table 4.5).

Table 4.5: Association of IKKα expression with clinicopathological features of breast cancer patients

Clinicopathological Features		Low Expression of IKKα Total number (N) =74	High Expression of IKKα Total number (N) =74	*P*-value
Age	≤ 50	21	16	**0.044**
	>50	17	20	
Histological Grade	II	14	21	0.814
	III	4	5	
Lymph node status	No metastasis	5	11	0.270
	Metastasis	10	10	
Progesterone Receptor	Positive	14	12	**0.021**
	Negative	8	18	

4.6.1 Association of IKKα Expression with Age

IKKα is a crucial protein involved in the immune regulation and aging directly affects the immune status of an individual. Thus correlation of IKKα expression with age was analyzed in IDC tissue samples. Patients were categorized into two age groups, one of less or equal to 50 years (≤50) and the other of above 50 year (Table 4.5). IKKα expression was low in 21 samples of ≤50 years and 17 samples of >50 years of age while the expression of IKKα was high in 16 tissue samples of ≤50 years and 20 samples of >50 years of age. Expression of IKKα was significantly low (p=0.044) in patients with age ≤50 years as compared to that of >50 years (Figure 4.19 and 4.20).

4.6.2 Association of IKKα Expression with PR Status

Aberrant activation of NF-κB provides mechanistic basis for breast cancers. (Ben-Neriah and Karin, 2011). Interaction between the hormonal receptors and NF-*κ*B are already reported therefore, in order to analyze association of IKKα with hormonal receptors, IHC was brought about (Van Der Burg and Van Der Saag, 1996). Here, IKKα expression was found to be low in 14 PR positive and 8 PR negative samples. While the expression of IKKα was high in 12 PR positive tissue samples and 18 samples with PR negative status (Figure 4.21 and 4.22). Low expression of IKKα was significantly associated (p=0.021) with PR positivity.

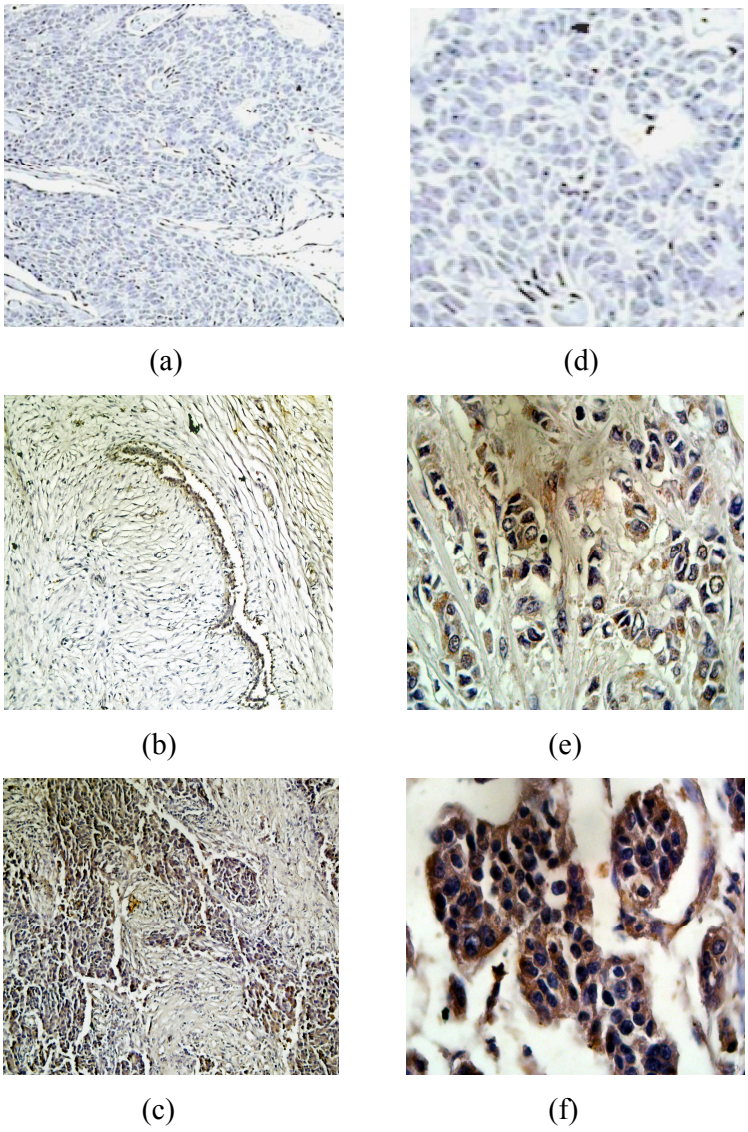

Figure 4.19: Immunohistochemical analysis of IKKα expression in invasive ductal carcinoma (IDC) patients of different age groups. Immunohistochemical staining with IKKα and histological comparison of IDC tissue samples was performed. Negative control (a,c), low expression of IKKα in patients of ≤50 years age (b,d) and high expression of IKKα in patients of >50 years of age (c,f) is shown. Images on left side (a, b, c) are captured at 40X magnification and on right side (d,e,f) are captured by 100X objective.

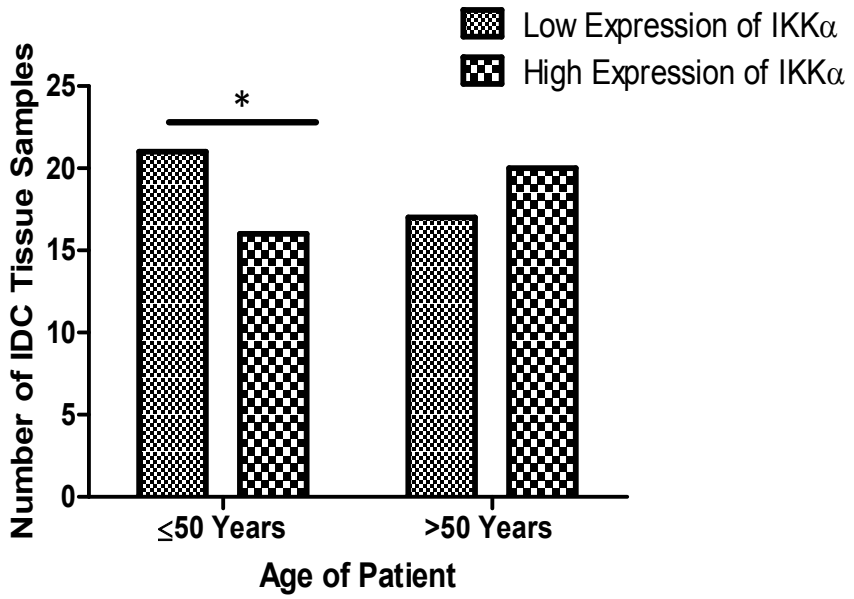

Figure 4.20: Histogram showing correlation of IKKα expression with age of IDC patients. Graph showing statistical comparison of IKKα expression in patients of ≤50 years (Total number (N)=37) and >50 years (N=37).

* Correlation is significant at the <0.05 level.

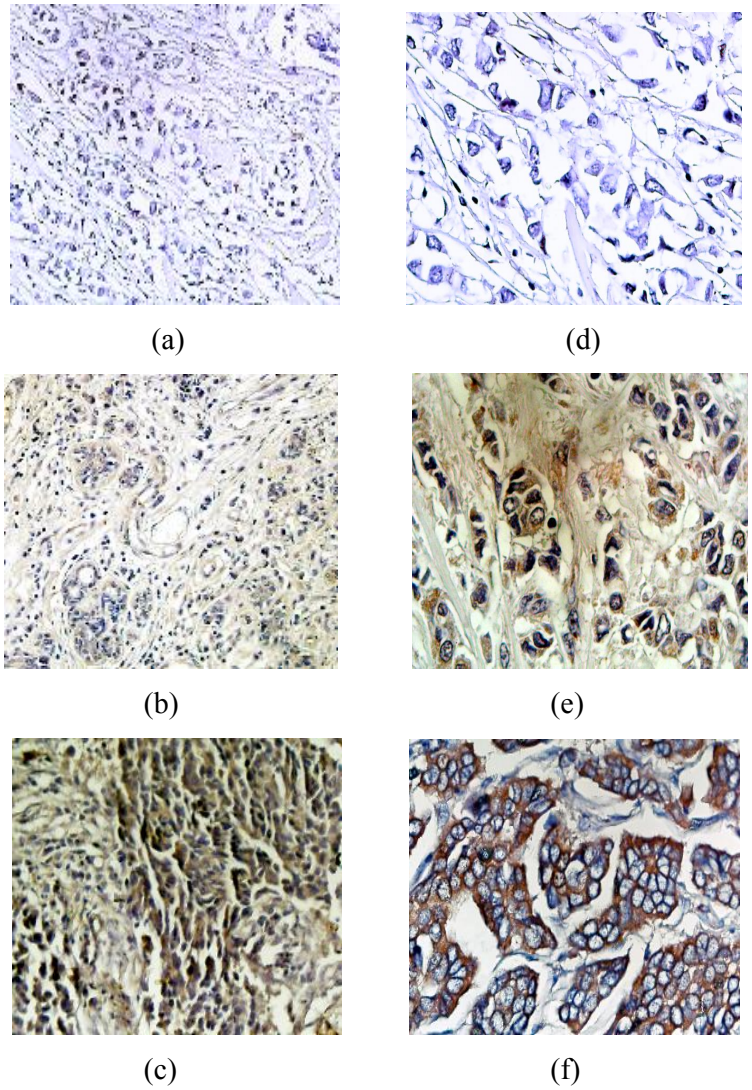

Figure 4.21: Immunohistochemical analysis of IKKα expression in invasive ductal carcinoma (IDC) patients with Progesterone receptor (PR) positive and negative status. Immunohistochemical staining with IKKα and histological comparison of IDC tissue samples was performed. Negative control (a,c), low expression of IKKα in PR positive tissue samples (b,d) and high expression of IKKα in PR negative tissue samples (c,f) is shown. Images on left side (a, b, c) are captured at 40 X magnification and on right side (d,e,f) are captured by 100X objective.

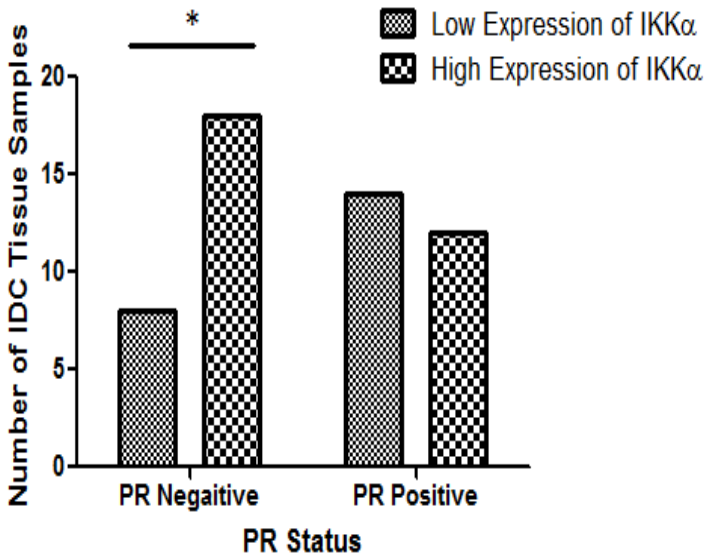

Figure 4.22: Histogram showing correlation of IKKα expression with PR status of IDC patients. Graph showing statistical comparison of IKKα expression in IDC patients with PR negative (Total number (N =26) and PR positive (N=26) status.

* Correlation is significant at the <0.02 level.

4.7 EVALUATION OF BREAST CANCER AWARENESS

Pakistan has highest incidence and mortality rate of breast cancer among Asian countries (Bhurgri *et al.*, 2000; Asif *et al.*, 2014). One of the leading causes of BC related mortality is the late diagnosis in Pakistan. Lack of public awareness about preventable risk factors and early warning signs is main cause of late diagnosis. Increase in survival rate of patients can be achieved by a significant approach of persuading awareness in population, better screening and in time detection of breast cancer at an initial stage. The dissemination of basic knowledge about breast cancer risk factors and early signs in Pakistani students was found to be unsatisfactory (Noreen *et al.*, 2015). The dissemination of basic knowledge about breast cancer risk factors and early signs in society can be carried out using a range of methods. One principally effective way is by educating young women while they are studying in educational institutes. No such study has ever been conducted in the Southern Punjab region of Pakistan. Therefore, as a side project this was intended to assess the awareness of the risk factors and early signs of breast cancer among the medical and non-medical students of university of southern Punjab, Pakistan (Noreen *et al.*, 2015).

4.7.1 Demographic characteristics

The survey included 566 female university students of university from southern Punjab, Pakistan. 326 non-medical students were aged between 20-28 years while 240 medical students ranged between 21-25 years. The mean age of non-the medical and medical participants was 23 years (SD±2.1) and 22 years (SD±1.3) respectively. Most of the students were Muslim. Majority of the non-medical; 199 (61%) and medical students; 182 (76%) were residents of urban areas. 281 (86%) non-medical and 214 (89%) medical students belonged to Punjabi families. Among non-medical girls 309 (95%) were unmarried and 17 (5%) were married. The majority of medical participants; 238 (99%) were unmarried and only 2 (1%) were married (Table 4.6).

Table 4.6: Demographic characteristics of the participants

		Non Medical Students N=326	Medical Students N=240
Characteristics		n (%)	n (%)
Age	Range (Years)	20-28	21-25
	Mean±SD	23±2.1	22±1.3
Religion	Muslims	321 (99)	238 (99)
	Non Muslims	5 (1)	2 (1)
Residency	Urban	199 (61)	182 (76)
	Rural	127 (39)	58 (24)
Ethnicity	Punjabi	281 (86)	214 (89)
	Sindhi	13 (4)	7 (3)
	Pathan	4 (1)	0 (0)
	Baloch	21 (6)	9 (4)
	Other	7 (3)	10 (4)
Marital Status	Unmarried	309 (95)	238 (99)
	Married	17 (5)	2 (1)

4.7.2 Source of Information

Most cited source of information by non medical students was television while medical students found their academic education to be the best source of information about breast cancer (Figure 4.23).

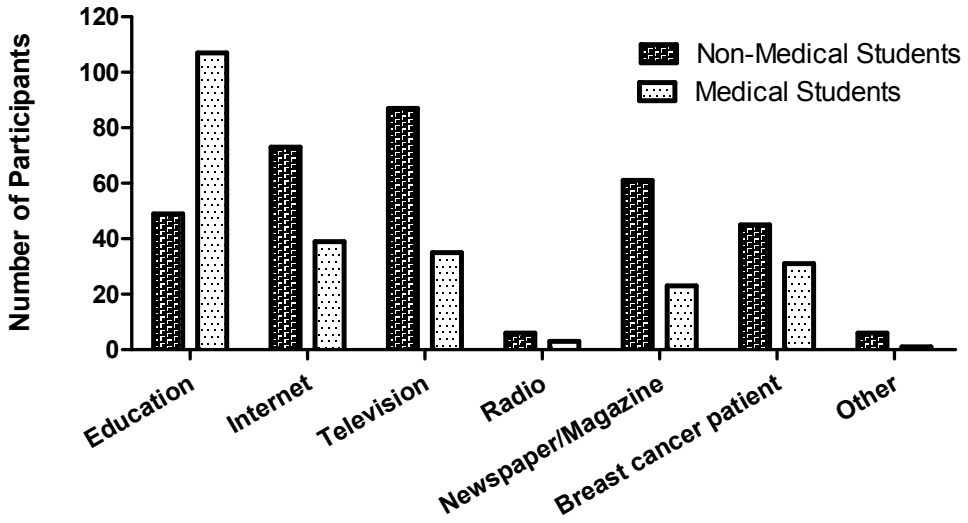

Figure 4.23: Histogram showing participant's source of information about breast cancer

4.7.3 Basic Knowledge about Breast Cancer

The next portion of the questionnaire contained questions about basic knowledge about breast cancer. Most of the non-medical 239 (73%) and medical 191 (80%) students knew that breast cancer is among the most prevalent cancers among women of Pakistan. But taken together across all the answers, knowledge of the students was not found to be satisfactory (less than 50% could give correct answer for most of the questions).

Only 71 (22%) non-medical and 67 (28%) medical students had awereness that breast cancer can also affect men. The majority of non-medical 260 (80%) and medical students i.e 199 (83) had an information about breast cancer treatment. 215 (90%) medical students had knowledge that breast cancer is not a contagious

disease. Their response was significantly better ($p<0.05$) than that of non-medical students as only 96 (29%) non-medical students had this information. 183 (56%) non-medical and 161 (67%) medical students were familiar with the possibility that a woman younger than 30 years can also develop breast cancer (Table 4.7).

4.7.4 Awareness of Early Warning Signs

The responses from medical students did not differ significantly ($p > 0.05$) from that of the non- medical ones about information regarding bloody discharge from nipple in a non-pregnant woman. Although the response of medical students to all other questions about noticeable indicators was significantly better ($p < 0.05$) than non-medical participants, but as a whole, the knowledge of both groups was inadequate. Most of the medical students (78%) knew that the pain in the breast does not always indicate the presence of breast carcinoma, but the presence of a lump in the breast can be a noticeable indicator (indicated by 90% medical students). Less than 35% students had knowledge about any potential indicative warning signs of breast cancer, for instance, change in the color or shape of nipple, change in skin color of the breast, bloody discharge from the nipple, the presence of a lump in the neck or armpit and asymmetry of the breasts (Table 4.8).

Table 4.7: Basic knowledge and perception about breast cancer

	Non Medical Students N=326			Medical Students N=240		
Questions	Corr. Ans. n (%)	Inco. Ans. n (%)	D/K n (%)	Corr. Ans. n (%)	Inco. Ans. n (%)	D/K n (%)
Is breast cancer one among the most prevalent cancers in women of Pakistan?	239 (73)	34 (10)	53 (16)	191 (80)	13 (5)	36 (15)
Can Breast cancer affect men?	71 (22)	99 (30)	155 (48)	67 (28)	96 (40)	77 (32)
Can breast cancer be treated?	260 (80)	19 (6)	47 (14)	199 (83)	22 (9)	19 (8)
Is breast cancer a contagious disease?	96 (29)	143 (44)	87 (27)	215 (90)	13 (5)	12 (5)
Can a woman younger than 30 years get breast cancer?	183 (56)	59 (18)	84 (26)	161 (67)	54 (23)	25 (10)

Note: Corr. Ans. = Correct Answer, Inco. Ans. = Incorrect Answer, D/K = Don't Know, N = Total number of participants. n = Number of participants.

Table 4.8: Awareness about early warning signs

	Non Medical Students N=326		Medical Students N=240		
Questions	Corr. Ans. n (%)	Inco. Ans. n (%)	Corr. Ans. n (%)	Inco. Ans. n (%)	p Value
Pain in breast	149 (46)	177 (54)	187 (78)	53 (22)	< 0.05
Change in skin color of breast (redness)	59(18)	267 (82)	73 (30)	167 (70)	< 0.05
Breast lump	137 (42)	189 (58)	216 (90)	24 (10)	< 0.05
Bloody discharge from nipple in a non-pregnant woman	28 (9)	298 (91)	29 (12)	211 (88)	> 0.05
Change in the color or shape of a woman's nipple	31 (10)	295 (90)	65 (27)	175 (73)	< 0.05
Lump in neck or armpit	50 (15)	276 (85)	84 (35)	156 (65)	< 0.05
Asymmetry of the breasts	37 (11)	289 (89)	59 (25)	181 (75)	< 0.05

Note: Corr. Ans. = Correct Answer, Inco. Ans. = Incorrect Answer, D/K = Don't Know, N = Total number of participants. n = Number of participants.

4.7.5 Awareness of Breast Cancer Risk Factors

Our results showed that there is a widespread lack of awareness among female students about potential risk factors associated with the breast cancer. Knowledge of the medical students about risk factors such as family history of breast cancer (85% gave correct answer), combined hormone therapy after menopause (61% gave correct answer), and the use of oral contraceptive (68% gave correct answer) was significantly better ($p < 0.05$) than that of non-medical students of the university.

98% of medical students knew that breastfeeding and giving birth to more than 5 children are not the risk factors for breast cancer. Interestingly, more non-medical participants (64%) were aware of the misconception that the use of tight bra can cause breast cancer than the medical students (43%). Diabetes was known to be a risk factor for breast cancer by 37% non-medical and only 5% of the medical students may be due to a general concept that diabetes is a risk for a number of other diseases (Table 4.9).

Table 4.9: Awareness of breast cancer risk factors

	Non Medical Students N=326		Medical Students N=240		
Questions	Corr. Ans. n (%)	Inco. Ans. n (%)	Corr. Ans. n (%)	Inco. Ans. n (%)	p Value
Older age (>45 years)	44 (13)	282 (87)	110 (46)	130 (54)	< 0.05
Less Physical activity	62 (19)	264 (81)	19 (8)	221 (92)	< 0.05
Use of tight bra for longer time	208 (64)	118 (36)	104 (43)	136 (57)	< 0.05
Being overweight / Obese	77 (24)	249 (76)	55 (23)	185 (77)	< 0.05
Consumption of fatty foods	40 (12)	286 (88)	31 (13)	209 (87)	> 0.05
Combined hormone therapy after menopause	53 (16)	273 (84)	146 (61)	94 (39)	< 0.05
Use of alcohol	68 (21)	258 (79)	73 (31)	167 (70)	< 0.05
Diabetes	119 (37)	207 (63)	13 (5)	227 (95)	< 0.05
Family history of breast cancer	102 (31)	224 (69)	205 (85)	35 (15)	< 0.05
Dense breast tissue	25 (8)	301 (92)	43 (18)	197 (82)	< 0.05
Early menarche (<12 years)	22 (7)	304 (93)	20 (8)	220 (92)	< 0.05
Late menopause (>55 years)	19 (6)	307 (94)	31 (13)	209 (87)	< 0.05
Oral contraceptive use	13 (4)	313 (96)	164 (68)	76 (32)	< 0.05
Past history of breast lumps	73 (22)	253 (78)	122 (51)	118 (49)	< 0.05
Smoking	66 (20)	260 (80)	46 (19)	194 (81)	> 0.05
Breast feeding	276 (85)	50 (15)	234 (98)	6 (2)	< 0.05

Chest radiation therapy	56 (17)	270 (83)	100 (46)	130 (54)	< 0.05
Having no children	19 (6)	307 (94)	23 (10)	217 (90)	> 0.05
Having more than 5 children	189 (58)	137 (42)	235 (98)	5 (2)	< 0.05
First childbirth after 30 years	22 (7)	304 (93)	17 (7)	223 (93)	> 0.05

Note: Corr. Ans. = Correct Answer, Inco. Ans. = Incorrect Answer, D/K = Don't Know, N = Total number of participants. n = Number of participants.

4.7.6 Individual Score of Participants

All the participants were categorized as poor, insufficient, satisfactory and good on basis of their score ranging from 1 to 32. Each successive category revealed difference of 25% increase in knowledge and awareness of breast cancer. Majority of non-medical students; 244 (75 %) and medical students; 131 (55 %) had insufficient knowledge (Table 4.10).

Table 4.10: Categorical distribution of students on basis of breast cancer awareness

Level of Knowledge	Score	Non-Medical Students N=326	Medical Students N=240
		n (%)	n (%)
Poor	≤ 8	73 (22)	11 (5)
Insufficient	≤ 16	244 (75)	131 (55)
Satisfactory	≤ 24	9 (3)	95 (40)
Good	≤ 32	0 (0)	3 (1)

Note: Total number of participants. n = Number of participants.

Chapter 5

DISCUSSION

Cancer is the consequence of uncontrolled division of abnormal cells with the propensity to invade other parts of the body. Cancer is found to be the most frequent cause of death (accounting for 13 % of total deaths) worldwide. In last few decades tremendous research in the field of cancer biology has been carried out to understand the mechanism involved in the cancer progression but still there is much to be achieved (Hoesel and Schmid, 2013). Slew of still unanswered questions cued that cancer is actually a complex disease (Hayden and Ghosh, 2012). Knowledge to date indicates that fortunately, one third of all cancers can be prevented and further one third can also be cured if diagnosed in time at an early stage (Parkin, 1994).

Cancer initiation, sustenance and progression are strictly controlled by proper regulation of molecular activities as a signaling cascade. Physiological, physical, or/and oxidative stress of the tumor microenvironment deregulates these pathways, leading to uncontrolled cell division, escape from immune activation, smoldering inflammation and subsequent development of cancer (Ben-Neriah and Karin, 2011). Cancer pathogenesis involves regulation of well documented NF-κB signaling pathway (Hanahan and Weinberg, 2011). Aberrant activation of NF-κB in tumor microenvironment leads to excessive inflammation and cancer progression. By shaping various aspects of innate and adaptive immunity, NF-κB transcription factors are known to play an important role in chronic inflammation and tumorogenesis.

Activity of NF-κB transcription factors is controlled by a group of non-covalently associated inhibitory molecules called IκB. (Whiteside and Israel, 1997). These inhibitors are phosphorylated and degraded by catalytic subunits of IKK complex; IKKα and IKKβ liberating NF-κB subunits to translocate to the nucleus where they bind to specific DNA targets as transcription factors to initiate transcription (Bonizzi and Karin, 2004; Hayden and Ghosh, 2012). NF-κB targets a number of genes involved in oncogenic cellular transformations (Figure 2.5). It fuels inflammation through production of COX-2 enzymes, inflammatory

cytokines such as TNFα, IL-1, IL-6 and IL-8, anti-apoptotic genes such as BCL-2, BCL-XL, CFLAR and cIAPs etc. (Basseres and Baldwin, 2006). IKKα and IKKβ have been reported to be involved in the endurance and development of cancers independent of NF-κB (Israel, 2010; Jiang *et al.*, 2010). One of the ways can be through their direct interaction with proto-oncogene such as c-Myc. IKKs interaction with c-Myc has been reported but not much detail is known about their interaction; therefore the main focus of the current study was to gain better insight into the molecular details and effects of the association between IKKs and c-Myc.

IKKα regulates transcription factors such as NF-*κ*B and E2F that are directly involved in the expressional regulation of a pleiotropic oncogenic Myc family of proteins (Wierstra and Alves, 2008; Levens, 2010). c-Myc belongs to the basic helix-loop-helix leucine zipper (bHLHLZ) containing family of transcription factors and is among the most well characterized and influential transcription factors as it is evidently involved in regulation of almost 15% of human genes (Xu *et al.*, 2010). Extensive research during last three decades manifested the crucial role of c-Myc protein in cell growth (Henriksson and Luscher, 1996), proliferation (Lemaitre *et al.*, 1996), apoptosis (Thompson, 1998), gene expression (Lee and Dang, 2006) and cancer metabolism (Miller *et al.*, 2012). Detailed structural analysis of IKKs and c-Myc are indicative of their interaction and effect of this co-operativity in oncogenic cellular transformation.

IKKα is reported to constitutively shuttle between cytoplasmic and nuclear region of the cell (Hoesel and Schmid, 2013). c-Myc being a transcription factor resides mainly inside the nucleus. Live cell microscopy through FRET analysis was used to study the interaction between IKKs and cMyc. FRET analysis indicated that c-Myc directly interacts with IKKα inside the nucleus (Figure 4.1 and 4.2). The association of IKKα with c-Myc was found to be more prominent as compared to IKKβ. In order to locate the interaction domain of IKKα with c-Myc, IKKα was truncated by cutting out HLH and LZ of IKKα. The lack of HLH and LZ domains resulted in disrupted interaction between IKKα and c-Myc (Figure 4.3 and 4.4). This is the first evidence showing the indispensable role of HLH and LZ domains in IKKα in the association between IKKα and c-Myc. The disrupted

association between truncated IKKα and c-Myc could be either because the c-Myc interacting domain is located on HLH/LZ domains or the lack of these domains might have caused conformational changes in the IKKα protein which may inhibit the c-Myc to bind with IKKα. c-Myc is already reported to heterodimerize with other HLH/LZ domain containing proteins such as Max (Blackwood and Eisenman, 1991). Thus the disrupted interaction between c-Myc and HLH/LZ domains lacking IKKα (Figure 4.3 and 4.4) seem to indicate that HLH/LZ domain of IKKα protein can possibly be site of interaction with HLH/LZ domain of c-Myc.

Interaction of c-Myc with IKKβ was comparatively less evident than that of with IKKα. Probable reason could be the presence of IKKβ in cytosolic region while IKKα is reported to shuttle between nucleus and cytosol (Birbach *et al.*, 2002). Presence of IKKα and c-Myc in the nuclear region leads to their co-localization in HEK 293 cells (Figure 4.3). However it was the first evidence of IKK interaction with c-Myc through FRET analysis in HEK 293 cells. The next question was to address the functional consequence of their interaction thus further study was conducted.

Proteins often interact to either promote or inhibit each other's function and activity. Therefore, the impact of interaction between IKKs and c-Myc was studied in cancer cell lines. Commonly, interacting proteins affect each other's transcriptional regulation (Yeh *et al.*, 2011) therefore it was intended to determine functional impact of IKKs and c-Myc interactions on each other at transcriptional and translational level. qPCR was carried out in order to find probable effect of this interaction on transcriptional level in HEK 293 cells. There was no significant effect of IKKs on transcription of c-Myc (Figure 4.5). These results are in line with a previous study carried out in breast cancer Michigan Cancer Foundation-7 (MCF7) cell line (Yeh *et al.*, 2011). Furthermore, over expression of c-Myc could also not result in any significant effect on transcription of IKKα (Figure 4.6) and IKKβ (Figure 4.7) in HEK 293 cells.

Results revealing no effect of c-Myc on the transcriptional expression of IKKα suggested that the interaction of both proteins could lead to some transcription-independent consequences in cancer cells. Therefore, in order to analyze the possible effect of c-Myc on protein expression and activity of IKKα, SDS-PAGE and western blot analysis was performed. Expression (Figure 4.8) and activity (Figure 4.9) of IKKα remained unaffected at translational level in response to the over expression of c-Myc in HEK 293 cells.

As c-Myc is a transcription factor thus IKKs interaction could have some effect on its transcriptional activity. Luciferase assay was performed in order to analyze the possible effect of IKKs on transcriptional activity of c-Myc in LNCaps. Basal expression of c-Myc is lower in LNCaps as compared to HEK-293 cells thus LNCaps were used to perform this experiment. Luciferase assay revealed notable decrease in the transcriptional activity of c-Myc in the presence of IKKα (Figure 4.10). This finding was further validated by using IKK inhibitors. For that cells were treated with inhibitor of IKK i.e. 10 μM Bay11-7082 for 12 hours (Yeh *et al.*, 2011). Increase in the transcriptional activity of c-Myc in presence of inhibitors confirmed the inhibitory effect of IKKα on transcriptional activity of c-Myc protein (Figure 4.11). Reduction in the transcriptional activity of c-Myc in the presence of IKKβ was also observed (Figure 4.12). Results were confirmed by treating cells with potent IKKβ inhibitor; 4 TPCA-1 for 12 hours. Increase in the transcriptional activity of c-Myc in the presence of inhibitor confirmed IKKβ to be responsible for indicated reduction (Figure 4.13).

FRET analysis indicated that HLH/LZ domain of IKKα protein can possibly be site of interaction with HLH/LZ domain of c-Myc which is indispensable for its ability to recognize the promoters of target genes and turn on gene expression. Results of leuciferase assay indicate that IKKs probably down regulate the transcriptional activity of c-Myc by virtue of their ability to compete with the HLH/LZ domain of c-Myc for binding to other transcriptional regulators. Moreover, this study also indicated that IKKα and IKKβ tend to keep tumor cell environment less aggressive by decreasing transcriptional activity of an oncoprotein; c-Myc.

Further support in this regard is lent by IKKα expression in BC biopsies. High IKKα expression was observed to be more frequent in non metastatic samples as compared to metastatic ones. Although this was statistically non-significant (P=0.270) but a trend was obvious and must be tested in large number of samples. IKKα, IKKβ and c-Myc are reported to be independently involved in the regulation of many genes related to cancer development. It seems as if c-Myc involved signaling pathway interacts with IKK involved signaling pathway to effect regulation of cancer cells. Further well defined studies are needed to characterize this interaction.

In order to go into the structural detail of c-Myc interaction with IKKs it was intended to perform some *in silico* studies. So far no model of c-Myc is available. Detailed analysis of c-Myc sequence revealed that many SNPs are reported in this protein which are most frequent type of polymorphisms found in human genome (Noreen *et al.*, 2012). Detailed information in this concern can be obtained using sequence and structure based tools of bioinformatics to select crucial and functional nsSNPs of c-Myc to get into deeply detailed research. In this study, some sequence and structural homology based algorithms were used to find those nsSNPs which can play a potential role in the structural and functional alteration of c-Myc proto-oncoprotein.

In Burkitt lymphoma samples the c-Myc2 proto onco-gene contained a cluster of four significant SNPs in 2nd and 3rd exon (Rabbitts *et al.*, 1983; Bhatia *et al.*, 1993). VAR_063384 (Glu39Asp) involves substitution of Glutamate (E) to Aspartate (D) at position 39. Both amino acids are medium sized and acidic with similar physico-chemical properties. VAR_063385 (Pro57Ser) involves substitution of medium sized and hydrophobic Proline (P) to small sized and polar Serine (S) at position 57. VAR_063386 (Pro59Ala) involves substitution of medium sized and hydrophobic Proline (P) to small sized and hydrophobic Alanine (A) at 59th amino acid. VAR_063387 (Asn86Thr) involves substitution Asparagine (N) to Threonine (T) at position 86. Both amino acid residues are of medium size and polar with similar physico-chemical properties. VAR_063385 (Pro57Ser) and VAR_063386 (Pro59Ala) at position 57 and 59 respectively are of more importance because these have an adjacent threonine residue at position 58.

Phosphorylation of serine at position 62 by extracellular signal regulated kinase (ERK) or CDK kinases increases Myc cellular stability. Then phosphorylation of threonine at position 58 is brought about by GSK3-β which triggers dephosphorylation of S62 by protein phosphatase 2A (PP2A) and subsequentely ubiquitin-mediated degradation is brought about through SCF-Fbwx7 (ubiquitin ligases) (Hann, 2006). Thus any variation in this domain can gain some biological importance affecting this series of phosphorylation, dephosphorylation and ubiquitinations.

Total 27 nsSNPs were found in the coding region along with two frame shift mutations; rs35631115 and rs67856294, among total 165 SNPs related to longest isoform of c-Myc protein (Table 4.7). As the SNPs associated with disease risk are generally nsSNPs; involving substitution of amino acids. These SNPs are likely to bring structural and functional changes in the protein. Evaluation of the possible role of nsSNP in the coding region was main aim of this study. One SNP (dbSNPid: rs4645960) found at position 175 has Gly [G] amino acid in wild type protein, which can be substituted either by Cys [C] or by Arg [R]. Both variants were considered separately to find if they have different effects. In order to find tolerant or deleterious effect of amino acid exchange on protein function, the SIFT algorithm (Ng and Henikoff, 2001) was used which is specialized in aligning homologous sequences, considers physical properties of amino acids and possible effects of naturally occurring nsSNPs on protein. Total 8 nsSNPs were found to be deleterious, out of which 2 had low confidence due to scarcity of available sequences for alignment (Table 4.2).

PolyPhen-2 server (Adzhubei et al., 2010) was used to forecast structural and functional effects of the SNPs of interest. PolyPhen searched for homologous protein sequences and 3D protein structures to predict the possible potential influence of variants on protein. Among total 27 variations, 7 were predicted to be probably damaging, one variant as possibly damaging while all others were considered to be benign (Table 4.3). Effect of four nsSNPs lying in coding region of c-Myc; rs114570780 (Tyr47His), rs150308400 (Cys148Tyr), rs137906262 (Leu159Ile) and rs200431478 (Ser362Phe) were predicted by SIFT algorithm to be intolerant and by PolyPhen-2 server to be probably damaging (Figure 4.17).

Four nsSNPs; rs4645959 (Asn26Ser), rs114570780 (Tyr47His), rs150308400 (Cys148Tyr) and rs137906262 (Leu159Ile) were located within N-terminal trans-activation domain of c-Myc1 containing 2 highly conserved domains called MBI and MBII. In c-Myc1 the MBI will range approximately 61-78 amino acids and MBII will span region of 144-158 amino acids (Figure 4.15). This region harboring MBI and MBII is essential for transactivation of target genes involved in process of apoptosis, cell proliferation and transformation (Wirtenberger *et al.*, 2005). In MBI domain a series of potential phosphorylations by MAPK, CDKs or GSK3β take place in cell cycle dependent manner.

These phosphorylation events regulate transcription and transformation. MBII encodes the hydrophobic part of the c-Myc protein. One Transcription activation domains (TAD) is located upstream of MBI and the other is upstream and overlapping region of MBII. A repression element is identified between the MBI and MBII domains while other overlaps MBII itself (Sakamuro and Prendergast, 1999). N-terminal trans-activation domain is also reported to interact with TBP, α-Tubulin, p107 as well as adaptor proteins; TRRAP, AMY-1, Bin1, Pam, and MM-1 (Beijersbergen *et al.*, 1994). Thus, interaction of these proteins and presence of sequences related to activation or repression activity in N-terminal trans-activation domain suggests its key role in apoptosis, chromatin modeling, cell cycle and transcriptional regulation (Sakamuro and Prendergast, 1999). Presence of genetic variants in this region can effect this regulation significantly.

The rs4645959 involves substitution of medium sized Asparagine (N) to small sized Serine (S) residue. According to SIFT Prediction this variant is intolerant (Tolerance index = 0.01). This nsSNP was reported to be putatively functional being capable of altering the secondary structure of protein. Heterozygous carriers of this variant among Polish and German individuals were observed to have an increased risk of familial breast cancer (Wirtenberger *et al.*, 2005). Two nsSNPs; rs141095253 (Pro397Leu) and rs145561065 (Leu434Phe) are found within while rs200431478 (Ser362Phe) is located near the C-terminal domain which spans 375-452 amino acid region of c-Myc1 (Figure 4.15). This C-terminal DNA binding segment contains BR-HLH-LZ domain. Another BR-HLH-

LZ domain containing protein; Max is identified as a closely related, obligate and physiological heterodimerization partener of c-Myc (Blackwood and Eisenman, 1991).

The HLH-LZ domains of both proteins form strong heterodimers and recognize the E-Box containing promoters to regulate transcriptional activation, cellular transformation, cell cycle progression and apoptosis (Amati *et al.*, 1993). Other than Max some proteins like Nmi, TFII-I, YY-1, AP-2, BRCA1 and Miz-1 are also reported to interact with C-terminal region of c-Myc. Based on these interactions, C-terminal region is supposed to control the access of the N-terminal trans-activation domain to specific genetic loci (Sakamuro and Prendergast, 1999). Presence of nsSNPs in C-terminal region can alter these interactions and consequently can lead to an impaired functioning of c-Myc protein in cell. However, when these SNPs were subjected to SNPeffect server to weigh up their possible effect on molecular phenotype of protein, most of the variations were neither predicted to affect aggregation tendency and amyloid propensity nor the chaperone binding tendency of c-Myc protein (Table 4.4).

It seems as, if these variations are really capable of bringing some damaging alterations to c-Myc, then these are certainly other than the aggregation tendency, amyloid propensity and the chaperone binding tendency of the protein as per prediction of SNPeffect. Chaperone binding tendency and protein stability of c-Myc was shown to be decreased by only Pro/Leu variation at position 397 (rs141095253). Further analysis can be performed using some other computational tools and availability of PDB structure can help in making more precise and reliable evaluations. Results of this study can be helpful and interesting especially to those epidemiologists who are interested in large-scale population based studies. Moreover, on basis of our predictions, the real effect of nsSNPs particularly rs114570780 (Tyr47His), rs150308400 (Cys148Tyr), rs137906262 (Leu159Ile) and rs200431478 (Ser362Phe) on c-Myc can be assured by conducting well defined in vitro and in vivo experimental studies.

The capability of IKKα to shuttle in and out of the cellular nuclei and cytoplasm and to interact with cMyc indicates an important role of IKKα in oncogenesis. Unfortunately, the data in this regard is somehow controversial yet

crucial for its potential therapeutic exploitation. IKKα has been reported to play pro-tumorogenic as well as anti-tumorogenic role. To explore the molecular details and ultimate role of IKKα one of the objectives included the expressional analysis of IKKα in breast cancer tissue samples and its correlation to clinicopathological features of Pakistani BC patients. In this study, Immunohistochemistry was carried out for in vivo expressional analysis of IKKα in 83 IDC tissue samples and its correlation to clinicopathological features of Pakistani BC patients was determined. Results of 9 samples were not predictable probably due to improper formalin fixation thus were excluded from the study. 74 remaining samples included 37 patients aged ≤50 years while 37 were of >50 years. 26 samples with PR positive status and 26 with PR negative status were stained and analyzed (Table 4.5). IKKα expression was categorized as low or high depending on percentage of IKKα positive cells in IDC tissue samples. The immune system is specialized to detect and correct the cell defects. Aging directly affects the immune status of an individual and damages immune competency. Undesirable changes in the immune system are evidently associated with aging. This down regulation of immune system increases the rate of cancer related morbidity and mortality in the elderly age (Wu and Meydani, 2014).

IKKα is a crucial protein involved in the immune regulation. In this study IKKα expression was found to be low in 21 samples taken from patients of ≤50 years and 17 samples of >50 years of age while the expression of IKKα was high in 16 tissue samples of ≤50 years and 20 samples of >50 years of age. Expression of IKKα was significantly low (p=0.044) in patients with age ≤50 years as compared to that of >50 years (Figure 4.19 and 4.20). Results indicate the association of aging with elevated expression of IKKα in patients of >50 years of age which may be indicative of poor prognosis.

Histological grade directly affects the propensity to invasion and metastasis of breast cancer. Low grade tumors morphologically resemble normal cells and are well differentiated while high grade tumors are highly undifferentiated. Highly undifferentiated cells are more likely to invade surrounding organs of the body causing metastasis and poor prognosis (cancer.gov, 2014). Metastasis is the hall mark of breast cancer. A cancer escapes

the immune surveillance by invading the lymph and blood vessels gaining ability to metastasize to distant organs of the body. However, IKKα expression was not found to be significantly associated with the histological tumor grade and lymph node metastasis. Possible reason for this finding can be less number of available samples. Further studies involving more number of samples can help to elucidate probable association of IKKα with histological tumor grade and lymph node metastasis in IDC.

PR and NF-κB are already reported to suppress each other's activity (Van Der Burg and Van Der Saag, 1996) and the regulation of NF-κB is brought about through IKKα, thus expressional analysis of IKKα in PR positive and negative samples was brought about in IDC samples. IKKα expression was found to be low in 14 PR positive and 8 PR negative samples. While the expression of IKKα was high in 12 PR positive tissue samples and 18 samples with PR negative status (Figure 4.21 and 4.22). Low expression of IKKα was significantly associated ($p=0.021$) with PR positivity. As PR and NF-κB are reported to suppress each other's activity (Van Der Burg and Van Der Saag, 1996) thus suppression of NF-κB in tumor microenvironment can be result of low expression of IKKα. May be decrease in level of IKKα helps to make cancer cell microenvironment less aggressive in PR positive BC patients as compared to PR negative. Thus, it seems to play a crucial role in proper regulation of NF-κB pathway and its association with PR status in BC patients.

While collecting BC biopsy samples, it was observed that a significant number of BC patients had a low literacy rate. Therefore, we hypothesized that one of the factors for increase in the incidence and mortality rate of breast cancer in Pakistan could be lack of awareness about BC and its late diagnosis (Bhurgri *et al.*, 2000; Asif *et al.*, 2014). BC is a health risk for women of all ethnic and social groups. Commonly Pakistani women are struck by it at a younger age and diagnosed at an advanced stage as compared to women of the western world (Rasheed, 2013). Late stage diagnosis usually involves metastasis and is hard to treat thus, reduces the survival rate. Likelihood of up to 5 years survival is about 85% among the cases diagnosed at an early stage while it reduces to just 10% among cases diagnosed at stage IV (Gilani *et al.*, 2003). Increase in survival rate

of patients can be achieved by a significant approach of persuading awareness in population, better screening and in time detection of breast cancer at an initial stage. The dissemination of basic knowledge about breast cancer risk factors and early signs in society can be carried out using a range of methods. One principally effective way is by educating young women while they are studying in educational institutes. Therefore, as a side project, this study aimed to assess the awareness of the risk factors and early signs of breast cancer among the medical and non-medical students of university of southern Punjab, Pakistan (Noreen *et al.*, 2015). Results show a widespread lack of knowledge regarding BC among non-medical and medical university students (Table 4.10). These results are in line with numerous studies conducted in other countries (Ahmed, 2010; Sait et al., 2010; Yadav and Jaroli, 2010; Al-Naggar et al., 2011; Sambanje and Mafuvadze, 2012; Kurtuncu et al., 2014). Although, the perception of medical students was comparatively better as compared to that of non-medical ones, but their overall knowledge was still generally insufficient. Level of knowledge regarding breast cancer did not differ among the respondents of different age, residency, religion, ethnicity or marital status.

This study highlighted the inadequate awareness about the potential risk factors among young women in Southern Punjab, Pakistan. Some BC risk factors are inevitable such as family history, diabetes, nature of the breast tissue, early menarche and late menopause etc., but some other factors such as diet, alcohol consumption, smoking, physical activity, hormone therapy and use of oral contraceptives can be controlled. The awareness regarding the healthy lifestyle adjustments can help to reduce the disease burden of society. Awareness of both the groups was poor concerning potential risk factors such as age, being obese, being physically inactive, intake of fatty food, diabetes, dense breast tissue, smoking, early menarche, late menopause, having no children and first childbirth after the age of 30 years. Crucial reproductive decisions are generally made at an age to which mostly university students belong, for example, time of first child's birth, use of oral contraceptives and period of breastfeeding etc. Only proper awareness can lead them to better decisions making and modification of lifestyle to decrease the risk of breast cancer.

This study shows a general lack of understanding among both groups of university students. These findings further highlight the need of immediate measures to increase awareness and thereby reduce the high morbidity and increasing mortality of women due to breast cancer. A proper platform should be established for health care providers and educationists. In this way they can educate women to be safe from preventable risk factors, and the early warning signs of disease. Internet social media, mass media, and non-government organizations (NGO) should step forward to foster the transfer of life-saving knowledge to as many people as possible. Special awareness programs, lectures, seminars, training courses and workshops should be conducted at educational institutes regularly. This will lead to in time diagnosis, cost effective treatment and a reduction of the burden that this killer disease places on society.

Conclusion

Cancer is a group of diseases involving abnormal cell growth with the propensity to invade or spread to other parts of the body. Cancer progression is principally regulated by NF-κB which is controlled by IKKs. IKKα, IKKβ and c-Myc are reported to be independently involved in the regulation of many genes related to cancer development. Physical interaction of IKKα with c-Myc and reduction of transcriptional activity of c-Myc by IKKs indicates interaction of c-Myc involving signaling pathways with IKK involving signaling pathways to effect regulation of cancer cells. Further well defined studies are needed to characterize this interaction. Results of *In Silico* study can be helpful and interesting especially to those epidemiologists who are interested in large-scale population based studies. Moreover, on basis of our predictions, the real effect of nsSNPs particularly rs114570780 (Tyr47His), rs150308400 (Cys148Tyr), rs137906262 (Leu159Ile) and rs200431478 (Ser362Phe) on c-Myc can be assured by conducting well defined in vitro and in vivo studies. Pakistan has highest incidence and mortality rate of breast cancer among Asian countries. Low expression of IKKα in PR positive tissue samples of IDC patients was observed.

This study shows a general lack of understanding about potential risk factors and early warning signs of breast cancer in general population. This awareness is necessary to decrease morbidity and mortality of breast cancer. These findings further highlight the need of immediate measures to increase awareness among people by establishing a proper platform for health care providers and educationists which can lead to the in time diagnosis, cost effective treatment and a reduction of the disease burden.

Chapter 6

REFERENCES

Adhikary, S. and Eilers, M., (2005). Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol, 6(8): 635-645.

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S. and Sunyaev, S. R., (2010). A method and server for predicting damaging missense mutations. Nat Methods, 7(4): 248-249.

Albanese, C., Wu, K., D'amico, M., Jarrett, C., Joyce, D., Hughes, J., Hulit, J., Sakamaki, T., Fu, M., Ben-Ze'ev, A., Bromberg, J. F., Lamberti, C., Verma, U., Gaynor, R. B., Byers, S. W. and Pestell, R. G. (2003). IKKalpha regulates mitogenic signaling through transcriptional induction of cyclin D1 via Tcf. Mol Biol Cell. 14(2): 585-599.

Al-Hussaini, H., Subramanyam, D., Reedijk, M. and Sridhar, S. S. (2011). Notch signaling pathway as a therapeutic target in breast cancer. Mol Cancer Ther. 10(1): 9-15.

Ali, S. A. (2012). The hedgehog pathway in breast cancer. Chin J Cancer Res. 24(4): 261-262.

Al-Naggar, R. A., Al-Naggar, D. H., Bobryshev, Y. V., Chen, R. and Assabri, A. (2011). Practice and barriers toward breast self-examination among young Malaysian women. Asian Pac J Cancer Prev. 12(5): 1173-1178.

Amati, B., Littlewood, T. D., Evan, G. I. and Land, H., (1993). The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. EMBO J, 12(13): 5083-5087.

Arnold, A. and Papanikolaou, A. (2005). Cyclin D1 in breast cancer pathogenesis. J Clin Oncol. 23(18): 4215-4224.

Asif, H. M., Sultana, S., Akhtar, N., Rehman, J. U. and Rehman, R. U. (2014). Prevalence, risk factors and disease knowledge of breast cancer in pakistan. Asian Pac J Cancer Prev. 15(11): 4411-4416.

Atalay, G., Cardoso, F., Awada, A. and Piccart, M. J. (2003). Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer. Ann Oncol. 14(9): 1346-1363.

Basseres, D. S. and Baldwin, A. S. (2006). Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene. 25(51): 6817-6830.

Baudino, T. A., Mckay, C., Pendeville-Samain, H., Nilsson, J. A., Maclean, K. H., White, E. L., Davis, A. C., Ihle, J. N. and Cleveland, J. L. (2002). c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev. 16(19): 2530-2543.

Baxter, F. O., Came, P. J., Abell, K., Kedjouar, B., Huth, M., Rajewsky, K., Pasparakis, M. and Watson, C. J. (2006). IKKbeta/2 induces TWEAK and apoptosis in mammary epithelial cells. Development. 133(17): 3485-3494.

Beijersbergen, R. L., Hijmans, E. M., Zhu, L. and Bernards, R., (1994). Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. EMBO J, 13(17): 4080-4086.

Beinke, S., Robinson, M. J., Hugunin, M. and Ley, S. C. (2004). Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105. Mol Cell Biol. 24(21): 9658-9667.

Ben-Neriah, Y. and Karin, M. (2011). Inflammation meets cancer, with NF-kappaB as the matchmaker. Nat Immunol. 12(8): 715-723.

Bertucci, F., Lagarde, A., Ferrari, A., Finetti, P., Charafe-Jauffret, E., Van Laere, S., Adelaide, J., Viens, P., Thomas, G., Birnbaum, D. and Olschwang, S. (2012). 8q24 Cancer risk allele associated with major metastatic risk in inflammatory breast cancer. PLoS One. 7(5): e37943.

Bhatia, K., Huppi, K., Spangler, G., Siwarski, D., Iyer, R. and Magrath, I. (1993). Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. Nat Genet. 5(1): 56-61.

Bhurgri, Y., Bhurgri, A., Hassan, S. H., Zaidi, S. H., Rahim, A., Sankaranarayanan, R. and Parkin, D. M. (2000). Cancer incidence in

Karachi, Pakistan: first results from Karachi Cancer Registry. Int J Cancer. 85(3): 325-329.

Birbach, A., Gold, P., Binder, B. R., Hofer, E., De Martin, R. and Schmid, J. A., (2002). Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. J Biol Chem, 277(13): 10842-10851.

Blackwood, E. M. and Eisenman, R. N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science. 251(4998): 1211-1217.

Bocker, W. (2002). [WHO classification of breast tumors and tumors of the female genital organs: pathology and genetics]. Verh Dtsch Ges Pathol. 86: 116-119.

Bonizzi, G. and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25(6): 280-288.

Byers, T., Graham, S., Rzepka, T. and Marshall, J. (1985). Lactation and breast cancer. Evidence for a negative association in premenopausal women. Am J Epidemiol. 121(5): 664-674.

Carayol, N. and Wang, C. Y. (2006). IKKalpha stabilizes cytosolic beta-catenin by inhibiting both canonical and non-canonical degradation pathways. Cell Signal. 18(11): 1941-1946.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Annu Rev Biochem. 56: 881-914.

Chariot, A. (2009). The NF-kappaB-independent functions of IKK subunits in immunity and cancer. Trends Cell Biol. 19(8): 404-413.

Chefetz, I., Holmberg, J. C., Alvero, A. B., Visintin, I. and Mor, G. (2011). Inhibition of Aurora-A kinase induces cell cycle arrest in epithelial ovarian cancer stem cells by affecting NFkB pathway. Cell Cycle. 10(13): 2206-2214.

Chen, J. J., Silver, D., Cantor, S., Livingston, D. M. and Scully, R. (1999). BRCA1, BRCA2, and Rad51 operate in a common DNA damage response pathway. Cancer Res. 59(7 Suppl): 1752s-1756s.

Chen, S. and Parmigiani, G. (2007). Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 25(11): 1329-1333.

Chen, Z. J. (2005). Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol. 7(8): 758-765.

Collaborative Group on Hormonal Factors in Breast Cancer (2002). Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease. Lancet. 360(9328): 187-195.

Cowling, V. H. and Cole, M. D. (2007). E-cadherin repression contributes to c-Myc-induced epithelial cell transformation. Oncogene. 26(24): 3582-3586.

Cowling, V. H., Chandriani, S., Whitfield, M. L. and Cole, M. D. (2006). A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation, and G2 arrest. Mol Cell Biol. 26(11): 4226-4239.

Dang, C. V., O'donnell, K. A., Zeller, K. I., Nguyen, T., Osthus, R. C. and Li, F., (2006). The c-Myc target gene network. Semin Cancer Biol, 16(4): 253-264.

Dave, S. S., Fu, K., Wright, G. W., Lam, L. T., Kluin, P., Boerma, E. J., Greiner, T. C., Weisenburger, D. D., Rosenwald, A., Ott, G., Muller-Hermelink, H. K., Gascoyne, R. D., Delabie, J., Rimsza, L. M., Braziel, R. M., Grogan, T. M., Campo, E., Jaffe, E. S., Dave, B. J., Sanger, W., Bast, M., Vose, J. M., Armitage, J. O., Connors, J. M., Smeland, E. B., Kvaloy, S., Holte, H., Fisher, R. I., Miller, T. P., Montserrat, E., Wilson, W. H., Bahl, M., Zhao, H., Yang, L., Powell, J., Simon, R., Chan, W. C. and Staudt, L. M., (2006). Molecular diagnosis of Burkitt's lymphoma. N Engl J Med, 354(23): 2431-2442.

Disis, M. L. (2010). Immune regulation of cancer. J Clin Oncol. 28(29): 4531-4538.

Donnelly, T. T., Al Khater, A. H., Al-Bader, S. B., Al Kuwari, M. G., Al-Meer, N., Malik, M., Singh, R., Chaudhry, S. and Fung, T. (2013). Beliefs and attitudes about breast cancer and screening practices among Arab women living in Qatar: a cross-sectional study. BMC Womens Health. 13: 49.

Donnelly, T. T., Al Khater, A. H., Al-Bader, S. B., Al Kuwari, M. G., Al-Meer, N., Malik, M., Singh, R., Chaudhry, S. and Fung, T., (2013). Beliefs and

attitudes about breast cancer and screening practices among Arab women living in Qatar: a cross-sectional study. BMC Womens Health, 13: 49.

Dunn, G. P., Old, L. J. and Schreiber, R. D. (2004). The three Es of cancer immunoediting. Annu Rev Immunol. 22: 329-360.

Eroles, P., Bosch, A., Perez-Fidalgo, J. A. and Lluch, A. (2012). Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer Treat Rev. 38(6): 698-707.

Escot, C., Theillet, C., Lidereau, R., Spyratos, F., Champeme, M. H., Gest, J. and Callahan, R. (1986). Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas. Proc Natl Acad Sci U S A. 83(13): 4834-4838.

Evans, J. P., Skrzynia, C., Susswein, L. and Harlan, M. (2005). Genetics and the young woman with breast cancer. Breast Dis. 23: 17-29.

Fallahzadeh, H., Momayyezi, M., Akhundzardeini, R. and Zarezardeini, S., (2014). Five year survival of women with breast cancer in yazd. Asian Pac J Cancer Prev, 15(16): 6597-6601.

Feldmann, G., Dhara, S., Fendrich, V., Bedja, D., Beaty, R., Mullendore, M., Karikari, C., Alvarez, H., Iacobuzio-Donahue, C., Jimeno, A., Gabrielson, K. L., Matsui, W. and Maitra, A. (2007). Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer Res. 67(5): 2187-2196.

Figueiredo, J. C., Knight, J. A., Cho, S., Savas, S., Onay, U. V., Briollais, L., Goodwin, P. J., Mclaughlin, J. R., Andrulis, I. L. and Ozcelik, H. (2007). Polymorphisms cMyc-N11S and p27-V109G and breast cancer risk and prognosis. BMC Cancer. 7: 99.

Forouzanfar, M. H., Foreman, K. J., Delossantos, A. M., Lozano, R., Lopez, A. D., Murray, C. J. and Naghavi, M. (2011). Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. Lancet. 378(9801): 1461-1484.

Ghosh, S., May, M. J. and Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 16: 225-260.

Gilani, G. M., Kamal, S. and Akhter, A. S., (2003). A differential study of breast cancer patients in Punjab, Pakistan. J Pak Med Assoc, 53(10): 478-481.

Gong, W. F., Zhong, J. H., Xiang, B. D., Ma, L., You, X. M., Zhang, Q. M. and Li, L. Q. (2013). Single nucleotide polymorphism 8q24 rs13281615 and risk of breast cancer: meta-analysis of more than 100,000 cases. PLoS One. 8(4): e60108.

Gregory, M. A., Qi, Y. and Hann, S. R. (2003). Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. J Biol Chem. 278(51): 51606-51612.

Gringhuis, S. I., Garcia-Vallejo, J. J., Van Het Hof, B. and Van Dijk, W. (2005). Convergent actions of I kappa B kinase beta and protein kinase C delta modulate mRNA stability through phosphorylation of 14-3-3 beta complexed with tristetraprolin. Mol Cell Biol. 25(15): 6454-6463.

Guo, Y., Fang, J., Liu, Y., Sheng, H. H., Zhang, X. Y., Chai, H. N., Jin, W., Zhang, K. H., Yang, C. Q. and Gao, H. J. (2011). Association between polymorphism rs6983267 and gastric cancer risk in Chinese population. World J Gastroenterol. 17(13): 1759-1765.

Gupta, S., Seth, A. and Davis, R. J. (1993). Transactivation of Gene-Expression by Myc Is Inhibited by a Point Mutation at the Map Kinase Phosphorylation Site. J Cell Biochem. 182-182.

Gustafson, W. C. and Weiss, W. A. (2010). Myc proteins as therapeutic targets. Oncogene. 29(9): 1249-1259.

Gustafson, W. C. and Weiss, W. A., (2010). Myc proteins as therapeutic targets. Oncogene, 29(9): 1249-1259.

Hanahan, D. and Weinberg, R. A., (2011). Hallmarks of cancer: the next generation. Cell, 144(5): 646-674.

Hann, S. R. (1995). Methionine deprivation regulates the translation of functionally-distinct c-Myc proteins. Adv Exp Med Biol. 375: 107-116.

Hann, S. R., (2006). Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. Semin Cancer Biol, 16(4): 288-302.

Hann, S. R., King, M. W., Bentley, D. L., Anderson, C. W. and Eisenman, R. N. (1988). A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell. 52(2): 185-195.

Hayden, M. S. and Ghosh, S., (2012). NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev, 26(3): 203-234.

Henriksson, M. and Luscher, B. (1996). Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res. 68: 109-182.

Henriksson, M., Bakardjiev, A., Klein, G. and Luscher, B. (1993). Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. Oncogene. 8(12): 3199-3209.

Herrmann, O., Baumann, B., De Lorenzi, R., Muhammad, S., Zhang, W., Kleesiek, J., Malfertheiner, M., Kohrmann, M., Potrovita, I., Maegele, I., Beyer, C., Burke, J. R., Hasan, M. T., Bujard, H., Wirth, T., Pasparakis, M. and Schwaninger, M. (2005). IKK mediates ischemia-induced neuronal death. Nat Med. 11(12): 1322-1329.

Hoesel, B. and Schmid, J. A. (2013). The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer. 12: 86.

Hsieh, C. C., Trichopoulos, D., Katsouyanni, K. and Yuasa, S. (1990). Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: associations and interactions in an international case-control study. Int J Cancer. 46(5): 796-800.

Hu, M. C., Lee, D. F., Xia, W., Golfman, L. S., Ou-Yang, F., Yang, J. Y., Zou, Y., Bao, S., Hanada, N., Saso, H., Kobayashi, R. and Hung, M. C. (2004). IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell. 117(2): 225-237.

Huber, M. A., Azoitei, N., Baumann, B., Grunert, S., Sommer, A., Pehamberger, H., Kraut, N., Beug, H. and Wirth, T. (2004). NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest. 114(4): 569-581.

Hunter, C. P. (2000). Epidemiology, stage at diagnosis, and tumor biology of breast carcinoma in multiracial and multiethnic populations. Cancer. 88(5 Suppl): 1193-1202.

Hunter, D. J., Colditz, G. A., Hankinson, S. E., Malspeis, S., Spiegelman, D., Chen, W., Stampfer, M. J. and Willett, W. C. (2010). Oral contraceptive use and breast cancer: a prospective study of young women. Cancer Epidemiol Biomarkers Prev. 19(10): 2496-2502.

Hynes, N. E. and Gullick, W. (2006). Therapeutic targeting of signal transduction pathways and proteins in breast cancer. J Mammary Gland Biol Neoplasia. 11(1): 1-2.

Israel, A., (2010). The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol, 2(3): a000158.

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

Jiang, R., Xia, Y., Li, J., Deng, L., Zhao, L., Shi, J., Wang, X. and Sun, B., (2010). High expression levels of IKKalpha and IKKbeta are necessary for the malignant properties of liver cancer. Int J Cancer, 126(5): 1263-1274.

Johansson, I., Killander, F., Linderholm, B. and Hedenfalk, I. (2014). Molecular profiling of male breast cancer - Lost in translation? Int J Biochem Cell Biol.

Karin, M. and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 18: 621-663.

Kelsey, J. L., Gammon, M. D. and John, E. M. (1993). Reproductive factors and breast cancer. Epidemiol Rev. 15(1): 36-47.

Kerlikowske, K., Cook, A. J., Buist, D. S., Cummings, S. R., Vachon, C., Vacek, P. and Miglioretti, D. L. (2010). Breast cancer risk by breast density, menopause, and postmenopausal hormone therapy use. J Clin Oncol. 28(24): 3830-3837.

Kucharczak, J., Simmons, M. J., Fan, Y. and Gelinas, C. (2003). To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene. 22(56): 8961-8982.

Kwak, Y. T., Li, R., Becerra, C. R., Tripathy, D., Frenkel, E. P. and Verma, U. N. (2005). IkappaB kinase alpha regulates subcellular distribution and turnover of cyclin D1 by phosphorylation. J Biol Chem. 280(40): 33945-33952.

Lambe, M., Hsieh, C., Trichopoulos, D., Ekbom, A., Pavia, M. and Adami, H. O. (1994). Transient increase in the risk of breast cancer after giving birth. N Engl J Med. 331(1): 5-9.

Lee, D. F. and Hung, M. C. (2008). Advances in targeting IKK and IKK-related kinases for cancer therapy. Clin Cancer Res. 14(18): 5656-5662.

Lee, L. A. and Dang, C. V., (2006). Myc target transcriptomes. Curr Top Microbiol Immunol, 302: 145-167.

Lee, S., Andrieu, C., Saltel, F., Destaing, O., Auclair, J., Pouchkine, V., Michelon, J., Salaun, B., Kobayashi, R., Jurdic, P., Kieff, E. D. and Sylla, B. S. (2004). IkappaB kinase beta phosphorylates Dok1 serines in response to TNF, IL-1, or gamma radiation. Proc Natl Acad Sci U S A. 101(50): 17416-17421.

Lemaitre, J. M., Buckle, R. S. and Mechali, M., (1996). c-Myc in the control of cell proliferation and embryonic development. Adv Cancer Res, 70: 95-144.

Levens, D., (2010). You Don't Muck with MYC. Genes Cancer, 1(6): 547-554.

Liu, B., Yang, Y., Chernishof, V., Loo, R. R., Jang, H., Tahk, S., Yang, R., Mink, S., Shultz, D., Bellone, C. J., Loo, J. A. and Shuai, K. (2007). Proinflammatory stimuli induce IKKalpha-mediated phosphorylation of PIAS1 to restrict inflammation and immunity. Cell. 129(5): 903-914.

Luo, J. L., Kamata, H. and Karin, M. (2005). IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. J Clin Invest. 115(10): 2625-2632.

Lutterbach, B. and Hann, S. R. (1997). Overexpression of c-Myc and cell immortalization alters c-Myc phosphorylation. Oncogene. 14(8): 967-975.

Macmahon, B., Cole, P., Lin, T. M., Lowe, C. R., Mirra, A. P., Ravnihar, B., Salber, E. J., Valaoras, V. G. and Yuasa, S. (1970). Age at first birth and breast cancer risk. Bull World Health Organ. 43(2): 209-221.

Mariani-Costantini, R., Escot, C., Theillet, C., Gentile, A., Merlo, G., Lidereau, R. and Callahan, R. (1988). In situ c-myc expression and genomic status of the

c-myc locus in infiltrating ductal carcinomas of the breast. Cancer Res. 48(1): 199-205.

Martin, M. (2006). Molecular biology of breast cancer. Clin Transl Oncol. 8(1): 7-14.

Mavaddat, N., Barrowdale, D., Andrulis, I. L., Domchek, S. M., Eccles, D., Nevanlinna, H., Ramus, S. J., Spurdle, A., Robson, M., Sherman, M., Mulligan, A. M., Couch, F. J., Engel, C., Mcguffog, L., Healey, S., Sinilnikova, O. M., Southey, M. C., Terry, M. B., Goldgar, D., O'malley, F., John, E. M., Janavicius, R., Tihomirova, L., Hansen, T. V., Nielsen, F. C., Osorio, A., Stavropoulou, A., Benitez, J., Manoukian, S., Peissel, B., Barile, M., Volorio, S., Pasini, B., Dolcetti, R., Putignano, A. L., Ottini, L., Radice, P., Hamann, U., Rashid, M. U., Hogervorst, F. B., Kriege, M., Van Der Luijt, R. B., Peock, S., Frost, D., Evans, D. G., Brewer, C., Walker, L., Rogers, M. T., Side, L. E., Houghton, C., Weaver, J., Godwin, A. K., Schmutzler, R. K., Wappenschmidt, B., Meindl, A., Kast, K., Arnold, N., Niederacher, D., Sutter, C., Deissler, H., Gadzicki, D., Preisler-Adams, S., Varon-Mateeva, R., Schonbuchner, I., Gevensleben, H., Stoppa-Lyonnet, D., Belotti, M., Barjhoux, L., Isaacs, C., Peshkin, B. N., Caldes, T., De La Hoya, M., Canadas, C., Heikkinen, T., Heikkila, P., Aittomaki, K., Blanco, I., Lazaro, C., Brunet, J., Agnarsson, B. A., Arason, A., Barkardottir, R. B., Dumont, M., Simard, J., Montagna, M., Agata, S., D'andrea, E., Yan, M., Fox, S., Rebbeck, T. R., Rubinstein, W., Tung, N., Garber, J. E., Wang, X., Fredericksen, Z., Pankratz, V. S., Lindor, N. M., Szabo, C., Offit, K., Sakr, R., Gaudet, M. M., Singer, C. F., Tea, M. K., Rappaport, C., Mai, P. L., Greene, M. H., Sokolenko, A., Imyanitov, E., Toland, A. E., Senter, L., Sweet, K., Thomassen, M., Gerdes, A. M., Kruse, T., Caligo, M., Aretini, P., Rantala, J., Von Wachenfeld, A., Henriksson, K., Steele, L., Neuhausen, S. L., Nussbaum, R., Beattie, M., Odunsi, K., Sucheston, L., Gayther, S. A., Nathanson, K., Gross, J., Walsh, C., Karlan, B., Chenevix-Trench, G., Easton, D. F. and Antoniou, A. C. (2012). Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the

Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol Biomarkers Prev. 21(1): 134-147.

Mavaddat, N., Peock, S., Frost, D., Ellis, S., Platte, R., Fineberg, E., Evans, D. G., Izatt, L., Eeles, R. A., Adlard, J., Davidson, R., Eccles, D., Cole, T., Cook, J., Brewer, C., Tischkowitz, M., Douglas, F., Hodgson, S., Walker, L., Porteous, M. E., Morrison, P. J., Side, L. E., Kennedy, M. J., Houghton, C., Donaldson, A., Rogers, M. T., Dorkins, H., Miedzybrodzka, Z., Gregory, H., Eason, J., Barwell, J., Mccann, E., Murray, A., Antoniou, A. C. and Easton, D. F. (2013). Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE. J Natl Cancer Inst. 105(11): 812-822.

Merkhofer, E. C., Cogswell, P. and Baldwin, A. S. (2010). Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha. Oncogene. 29(8): 1238-1248.

Meyer, A., Schurmann, P., Ghahremani, M., Kocak, E., Brinkhaus, M. J., Bremer, M., Karstens, J. H., Hagemann, J., Machtens, S. and Dork, T. (2009). Association of chromosomal locus 8q24 and risk of prostate cancer: a hospital-based study of German patients treated with brachytherapy. Urol Oncol. 27(4): 373-376.

Meyer, N. and Penn, L. Z., (2008). Reflecting on 25 years with MYC. Nat Rev Cancer, 8(12): 976-990.

Miller, D. M., Thomas, S. D., Islam, A., Muench, D. and Sedoris, K. (2012). c-Myc and cancer metabolism. Clin Cancer Res. 18(20): 5546-5553.

Mukherjee, S. and Conrad, S. E. (2005). c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem. 280(18): 17617-17625.

Murphy, C. G. and Moynahan, M. E. (2010). BRCA gene structure and function in tumor suppression: a repair-centric perspective. Cancer J. 16(1): 39-47.

Nabel, G. J. and Verma, I. M. (1993). Proposed NF-kappa B/I kappa B family nomenclature. Genes Dev. 7(11): 2063.

Nesbit, C. E., Grove, L. E., Yin, X. and Prochownik, E. V. (1998). Differential apoptotic behaviors of c-myc, N-myc, and L-myc oncoproteins. Cell Growth Differ. 9(9): 731-741.

Ng, P. C. and Henikoff, S., (2001). Predicting deleterious amino acid substitutions. Genome Res, 11(5): 863-874.

Noreen, M., Murad, S., Furqan, M., Sultan, A. and Bloodsworth, P., (2015). Knowledge and Awareness about Breast Cancer and its Early Symptoms among Medical and Non-Medical Students of Southern Punjab, Pakistan. Asian Pac J Cancer Prev, 16(3): 979-984.

Noreen, M., Shah, M. A., Mall, S. M., Choudhary, S., Hussain, T., Ahmed, I., Jalil, S. F. and Raza, M. I., (2012). TLR4 polymorphisms and disease susceptibility. Inflamm Res, 61(3): 177-188.

Noto, H., Goto, A., Tsujimoto, T., Osame, K. and Noda, M. (2013). Latest insights into the risk of cancer in diabetes. J Diabetes Investig. 4(3): 225-232.

Okobia, M. N., Bunker, C. H., Okonofua, F. E. and Osime, U. (2006). Knowledge, attitude and practice of Nigerian women towards breast cancer: a cross-sectional study. World J Surg Oncol. 4: 11.

Osipo, C., Patel, P., Rizzo, P., Clementz, A. G., Hao, L., Golde, T. E. and Miele, L. (2008). ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor. Oncogene. 27(37): 5019-5032.

Os'kina, N. A., Boiarskikh, U. A., Lazarev, A. F., Petrova, V. D., Ganov, D. I., Tonacheva, O. G., Lifshits, G. I. and Filipenko, M. L. (2012). [Association of chromosome 8q24 variants with prostate cancer risk in the Siberian region of Russia and meta-analysis]. Mol Biol (Mosk). 46(2): 234-241.

Papelard, H., De Bock, G. H., Van Eijk, R., Vliet Vlieland, T. P., Cornelisse, C. J., Devilee, P. and Tollenaar, R. A. (2000). Prevalence of BRCA1 in a hospital-based population of Dutch breast cancer patients. Br J Cancer. 83(6): 719-724.

Park, K. J., Krishnan, V., O'malley, B. W., Yamamoto, Y. and Gaynor, R. B. (2005). Formation of an IKKalpha-dependent transcription complex is

required for estrogen receptor-mediated gene activation. Mol Cell. 18(1): 71-82.

Parkin, D. M. (1994). Cancer in developing countries. Cancer Surv. 19-20: 519-561.

Pei, Y. L., Zhang, H. L. and Han, H. G. (2013). Polymorphism of 8q24 rsl3281615 and breast cancer risk : a meta-analysis. Tumour Biol. 34(1): 421-428.

Perkins, N. D. and Gilmore, T. D. (2006). Good cop, bad cop: the different faces of NF-kappaB. Cell Death Differ. 13(5): 759-772.

Pomerantz, M. M., Ahmadiyeh, N., Jia, L., Herman, P., Verzi, M. P., Doddapaneni, H., Beckwith, C. A., Chan, J. A., Hills, A., Davis, M., Yao, K., Kehoe, S. M., Lenz, H. J., Haiman, C. A., Yan, C., Henderson, B. E., Frenkel, B., Barretina, J., Bass, A., Tabernero, J., Baselga, J., Regan, M. M., Manak, J. R., Shivdasani, R., Coetzee, G. A. and Freedman, M. L. (2009). The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. Nat Genet. 41(8): 882-884.

Prall, O. W., Rogan, E. M. and Sutherland, R. L. (1998). Estrogen regulation of cell cycle progression in breast cancer cells. J Steroid Biochem Mol Biol. 65(1-6): 169-174.

Pulverer, B. J., Fisher, C., Vousden, K., Littlewood, T., Evan, G. and Woodgett, J. R. (1994). Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. Oncogene. 9(1): 59-70.

Rabbitts, T. H., Hamlyn, P. H. and Baer, R., (1983). Altered nucleotide sequences of a translocated c-myc gene in Burkitt lymphoma. Nature, 306(5945): 760-765.

Ramensky, V., Bork, P. and Sunyaev, S., (2002). Human non-synonymous SNPs: server and survey. Nucleic Acids Res, 30(17): 3894-3900.

Rasheed, R., (2013). Breast cancer. J Coll Physicians Surg Pak, 23(10): 766-767.

Rocha, S., Martin, A. M., Meek, D. W. and Perkins, N. D. (2003). p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. Mol Cell Biol. 23(13): 4713-4727.

Rothwarf, D. M., Zandi, E., Natoli, G. and Karin, M. (1998). IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature. 395(6699): 297-300.

Russo, J. and Russo, I. H. (1994). Toward a physiological approach to breast cancer prevention. Cancer Epidemiol Biomarkers Prev. 3(4): 353-364.

Sakamuro, D. and Prendergast, G. C., (1999). New Myc-interacting proteins: a second Myc network emerges. Oncogene, 18(19): 2942-2954.

Sato, T., Neilson, L. M., Peck, A. R., Liu, C., Tran, T. H., Witkiewicz, A., Hyslop, T., Nevalainen, M. T., Sauter, G. and Rui, H. (2011). Signal transducer and activator of transcription-3 and breast cancer prognosis. Am J Cancer Res. 1(3): 347-355.

Savas, S., Kim, D. Y., Ahmad, M. F., Shariff, M. and Ozcelik, H., (2004). Identifying functional genetic variants in DNA repair pathway using protein conservation analysis. Cancer Epidemiol Biomarkers Prev, 13(5): 801-807.

Schmid, J. A. and Birbach, A. (2008). IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. Cytokine Growth Factor Rev. 19(2): 157-165.

Sen, R. and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell. 46(5): 705-716.

Shih, V. F., Tsui, R., Caldwell, A. and Hoffmann, A. (2011). A single NFkappaB system for both canonical and non-canonical signaling. Cell Res. 21(1): 86-102.

Sil, A. K., Maeda, S., Sano, Y., Roop, D. R. and Karin, M. (2004). IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature. 428(6983): 660-664.

Singhi, A. D., Cimino-Mathews, A., Jenkins, R. B., Lan, F., Fink, S. R., Nassar, H., Vang, R., Fetting, J. H., Hicks, J., Sukumar, S., De Marzo, A. M. and Argani, P. (2012). MYC gene amplification is often acquired in lethal distant breast cancer metastases of unamplified primary tumors. Mod Pathol. 25(3): 378-387.

Smyth, M. J., Dunn, G. P. and Schreiber, R. D. (2006). Cancer immunosurveillance and immunoediting: the roles of immunity in

suppressing tumor development and shaping tumor immunogenicity. Adv Immunol. 90: 1-50.

Sole, X., Hernandez, P., De Heredia, M. L., Armengol, L., Rodriguez-Santiago, B., Gomez, L., Maxwell, C. A., Aguilo, F., Condom, E., Abril, J., Perez-Jurado, L., Estivill, X., Nunes, V., Capella, G., Gruber, S. B., Moreno, V. and Pujana, M. A. (2008). Genetic and genomic analysis modeling of germline c-MYC overexpression and cancer susceptibility. BMC Genomics. 9: 12.

Spotts, G. D., Patel, S. V., Xiao, Q. and Hann, S. R. (1997). Identification of downstream-initiated c-Myc proteins which are dominant-negative inhibitors of transactivation by full-length c-Myc proteins. Mol Cell Biol. 17(3): 1459-1468.

Takatsuno, Y., Mimori, K., Yamamoto, K., Sato, T., Niida, A., Inoue, H., Imoto, S., Kawano, S., Yamaguchi, R., Toh, H., Iinuma, H., Ishimaru, S., Ishii, H., Suzuki, S., Tokudome, S., Watanabe, M., Tanaka, J., Kudo, S. E., Mochizuki, H., Kusunoki, M., Yamada, K., Shimada, Y., Moriya, Y., Miyano, S., Sugihara, K. and Mori, M. (2013). The rs6983267 SNP is associated with MYC transcription efficiency, which promotes progression and worsens prognosis of colorectal cancer. Ann Surg Oncol. 20(4): 1395-1402.

Tan, M. H., Mester, J. L., Ngeow, J., Rybicki, L. A., Orloff, M. S. and Eng, C. (2012). Lifetime cancer risks in individuals with germline PTEN mutations. Clin Cancer Res. 18(2): 400-407.

Thompson, E. B., (1998). The many roles of c-Myc in apoptosis. Annu Rev Physiol, 60: 575-600.

Tomlinson, I., Webb, E., Carvajal-Carmona, L., Broderick, P., Kemp, Z., Spain, S., Penegar, S., Chandler, I., Gorman, M., Wood, W., Barclay, E., Lubbe, S., Martin, L., Sellick, G., Jaeger, E., Hubner, R., Wild, R., Rowan, A., Fielding, S., Howarth, K., Silver, A., Atkin, W., Muir, K., Logan, R., Kerr, D., Johnstone, E., Sieber, O., Gray, R., Thomas, H., Peto, J., Cazier, J. B. and Houlston, R. (2007). A genome-wide association scan of tag SNPs

identifies a susceptibility variant for colorectal cancer at 8q24.21. Nat Genet. 39(8): 984-988.

Trichopoulos, D., Macmahon, B. and Cole, P. (1972). Menopause and breast cancer risk. J Natl Cancer Inst. 48(3): 605-613.

Tu, Z., Prajapati, S., Park, K. J., Kelly, N. J., Yamamoto, Y. and Gaynor, R. B., (2006). IKK alpha regulates estrogen-induced cell cycle progression by modulating E2F1 expression. J Biol Chem, 281(10): 6699-6706.

Van Der Burg, B. and Van Der Saag, P. T., (1996). Nuclear factor-kappa-B/steroid hormone receptor interactions as a functional basis of anti-inflammatory action of steroids in reproductive organs. Mol Hum Reprod, 2(6): 433-438.

Velasco-Velazquez, M. A., Li, Z., Casimiro, M., Loro, E., Homsi, N. and Pestell, R. G. (2011). Examining the role of cyclin D1 in breast cancer. Future Oncol. 7(6): 753-765.

Vennstrom, B., Sheiness, D., Zabielski, J. and Bishop, J. M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol. 42(3): 773-779.

Vita, M. and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol. 16(4): 318-330.

Walsh, T. and King, M. C. (2007). Ten genes for inherited breast cancer. Cancer Cell. 11(2): 103-105.

Wang, Y. P., Zhang, J., Zhu, H. Y., Qian, C. L., Liu, H., Ji, F. and Shen, Z. Y. (2014). Common variation rs6983267 at 8q24.1 and risk of colorectal adenoma and cancer: evidence based on 31 studies. Tumour Biol. 35(5): 4067-4075.

Waterfield, M., Jin, W., Reiley, W., Zhang, M. and Sun, S. C. (2004). IkappaB kinase is an essential component of the Tpl2 signaling pathway. Mol Cell Biol. 24(13): 6040-6048.

Whiteside, S. T. and Israel, A., (1997). I kappa B proteins: structure, function and regulation. Semin Cancer Biol, 8(2): 75-82.

Wierstra, I. and Alves, J., (2008). The c-myc promoter: still MysterY and challenge. Adv Cancer Res, 99: 113-333.

Wirtenberger, M., Hemminki, K., Forsti, A., Klaes, R., Schmutzler, R. K., Grzybowska, E., Bermejo, J. L., Wappenschmidt, B., Bugert, P., Butkiewicz, D., Pamula, J., Pekala, W., Zientek, H., Bartram, C. R. and Burwinkel, B. (2005). c-MYC Asn11Ser is associated with increased risk for familial breast cancer. Int J Cancer. 117(4): 638-642.

Wu, D. and Meydani, S. N., (2014). Age-Associated Changes in Immune Function: Impact of Vitamin E Intervention and the Underlying Mechanisms. Endocr Metab Immune Disord Drug Targets.

Wu, R. C., Qin, J., Hashimoto, Y., Wong, J., Xu, J., Tsai, S. Y., Tsai, M. J. and O'malley, B. W. (2002). Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. Mol Cell Biol. 22(10): 3549-3561.

Xu, J., Chen, Y. and Olopade, O. I., (2010). MYC and Breast Cancer. Genes Cancer, 1(6): 629-640.

Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J. and Israel, A. (1998). Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell. 93(7): 1231-1240.

Yang, W., Shen, J., Wu, M., Arsura, M., Fitzgerald, M., Suldan, Z., Kim, D. W., Hofmann, C. S., Pianetti, S., Romieu-Mourez, R., Freedman, L. P. and Sonenshein, G. E., (2001). Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. Oncogene, 20(14): 1688-1702.

Yasui, Y. and Potter, J. D. (1999). The shape of age-incidence curves of female breast cancer by hormone-receptor status. Cancer Causes Control. 10(5): 431-437.

Yeh, P. Y., Lu, Y. S., Ou, D. L. and Cheng, A. L. (2011). IkappaB kinases increase Myc protein stability and enhance progression of breast cancer cells. Mol Cancer. 10: 53.

Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M. and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha

and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 91(2): 243-252.

Zeng, Q., Li, S., Chepeha, D. B., Giordano, T. J., Li, J., Zhang, H., Polverini, P. J., Nor, J., Kitajewski, J. and Wang, C. Y. (2005). Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell. 8(1): 13-23.

Zhu, K., Caulfield, J., Hunter, S., Roland, C. L., Payne-Wilks, K. and Texter, L. (2005). Body mass index and breast cancer risk in African American women. Ann Epidemiol. 15(2): 123-128.

Zhu, Y., Spitz, M. R., Amos, C. I., Lin, J., Schabath, M. B. and Wu, X., (2004). An evolutionary perspective on single-nucleotide polymorphism screening in molecular cancer epidemiology. Cancer Res, 64(6): 2251-2257.

Zimmerman, K., Legouy, E., Stewart, V., Depinho, R. and Alt, F. W. (1990). Differential regulation of the N-myc gene in transfected cells and transgenic mice. Mol Cell Biol. 10(5): 2096-2103.

Detail of Tissue Samples of invasive ductal carcinoma (IDC) patients

S. No.	Age	PR Status	Histological Grade	Lymph Node Invasion	IKKα Staining
s1	50	Negative	II	Metastasis	Low
2	65	Negative			Unpredictable
3	60	Positive			Low
4	62	Negative			High
5	72	Positive			Low
6	47	Positive			High
7	59	Positive			Low
8	60	Negative	II	Metastasis	High
9	61	Positive	II	Metastasis	Low
10	58	Negative	III	Metastasis	High
11	61	Positive	III	Metastasis	High
12	50	Negative	III	Metastasis	Low
13					Unpredictable
14	40	Negative	II	Metastasis	High
15	35	Negative	II	Metastasis	Low
16	45	Negative			Low
17	32	Negative	II		High
18	58	Negative			Unpredictable
19	60	Negative	II	Metastasis	High
20	51	Negative	II		High
21	64	Positive			High
22	37	Positive	II		Low
23	50	Negative	II	Metastasis	Low
24	42	Positive	II	No Metastasis	Low

25	45	Negative			Unpredictable
26	60	Positive	II	No Metastasis	Low
27	50	Negative	II	Metastasis	Low
28	71				Low
29	50	Negative	II	No Metastasis	High
30					Unpredictable
31	67	Negative			Unpredictable
32	71	Positive			High
33	40	Negative	III		Low
34	66				Unpredictable
35	65	Positive		Metastasis	Low
36	42	Negative	II		Low
37	55	Negative			High
38	50	Negative	II	No Metastasis	High
39	35	Positive	II		Low
40	53	Positive	II		High
41	70	Negative			High
42	48	Negative	II	Metastasis	High
43	33	Negative			High
44	53	Negative	II	No Metastasis	High
45	42	Negative			High
46	50				Low
47	48	Positive		No Metastasis	High
48	50	Positive	II		High
49	55	Negative	II		High
50	64	Positive			Low

51	30	Positive			Low
52	46				Low
53	48	Positive	II		Low
54	80				Low
55	32	Positive			Low
56	58	Positive			Low
57	61				Unpredictable
58	51	Positive	II	Metastasis	High
59	45	Positive	II	No Metastasis	High
60	50				Unpredictable
61	44	Positive			High
62	68			Metastasis	Low
63	57				Low
64	62				Low
65	71	Positive	II		High
66	53				Low
67	36				Low
68	51				Low
69	43	Negative	III	Metastasis	High
70	57	Negative	III	No Metastasis	High
71	55	Positive	III	No Metastasis	High
72	40		II	No Metastasis	Low
73	36		II	No Metastasis	Low
74	60		II	No Metastasis	High
75	45		II	Metastasis	High

76	59		III	Metastasis	Low
77	34		II	No Metastasis	High
78	70		III	Metastasis	Low
79	25		II	No Metastasis	High
80	34				Low
81	73		II	Metastasis	High
82	52		II	No Metastasis	High
83	35		II	No Metastasis	Low