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**MUTANT P53 MEDIATED CELL MOTILITY IN H1299 LUNG CANCER
CELLS**

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

2-mercaptoethanol	2-hydroxyethylmercaptan; β -mercaptoethanol
ATM	Ataxia Telangiectasia Mutated
BAD	Bcl-2-associated Death Promotor
BCA	Bicinchoninic Acid
Bcl	B- Cell Lymphoma
BM	Basement Membrane
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CDK	Cyclin Dependent Kinase
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GSK3 β	Glycogen Synthase Kinase 3 β
I κ B	Inhibitor of κ B
MeOH	Methanol
MDM	Murine Double Minute
MMP	Matrix Metalloprotease
mRNA	Messenger RNA
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NF- κ B	Nuclear Factor- κ B

NSCLC	Non- Small Cell Lung Cancer
NTC	Non-targeting Control
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet- Derived Growth Factor
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Real-time PCR
RB	Retinoblastoma
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCLC	Small-cell Lung Cancer
SDS	Sodium Dodecyl Sulphate
shRNA	Short Hairpin RNA
TCF	T-cell Factor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factors
TRAF	TNF Receptor Associated Factor
Tris	Tris (Hydroxymethyl) Methylamine
TTBS	Tween-Tris-Buffered Saline
Tween 20	Polyoxyethylene(20)sorbitan Monolaura
uPA	Urokinase-type Plasminogen Activator

Abstract

MUTANT P53 MEDIATED CELL MOTILITY IN H1299 LUNG CANCER CELLS

By Mi-Yon Choi, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Major Director: Dr. Andrew Yeudall
Philips Institute, VCU School of Dentistry

Studies have shown that gain-of- function mutant p53, AKT, and NFκB promote invasion and metastasis in tumor cells. Signals transduced by AKT and p53 are integrated via negative feedback between the two pathways. Tumor derived p53 was also indicated to induce NFκB gene expression. Due to the close relationship between p53/AKT and p53/NFκB, we hypothesized that AKT and NFκB can enhance motility in cells expressing mutant p53. Effects on cell motility were determined by scratch assays. CXCL5- chemokine is also known to induce cell motility. We hypothesized that enhanced cell motility by AKT and NFκB is mediated, in part, by CXCL5. CXCL5 expression levels in the presence and absence of inhibitors were determined by qRT-PCR. We also hypothesized that gain-of-function mutant p53 contributes to the activation of AKT. The

effect of mutant p53 on AKT phosphorylation was investigated with a Ponasterone A-inducible mutant cell line (H1299/R175H) and vector control. These results indicated that AKT and NF κ B enhance motility in cells expressing mutant p53 and this enhanced motility is, in part, mediated by CXCL5. However, AKT phosphorylation was independent of mutant p53.

Introduction

Cancer

Each year, cancer is responsible for one-fifth of deaths in the United States and 100- 350 deaths per 100,000 people worldwide. Cancer is due to malfunction of mechanisms that regulate the growth and proliferation of cells, which transforms normal cells into malignant cancer cells. Almost all cancers are due to genetic damage accompanied by influences of tumor-promoting chemicals, hormones, and sometimes virus (Lodish, 2004). Several observations indicated that the genomes of tumor cells are invariably altered at multiple sites, suffering disruptions like point mutations and change in chromosome complement (Kinzler and Vogelstein, 1996). Six fundamental cellular properties are altered in cancer cells that collectively dictate malignant growth. These six hallmarks of cancer are self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis (Hanahan & Weinberg, 2000). Most human cancers share these six properties.

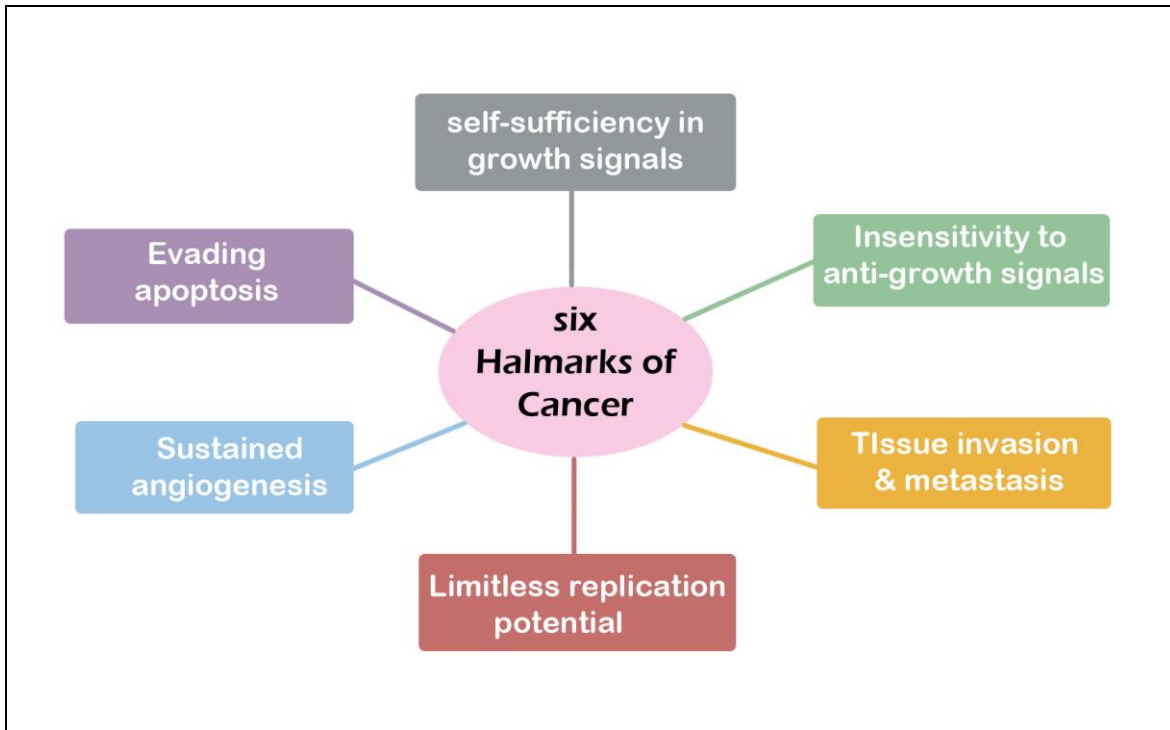


Figure 1. Six Properties of Cancer

In normal cells, mitogenic growth signals are required to shift from a quiescent state into a proliferative state. However, cancer cells can generate their own growth signals (e.g. PDGF and TGF α) to mimic the normal growth signals, creating autocrine stimulation (Fedi et al., 1997). Antiproliferative signals, including soluble growth inhibitors and immobilized inhibitors, found in normal tissue are required to maintain cellular quiescence and tissue homeostasis. Absence of these antiproliferative signals causes quiescent (G_0 state) cells to enter into the G_1 phase of the cell cycle. Based on the environmental signals, cells in G_1 phase either proliferate, enter into a quiescent state, or enter into an irreversible postmitotic state (cell differentiation) (Hanahan & Weinberg, 2000). These antiproliferative mechanisms can be disrupted in a variety of ways in different types of human cancer (Lodish, 2004). However, cell proliferation depends on more than avoidance

of antiproliferative signals. Cells undergoing multiplication can be instructed to enter into the irreversible postmitotic state (terminal differentiation). Cancer cells can reverse this process by overexpressing oncoproteins, such as c-Myc, to impair differentiation and promote growth (Kinzler and Vogelstein, 1996).

During normal development, many cells are designated for programmed cell death, also known as apoptosis. In response to abnormalities, such as DNA damage, hypoxia, and signaling imbalance by oncogene activity, cells can trigger apoptosis (Lodish, 2004). The rate of apoptosis also contributes to the ability of tumor cell populations to expand in number. If cells survive with abnormalities by avoiding apoptosis, they will continue to grow in number and may contribute to tumor formation. In cancer cells, apoptosis can be avoided in two ways; over expression of antiapoptotic proteins (e.g. Bcl-2, Bcl-XL, and Bcl-W) (Hueber et al., 1997) or inactivation of tumor suppressor genes (e.g. p53) (Symonds et al., 1994). Both mechanisms contribute to rapidly growing tumors with low numbers of apoptotic cells.

All cells in a tissue reside within 100 μ m of a capillary blood vessel to obtain the oxygen and nutrients which are crucial for cell function and survival (Hanahan and Weinberg, 2000). Just like normal cells, tumor cells also require recruitment of new blood vessels in order for them to grow to a large mass. Without a blood supply, tumor cell growth is limited to about 10^6 cells due to division of cells outside the mass balanced by death from the center. These small localized tumors are called benign and closely resemble normal cells, without causing any threat to their host (Lodish, 2004). Previous studies have shown that angiogenesis, development of new vessels, was found to be

activated prior to becoming a full-blown tumor. Tumors activate the “angiogenic switch” by changing the balance of angiogenesis inducers and counteracting inhibitors (Hanahan & Folkman, 1996). Angiogenic growth factors that stimulate formation of new vessels include bFGF, TGF α , and VEGF. The presence of an adjacent blood vessel also facilitates the process of metastasis which functions in migration and invasion of distant tissues (Folkman, 2002). 90% of human cancer death is caused by this metastatic property of cancer cells. Attracted by signals secreted by macrophages and other cells, cancer cells can leave the primary tumor site and reach the blood vessels. These tumor cells penetrate vessel walls by extending “invadopodia,” which produce different proteases to open up a pathway (Yamaguchi et al., 2005). Once they reach the bloodstream, these cancer cells are free to move out and colonize in the adjacent tissue, as long as they acquire properties mentioned above.

Lung Cancer

Lung cancer is the leading cause of cancer death in the world. Approximately 1.2 million deaths were reported to be due to lung cancer each year and it is still on the rise. It has been shown that the most well-known cause is tobacco use and is responsible for about 85% of lung cancer. Cigarettes contain about 50 known lung carcinogens, which can induce genomic damage and alter the defense mechanisms of lung epithelium leading to lung cancer (Li, 2009). However, other causes have been reported, such as occupational exposures, infections and genetics (De Matteis, 2008). Although it is a leading cause of death world-wide, it has been shown that the rate of lung cancer is decreasing in the

developed countries, mainly due to an effort to decrease smoking. The histological subtype of lung cancer has also shifted from squamous cell carcinoma and small cell lung cancer to that of adenocarcinoma. Unfortunately, the rate of tobacco use and lung cancer still remains high in developing countries. Previous studies have also reported that women are more prone to lung cancer than man. There is no conclusive evidence why more women are affected; however, it has been shown that women are less successful in smoking cessation. Men and women are different physiologically, psychologically, and behaviorally and it may be necessary to clearly understand the differences and reasons for women in initiating smoking as well as quitting smoking in order to decrease the rate of lung cancer in women (Toh, 2009).

Lung cancer can be categorized into small- cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is a subtype with a neuroendocrine phenotype, whereas NSCLC includes adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma (Sekido et al., 2003). Tobacco carcinogens were suggested to cause p53 mutations in lung. P53 is a tumor suppressor protein that regulates cell-cycle check point signals, which cause the cell to undergo either G₁ arrest, DNA repair, or apoptosis (Levine, 1997). P53 mutations in lung cancer correlate with tobacco smoking and usually consist of the G-T transversions expected from tobacco smoke carcinogens (Greenblatt et al., 1994). About 70%-80% of the somatic mutations of p53 found in lung cancer are missense and prolong the half-life of the mutant p53 protein (Sekido et al., 2003). Alteration of p53 is observed in 75%-100% of SCLCs and 47% of NSCLCs. In NSCLCs, p53 alteration can be seen in 51% of squamous-cell carcinomas, 54% of large-cell carcinomas and 39% of

adenocarcinomas (Tammemagi et al., 1999). Other pathways controlling the G₁ to S phase transition of the cell cycle are also shown to be affected in lung cancer, such as p16- cyclin D1- CDK4- RB pathway. P16 mutations have been observed in 30%-70% of NSCLCs, whereas inactivation of RB is the preferential mechanism in SCLC. RB inactivation occurs in ~90% of SCLCs and 15%-30% of NSCLCs (Sekido et al., 1998).

P53 Protein

p53 is a stress response protein that functions primarily as tetrameric transcription factor which regulates genes that control progression through the cell cycle or that initiate apoptosis (Brosh and Rotter, 2009). In response to stress signals, such as DNA damage, UV radiation, and hypoxia, wild-type p53 induces growth arrest, DNA repair, or apoptosis (Levine, 1997). In normal resting cells, p53 is extremely unstable and is kept at low level by murine double minute 2 (MDM2) protein. MDM2 binds to p53 protein and induces addition of ubiquitin molecules, targeting p53 for proteosomal degradation (Lodish, 2004). However, under cellular stress, the p53 protein is rescued from degradation and accumulates to high levels (Boehme and Blattner, 2009). Under stress, cells activate ATM or ATR, serine-threonine kinases that phosphorylate MDM2, leading to increase in p53 concentration. Activated ATM also stabilizes p53 by phosphorylation, permitting p53-activated expression of genes to induce cell cycle arrest in G₁, promote apoptosis, or participate in DNA repair. Among the genes activated by p53 are pro-apoptotic proteins such as Bax (Lane, 2005).

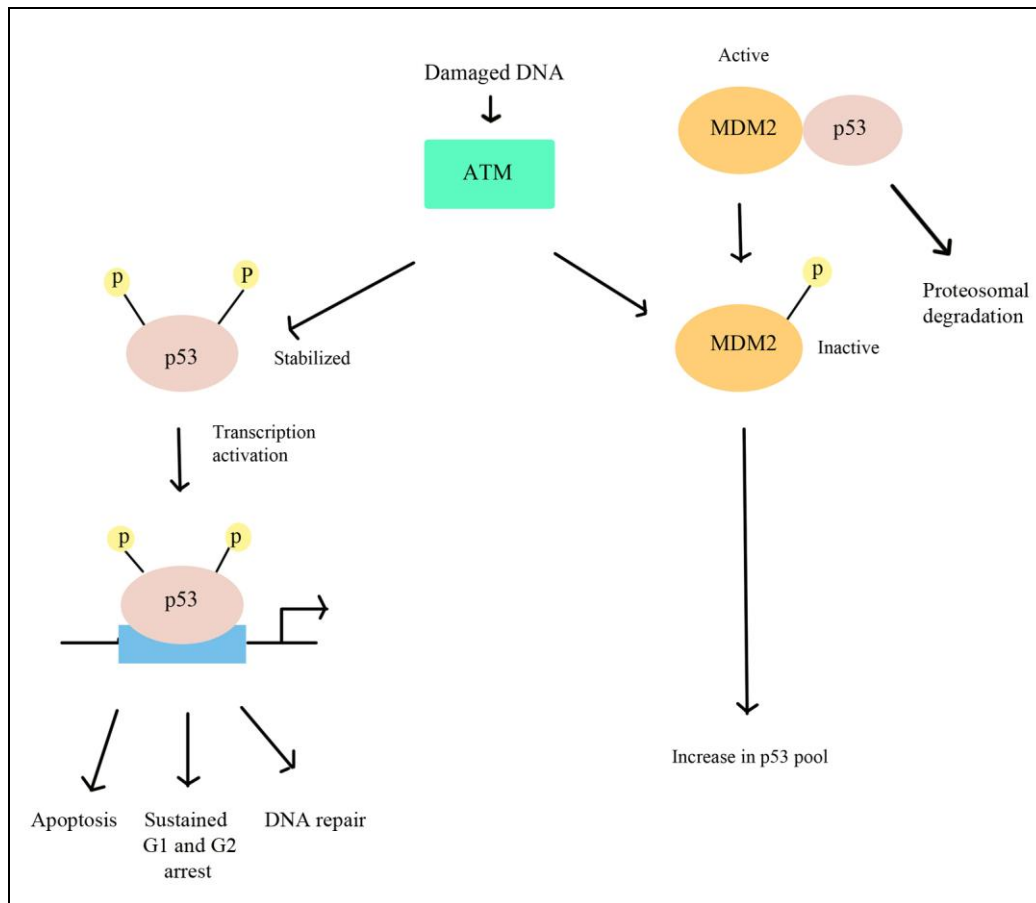


Figure 2. P53 Activation in Response to DNA Damage

P53 and Cancer

P53 is one of the most commonly mutated genes and it is seen in greater than 50% of all human cancers (Harris, 1996). The majority of cancer associated mutations in p53 are monoallelic missense mutations within the central DNA-binding domain, which usually lead to expression of highly stable mutant protein, incapable of activating wild-type p53 target genes. Besides losing their wild-type activity, many p53 mutants also function as dominant negative proteins that inactivate wild-type p53 expression from the

remaining wild-type allele by hetero-oligomerization. However, an increasing number of studies suggest that many p53 mutants not only lose their tumor suppressive function, but also gain new oncogenic properties that are independent of wild-type p53, known as 'gain-of-function' (Brosh and Rotter, 2009). P53 null cells transfected to express mutant p53 have a growth advantage compared to vector control (Scian et al, 2004). Other studies have shown that mutant p53 mediates upregulation of genes that are important in cell survival such as ASNS, hTERT, and NF κ B2 (Scian et al, 2004) (Scian et al, 2005). Mutant p53s also gain new oncogenic properties by interacting with p73 and p63, which are known as p53 family members. Mutant p53 binding with p73 and p63 consequently inhibits their ability to induce cell-cycle arrest (Brosh and Rotter, 2009).

AKT

AKT, also known as protein kinase B (PKB), is a serine/threonine kinase that regulates many biological processes, such as proliferation and apoptosis. Activated AKT can directly phosphorylate several components of the cell-death machinery, such as BAD, caspase-9, and FKHR. BAD is a member of the Bcl2 family of proteins that promotes cell death by forming hetero-dimers with the survival factor BCL-X. AKT phosphorylation of BAD prevents BAD/BCL-X interaction, restoring BCL-X's anti-apoptotic function (Datta et al., 1997). Caspase-9, a pro-apoptotic protease, also gets inactivated through direct phosphorylation by AKT (Cardone et al., 1998). AKT can also influence cell survival by indirectly affecting two central regulators of cell death, nuclear factor κ B (NF- κ B) and p53. NF- κ B is a transcription factor complex that promotes cell survival in response to

apoptotic stimuli. AKT positively affects NF- κ B activity by phosphorylation and activation of I κ B kinase, which induces degradation of I κ B. Dissociation of NF κ B from I κ B allows NF κ B to translocate to the nucleus and activate target genes (Kane et al., 1999). AKT can also phosphorylate MDM2, which enhances the translocation of MDM2 to the nucleus. Increased MDM2 translocation to the nucleus will increase p53 degradation which, in turn, promotes cell survival (Mayo and Donner, 2001).

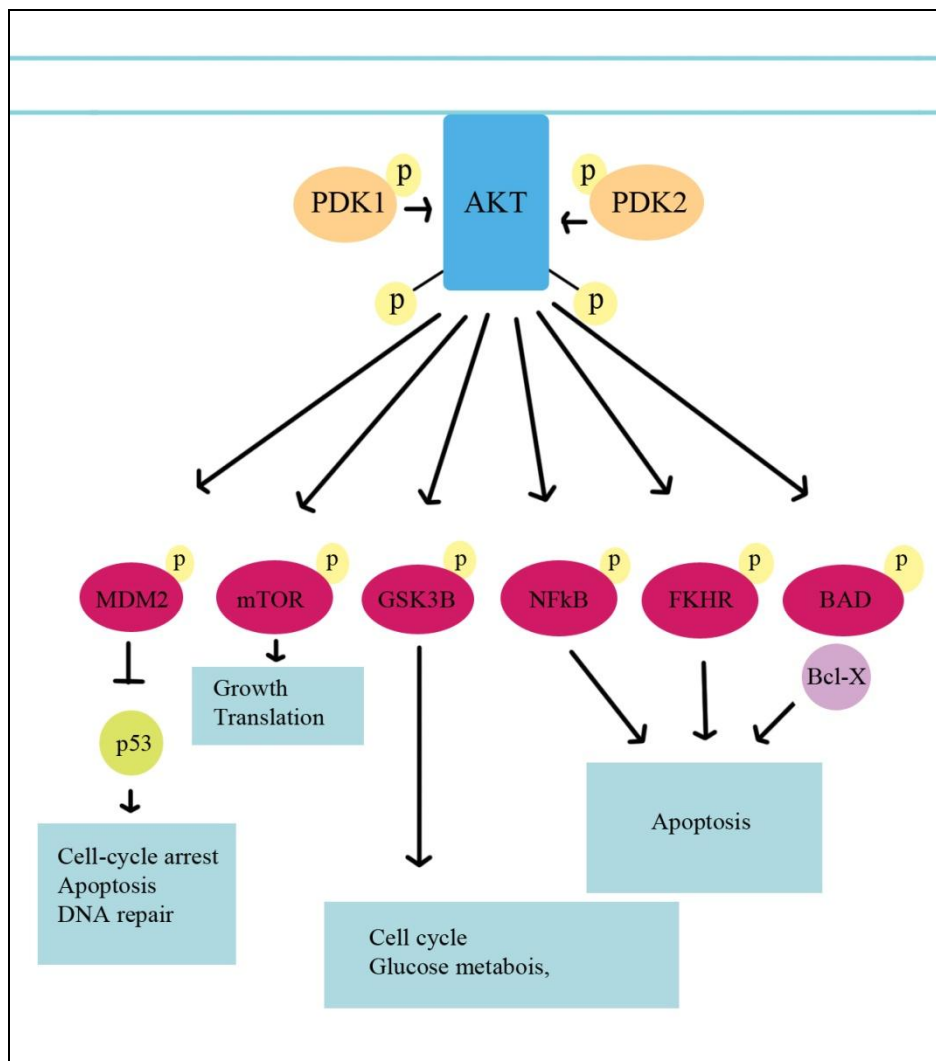


Figure 3. AKT/PI3K Signaling Pathway

Nuclear Factor- κ B

Nuclear factor- κ B protein complex is a ubiquitous transcription factor that controls the expression of genes involved in immune responses, apoptosis, and cell cycle. The mammalian NF κ B family consist of 5 members; NF κ B1 (p50/p105), NF κ B2 (p52/p100), c-Rel, RelA (p65) and RelB (Lee et al., 2007). NF- κ B protein exists in unstimulated cells as homo- or heterodimers bound to I κ B protein, an inhibitory factor of NF- κ B. NF- κ B has a highly conserved 300 amino acid Rel homology domain that is located near N-terminus of the protein. This highly conserved domain is responsible for DNA binding, dimerization, and interaction with I κ B protein (Baldwin, 1996). Dimerization with I κ B sterically blocks NF- κ B's nuclear localization sequence, causing cytoplasmic localization of the protein complex (Siebenlist et al., 1994). When external stimuli, such as infectious agents or inflammatory cytokines, are present, I- κ B kinase becomes activated and phosphorylates two critical serine residues in the N- terminal regulatory domain of I κ B. This phosphorylation by I κ B kinase targets I κ B for rapid polyubiquitination and subsequent degradation by the proteosome (Senftleben and Karin, 2002). Dissociation from I κ B allows NF- κ B to expose nuclear- localization sequence and translocate into nucleus. In the nucleus, NF- κ B regulates the transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules and pro/antiapoptotic proteins (Lodish, 2004).

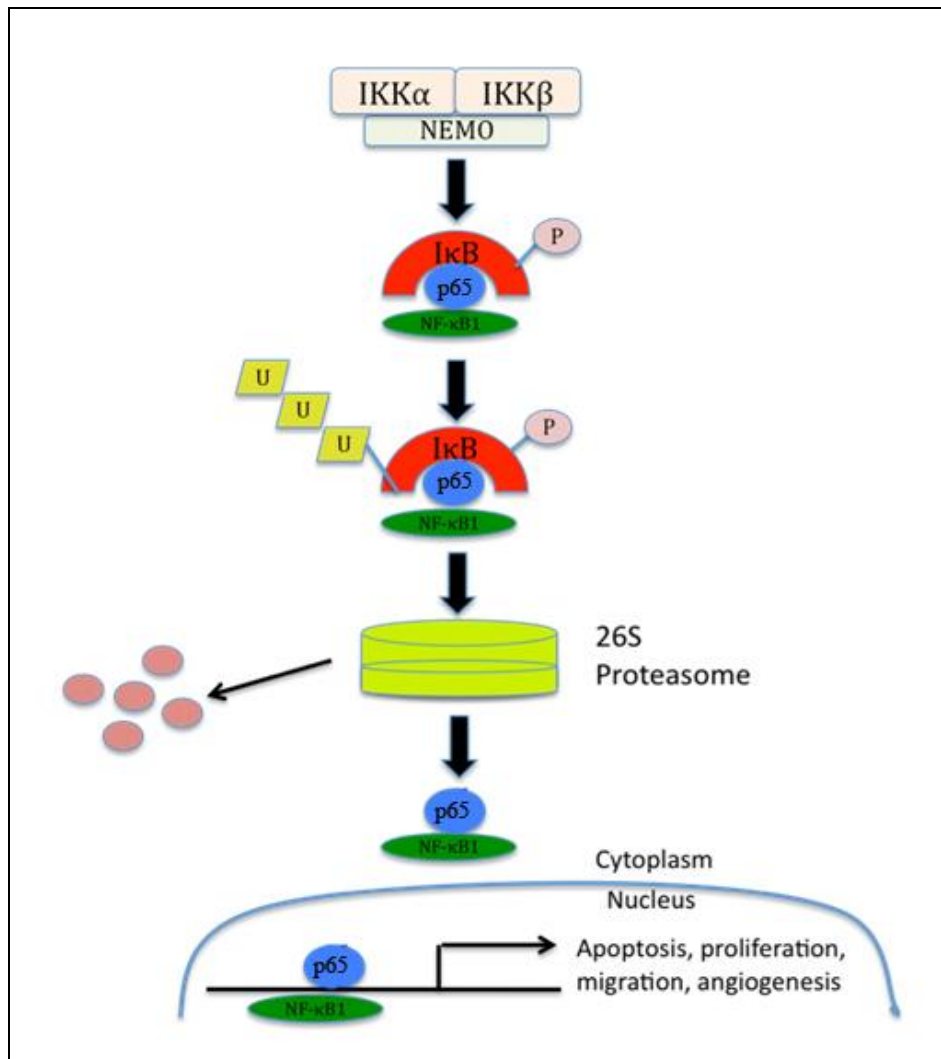


Figure 4. NF-κB Signaling Pathway

NF-κB is continuously activated in a number of solid tumors. Some of the reasons for sustained NF-κB activity include abnormal IκB activity and shorter half-life of IκB (Garg and Aggarwal, 2002). Several oncogenes, including ras and myc, require NF-κB to induce abnormal cell proliferation and tumorigenesis (Jong et al., 2000).

NFκB and Cancer

Studies have shown that NFκB is activated in a number of hematologic and solid tumors. The activation of NFκB is necessary for the transforming ability of several cellular oncoproteins (Lee et al., 2007). Several oncogenes, such as ras, bcr-abl and myc, can activate NFκB. NFκB is required for the H-ras oncogene to induce cell proliferation and tumorigenesis (Jong et al., 2000). NFκB suppresses apoptosis by enhancing the expression of multiple anti-apoptotic proteins and interferes with the expression or activity of proapoptotic proteins. The anti-apoptotic proteins regulated by NFκB include inhibitor of apoptosis proteins (IAPs) 1 and 2, Bcl-X_L, Bfl-1, TNF receptor associated factor 1 (TRAF) and TRF2 (Kucharczak et al., 2003). Proapoptotic genes that are upregulated by NFκB include Fas/CD95, FasL, death receptor 4 (DR4) and DR5 (Lee et al, 2007). NFκB is also indicated to destabilize p53 by enhancing MDM2 expression, interfering with p53 induced apoptosis (Tergaonkar et al., 1998). NFκB plays an important role in tumor invasion and metastasis by regulating matrix metalloproteinase 2 (MMP2), MMP9, and serine protease urokinase-type plasminogen activator (uPA) (Lee et al., 2007). Intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, that are associated with metastasis, are also regulated by NFκB (Johnson, 1999). NFκB also promotes cell cycle progression by regulating expression of genes, such as cyclin D1, D2,D3 and c-myc, that are involved in the cell cycle machinery (Lee et al., 2007).

Chemokines

Chemokines are a superfamily of small cytokine-like molecules that induce directed chemotaxis in nearby responsive cells. They function mainly as chemoattractants

for leukocytes, monocytes, neutrophils, and other effector cells from the bloodstream to sites of infection. Some chemokines are also identified as key regulators of lymphocyte development, migration, and angiogenesis. Chemokines interact with seven transmembrane-spanning G-protein-coupled receptors (GPCR) and they can be released by many different types of cells (Janeway, 2005). Chemokines are subclassified into four subfamilies; CC, CXC, XC, and CX3C (Zlotnik and Yoshie, 2000). The CXC chemokines are further subdivided into ELR⁺ and ELR⁻ based on the presence or the absence of the motif glu-leu-arg (ELR) preceding the CXC sequence (Raman et al., 2007). ELR⁺ CXC chemokines, such as CXCL5 and CXCL8, induce the migration of neutrophils, and stimulate endothelial cell migration during angiogenesis, whereas ELR⁻ CXC chemokines target lymphocytes (Frederick and Clayman, 2001). The CC chemokines act on subsets of dendritic cells, lymphocytes, macrophages, eosinophils, and natural killer cells. However, CC chemokines do not stimulate neutrophils (Raman et al, 2007).

The secretion of the chemokines and the cell surface expression of the chemokine receptors on tumor cells play critical roles in facilitation of tumor growth and/ or metastasis (Raman et al, 2007). Studies have shown that CXC chemokines play a most extensive role in angiogenesis out of the four families of chemokines. Early evidence suggested that ELR⁺ CXC chemokines, such as CXCL5 and CXCL8, stimulate endothelial cell migration, proliferation, and angiogenesis in vivo (Raman et al, 2007). However, recent studies have shown that the ELR⁻ chemokine CXCL12 and its receptor CXCR4 can both increase angiogenesis and play an important role in metastatic progression (Orimo and Weinberg, 2006).

Hypothesis

AKT and NF κ B promote motility and CXCL 5 expression in cells expressing gain-of-function mutant p53. P53 also promotes the phosphorylation of AKT.

Aims of Current Studies

- To determine the effect of AKT on motility in cells expressing gain-of-function p53.
- To determine the effect of AKT on chemokine expression in cells expressing gain-of-function p53.
- To determine the effect of gain-of-function mutant p53 on AKT phosphorylation
- To determine the effect of NF κ B on motility in cells expressing gain-of-function p53.
- To determine the effect of NF κ B on chemokine expression in cells expressing gain-of-function p53.

Materials and Methods

Cell Culture

H1299 lung cancer cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (4500 mg/L D-Glucose, L-Glutamine and 110 mg/L sodium pyruvate) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and penicillin-streptomycin (10 µg/ml) (Invitrogen, Carlsbad, CA) as an antibiotic. The cells were maintained in a 37°C humidified incubator with 90% air and 10% CO₂.

Cell Line

To examine the gain-of-function effect of mutant p53, H1299 cells were used. H1299 is a p53-null human lung carcinoma cell line. Stable cell lines were generated by transfecting H1299 cells using the calcium phosphate method with either pCMV- Neo Bam p53-D281G or vector alone (Scian et al., 2004). Several clones from each transfection were expanded into cell lines. Cell lines that were selected for these experiments were; H1299-control 5 (HC -5), H1299-p53-D281G, H1299-p53-R273H, H1299-p53-R175H, H1299-p53-H179L, H1299-p53-R248W (Scian et al, 2004).

Antibodies

Antibody recognizing phospho-Akt (Ser473) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibody for Akt1 was purchased from BD Biosciences, Pharmingen (Mississauga, ON Canada). p53 (DO-1) monoclonal antibody (sc-126) was purchased from Santa Cruz Biotechnologies, Inc., (Santa Cruz, CA). Antibody that recognizes actin (sc-1616) was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-goat, anti-rabbit and anti-mouse secondary antibodies were obtained from MP Biomedical (Aurora, OH).

Primary Antibody	Supplier	Dilution
P53 (DO-1)	Santa Cruz Biotechnology, Santa Cruz, CA	1:1000
Phospho-AKT	Cell Signaling Technology, Inc. Danvers, MA	1:1000
AKT1	BN Biosciences, Pharmingen Mississauga, ON Canada	1:1000
Actin	Santa Cruz Biotechnology, Santa Cruz, CA	1:1000

Table 1: Primary Antibodies for Western Blot

Secondary Antibody	Supplier	Dilution
Horseradish peroxidase0conjugated anti-goat	MP Biomedical Aurora, OH	1:10,000
Anti- rabbit	MP Biomedical Aurora, OH	1:15,000
Anti- mouse	MP Biomedical Aurora, OH	1:10,000

Table 2: Secondary Antibodies for Western Blot

Inhibitors

AKT inhibitor from Calbiochem (Cat # 124005) (Darmstadt, Germany) was used at 20 μ M working concentration. NF- κ B inhibitor from Calbiochem (Cat # 4814056) (Darmstadt, Germany) was prepared in DMSO as a stock solution of 2mM and used at 2 μ M.

Protein Extraction.

Cells were cultured to ~90% confluence on a 60 mm plate. Medium was aspirated from the plate and the cells were washed with PBS. After the PBS was aspirated, 100 μ l of lysis buffer (1M HEPES pH 7.5, 0.5M EGTA pH 8.0, 100mM β -glycerophosphate, 5 mL NP-40 lysis buffer, 2M MgCl₂) was added to each plate. 0.1M PMSF (10 μ l/ml LB), leupeptin (1 μ l/ ml LB), aprotinin (1 μ l/ml LB), 1M DTT (1 μ l/ml LB), and 1M orthovanadate (2 μ l/ml LB) were added to the lysis buffer before it was added to the plates. Plates were placed on ice for 10 minutes with occasional rocking to distribute the lysis buffer evenly. Cells were then scraped with a cell lifter to one edge of the plate and the lysates were transferred to a 1.5 ml microcentrifuge tube. The lysates were centrifuged at 10,400 x g at 4 $^{\circ}$ C for 10 min and the supernatant was carefully transferred to a new tube, discarding the pellet. The lysates were quantified spectrophotometrically, using the Biorad BCA Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA) at 600 nm and were compared to a standard curve of known quantities of bovine serum albumin (BSA). Protein was stored at -20 $^{\circ}$ C.

Western Blot

30-80 μ g of protein lysates were loaded onto 10% denaturing polyacrylamide gels (Sambrook & Russell, 2001). Lysates were resolved for 1.5h at 120 V in 1X SDS-PAGE running buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 70 mM EDTA, 2% SDS). The protein was then transferred from gel to PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA) for 16-20 h in 1x transfer buffer (20mM pH 7.9, 100mM NaCl, 70mM EDTA, and 20% MeOH). The membrane was placed in 100% MeOH for several minutes and was air dried completely. The membrane was then re-wet in 100% MeOH, for several minutes and was rinsed in T-TBS (0.05% Tween-TBS). 5% non-fat dried milk in T-TBS was used to block the membrane for 1h at room temperature on the shaker. The membrane was rinsed once with T-TBS and was placed in the primary antibody solution. To probe for p53, AKT and actin, the primary antibody was diluted to 1:1000 in 5% milk in T-TBS and was incubated in room temperature on the shaker for 1 h. To probe for p-AKT, the primary antibody was diluted to 1:1000 in 5% BSA in T-TBS at 4°C on the shaker overnight. After the primary antibody, the membrane was washed with T-TBS 6 times for 3 min each. Secondary antibody for p53 (anti-mouse) and actin (anti-goat) was diluted to 1:10,000 in 5% milk in T-TBS and was incubated at room temperature on the shaker for 1h. Secondary antibody for p-AKT (anti-rabbit) and AKT (anti-rabbit) was diluted to 1:15,000 in 5% milk and was incubated at room temperature on the shaker for 1 h. After the secondary antibody, membranes were washed with T-TBS, 6 times for 3min each. The membrane was incubated with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences Inc., Boston, MA) for 1 min at room temperature and was exposed to x-ray film.

Scratch Assay

Cells were plated in triplicate in 12-well plates (Greiner Bio-One, Monroe, NC) at a concentration of 5×10^5 cells/well. After the cells were grown to 100% confluence, medium was aspirated from each well, and cells were washed with PBS. Each well was scratched once vertically with the tip of a sterile pipette tip. The width of scratch was measured in three different locations of each well using a light microscope and AxioVision software. The plates were then incubated at 37° C with 90% air and 10% CO₂. After 10-16 hours of incubation, the width of the scratch was measured again at the same location. The distance cells migrated into the scratch was calculated as a function of time in order to obtain the motility rate.

RNA Extraction

Cells were plated on 60 mm round plates and cultured in DMEM with 10% FBS to 70-80% confluence. Medium was removed and cells were incubated in 1 ml of TRIzol (Invitrogen) for 5 min at room temperature. Contents were transferred to the sterile 1.5 ml microcentrifuge tube and 200 μ l chloroform per 1 mL TRIzol was added. The tube was shaken vigorously for 15 sec and was centrifuged at 12,000 x g for 15 min at 4° C. The upper aqueous phase was carefully extracted and transferred to new sterile microcentrifuge tube. To precipitate RNA 500 μ L of isopropyl alcohol per 1 mL TRIzol was added to the aqueous phase and was inverted several times to mix. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4° C.

Supernatant was removed and 1 mL of 75% ethanol per 1 mL TRIzol was added to wash the RNA. The sample was mixed by vortex and was centrifuged at 12,000 x g for 10 min at 4° C. After the wash, ethanol was removed and the RNA pellet was air-dried for 5-10 min. The RNA pellet was then dissolved in RNase-free H₂O by gently pipetting and was incubated for 10 min at 55 to 60° C. RNA was stored at -80° C until use.

Agarose Gel Electrophoresis

To test the quality of RNA, 1% agarose gel was prepared with 0.5 g agarose (ISC BioExpress, Kayesville, UT) in 50 mL 1x TAE buffer (40 nM Tris, 20 mM glacial acetic acid, 10 mM EDTA pH 8.0). 1 µg RNA sample was loaded with 6x Ficoll loading buffer, and run on the gel for 20-30 minutes at 120V. 5µL Hyperladder marker I (Bioline, Taunton, MA) was also loaded on the gel. The RNA was visualized by UV transillumination.

Reverse Transcription

2 µg RNA was added to a nuclease-free microcentrifuge tube, with 1 µL of 100µM oligo(dT), 1 µL 10mM dNTP, 1 µL oligo(dT) primer, and sterile, distilled nuclease-free H₂O up to 13 µL in total. The mixture was incubated for 5 min at 65° C and was chilled on ice for 5 min. After briefly centrifuging, 4 µl 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3) and 2 µL 0.1 M DTT were added. The contents were mixed gently, and incubated in a 42° C water bath for 2 min. 1 µL (200 u/µL) SuperScript II RT (Invitrogen, Carlsbad, CA) was added and mixed with gentle pipetting. The mixture was centrifuged briefly to collect the contents at the bottom of the tube and was incubated in a 42° C water bath for

50 min. The reaction was inactivated by heating at 70°C for 15 min. cDNA was stored at -20°C until use.

Nucleic Acid Quantification

Nucleic acids were quantified using the Thermo Scientific NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). 1 µL ddH₂O was loaded as a blank, and subsequently 1 µl sample was loaded in order to measure nucleic acid concentration.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an ABI 7500 Fast System (Applied Biosystems, Rockville, MD) and a SYBR green-based procedure. A master mix containing 5% primers, 35% ddH₂O and 50% Absolute SYBR Green ROX Mix (ABgene, Epsom, Surrey, UK) was made for each gene target to be analyzed. 9 µl of master mix was added to each well of the 96-well plate (Applied Biosystems, Foster City, CA) to get the desired target to a total volume of 10 µl. 1 µl of cDNA was added to each triplicate well and 1 µl of ddH₂O was added to one well as a non-template control (NTC). 1 µL of standards was also added to provide a standard curve. To generate standards, serial dilutions were made using previously generated PCR products. The 96-well plate was then sealed with optical cover and inserted into the 7500 Fast Real-Time PCR System and the qRT-PCR was carried out using the SYBR green-protocol.

MTT Assay

Cells were plated at 2×10^4 cells per well in triplicate in a 12-well plate and allow to grow for 24 h. 100 μ L of MTT reagent (3-(4,5-Dimethylthiazolyl-2)-2,5-Diphenyl Tetrazolium Bromide in PBS) (MP Biomedicals Inc., Solon, OH) was added to each well, and the cells were incubated for 4 h at 37°C in 90% air and 10% CO₂. The medium and reagent were removed from each well, and the formazan crystals were solubilized in 1 mL of MTT solubilization buffer (0.01M HCl with 10% SDS) and incubated overnight at 37°C. Absorbance was measured spectrophotometrically at 570 nm. The MTT solubilization buffer was used as the blank measurement.

Statistical Analysis

Data were analyzed using Microsoft Excel and Graphpad QuickCalcs software (<http://www.graphpad.com/quickcalcs/>). Paired T-tests were performed, and p-values were compared. An α -value of 0.05 was considered statistically significant. Error bars on the figures represent the standard error of the mean.

Results

Effect of Mutant p53 on Cell Motility

Previous studies have shown that cells expressing mutant p53 possess a selective growth advantage over those expressing wild-type p53 (Levine, 1997). It is also known that mutant p53 promotes cell proliferation (Scian et al., 2004). Our goal was to examine gain-of-function mutant p53 and its role in cell motility. To examine the gain-of-function effect of mutant p53 on cell motility, we used the stably transfected H1299 cell lines. H1299 is a human lung cancer cell devoid of endogenous p53. These cells were transfected to stably express different mutant p53; R248W, R175H, D281G, H179L, H273H (Scian et al., 2004). To confirm the stable mutant p53 expression we prepared protein lysates from each cell line and performed western blot. As expected (Figure 5), all of the transfected cell lines stably express mutant p53. We can also see that the HC5 control cell line transfected with empty vector does not express p53. To examine if mutant p53 plays a role in cell motility, scratch assays were performed. After cells were grown to 100% confluence, a scratch was made using sterile pipette tip. The width of the scratch was measured using a light microscope and was noted as 0 h. After allowing the cells to migrate for 10 h under standard conditions, the width was measured at the same location and the rates were calculated (Figure 6). Compared to the vector control (HC5), most cell lines expressing mutant p53 had a higher motility rate (Figure 6). However, the motility

rate varied between cell lines expressing different mutants. D281G showed the highest rate ($\sim 70\mu\text{m/hr}$), whereas the rate of R248W was close to that of the vector control. Cells expressing p53-D281G and H179L were selected for the further experiments.

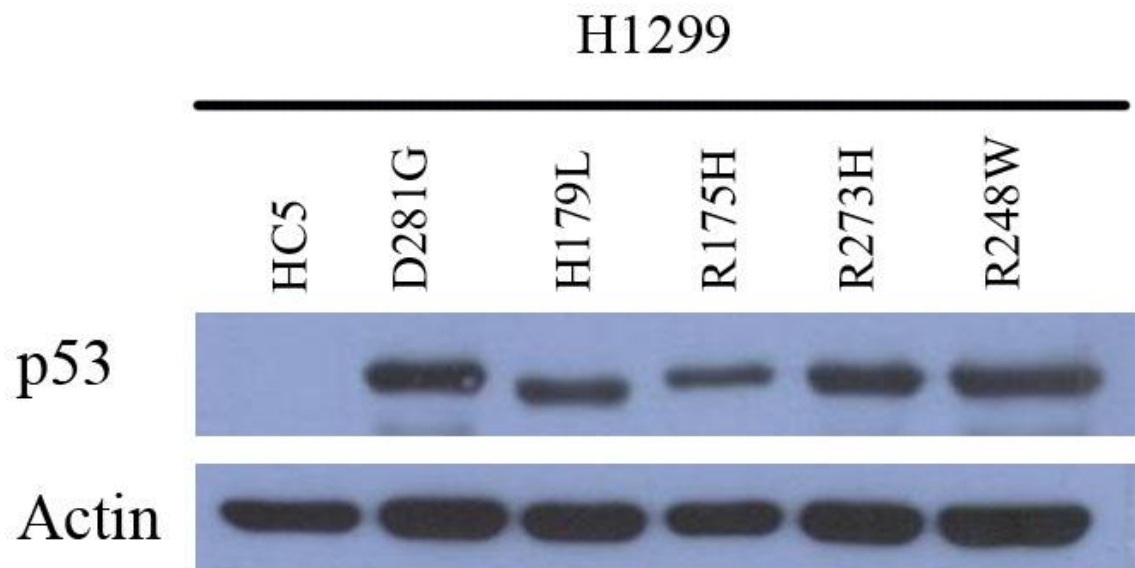


Figure 5: P53 Expression in H1299/D281G, H179L, R175H, R273H, R 248W, and Control Cells. Cells were counted and plated in a 6-well plate and were allowed to grow over night. Lysates were collected as described in ‘Methods,’ and were analyzed for mutant p53 expression using western blot. Actin was used as a loading control.

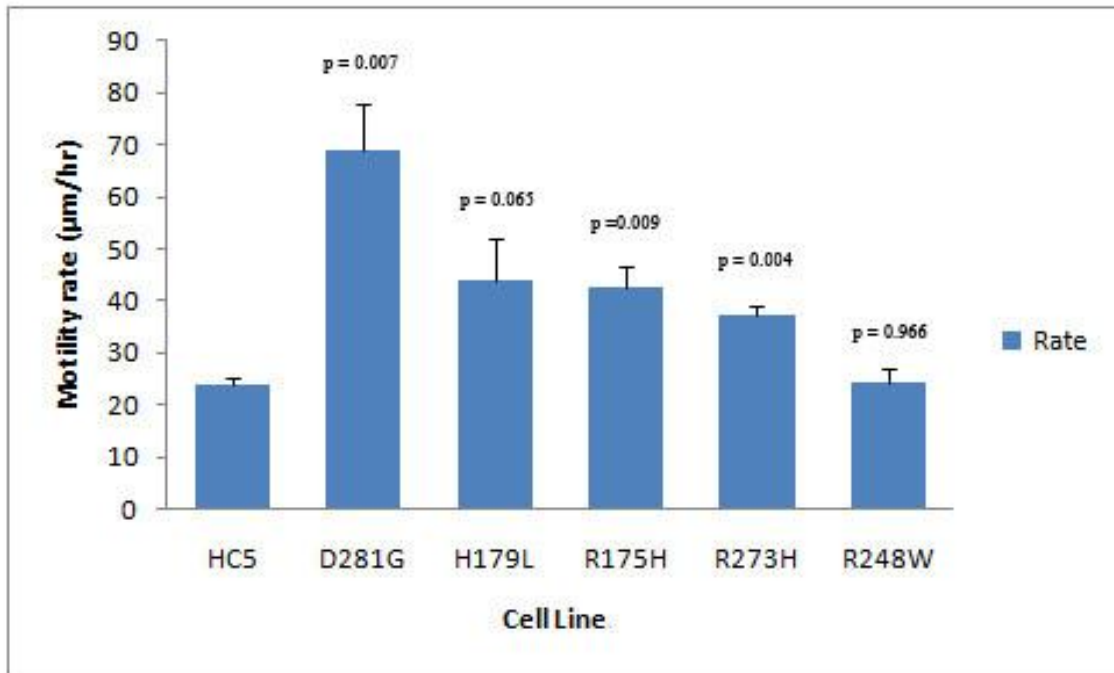


Figure 6: Expression of Mutant p53 Affects Cell Motility. Cells were counted and plated in triplicate in 12-well plates and incubated under standard conditions for overnight. Scratch assay was performed as described in ‘Methods,’ and width was measured using a light microscope.

AKT Affects Cell Motility in Cells Expressing Mutant p53

To examine the effect of AKT on cell motility in mutant p53 cells, a chemical AKT inhibitor was used. MTT assay was performed to determine the appropriate concentration of AKT inhibitor to use without being toxic to the cells (Figure 7). Both of the p53 mutant expressing cell lines (D281G and H179L) and vector control were viable in 1 μ M and 20 μ M concentration of AKT inhibitor. 20 μ M concentration of AKT inhibitor was used in our experiment to maximize the inhibitory effect. After selecting the proper concentration to use, scratch assays were performed to examine the effect of AKT on cell motility in the presence and absence of AKT inhibitor. Cells were pre-incubated with AKT inhibitor for 24 h prior to scratch. As a control, cells were incubated in DMSO in an equivalent volume to AKT inhibitor. Figure 8 shows that the cells in the absence of AKT inhibitor migrate at a faster rate compared to cells in the presence of AKT inhibitor. A similar trend can be seen in both mutant P53-expressing cell lines; D281G and H179L.

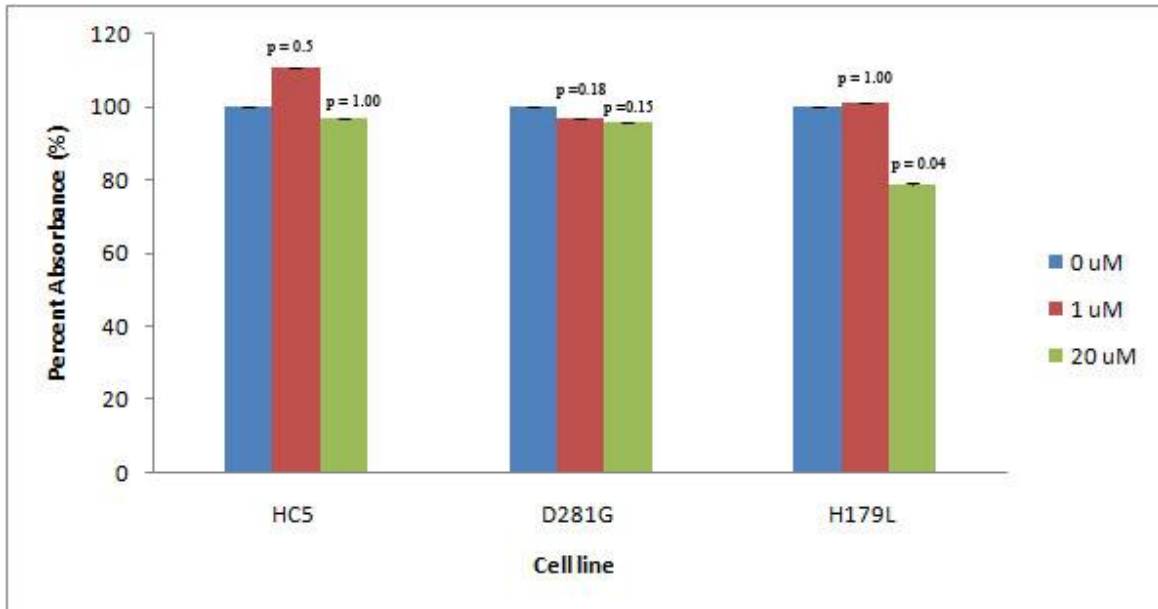


Figure 7: Effects of AKT Inhibitor on Cell Growth on Cells Expressing p53 Mutants.

Cells were counted and were plated in triplicate in 12-well plates and incubated in the indicated concentrations of AKT inhibitor. After 24 hours of incubation MTT assay was performed as described in 'Methods.' Absorbance was measured in a spectrophotometer at 570 nm.

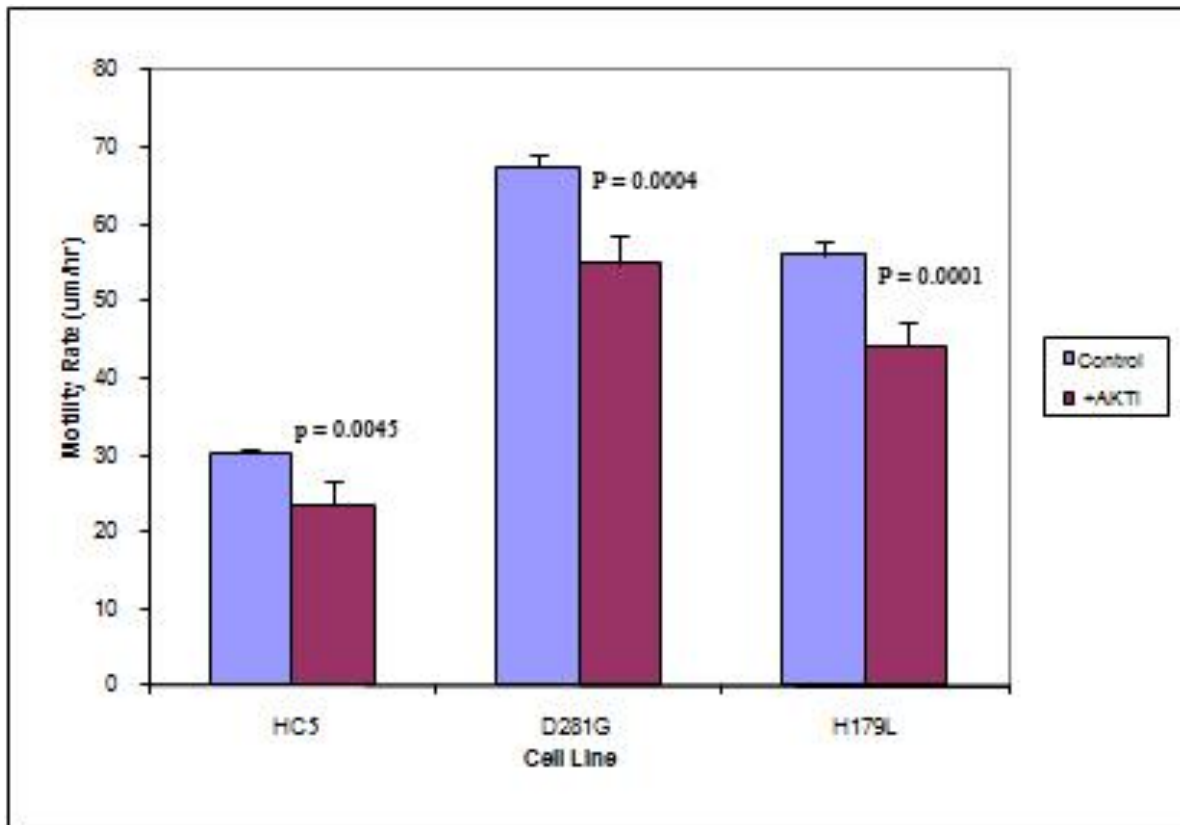


Figure 8: Effect of AKT Inhibitor on Cell Motility in Cells Expressing p53 Mutants.

Cells were counted and plated in triplicate in 12-well plates in the presence and in the absence of AKT inhibitor (20 µM). After 24 hours of incubation, scratch assays were performed as described in 'Methods,' and width was measured using light microscope.

Effect of AKT on CXC-Chemokines

Previous studies using H1299 cells expressing mutant p53 D281G indicated that inhibition of CXCL5 expression results in decreased cell motility (Yeudall et al., submitted). Our data had shown that cell motility was reduced by inhibiting AKT (Figure 8). It is possible that the effect of AKT on cell motility is mediated by CXC-chemokines. To investigate the effect of AKT on CXC-chemokine expression, qRT-PCR was performed using H1299 cells expressing D281, H179L, and vector control. Cells were pre-incubated with AKT inhibitor for 24 h prior to RNA extraction. After performing reverse transcription, qRT-PCR was performed on the cDNA template using CXCL5-specific primers. Figure 9 shows that in the presence of AKT inhibitor, CXCL5 expression is reduced in both of the mutant cell line (D281G and H179L).

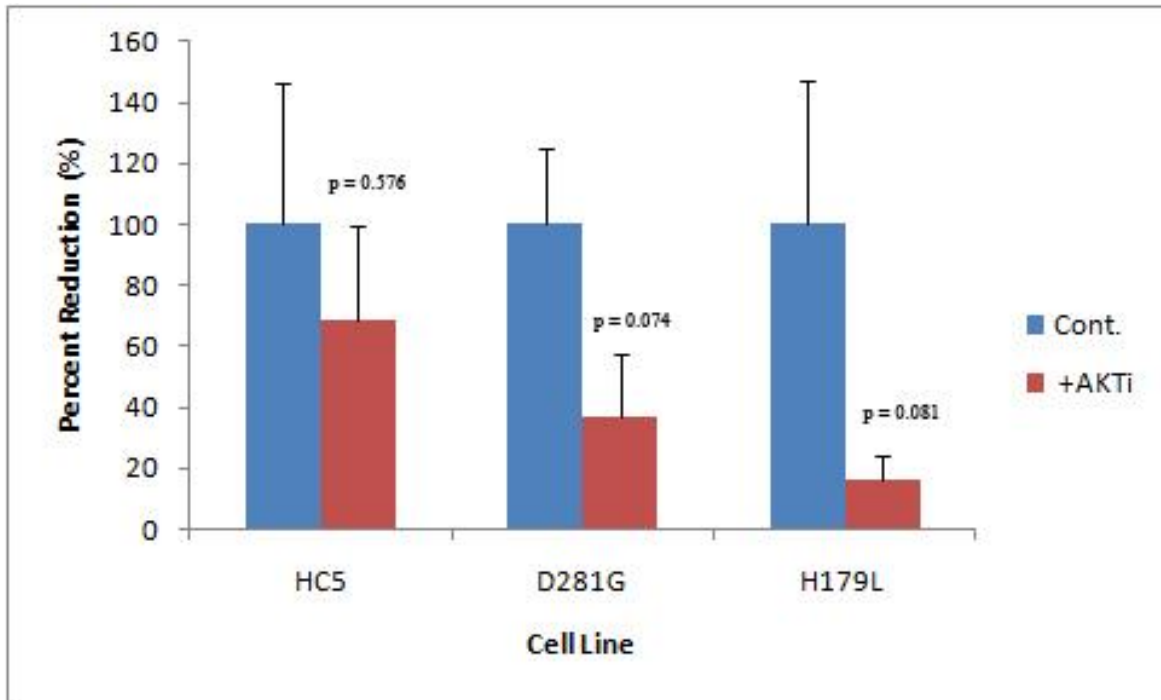


Figure 9: Effect of AKT Inhibitor on CXCL5 Expression. RNA was extracted and reverse-transcription was performed to create cDNA as described in ‘Methods’. cDNA was quantified by qRT-PCR. The relative expression ratio represents expression of CXCL5 to the internal standard, γ -tubulin.

Effect of p53 on AKT Phosphorylation

Studies have shown that activated AKT can directly phosphorylate components of the cell-death machinery, such as BAD and caspase-9 (Datta et al., 1997). In the presence of mutant p53, tumor suppressor function is lost and cell survival increases (Brosh and Rotter, 2009). It is possible that the cells expressing mutant p53 possess a selective growth advantage through effects on AKT. To investigate the effect of p53 on AKT phosphorylation, H1299 cells containing Ponasterone A- inducible, mutant p53-R175H were used. In the presence of Ponasterone A, mutant p53-R175H is expressed. Cells were incubated in the presence and in the absence of Ponasterone A over the following time course; 24 h, 48 h, 72 h, 96 h, and 120 h. 100% ethanol (solvent for Ponasterone A) was used as a control for the uninduced cells. Lysates were collected and western blotting was performed to determine p53, phosphorylated AKT and total AKT expression. Figure 10 shows that only the cells that were exposed to Ponasterone A expressed mutant p53. Expression of phosphorylated AKT and the total amount of AKT both increased over time up to 96 h in the induced cells. However, we also noticed that expression of phosphorylated AKT and total AKT in uninduced cells increased over time in a similar manner. We carried out a similar experiment using the control cell that contained empty vector which does not express mutant p53. Figure 11 shows the increase in phosphorylated AKT and total AKT expression over time in both induced and uninduced control cells. Actin was used as a loading control.

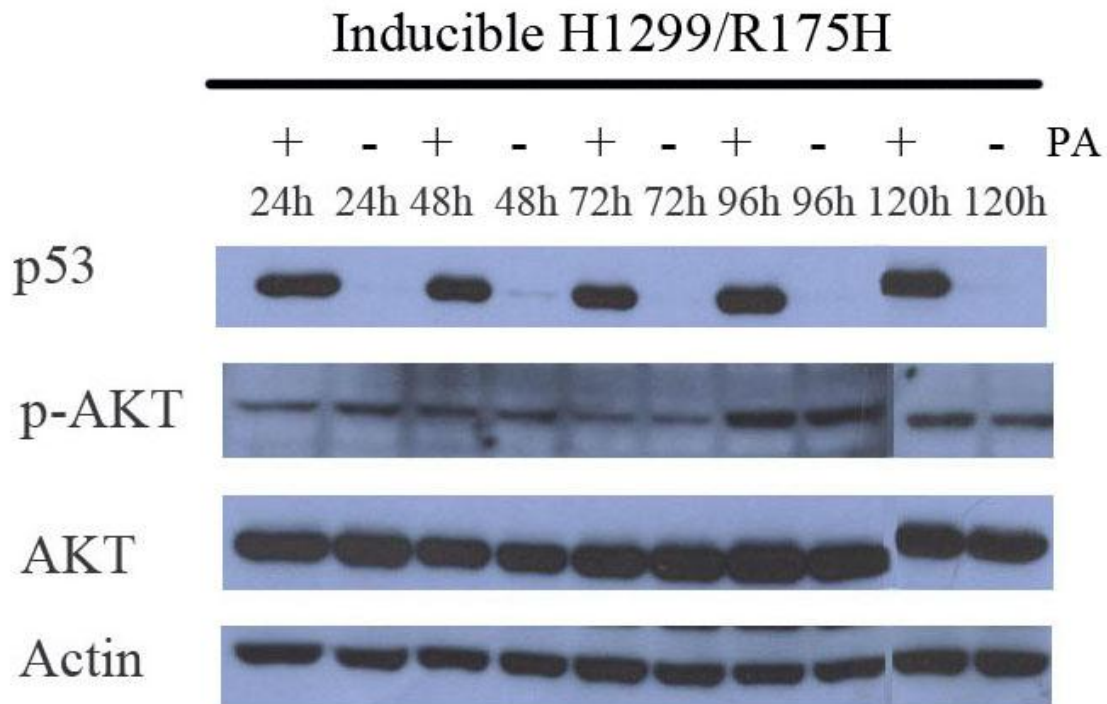


Figure 10: P53, Phosphorylated AKT and Total AKT Expression in Cells Expressing Mutant p53. Cells were counted and plated in 60 mm round plates and were allowed to grow in the absence (-) or presence (+) of Ponasterone A for the indicated times. Lysates were collected as described in ‘Methods,’ and were analyzed for mutant p53, phosphorylated AKT and total AKT expression using western blot. Actin was used as a loading control.

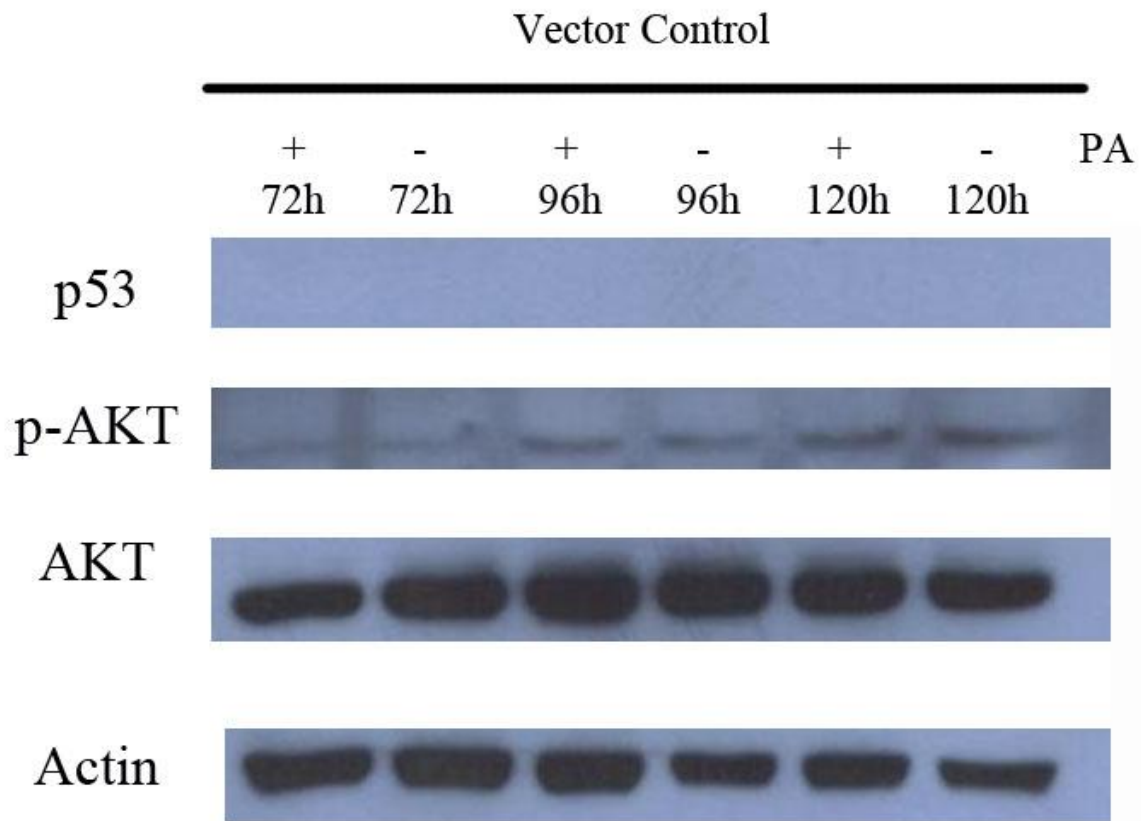


Figure 11: P53, Phosphorylated AKT, and Total AKT Expression in Vector Control.

Cells were counted and plated in 60 mm round plates and were allowed to attach overnight. Cells were grown in the absence (-) or presence (+) of Ponasterone A for the indicated times. Lysates were collected as described in 'Methods,' and were analyzed for mutant p53, phosphorylated AKT and total AKT expression using western blot. Actin was used as a loading control.

NFκB Promotes Cell Motility in Cells Expression Mutant p53

NFκB is known to regulate the transcription of diverse genes encoding growth factors and pro/antiapoptotic proteins. Our goal was to explore the effect of NFκB on the motility of cells expressing mutant p53. As in studies using the AKT inhibitor, H1299/D281G and H1299/H179L were used for this experiment. MTT assay was performed to determine the appropriate concentration of AKT inhibitor to use without being toxic to the cells (Figure 12). Both of the p53 mutant cell lines (D281G and H179H) and vector control showed that cells were viable in all three concentration of NFκB inhibitor (20 nM, 200 nM, and 2 μM). 2 μM concentration of NFκB inhibitor was used in our experiment to maximize the inhibitory effect. We performed a western blot to determine if the inhibitor is working as expected. We can see that in the presence of NFκB inhibitor, expression of phosphorylated NFκB is reduced compared to the controls (Figure 13). After selecting the proper concentration to use, scratch assays were performed to examine the effect of NFκB on motility in cells expressing p53 mutants. Scratch assays were carried out in the presence and absence of NFκB inhibitor. Figure 14 shows that the cells in absence of NFκB inhibitor migrate at a faster rate compared to cells in the presence of NFκB inhibitor. A similar trend can be seen in both of the p53 mutant cell lines and vector control, although the magnitude of the reduction was less in control cells.

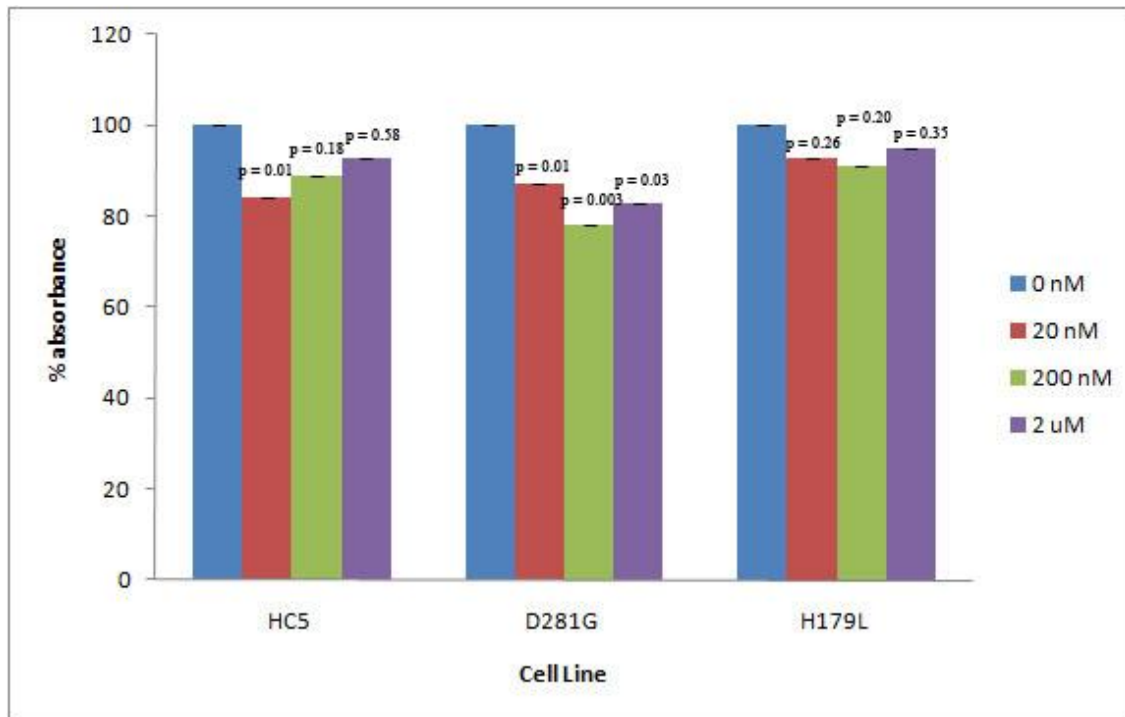


Figure 12: Effects of NFκB Inhibitor on Growth of Cells Expressing p53 Mutants.

Cells were counted and were plated in triplicate in 12-well plates and incubated in different concentrations of NFκB inhibitor as indicated. After 24 hours of incubation MTT assay was performed as described in 'Methods.' Absorbance was measured in a spectrophotometer at 570 nm.

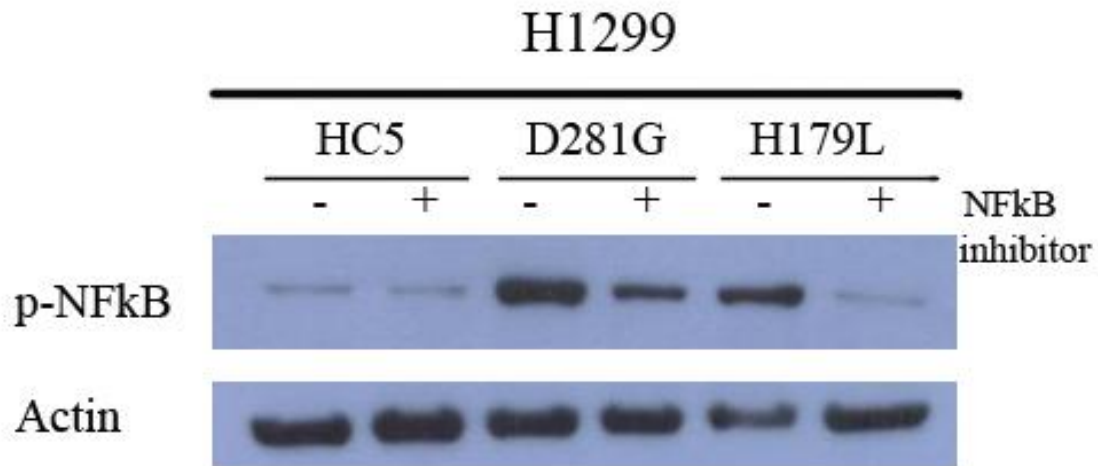


Figure 13: Phosphorylated NFκB Expression in H1299/D281G, H179L and Vector Control. Cells were counted and plated in a 6-well plate in the presence and absence of NFκB inhibitor and were allowed to incubate for 24 hours. Lysates were collected as described in ‘Methods,’ and were analyzed for phosphorylated NFκB expression using western blot. Actin was used as a loading control.

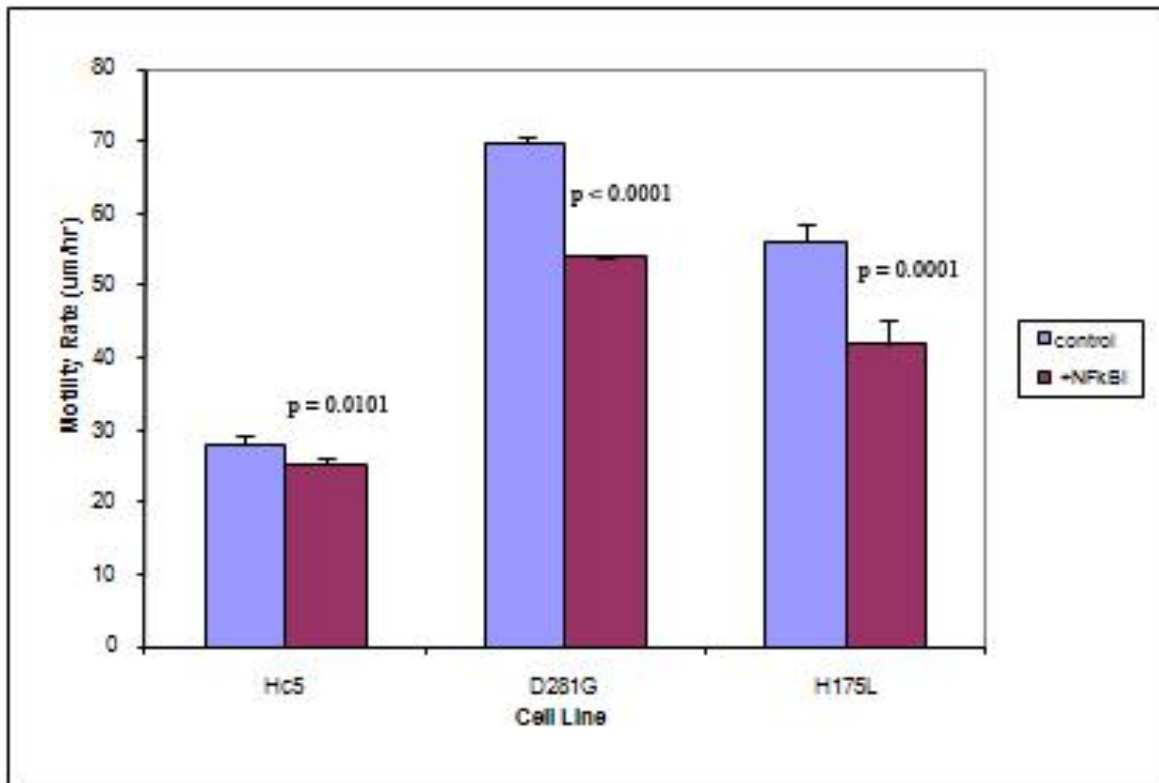


Figure 14: Effect of NFκB Inhibitor on Motility of Cells Expressing p53 Mutants.

Cells were counted and plated in triplicate in 12-well plates in the presence and absence of NFκB inhibitor (2 µM). After 24 hours of incubation, scratch assays were performed as described in 'Methods,' and width was measured using light microscope.

Effect of NFκB on CXC-Chemokines

It has been suggested that NFκB and CXC-chemokines enhance the migratory phenotype in cells expressing mutant p53 (Figure 14) (Miyazaki et al., 2006). It is possible that the effect of NFκB on cell motility is mediated by CXC-chemokines. To investigate the effect of NFκB on CXC-chemokine expression, qRT-PCR was performed. H1299 cells expressing mutant p53 D281G, H179L and vector control were pre-incubated in the presence and absence of NFκB inhibitor for 24hours prior to RNA extraction. After performing reverse transcription, qRT-PCR was performed on the cDNA template using CXCL5-specific primers. Figure 15 shows that in the presence of AKT inhibitor, CXCL5 expression is reduced in both of the mutant p53-expressing cell lines (D281G and H179L).

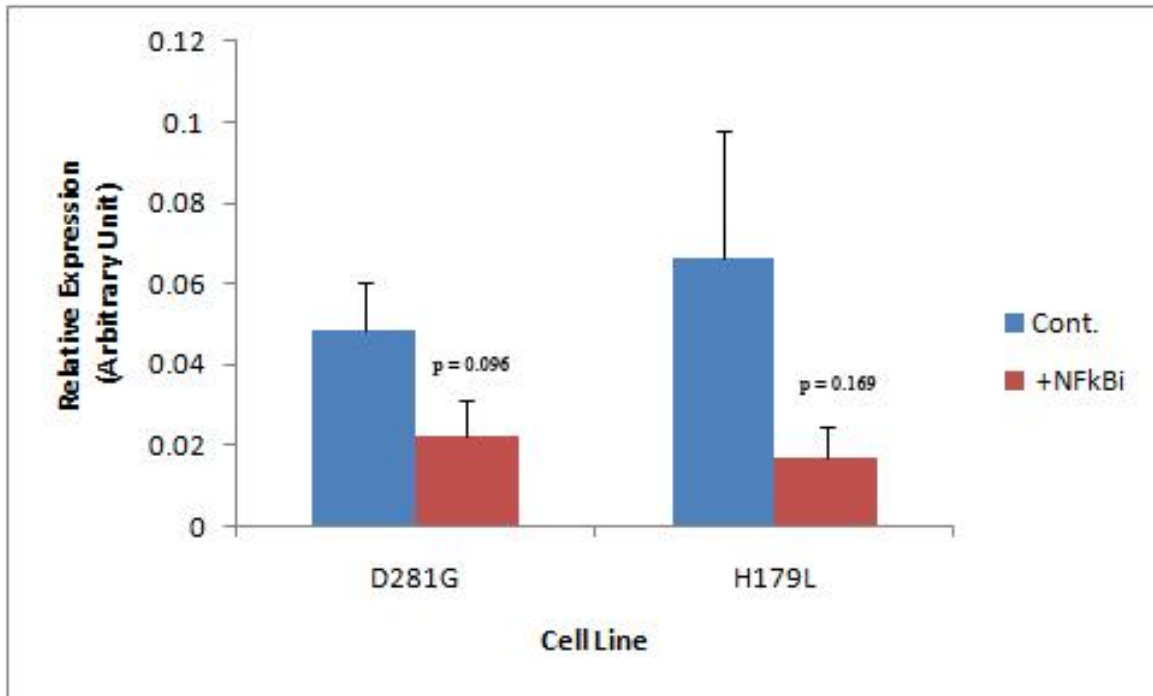


Figure 15: Effect of NFκB Inhibitor on CXCL5 Expression. RNA was extracted and reverse-transcription was performed to create cDNA as described in ‘Methods’. cDNA was quantified by qRT-PCR. The relative expression ratio represents expression of CXCL5 to the internal standard, γ -tubulin.

Discussion

Aims of Current Studies

The aims of this study were to determine the effect of AKT and NF κ B on cell motility and CXCL5 expression in cells expressing gain-of function mutant p53. We also examined the effect of mutant p53 on AKT phosphorylation.

Mutant p53 Enhances Cell Motility

Studies had shown that mutant p53 not only has a dominant negative effect over wild- type p53, but can also gain new oncogenic properties that are independent of wild- type p53 (Brosh and Rotter, 2009). Elevated expression of gain-of-function p53 mutants enhances the invasive phenotype (Dong et al., 2009) and mediates cell survival (Wee et al., 2009). It was also indicated that wild-type p53 regulates cell motility by suppressing phosphatidylinositide 3- OH kinase and Rac 1 signaling pathways (Guo et al., 2004). Therefore, our focus was to determine the effect of gain-of-function p53 mutants on cell motility, which is a key property in metastatic cells. P53 null H1299 human lung cancer cells were previously transfected to express different mutant p53 proteins; R248W, R175H, D281G, H179L, H273H (Scian et al., 2004). To examine if mutant p53 plays a role in cell motility, scratch assays (wound closure) were performed. We found that most cells that stably express p53 mutants showed a higher motility rate compared to HC5

(vector control) cells. This indicates that mutant p53 expressed in H1299 cell contributes to enhanced motility.

AKT Promotes Motility in Cells Expressing p53 Mutants

AKT has been implicated as a major factor in many types of cancer. AKT regulates many biological processes, such as proliferation and apoptosis (Vivanco and Sawyers, 2002). Other studies have shown that AKT can be activated downstream of G-protein coupled receptors, and plays an important role in angiogenesis (Sodhi et al., 2004). Recent studies suggest that AKT plays an important role in metastasis by effecting epithelial-to-mesenchymal transition (EMT). EMT is the process where epithelial cells become migratory and invasive by detaching from their normal surroundings and adopt the phenotype of mesenchymal cells. Common features of tumor EMT include remodeling of the cytoskeleton, reduced cell-to-cell adhesion, and increased motility and invasiveness (Sheng et al., 2009). Signals, such as TGF- β , FGF, and oncogenic proteins, lead to increased EMT by switching on the transcription factors that down-regulate E-cadherin, increase β -catenin, and promote Rac and Rho-dependent cell motility (Thiery, 2002). AKT is thought to influence the transcriptional activity of β -catenin by directly phosphorylating it on Ser-552 (Fang et al., 2007). In addition, AKT also down-regulates GSK-3 β which triggers the degradation of β -catenin. β -catenin in complex with E-cadherin plays a role in cell-to-cell adhesion; however this function is lost when β -catenin disassociates from E-cadherin (Sheng et al., 2009). Accumulated β -catenin in the nucleus causes increased proliferation and carcinogenesis by β -catenin interacting with T cell

Factor (TCF) (Du et al., 2009). Other studies have shown that AKT can promote basement membrane (BM) degradation and extracellular matrix remodeling (EMC) by AKT downstream effectors such as matrix metalloproteinase-9 (MMP-9) and urokinase-type plasminogen activator (uPA) (Sheng et al., 2009). It is possible that mutant p53 enhances tumor progression by means of the AKT signaling pathway. Here, scratch assays showed that cells incubated with AKT inhibitor had decreased motility compared to cells without AKT inhibitor. This suggests that effect of gain-of-function mutant p53 on cell motility is partly due to the activation of AKT.

AKT Phosphorylation Can Occur Independently of p53 Mutation

The p53 tumor suppressor protein and AKT play important roles in the transduction of proapoptotic and anti-apoptotic signals, respectively (Lim et al., 2009; Vivanco and Sawyers, 2002). Signals transduced by AKT and p53 are integrated via negative feedback between the two pathways (Gottlieb et al., 2002). The p53-AKT network was predicted to have all-or-none switching behavior between an anti-apoptotic state (low p53 and high AKT levels) and a pro-apoptotic state (high p53 and low AKT levels) (Wee et al., 2009). AKT is thought to influence activity of p53 through phosphorylation of the p53-binding protein, MDM2. MDM2 is a negative regulator of p53 that targets p53 for degradation through ubiquitination. AKT mediated phosphorylation of MDM2 allows MDM2 to translocate more efficiently to the nucleus, resulting in enhanced degradation of p53 (Zhou et al., 2001). AKT is also known to function in cell survival by directly phosphorylating pro-apoptotic proteins, such as BAD and caspase-9 (Datta et al., 1997; Cardone et al.,

1998). Because of the close connection between AKT and p53, it is possible to suspect that mutant p53 mediates the activity of AKT and its role in cell survival. To explore this possibility, we used the Ponasterone A- inducible mutant p53 R175H. We observed the expression of phosphorylated AKT and the total amount of AKT increased over time up to 96 hours in Ponasterone A induced cells. However, cells in the absence of Ponasterone A also showed increase in phosphorylated AKT and total AKT expression as well. Further study using the control cell line showed that phosphorylated AKT and total AKT expression increased over time in both induced and uninduced control cells. It has been documented that mutant p53 induces AKT phosphorylation in endometrial cell using the R175H mutant (Dong et al., 2009). However, our studies indicated that the phosphorylation of AKT is independent of mutant p53 R175H or Ponasterone A in H1299 lung cancer cells.

NFκB Promotes Cell Motility in Cells Expressing p53 Mutants

NFκB has been suggested to play a role in regulation of apoptosis, invasion, and angiogenesis in tumor cells (Lee et al., 2007). Previous studies have shown that tumor derived p53 mutants can induce NFκB2 gene expression (Scian et al., 2005). It is also known that NFκB regulates the transcription of diverse genes encoding cytokines, growth factors, and cell adhesion molecules (Lodish, 2004). Studies have shown that NFκB plays a substantial role in EMT and promotes cell migration and invasiveness (Tobar, 2010). During EMT, cancer cells lose expression of proteins that promotes cell-to-cell adhesion such as E-cadherin, acquire mesenchymal markers such as vimentin, and increase protease

expression to allow cells to enhance their ability for migration and invasion (Min et al., 2008). One of the key responses in EMT is ECM degradation (Thiery, 2002), which is one of the hallmarks of tumor invasiveness (Hanahan and Weinberg, 2000). MMP-9 and uPA are two classes of proteases that are involved in ECM degradation and have been implicated as a critical requirement in tumor spreading during metastasis (Kim et al., 1998). It has been indicated that TGF- β induces proteases by ROS-mediated NF κ B activation (Tobar et al., 2010). NF κ B acts as transcriptional factor for MMP-9 and uPA which possess NF κ B elements in their promoter regions (Reuning et al., 1999; Yan and Boyd, 2007). In addition, NF κ B represses expression of the tumor suppressor maspin, which inhibits uPA activation and metastasis (Luo et al., 2007). Our goal was to determine whether NF κ B contributes to increased cell motility in mutant p53 cell lines. Our scratch assay results show that cells incubated in the presence of the NF κ B inhibitor had decreased motility compared to controls without NF κ B inhibitor. This indicates that gain-of-function mutant p53 effects on cell motility are due in part to the enhanced NF κ B activity.

Inhibition of AKT or NF κ B Reduce CXCL5-Chemokine Expression

Chemokines functions mainly as chemoattractants for leukocytes, monocytes, neutrophils, and other effector cells (Gerber et al., 2009). The secretion of the chemokines and the cell surface expression of the chemokine receptors on tumor cells play critical roles in facilitation of tumor growth and/ or metastasis. Evidence suggests that ELR⁺ CXC chemokines, such as CXCL5 and CXCL8, stimulate cell migration, proliferation, and angiogenesis (Raman et al, 2007). It was also suggested that the ELR⁻ chemokine

CXCL12 and its receptor CXCR4 both play an important role in metastatic progression (Orimo and Weinberg, 2006). Previous studies have shown that CXC chemokines, such as CXCL5, CXCL8, and CXCL12, can be upregulated by mutant p53 protein. Inhibition of CXCL5 reduces cell motility in mutant p53 expressing cell (Yeudall et al., submitted). Our studies suggest that AKT and NF κ B also play a role in cell motility. Inhibition of AKT or NF κ B decreases cell motility in gain-of-function mutant p53 expressing cells. We also investigated the effects of AKT and NF κ B on CXCL5 expression in cells expressing mutant p53. QRT-PCT results indicate that induced cell motility by AKT or NF κ B may be mediated, in part, by increased CXCL5 expression. Previous studies have shown that expression of CXCL5 in vector control cells is low compared to gain-of-function mutant p53 cells (Yeudall et al., submitted), however here our data showed that the CXCL5 expression is higher. We suspect that high expression of CXCL5 in vector control is due to DMSO that was used as a solvent. Further studies can be performed comparing CXCL5 expression of cells in the presence and absence of DMSO in vector control cells.

Future studies

In this study, we have shown that gain-of-function mutant p53 can induce cell motility. We also showed that AKT and NFκB play roles in cell motility in gain-of-function mutant p53 expressing cells. Inhibition of AKT or NFκB reduced cell motility in cells expression mutant p53. Regulation of cell motility by AKT and NFκB are, in part, mediated by CXCL5-chemokines. To confirm the effects of AKT and NFκB on CXCL5 expression, luciferase reporter assays can be performed to determine chemokine promoter activity in the presence and in absence of inhibitors. We can also expand the motility study by using CXCL5-chemokine knock down or over expressed cells in presence and absence of inhibitors.

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Literature Cited

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