Cancer Therapy Vol 3, 515-524, 2005

Growth inhibition of head and neck squamous cell carcinoma by imatinib mesylate (Gleevec)

Research Article

Hyung Ro Chu[1], Weg M. Ongkeko[2], Amilcar Diaz[3], Xabier Altuna[4], Joe Aguilera[5], Robert A. Weisman[2] and Jessica Wang-Rodriguez[6]

[1]Department of Otolaryngology-HNS, Kangnam Sacred Heart Hospital, Hallym University, Seoul, Korea
[2]Department of Surgery, Division of Head and Neck Surgery, University of California, San Diego, California
[3]Department of Pathology, University of California, San Diego, California
[4]Servicio de Otorrinolaringología, Hospital Donostia. San Sebastián, Spain
[5]University of California, San Diego, Moore's Cancer Center
[6]Department of Pathology, University of California, San Diego and VA San Diego Healthcare System, San Diego, California

***Correspondence:** Jessica Wang-Rodriguez, M.D., Department of Pathology, University of California, San Diego, VA San Diego Healthcare System, 3350 La Jolla Village Dr. (113), San Diego, CA 92161, USA; Telephone: (858) 552-8585 ext. 7734; Fax: (858) 552-4370; Email: jwrodriguez@ucsd.edu

Key words: Gleevec, Imatinib mesylate, Akt, tyrosine kinase, serine threonine kinase, head and neck cancer

Abbreviations: acute myelogenous leukemia, (AML); gastrointestinal stromal tumors, (GIST); head and neck squamous cell carcinoma, (HNSCC); phophoinositide 3-kinases, (PI3Ks); phosphoinositides, (PIs); phosphorylated Akt, (p-Akt); platelet-derived growth factor receptor, (PDGFR); pleckstrin homology, (PH); protein tyrosine kinases, (PTKs); receptor tyrosine kinases, (RTKs); small-cell lung cancer, (SCLC); tyrosine kinases, (TKs)

Received: 4 October 2005; Accepted: 13 October 2005; electronically published: October 2005

Summary

Imatinib mesylate (Gleevec) is known to exert anti-growth effects through many protein tyrosine kinases (PTKs) such as platelet-derived growth factor receptor (PDGFR), c-Kit, and c-abl. The serine/threonine kinase Akt, or protein kinase B (PKB), was described to be the downstream effector of many PTKs and inhibits apoptosis in cancer cells. The purpose of this study was to examine the expression of these PTKs and Akt in head and neck squamous cell carcinoma (HNSCC) after exposure to Imatinib and to determine if this drug will inhibit the growth of HNSCC cells at clinically relevant doses. Imatinib was introduced into cultures of the squamous cell lines UMSCC10B, HN12 and HN30 at clinically used concentrations. Protein tyrosine kinases, PDGFR, c-Kit, and c-Abl, were evaluated by Western blot. Cell viability was assessed by clonogenic survival analysis. HNSCC tissue samples were stained for PDGFR, c-Kit and phosphorylated Akt (p-Akt). Akt kinase activity was measured in the presence or absence of Imatinib. In addition, Akt phosphorylation following Imatinib treatment was assessed using Western blot. Akt siRNA was used as the positive control for complete inhibition of Akt. Colony forming efficiency decreased with an increase in concentration of Imatinib. Three μM of Imatinib completely reduced cell viability in HN12 and HN30 and 10 μM in UMSCC10B. Immunohistochemistry confirmed high expression of PDGFR, c-Kit, and p-Akt in human HNSCC tissues. Expression of PDGFR, c-Kit, and c-Abl in the HNSCC cell lines did not change after Imatinib treatment. Akt kinase activity was significantly inhibited with increasing concentration of Imatinib in HNSCC cells, and near complete dephosphorylation of Akt was observed at 6 μM of Imatinib in the UMSCC10B and HN30 cell lines. Akt SiRNA alone, however, significantly reduced the cell viability by approximately 1/3. Imatinib at clinically relevant concentrations caused a dose dependent decrease in HNSCC survival with a complete inhibition of growth at the highest concentrations tested. The decreased cell survival may be related to the inhibition of PTKs and a reduction of Akt kinase activity but was not due to inhibition of Akt alone.

I. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant neoplasm in the world today (Jemal et al, 2004). The overall 5-year survival rate for patients is less than 50%, among the lowest of the major cancer types, and has not improved during the last

decade (Jemal et al, 2004). The lack of progress in head and neck oncology emphasizes the importance of molecular genetic studies that correlate with tumor biology. The molecular alterations observed in HNSCC are mainly due to oncogene activation and tumor suppressor gene inactivation, leading to deregulation of cell proliferation. Understanding the molecular mechanisms of the tumor biology will aid in identifying targets for treatment.

Protein-tyrosine kinases (PTKs) are important regulators of intracellular signal-transduction pathways mediating cell development, growth, and multicellular communication. Their activity is normally tightly controlled and regulated. Perturbation of PTK signaling by mutations and other genetic alterations results in deregulated kinase activity and malignant transformation (Blume-Jensen and Hunter, 2001). Protein kinases were thought to be poor therapeutic targets because of their ubiquitous nature and crucial role in many normal physiologic processes.

The advent of Gleevec (STI-571, imatinib, CGP 57148; Novartis Pharmaceuticals, Basel, Switzerland) demonstrated that kinases could be clinically useful drug targets for treating certain types of cancer. Imatinib is a PTK inhibitor, which selectively suppresses the activity of c-Abl, Bcr-Abl, platelet-derived growth factor receptor (PDGFR), and c-Kit (Buchdunger et al, 1996; Druker et al, 1996; Heinrich et al, 2000; Capdeville et al, 2002; Roskoski, 2003). This compound is now standard treatment for chronic myelogenous leukemia (CML) (Druker et al, 2001; Kantarjian et al, 2002; Kurzrock et al, 2003). Human CML and some forms of Philadelphia chromosome-positive acute lymphocytic leukemia are characterized by reciprocal translocation between chromosomes 9 and 22. This translocation results in the fusion of two cellular genes, Abl and Bcr, resulting in the formation of a chimeric gene termed Bcr-Abl (Shore et al, 1990). Imatinib occupies the nucleotide-binding pocket of Bcr-Abl protein and blocks access to ATP thereby preventing phosphorylation of any substrate (Druker et al, 1996; Kurzrock et al, 2003) and inducing apoptosis (Druker et al, 1996; Kantarjian et al, 2002; Kurzrock et al, 2003).

Imatinib is not specific for Bcr-Abl, and its action extends to c-Kit and PDGFR (Heinrich et al, 2000; Pietras et al, 2001, 2002). The c-Kit proto-oncogene is a 145 kD transmembrane glycoprotein and a number of the receptor PTK subclass III family that includes receptors for PDGF, macrophage colony-stimulating factor, and flt3 ligand (Yarden et al, 1987, Small et al, 1994). Its ligand, alternatively known as stem cell factor, mast cell growth factor, or steel factor, is an early growth factor that supports the growth and differentiation of multiple hematopoietic lineages (Besmer, 1991; Ashman, 1999). In addition to its importance in normal cellular physiologic activities, c-Kit plays a role in biologic aspects of certain human cancers, including germ cell tumors, mast cell tumors, gastrointestinal stromal tumors (GIST), small-cell lung cancer (SCLC), melanoma, breast cancer, acute myelogenous leukemia (AML), and neuroblastoma (Besmer, 1991; Hibi et al, 1991; Beck et al, 1995; DiPaola et al, 1997; Hirota et al, 1998; Ashman, 1999; Tian et al, 1999; Heinrich et al, 2000). Imatinib has been recently approved for the treatment of c-Kit-positive advanced and/or surgically unresectable GISTs and is undergoing trials for the treatment of SCLC.

Autocrine PDGFR stimulation is found in several human tumor types, including dermatofibroma protuberans, giant cell fibroblastoma, and glioblastoma, each of which responds to Imatinib with inhibition of growth and apoptosis in vitro and in xenograft models (Buchdunger et al, 2000; Sjoblom et al, 2001).

A significant advantage of Imatinib is that it is effective when administered orally. Many anticancer drugs are effective only when injected intravenously. In contrast to other chemotherapy drugs, side effects from Imatinib are mild. The most common side effects are mild nausea, edema, myalgias, arthralgias, diarrhea, and skin rash which occur in about 10% of patients (Druker et al, 2001; Kurzrock et al, 2003).

In a preliminary study, we found that PDFGR, c-Kit, and c-Abl were highly expressed in cultured squamous cell lines by Western blot analysis. For stimuli that induce PTK activity in cells almost invariably induce subsequent activity of proteins with Src homology domains, such as phophoinositide 3-kinases (PI3Ks) which in turn generate inositol phospholipids (Stephens et al, 1993; Vanhaesebroeck and Alessi, 2000). These lipids and the protein kinase are mostly likely activated by the protein kinase Akt (also known as PKB), and trigger a cascade of responses, from cell growth and proliferation to survival and motility that lead to tumor progression (Vanhaesebroeck and Alessi, 2000; Blume-Jensen and Hunter, 2001; Amornphimoltham et al, 2004). Most of the growth factors activate Akt through a PI3K signaling pathway-dependent mechanism (Cross et al, 1995; Franke et al, 1995), but a few PI3K-independent mechanisms also have been reported (Moule et al, 1997; Filippa et al, 1999). Because these mechanisms may be involved in the action of Imatinib against cancer cells, we designed a study to investigate the effect of Imatinib in human HNSCC where PTKs such as PDGFR and c-Kit are expressed, and to examine its effect on the activation of Akt as a probable mechanism of action.

II. Materials and Methods

A. Reagents

Imatinib (Novartis, Basel, Switzerland) was dissolved in Me_2SO_4 (DMSO) to a stock concentration of 3 mM and stored at -20°C. Dilutions for all experiments were freshly made before use.

B. Cell lines and tissue culture

Human HNSCC cell lines UMSCC10B, HN12, and HN30, were used in this study. UMSCC10B was supplied by Dr. Tom Carey form University of Michigan. HN12 and HN30 cell lines were obtained form Dr. J.S. Gutkind, Oral and Pharyngeal Cancer Branch, National Institute of Craniofacial and Dental Research, National Institutes of Health, Bethesda, MD. All cell lines were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 10,000 unit/ml penicillin G sodium, 10 mg/ml streptomycin sulfate (Invitrogen

Co., Carlsbad, CA), and L-glutamine (Invitrogen Co.) at 37°C in 5% CO_2.

C. Western blot analysis for PDGFR, c-Kit, and c-Abl

After twenty-four hours of achieving cell confluence, 5 ml of 6 μM of Imatinib was added to each plate in duplicate for 24 hours at 37 °C, and 5 ml of DMSO was added to the control plate. Cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. Cell lysates were separated on 10% NuPAGE® Novex Bis-Tris Gels (Invitrogen Co.) and transferred electrophoretically to a nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories, Hercules, CA). Membranes were incubated for one hour at room temperature with primary antibodies in the following concentrations: anti-PDGFR- antibody (1:200 dilution), anti-PDGFR- antibody (1:200), anti-c-Kit antibody (1:200), and anti-c-Abl antibody (1:100; all from Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary antibodies (1:2,000 dilution anti-rabbit antibodies for PDGFR- , PDGFR- , and c-Kit; 1:2,000 antimouse for c-Abl) were added. Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Pierce, Rockford, IL).

D. Clonogenic survival analysis

Cultures were trypsinized to generate a single cell suspension, which was seeded onto 50 mm tissue culture plates at a density of 700 cells/plate in triplicate. To assess the effect of Imatinib on cell proliferation, the HNSCC cell lines were incubated for a total of 8 days in various concentrations (0, 0.5, 1, 3, 6, and 10 μM) of Imatinib which was renewed on day 4. Control plates received DMSO only. Eight days after seeding, colonies were stained with crystal violet, and the number of colonies containing at least 50 cells was counted using AlphaImager 2200 and 1220 software (Alpha Innotech Co., San Leandro, CA). All of the assays were performed in triplicate and were repeated at least twice.

In a separate experiment, Akt siRNA (SignalSilence™ siRNA kit; Cell Signaling Technology) at a concentration of 100 nM was added to UMSCC10B without the addition of Imatinib and incubated for 10 days. A repeat dose of Akt siRNA was added on day 4. Colonies were counted using the above method at the end of the 10th day.

E. HNSCC specimens and immunohistochemical analysis

Immunohistochemical studies on archived, formalin fixed, paraffin embedded HNSCC tissue samples were performed using polyclonal rabbit antibodies against PDGFR (Santa Cruz Biotechnology) and c-Kit (Santa Cruz Biotechnology). A mouse monoclonal antibody against p-Akt (Ser 473) (Cell Signaling Technology, Beverly, MA) was used for phosphorylated Akt. Negative control slides were from the same tissue but without the addition of the primary antibodies. As normal controls, 5 specimens each of normal tonsils, uvula, and laryngeal mucosa were included.

The tissue sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by steam heating with 1x DAKO Target Retrieval solution. The sections were then allowed to cool to room temperature in the solution. The endogenous peroxidase was removed by 3% H_2O_2. Non-specific binding of biotin and avidin was abolished by blocking solution for 30 minutes (Protein Block Serum-Free, DAKO, Carpinteria, CA). The background staining was reduced with incubation of goat serum (1:20 dilution) for 60 minutes. Primary antibodies were placed on slides and incubated for 1 hour at room temperature in the case of PDGFR and c-Kit and overnight at room temperature in the case of p-Akt. Secondary antibodies conjugated with Strepavidine/HRP (LSAB2, DAKO) were used. The slides were washed and antibody complex visualized by 3,3'-diaminobenzidine (DAB, DAKO). The nuclei were counterstained by Gill's II hematoxylin. Immunoactivity in the tissues was estimated by counting the number of positive cells per 1,000 tumor cells. Cases were considered positive if more than 20% of the tumor cells were staining.

F. Akt kinase activity assay

Equal numbers of cells were seeded into 3 cell culture plates (100 mm). After allowing cells to attach overnight, Imatinib or DMSO was added at concentrations of 0, 5, and 10 μM. Cells were harvested after 24 hours for Akt kinase assay (nonradioactive, Cell Signaling Technology). The harvested cell pellet was lysed with 1 mM phenylmethylsufonyl fluoride (PMSF) and 0.5 ml of cell lysis buffer. Cell lysates (supernatant) were prepared after sonication and centrifugation for 10 minutes at 4°C. The total protein of each cell line was standardized using a BCA protein assay reagent kit (Pierce, Rockford, IL). Immobilized Akt monoclonal antibody (Cell Signaling Technology, Beverly, MA) was added to an equal amount (200 μl) of each cell lysate and incubated overnight at 4°C in rocker. The immunoprecipitate was washed twice each with cell lysis buffer and kinase buffer containing 25 mM Tris (pH 7.5), 5 mM -glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. The pellet was re-suspended in 50 μl of kinase buffer, 10 mM of ATP, and 1 μg of GSK-3 fusion protein. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 μl 3% SDS buffer. Samples were loaded on 10% NuPAGE® Novex Bis-Tris Gels (Invitrogen Co.) and transferred electrophoretically to a nitrocellulose membrane. The volume of sample per well on gel was adjusted appropriately according to the total protein amount of each sample that was determined by BCA protein assay. The membrane was incubated for 2 hours with blocking buffer that contained PBS and 5% nonfat dry milk. GSK-3 phosphorylation was detected using phosphopho-GSK-3 / (Ser21/9) antibody (1:1,000 dilution) and horseradish peroxidase-conjugated anti-rabbit antibody by Western blot. To assess the level of expression of p-GSK, SeeBlue Plus2® (Invitrogen Co.) was used as a standard ladder.

G. Akt siRNA as a positive control

To investigate the mechanism of action of Imatinib on Akt, we compared the expression of Akt and p-Akt after exposure to Imatinib, using Akt siRNA (SignalSilence™ siRNA kit; Cell Signaling Technology) as the positive control. The three HNSCC cell lines were cultured in three 50 mm culture plates and allowed to grow to less than 50% confluence. Then the cells were incubated with either Akt siRNA or 6 μM of Imatinib for 24 hours. The expression was compared to the control samples that were exposed to Transfection reagent (SignalSilence™ siRNA kit component; Cell Signaling Technology).

For cells incubated with Akt siRNA, two sets of 1 ml serum-free media were prepared in sterile microfuge tubes. 20 μl of Transfection reagent were added to the each tube, followed by incubation for 10 minutes in room temperature. Akt siRNA was added at a concentration of 100 nM and was incubated for another 10 minutes at room temperature. This mixture was added to one set of the culture plates and agitated for 30 seconds. The contents of the other microfuge tube were added to another plate as a control. The three cell lines were exposed to either Akt

siRNA or Imatinib at 6ÌM for 24 hours. Scramble siRNA was used as a negative control.

After incubation with Imatinib or Akt siRNA, cells were harvested and centrifuged at 2,000 rpm. Protein was extracted from cells using lysis buffer containing protease inhibitor and was separated using 10% NuPAGE® Novex Bis-Tris Gels and blotted onto a nitrocellulose membrane. The membrane was probed using anti-p-Akt antibody (1:100; Cell signaling Technology), anti-Akt1 antibody (1:100; Santa Cruz Biotechnology), and anti- -actin antibody (1:5,000; Sigma-Aldrich Inc.), followed by 1-hour room temperature incubation with the following secondary antibodies: anti-mouse antibody (1:5,000) for p-Akt; anti-goat antibody (1:10,000) for Akt1; and anti-mouse antibody for actin (1:2,000). Positive protein signals were visualized with the enhanced chemiluminescence detection system.

III. Results

A. Expression of TKs in HNSCC cell lines

By Western Blot analysis, human HNSCC cell lines, UMSCC10B, HN12, and HN30, were shown to express PDGFR- , PDGFR- , c-Kit, and c-Abl (**Figure 1**). Exposure of HNSCC cells to 6 μM Imatinib for 24 hours has no apparent effect on the quantitative expressions of these PTKs.

B. Inhibition of cell growth by Imatinib at clinically relevant concentrations

The effect of Imatinib on three HNSCC cell lines was measured by means of clonogenic survival analysis. A near-complete growth inhibition in HN12 and HN30 cells was observed when treated with 3 μM Imatinib, whereas UMSCC10B cells exhibited a similar response at a concentration of 10 μM (**Figure 2**).

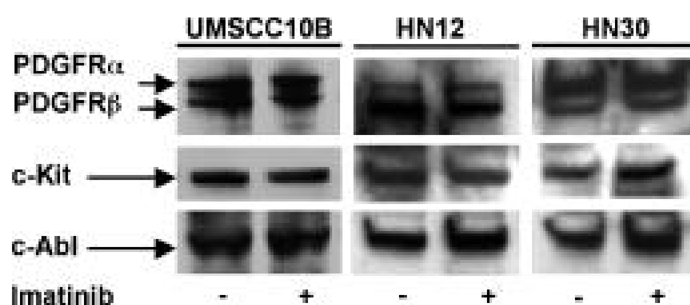

Figure 1. Western blot analysis of PDGFR- , PDGFR- , c-Kit, and c-Abl in UMSCC10B, HN12, and HN30 cells. The protein tyrosine kinases were present in all three cell lines, but their expression was not changed after 24 hours of Imatinib treatment at 6 μM.

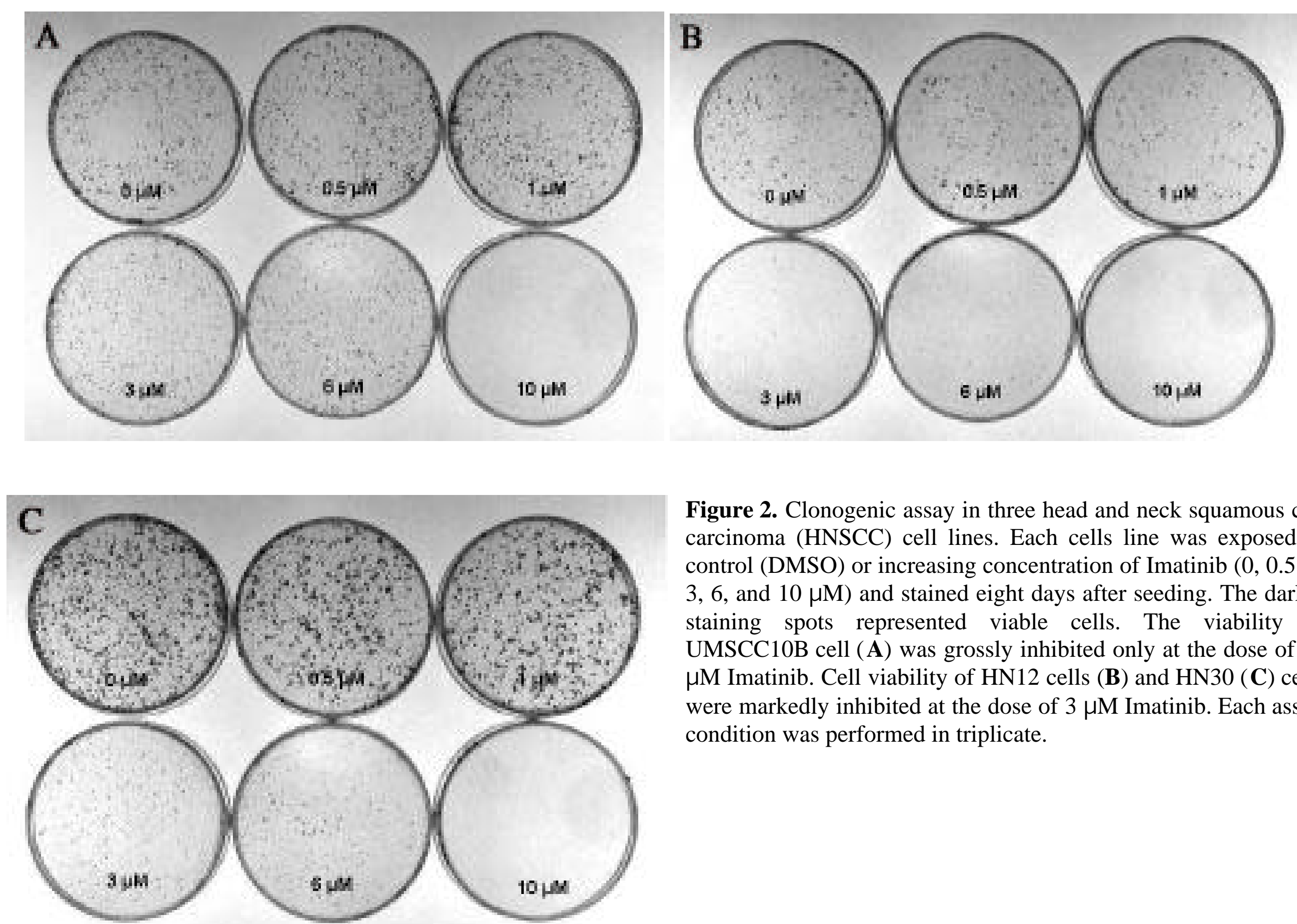

Figure 2. Clonogenic assay in three head and neck squamous cell carcinoma (HNSCC) cell lines. Each cells line was exposed to control (DMSO) or increasing concentration of Imatinib (0, 0.5, 1, 3, 6, and 10 μM) and stained eight days after seeding. The darker staining spots represented viable cells. The viability of UMSCC10B cell (**A**) was grossly inhibited only at the dose of 10 μM Imatinib. Cell viability of HN12 cells (**B**) and HN30 (**C**) cells were markedly inhibited at the dose of 3 μM Imatinib. Each assay condition was performed in triplicate.

The concentration of 3 to 6 μM can be achieved by once daily administration of Imatinib orally in patients (4). Consistent with the results from viability staining assays, the quantitative analyses of colony forming efficiency demonstrated that Imatinib exerted a significant inhibitory effect on cell growth of HNSCC cell lines (**Figure 3**).

Akt siRNA alone at a concentration of 100 nM for 10 days achieved approximately 33% inhibitory effect on the cell colony. The number of colonies in the control cells was 459+/-31. Cells treated with Akt SiRNA had a reduction of colonies to 300+/-54 (p=0.01, **Figure 4**).

C. Immunohistochemical study

Immunohistochemistry was performed on 37 HNSCC tumors and 5 controls. **Figure 5** shows a representative example of immunohistochemical staining for (A) PDGFR, (B) c-Kit, and (C) p-Akt. Positive staining is located in the cytoplasm of PDGFR and c-Kit, and in the cytoplasm and nucleus for the phospho-Akt. PDGFR was positive in 36 of 37 cancer samples (97.3%) as well as all 5 control specimens. The proportion of positive staining for c-Kit and p-Akt were 70.3% (26 of 37) and 67.6% (25 of 37), respectively. In contrast, the control cases showed no expression in p-Akt and c-Kit (p=0.07).

D. Effect of Imatinib on Akt kinase activity

HNSCC cells in the presence of Imatinib inhibited Akt kinase activity in a dose-dependent fashion (**Figure 6**). Imatinib at a concentration of 5 μM inhibited the Akt kinase by approximately 50% in these cell lines. At a concentration of 10 μM, all three cell lines revealed further reduction, but not complete absence of Akt kinase activity.

E. Akt SiRNA vs. Imatinib effect on phosphorylated Akt

Figure 7 showed the presence of Akt1 and phosphorylated Akt (p-Akt) in cells after exposure to 6 uM of Imatinib vs. Akt SiRNA for 24 hours. Both Akt siRNA and Imatinib caused a near-complete dephosphorylation of Akt, as evidenced by a significant reduction in p-Akt expression, in all three cell lines, to levels that were comparable to those seen after exposure to Akt SiRNA in UMSCC10B cells. The expression of Akt1 remained present after Imatinib treatment, although there appeared be a reduction of Akt1 in UMSCC10B after exposure to Imatinib. In contrast, Akt siRNA caused marked reduction of both Akt1 and the p-Akt.

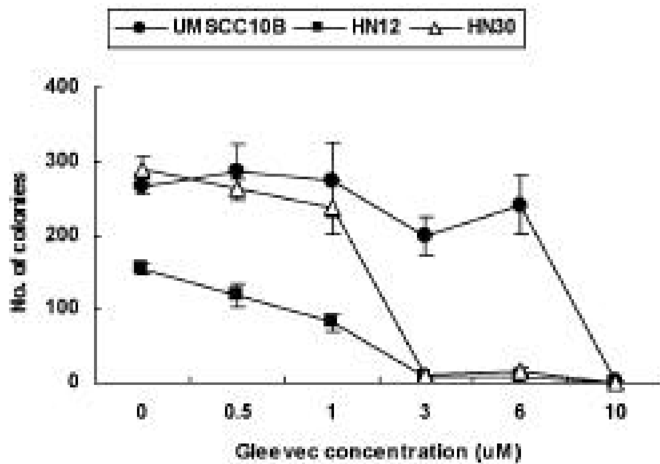

Figure 3. Effect of Imatinib on tumor cell viability. Colony-forming efficiency was determined 8 days later. The colonies were counted if they had equal or greater than 50 cells. Each value represented the mean ± standard deviation for three independent experiments.

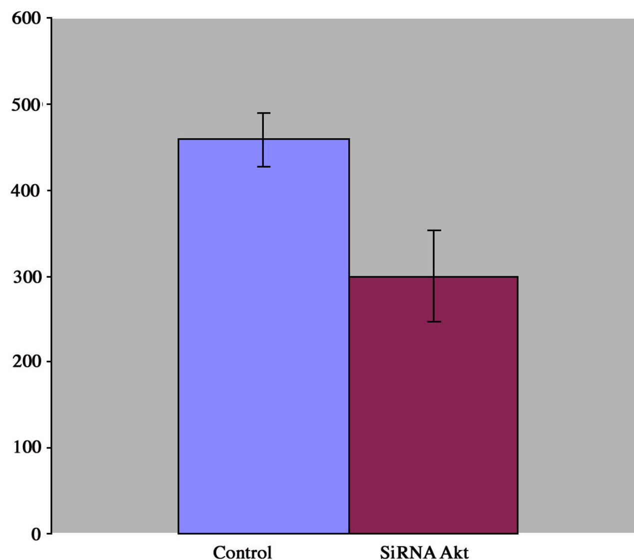

Figure 4. Clonogenic assay on cell UMSCC10B after 10 days of incubation with 100 nM Akt SiRNA. The number of colonies counted in control cells was significantly higher than that treated with Akt SiRNA (p=0.01).

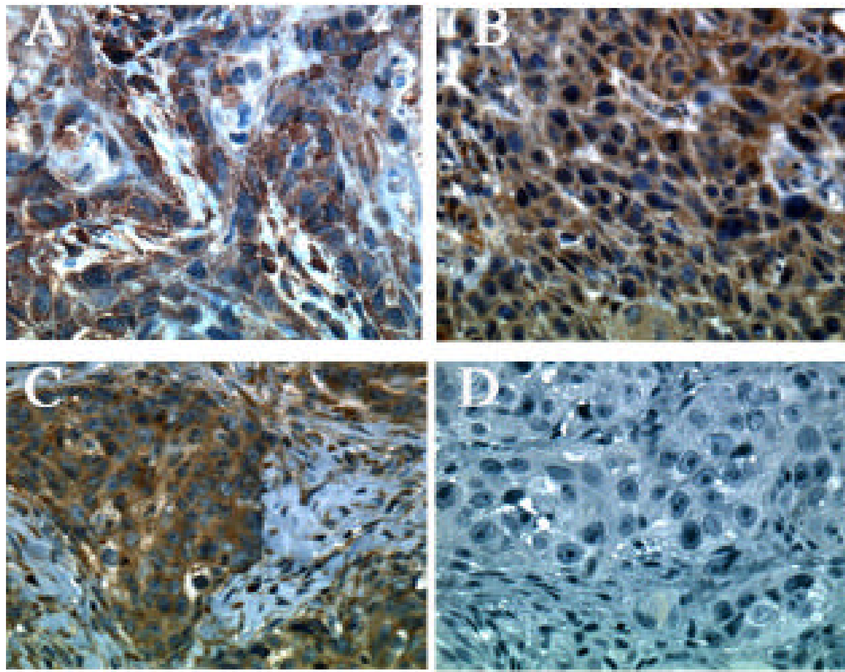

Figure 5. Immunohistochemical detection for PDGFR (**A**), c-Kit (**B**), and phospho-Akt (**C**) and negative control (**D**). Positive staining indicated the presence of p-Akt. Positive staining was observed mainly in the cytoplasm and evenly distributed within the tumor. p-Akt is a phosphorylated form of Akt. All images were photographed at 400X magnification..

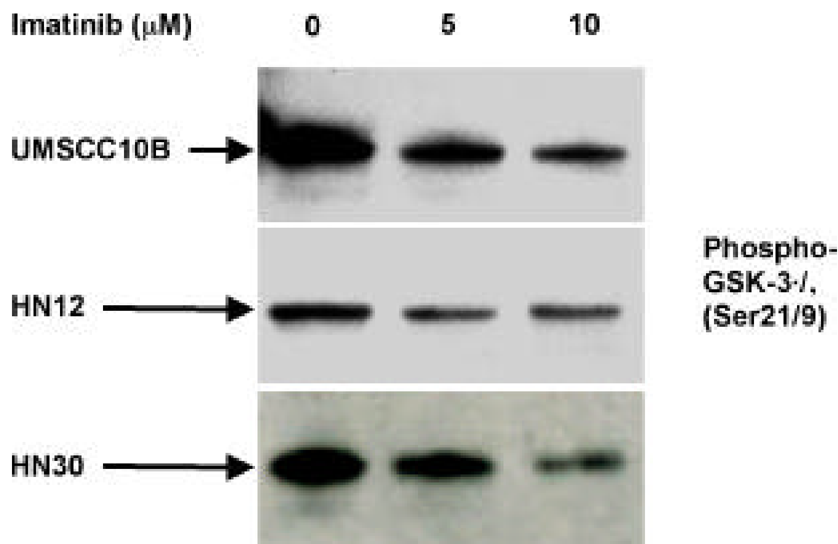

Figure 6. Analysis of Akt kinase activity in Imatinib-treated head neck squamous cell carcinoma cell lines. Cells were exposed to 0, 5, and 10 μM of Imatinib for 24 hours and lysed for immunoblot analysis. Kinase assay was performed using GSK-3 fusion protein as a substrate. The protein levels of phosphorylated GSK reflect the Akt kinase activity. Akt kinase was reduced by approximately 50% at 5μM of Imatinib in all three cell lines, and approximately by 75% at 10μM in UMSCC10B and HN30 cells.

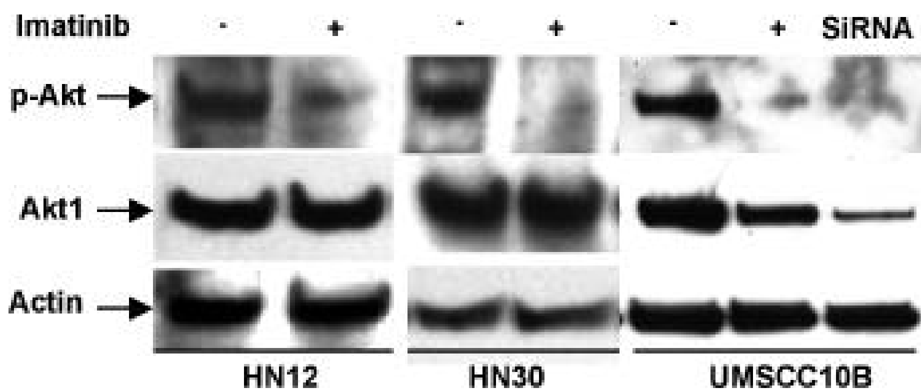

Figure 7. Imatinib or Akt SiRNA-dependant suppression in Akt expression. Western blots showed phosphorylated Akt (p-Akt), Akt1, and -actin from three HNSCC cell lines. The three cell lines were treated with Imatinib at 6 μM for 24 hours and separate UMSCC10B cells were treated with 100 nM Akt SiRNA for 24 hours as positive control. The expressions of Akt1 were not significantly altered with Imatinib treatment, with the exception of UMSCC10B, that showed approximately 50% reduction in Akt1 expression. The presence of p-Akt was severely reduced to a level similar to the effect of Akt SiRNA.

IV. Discussion

In the current study, we found that HNSCC cell lines as well as human HNSCC's expressed high levels of PDGFR, c-Kit, and c-Abl tyrosine kinases (TKs). The receptor tyrosine kinases (RTKs), PDGFR and c-Kit, and a non-receptor thyrosine kinase, c-Abl, are the main targets for Imatinib (Buchdunger et al, 2000; Vlahovic and Crawford, 2003). Since Imatinib has proven to be an effective inhibitor for various tyrosine kinases, we hypothesized that this tyrosine kinase inhibitor could also inhibit the proliferation of HNSCC cells.

The clonogenic analysis showed a significant inhibition of cell viability in HN12 and HN30 cells at a concentration of 3 μM Imatinib, which was the serum level achieved by the typical dosage of 300mg Imatinib taken orally in the once daily for CML treatment (Roskoski, 2003).

Though the activity of these target kinases are presumably lowered by Imatinib, the protein levels of PDGFR, c-Abl and c-Kit expression in HNSCC cells were not altered following Imatinib treatment. Because of its critical role in the downstream-signaling pathway of PTKs such as PDGFR and c-Kit, we proceeded to measure Akt kinase activity following the treatment of Imatinib. We hypothesized that Imatinib would inhibit cancer cell proliferation either through inhibition of PI3K/Akt or PI3K-independent Akt pathway. Akt is a 57 kD serine/threonine kinase with pleckstrin homology (PH) domain that preferentially binds phophatidylinositol (Blume-Jensen and Hunter, 2001; Roskoski, 2003) diphosphate (PtdIns(3,4)P_2) and PtdIns(3,4,5)P_3 over other phosphoinositides (PIs) (James et al, 1996; Stephens et al, 1998; Vanhaesebroeck and Alessi, 2000). Stimuli that induce PTK activity in cells almost invariably lead to the generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Stephens et al, 1993).

Akt itself has a pivotal role in cell cycle progression, differentiation of smooth muscle cells, angiogenesis, inhibition of apoptosis, and cell growth (Franke et al, 1995; Brennan et al, 1997; Muise-Helmericks et al, 1998; Hayashi et al, 1999; Ozes et al, 1999; Verdu et al, 1999; Okano et al, 2000). Akt also plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis (Blume-Jensen and Hunter, 2001; Vivanco and Sawyers, 2002). Akt promotes cell survival through downstream signaling. Phosphorylation of Akt contributes to the activation of Akt (Kohn et al, 1996). To date, there are at least seven signaling pathways downstream of Akt (McCormick, 2004). Proteins that encourage apoptosis, such as BAD, caspase-9, are among the first targets of Akt to be identified. In addition, Akt triggers other cell-death regulators such as IKK , the forkhead transcription factors, Mdm2 and Yap (Basu et al, 2003; Wendel et al, 2004). Such association has been shown in several cancer types such as breast, pancreatic, and ovarian cancer (Cheng et al, 1992, 1996; Ballacosa et al, 1995).

The involvement of Akt in human HNSCC has been demonstrated previously in a number of in vitro and in vivo studies. For example, Amornphimoltham et al. (Amornphimoltham et al, 2004) demonstrated that activation of Akt could be detected in head and neck tumors with a pattern of expression and localization correlating with the progression of lesions. Grille et al. (Grille et al, 2003) reported that activation of the Akt pathway led to epithelial-mesenchymal transition (EMT) and invasion in HNSCC cells. In this study, we also found a high percentage of p-Akt expression in human HNSCC by immunohistochemistry.

Previous studies have demonstrated that Imatinib activity is mediated in part through direct inhibition of the Akt signaling pathway (Matei et al, 2004; Appel et al, 2005). In this study, we also demonstrated the inhibitory effect of Imatinib on Akt in the HNSCC cells, mainly in the phosphorylated, comparable to that of Akt siRNA. When used Akt siRNA as a positive control to completely block Akt, we observed that Imatinib at 6Ì remarkably inhibits phospho-Akt in all three cells to a similar level as to when cells were exposed to Akt siRNA. However, the near absence of p-Akt did not correlate with the growth inhibition in UMSCC 10B cells, which required a higher dose (10μM) of Imatinib. We concluded that Imatinib-induced growth inhibition was not due to the blocking of Akt phosphorylation alone. Furthermore, different cell lines with different genomic instability may have unpredictable sensitivity to Imatinib.

In addition, directly incubating the UMSCC10B cells with the Akt siRNA showed significant growth inhibition, but failed to demonstrate the growth inhibitory effect similar to that of Imatinib. Therefore, inactivation of Akt alone did not appear to account for the action of Imatinib. This growth inhibition may involve other PTKs, perhaps in combination with Akt or Akt independent pathways. However, in the absence of additional information on other signal transduction pathways mediating cell proliferation and multicellular communication, it is difficult to present an exact molecular mechanism of growth inhibition of HNSCC by Imatinib. We expect that Imatinib can inhibit cancer cell proliferation at more than one level. We are actively investigating other TKs that maybe modulated by Imatinib.

In contrast to CML, in which one gene mutation drives cancer progression (Sawyers, 1999), most solid tumors are thought to be the result of several genetic alterations (Hanahan and Weinberg, 2000). Our study shows that a high percentage of human HNSCC tissue expressed PDGFR, c-Kit, and p-Akt. Drugs like Imatinib, which target PTKs and possibly Akt in cancers that over-express them, may prove to be useful as single agent, or in combination with other types of therapy, especially since their toxicity profile is very acceptable.

In summary, we have demonstrated that at concentrations achieved in serum by oral administration of standard therapeutic doses, Imatinib caused significant inhibition of cell proliferation in three HNSCC cell lines. The mechanism of this inhibition may involve many PTKs with or without inactivation of Akt. This study serves as the first step in exploring the role of Imatinib in the treatment of HNSCC. The marked inhibition of Imatinib on proliferation and survival of HNSCC cell lines justifies a similar study in animal models. Because of the low toxicity of Imatinib, the data from this study can also support preliminary clinical trials of this drug as an

adjunct to other treatment modalities, such as surgery, radiation therapy and chemotherapy in HNSCC.

References

Amornphimoltham P, Sriuranpong V, Patel V, Benavides F, Conti CJ, Sauk J, Sausville EA, Molinolo AA, Gutkind JS (**2004**) Persistent activation of the Akt pathway in head and neck squamous cell carcinoma: a potential target for UCN-01. **Clin Cancer Res** 10(12 Pt 1), 4029-37

Appel S, Rupf A, Weck MM, Schoor O, Brummendorf TH, Weinschenk T, Grunebach F, Brossart P (**2005**) Effects of Imatinib on Monocyte-Derived Dendritic Cells Are Mediated by Inhibition of NF- B and Akt Signaling Pathways. **Clin Cancer Res** 11, 1928-1940.

Ashman L (**1999**) The biology of stem cell factor and its receptor C-kit. **Int J Biochem Cell Biol** 31, 1037-51.

Basu S, Totty NF, Irwin MS, Sudol M, Downward J (**2003**) Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. **Mol Cell** 11, 11-23.

Beck D, Gross N, Brognara CB, Perruisseau G (**1995**) Expression of stem cell factor and its receptor by human neuroblastoma cells and tumors. **Blood** 86, 3132-8.

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, et al (**1995**) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. **Int J Cancer** 64, 280-5.

Besmer P (**1991**) The kit ligand encoded at the murine Steel locus: a pleiotropic growth and differentiation factor. **Curr Opin Cell Biol** 3, 939-46.

Blume-Jensen P, Hunter T (**2001**) Oncogenic kinase signalling. **Nature** 411, 355-65.

Brennan P, Babbage JW, Burgering BM, Groner B, Reif K, Cantrell DA (**1997**) Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. **Immunity** 7, 679-89.

Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB (**2000**) Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. **J Pharmacol Exp Ther** 295, 139-45.

Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, Lydon NB (**1996**) Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. **Cancer Res** 56, 100-4.

Capdeville R, Buchdunger E, Zimmermann J, Matter A (**2002**) Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. **Nat Rev Drug Discov** 1, 493-502.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR (**1992**) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. **Proc Natl Acad Sci U S A** 89, 9267-71.

Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK, Testa JR (**1996**) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. **Proc Natl Acad Sci U S A** 93, 3636-41.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (**1995**) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. **Nature** 378, 785-9.

DiPaola RS, Kuczynski WI, Onodera K, Ratajczak MZ, Hijiya N, Moore J, Gewirtz AM (**1997**) Evidence for a functional kit receptor in melanoma, breast, and lung carcinoma cells. **Cancer Gene Ther** 4, 176-82.

Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL (**2001**) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. **N Engl J Med** 344, 1031-7.

Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB (**1996**) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. **Nat Med** 2, 561-6.

Filippa N, Sable CL, Filloux C, Hemmings B, Van Obberghen E (**1999**) Mechanism of protein kinase B activation by cyclin AMP-dependent protein kinase. **Mol Cell Biol** 19, 4989-5000.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN (**1995**) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. **Cell** 81, 727-36.

Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, Lee-Kwon W, Donowitz M, Tsichlis PN, Larue L (**2003**) The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. **Cancer Res** 63, 2172-8.

Hanahan D, Weinberg RA (**2000**) The hallmarks of cancer. **Cell** 100, 57-70.

Hayashi K, Takahashi M, Kimura K, Nishida W, Saga H, Sobue K (**1999**) Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. **J Cell Biol** 145, 727-40.

Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ (**2000**) Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. **Blood** 96, 925-32.

Hibi K, Takahashi T, Sekido Y, Ueda R, Hida T, Ariyoshi Y, Takagi H, Takahashi T (**1991**) Coexpression of the stem cell factor and the c-kit genes in small-cell lung cancer. **Oncogene** 6, 2291-6.

Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y (**1998**) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. **Science** 279, 577-80.

James SR, Downes CP, Gigg R, Grove SJ, Holmes AB, Alessi DR (**1996**) Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-triphosphate without subsequent activation. **Biochem J** 315, 709-13.

Jemal A, Clegg LX, Ward E, Ries LA, Wu X, Jamison PM, Wingo PA, Howe HL, Anderson RN, Edwards BK (**2004**) Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. **Cancer** 101, 3-27.

Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ; American Cancer Society (**2004**) Cancer Statistics. **CA Cancer J Clin** 54, 8-29.

Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E; International STI571 CML Study Group (**2002**) Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. **N Engl J Med** 346, 645-52.

Kohn AD, Takeuchi F, Roth RA (**1996**) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. **J Biol Chem** 271, 21920-6.

Kurzrock R, Kantarjian HM, Druker BJ, Talpaz M (**2003**) Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. **Ann Intern Med** 138, 1031-7.

Matei D, Chang DD, Jeng MH. (**2004**) Imatinib Mesylate (Gleevec) Inhibits Ovarian Cancer Cell Growth through a Mechanism Dependent on Platelet-Derived Growth Factor Receptor- and Akt Inactivation. **Clin Cancer Res** 10, 681-690.

McCormick F (**2004**) Cancer: survival pathways meet their end. **Nature** 428, 267-9.

Moule SK, Welsh GI, Edgell NJ, Foulstone EJ, Proud CG, Denton RM (**1997**) Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and -adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. **J Biol Chem** 272, 7713-9.

Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N (**1998**) Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. **J Biol Chem** 273, 29864-72.

Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H (**2000**) Akt/protein kinase B isoform are differentially regulated by epidermal growth factor stimulation. **J Bio Chem** 275, 30934-42.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB (**1999**) NF- B activation by tumor necrosis factor requires the Akt serine-threonine kinase. **Nature** 401, 82-5.

Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A (**2001**) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. **Cancer Res** 62, 5476-84.

Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A (**2002**) Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. **Cancer Res** 62, 5476-84.

Roskoski R Jr (**2003**) STI-571: an anticancer protein-tyrosine kinase inhibitor. **Biochem Biophys Res Commun** 309, 709-17.

Sawyers CL (**1999**) Chronic myeloid leukemia. **N Engl J Med** 340, 1330-40.

Shore SK, Bogart SL, Reddy EP (**1990**) Activation of murine c-abl protooncogene: effect of a point mutation on oncogenic activation. **Proc Natl Acad Sci U S A** 87, 6502-6.

Sjoblom T, Shimizu A, O'Brien KP, Pietras K, Dal Cin P, Buchdunger E, Dumanski JP, Ostman A, Heldin CH (**2001**) Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. **Cancer Res** 61, 5778-83.

Small D, Levenstein M, Kim E, Carow C, Amin S, Rockwell P, Witte L, Burrow C, Ratajczak MZ, Gewirtz AM, et al (**1994**) STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD^{34+} human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. **Proc Natl Acad Sci U S A** 91, 459-63.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT (**1998**) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-triphosphate-dependent activation of protein kinase B. **Science** 279, 710-4.

Stephens LR, Jackson TR, Hawkins PT (**1993**) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5) -triphosphate: a new intracellular signalling system? **Biochem Biophys Acta** 1179, 27-75.

Tian Q, Frierson HF Jr, Krystal GW, Moskaluk CA (**1999**) Activating c-kit gene mutations in human germ cell tumors. **Am J Pathol** 154, 1643-7.

Vanhaesebroeck B, Alessi DR (**2000**) The PI3K-PDK1 connection: more than just a road to PKB. **Biochem J** 346, 561-76.

Verdu J, Buratovich MA, Wilder EL, Birnbaum MJ (**1999**) Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. **Nat Cell Biol** 1, 500-6.

Vivanco I, Sawyers CL (**2002**) The phosphatidylinositol 3-kinase-AKT pathway in human cancer. **Nat Rev Cancer** 2, 489-501.

Vlahovic G, Crawford J (**2003**) Activation of tyrosine kinases in cancer. **Oncologist** 8, 531-8.

Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J, Lowe SW (**2004**) Survival signalling by Akt and elF4E in oncogenesis and cancer therapy. **Nature** 428, 332-7.

Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A (**1987**) Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. **EMBO J** 6, 3341-51.

Jessica Wang-Rodriguez