12-2013

Radiosensitization of Head & Neck Carcinoma Cells by Linifanib, A Receptor Tyrosine Kinase Inhibitor

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School of Medicine
in conjunction with the
Faculty of Graduate Studies

Radiosensitization of Head & Neck Carcinoma Cells by Linifanib, A Receptor Tyrosine Kinase Inhibitor

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Pharmacology

December 2013

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Saied Mirshahidi and Dr. Chien-Shing Chen for giving me the opportunity to study under their supervision and work on understanding the function of Linifanib (ABT-869), a multi-receptor tyrosine kinase inhibitor. They have spent countless time checking my work, providing suggestions and taught me how to think critically to become a good scientist, which is very important for my future career. I also thank them for all the generous support, passionate endeavors that they have passed on and invested in me during my PhD studies.

I also thank my committee members, Dr. Gridley, Dr. Wall and Dr. Zhang, for their help and advice. Their valuable opinions have helped me strengthen my research. Many appreciations go to my lab colleague, Rosalia de Necochea-Campion, for sharing her perspectives and experimental experiences. Also thank Mr. Celso Perez for his excellent assistance with my radiation experiments and other people who have helped me, including members in Department of Radiation Medicine and Dr. Lubo Zhang's lab members. It was my pleasure to meet you all and have you around.

Finally, I would like to convey my deepest appreciation to my parents and my family for their continuous support and love during my PhD journey. This has been a great dream I aspire to throughout my life.

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ABBREVIATIONS

AA	Antiangiogenic agent
AIF	Apoptosis inducing factor
AKT	Protein kinase B
CA	Carbonic anhydrase
CI	Confidence interval
CCRT	Concurrent chemoradiation
COX	Cyclooxygenase
DSB	Double strand break
EBV	Epstein-Barr virus
EGF/R	Epidermal growth factor/receptor
EMT	Epithelial-to-mesenchymal transition
ERCC	Excision-repair cross-complementing protein
FGF	Fibroblast growth factor
FHX	5-fluorouracil, hydroxyurea, radiotherapy
FLK	Fetal liver kinase
FLT	Fms-like tyrosine kinase
GLUT	Glucose transporter
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor

IGRT	Image-guided radiotherapy
IL-6	Interleukin-6
IMRT	Intensity modulated radiotherapy
KDR	Kinase-insert domain receptor
Kit	Stem cell factor receptor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NPC	Nasopharyngeal carcinoma
mTOR	Mammalian target of rapamycin
PDGF/R	Platelet-derived growth factor/receptor
PI3K	Phosphoinositide 3-kinase
PLGF	Placental growth factor
RT	Radiation therapy
STAT	Signal transducer and activator of transcription
TKI	Tyrosine kinase inhibitor
uPA	Urokinase plasminogen activator
VEGF/R	Vascular endothelial growth factor/receptor
XRCC	X-ray repair cross-complementing protein

ABSTRACT OF THE DISSERTATION

Radiosensitization of Head & Neck Carcinoma Cells by Linifanib, A Receptor Tyrosine Kinase Inhibitor

Heng-Wei Hsu

Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, December 2013
Dr. Saied Mirshahidi & Dr. Chien-Shing Chen

Tumor angiogenesis is a hallmark of advanced cancers and promotes invasion and metastasis. Over 90% of head and neck squamous cell carcinomas (HNSCC) express angiogenic factors such as vascular endothelial growth factor (VEGF). Since radiotherapy is one of the most commonly used treatments for HNSCC, it is imperative to identify the interactions between antiangiogenic therapy and radiotherapy, and to develop combination therapy to improve clinical outcome. The mechanisms between antiangiogenic agents and ionizing radiation are complicated and involve many interactions between the vasculature, tumor stroma and tumor cells. The proliferation and metastasis of tumor cells rely on angiogenesis/blood vessel formation. Rapid growing tumors will cause hypoxia, which up-regulates tumor cell survival factors, such as VEGF and hypoxia-inducing factor-1α (HIF-1α), giving rise to more tumor proliferation, angiogenesis and increased radioresistance. Thus, agents that target new tumor vessel formation can modulate the tumor microenvironment to improve tumor blood flow and oxygenation, leading to enhanced radiosensitivity.

Signal transducer and activator of transcription 3 (STAT3), is a potential modulator of VEGF expression and regulates cell-cycle progression, angiogenesis, metastasis and apoptosis. Approximately 80% of HNSCC exhibit up-regulation of

STAT3 expression, which theoretically mediates radio-resistance and chemo-resistance. Therefore, inhibition of STAT3 may render tumor cells growth arrest and/or apoptosis. Recently it has been discovered that DNA damage can induce the expression and secretion of interleukin-6 (IL-6), resulting in the activation of STAT3 signaling pathway. Therefore, by inhibiting STAT3, one can also inhibit DNA damage repair and induce apoptosis in tumor cells.

In this project, we tested the feasibility of Linifanib (ABT-869), a multi-receptor tyrosine kinase inhibitor of VEGF and platelet derived growth factor (PDGF) receptor families, on radio-sensitization of HNSCC. The results show that Linifanib (ABT-869) can induce an antitumor effect and radio-sensitize HNSCC cells via inhibition of STAT3 signaling pathway. Combining antiangiogenic targeted agent such as Linifanib (ABT-869) with radiation to enhance tumor killing and apoptosis may provide a novel therapeutic strategy and improve efficacy of radiation against HNSCC in the future.

CHAPTER ONE

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), including cancers of the oral cavity, oropharynx, hypopharynx, pharynx and larynx, is the sixth most common cancer worldwide with approximately 600,000 new cases diagnosed each year (Saman, 2012). The risk factors are tobacco and alcohol consumption (Saman, 2012), human papillomavirus (HPV) (Gillison et al., 2012; Kundu and Nestor, 2012), Epstein-Barr virus (EBV) (Lui et al., 2009; Ho et al., 2013), areca nut (Tseng et al., 2012), and dietary factors, like higher red meat consumption (Saman, 2012). Two-third of patients are presented with advanced disease, a combined modality treatment with surgery, radiation therapy and chemotherapy is current standard of care (Forastiere et al., 2001). Surgery can be performed if complete tumor resection is possible (Bonner et al., 2006), however the majority of patients with advanced stage HNSCC are inoperable. The most frequent treatment is to combine chemotherapeutic agents with radiation (Pan et al., 2009). Although concurrent chemo-radiation protocols are effective in treating HNSCC, treatment outcomes vary considerably and cytotoxic side effects are significant (Yin et

al., 2011). In addition, tumor control and survival are still unsatisfactory. Even those who have achieved complete remission have a reported local recurrences incidence of 50% to 60%, and distant metastases develop in 20% to 30% of cases, with the 5-year overall survival rate less than 50% (Sahu and Grandis, 2011).

Recent studies have focused on the use of novel molecule-targeting agents as they have non-overlapping side effects and can be incorporated with existing treatment modality of HNSCC to improve outcome. Targeting epidermal growth factor receptor (EGFR) becomes a rational approach for HNSCC treatment since higher expression of EGFR has been associated with resistance to radiation and/or chemotherapy (Bonner et al., 2006; Vermorken et al., 2008). Cetuximab, a monoclonal antibody against EGFR, is an FDA-approved targeted agent for the treatment of advanced HNSCC (Kundu and Nestor, 2012; Vincenzi et al., 2010). Combination of cetuximab and radiation improves the overall survival in patients with locally advanced HNSCC, compared to radiation alone (49 months versus 29.3 months, P=0.03) (Bonner et al., 2006; 2010). In order to provide personalized medicine and continue to improve outcome, other novel targeting strategies are needed. In the past 5 years, antiangiogenic therapies have seen a rapid ascent into mainstream clinical practice. Since angiogenesis is a hallmark of advanced

and metastatic cancers, combining anti-angiogenic agents and radiation seems to be feasible, and warrants further investigation.

The Role of Angiogenesis in Tumor Growth and Metastasis

Angiogenesis is defined as the process of forming new blood vessels to support tissue growth. It involves endothelial cell differentiation, proliferation, migration and cord formation, which lead to tubulogenesis to form vessels (Rahimi, 2006). Four decades ago, Judah Folkman was the first to demonstrate that angiogenesis is important for the growth and survival of tumor cells (Folkman, 1971). The relationship between angiogenesis and tumor growth suggests that both tumor cells and their supporting endothelial cells are potential targets for cell killing and should be considered when planning cancer treatment (Menard and Camphausen, 2002). Also, solid tumors will not grow larger than 2 to 3 mm in diameter in the absence of new blood vessels, and require angiogenesis to metastasize (Folkman, 1995). Vascular supply is an essential component of the progressive growth of solid tumors because cells in solid tumors, must receive oxygen and other nutrients to grow (Folkman, 1976). The "tumor cord" model implied that hypoxic cells exist in a state of oxygen and nutrient starvation at the limits of the diffusion range of oxygen, and it was hypothesized that tumor cells could proliferate and

grow only if they were close to a supply of oxygen from tumor stroma. To increase in size beyond this passive diffusion-limited state, the growing tumor mass must acquire new blood vessels. A switch to the angiogenic phenotype allows the tumor to expand rapidly. This so-called "angiogenic switch" (Hanahan and Folkman, 1996) is regulated by environmental factors and by genetic alterations that act to either up-regulate proangiogenic factors (*i.e.,* VEGF and bFGF) and transforming growth factors (TGF-α and TGF-β) and/or downregulate inhibitors of angiogenesis, *i.e.*, angiostatin, endostatin, thrombospondin, and IFN-α (Los, 2011). Meanwhile, secretion of proteolytic enzymes, including matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), can break down extracellular matrix and basement membranes and allow endothelial cells to migrate and organize themselves into pericyte supported tubules, eventually, tumor cells metastasize out (von Tell et al., 2006). A key feature of these new tumor vessels is that they are structurally abnormal and differ in their behavior from normal blood vessels. These incomplete endothelial lining and interrupted basement membranes result in an increased vascular permeability with extravasation of blood plasma and of red blood cells expanding the interstitial fluid space and drastically increasing the hydrostatic pressure (interstitial fluid pressure, IFP) in the tumor interstitium (Vaupel, 2004). Such "leaky" and inefficient tumor vessels deliver less blood, oxygen, nutrients, and ultimately

anticancer drugs to the tumor, increasing hypoxic conditions and thereby keeping the angiogenesis cascade continuously active (Bergers and Benjamin, 2003).

Hypoxic condition ($pO_2 < 2.5$ mmHg) can lead to elevated activity of DNA-repair enzymes and resistance-related proteins, increased transcription of growth factors, and genomic changes (genomic instability leading to clonal heterogeneity and selection of resistant clonal variants, like cancer stem cells) (Vaupel, 2004). This is the most potent stimulus for induction of VEGF, which occurs by activation of Src kinase (Park et al., 2012). Src kinase activation leads to an increase in HIF-1α and consequent upregulation of VEGF expression (Dal Monte et al., 2011). Other growth factors stimulating VEGF production include insulin-like growth factor (IGF)-I and -II, EGF, and PDGFs. There are some signaling pathways related to the up-regulation of VEGF, like PI3K/AKT, RAS/MAPK and STAT pathways (Jasinghe et al., 2008; Wong et al., 2009; Zhou et al., 2009). Although VEGF causes a large increase in blood vessel formation, these vessels are immature, tortuous and leaky. The formation of thicker, more stable vessels requires encapsulation by pericytes that is driven by PDGFR-β signaling. In other words, PDGFR-β can support perivascular cells to maintain tumor vasculature formation (Albert et al., 2006). Low perfusion rates and hypoxia may then coexist with high nonfunctional vascular density, creating hypoxic regions. In these regions of hypoxia, endothelial cells

may up-regulate survival factors to maintain their integrity and prevent apoptosis (Eberhard et al., 2000). Thus, so-called "angiogenic hot spots" or localized regions of intense angiogenesis may be created and may be associated with failure of radiotherapy (Koukourakis et al., 2001).

Vascular Endothelial Growth Factor (VEGF) and Its Receptors: The Role in HNSCC

VEGF plays a central role in the formation of new blood vessels and its importance in HNSCC has been well established (Moriyama et al., 1997). The VEGF family of proteins consists of seven ligands, including VEGF A-E and placenta growth factor (PLGF) 1 and 2 (Ferrara et al., 2003). PLGF, VEGF-A and VEGF-B are known to bind VEGFR-1. VEGF-A, VEGF-C and VEGF-D are known to bind VEGFR-2 (Dorsey and Agulnik, 2013; Tammela et al., 2005). VEGF-C and VEGF-D also bind to VEGFR-3, which is expressed by lymphatic endothelial cells and hematopoietic progenitor cells (Achen et al., 2006; Jussila and Alitalo, 2002). VEGFR-1/FLT-1 (fms-like tyrosine kinase) and VEGFR-2/KDR/FLK-1 (fetal liver kinase) are primarily involved in angiogenesis. Previous reports show that among VEGF family proteins, VEGF-A is the most common and can bind to two receptor tyrosine kinases, VEGFR-1 and VEGFR-2,

promoting endothelial cell differentiation, migration, survival and induction of matrix metalloproteinase (MMPs) (Jain, 2005; Rahimi, 2006). VEGFR-1 is more involved in the development of the vascular system during angiogenesis. VEGFR-2 is the predominant mediator of the angiogenic functions attributed to VEGF that exerts its mitogenic, chemotactic, and vascular permeabilizing effects on endothelial cells (Christopoulos et al., 2011). It also activates signaling pathways such as PI3K/AKT and Ras/MAPK pathways to help with endothelial cell proliferation and survival (Ferrara et al., 2003). Meanwhile, tumor cells and stromal cells, like endothelial cells and fibroblasts, can produce VEGF. Through a paracrine loop, tumor cell VEGF can increase endothelial cell survival (Harmey and Bouchier-Hayes, 2002). Since VEGF and PDGF receptors, as well as their ligands, are highly expressed in HNSCC, over-expression of PDGF enhances tumor formation by stimulating VEGF expression in neovessels and by attracting vessel-associated pericytes (Guo et al., 2003). Dual inhibition of VEGF and PDGF can markedly decrease angiogenesis and inhibit tumor growth *in vitro* and *in vivo* (Choong et al., 2010; Erber et al., 2004). Therefore, these could be good targets to inhibit angiogenesis for the treatment of HNSCC.

Signal Transducer and Activator of Transcription 3 (STAT3): The Role in HNSCC

STAT3 is a multi-fuctional oncogenic transcription factor, present in a number of different cancer cells including head and neck cancers (Buettner et al., 2002; Leong et al., 2003; Yin et al., 2010). It is activated by tyrosine phosphorylation via upstream receptor that binds to growth factors such as EGF, VEGF, PDGF and interleukin-6 (IL-6) (Garg et al., 2005). Approximately 80% of HNSCC exhibit up-regulation of STAT3 expression, which implies it may mediate radio-resistance and chemo-resistance (Greten et al., 2002; Real et at., 2002).

Constitutive activation of STAT3 in HNSCC is caused by diverse signal transduction pathways. The frequent activation of TGF-α/EGFR and IL-6/gp130/Jak can upregulate STAT3 expression in HNSCC (Sriuranpong et al., 2003). Src also plays causative roles in STAT3 upregulation. Tobacco and EBV infection activate STAT3 in oral keratinocyte and nasopharyngeal epithelial cells. STAT3 up-regulates the levels of anti-apoptotic proteins cyclinD1 and c-myc, which result in abnormal proliferation (Masuda et al., 2002). Other anti-apoptotic proteins, like Bcl-2, Bcl-XL and Survivin, are also targets of STAT3. Overexpressions of these proteins promote cell growth and increase chemoradiation resistance. Moreover, it is proposed that these ani-apoptotic

proteins protect DNA-damaged cancer stem cells from elimination by apoptosis and thereby allow them to expand clonally (Gerl and Vaux, 2005; Yu and Jove, 2004). In tumor cells, STAT3 is a positive modulator of VEGF production and secretion, in turn; VEGF activates STAT3 in endothelial cells, which enhance endothelial cell migration, vessel formation and metastasis. As for innate and adoptive immune responses, STAT3 is a negative modulator. Tumor cells expressing constitutively active STAT3 decrease markedly the level of inflammatory cytokines, like TNF-α, IFN-γ, RANTES and IP-10, therefore inhibit both acute and adoptive immune responses (Jewett et al., 2006; Wang et al., 2004). Furthermore, these tumor cells significantly increase the level of immunosuppressive cytokines and growth factors like VEGF and IL-10, which inhibit the functions of dendritic cells, natural killer cells and cytotoxic T-lymphocytes. As a result, tumor cells with constitutive STAT3 activation develop the state of "immune evasion" (Jewett et al., 2006). Recent findings also suggest that STAT3 is involved in the process of epithelial-to-mesenchymal transition (EMT), thus, tumor cells acquire the ability to migrate and metastasize (Christofori, 2006; Thiery, 2002). Taken together, STAT3 orchestrates HNSCC tumor development and progression (Masuda et al., 2010). (Figure 1)

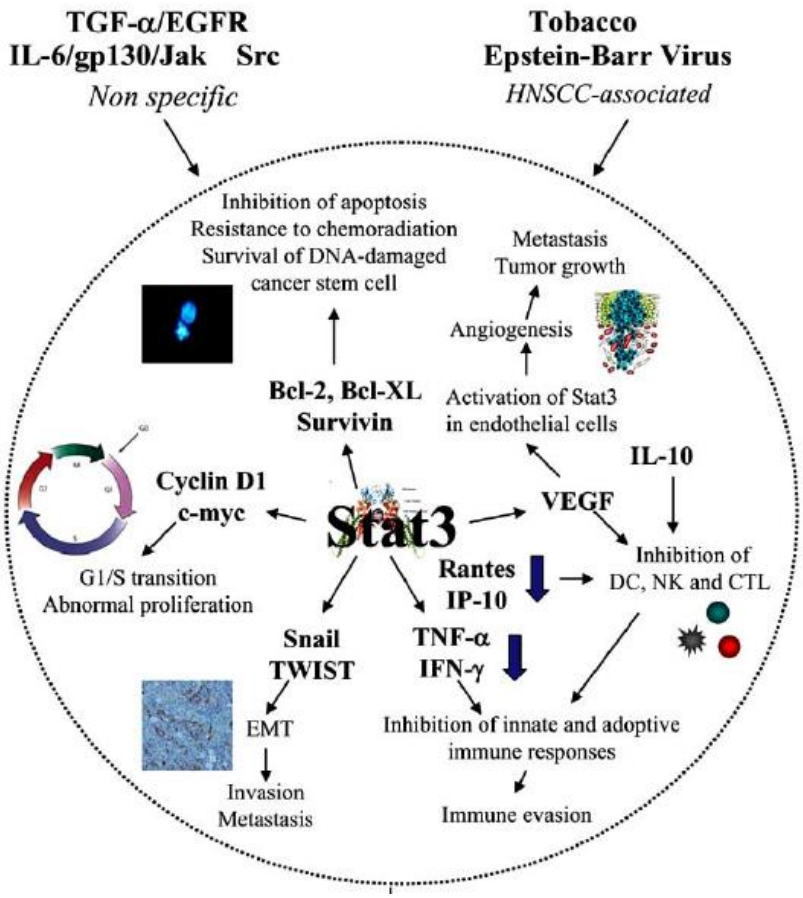

Figure 1. A proposed mechanism by which HNSCC is addicted to STAT3. Constitutive activation of STAT3 in HNSCC is caused by diverse signal transduction pathways, therefore, STAT3 is like the Achilles' heel of HNSCC and can orchestrate tumor development and progression. Current Cancer Drug Targets Masauda M. 2010, 10, 117-126.

Radiation and Hypoxia

Radiation-induced DNA double strand breaks trigger cell cycle arrest and cell death by apoptosis and/or necrosis. Oxygen is known to be a potent radiosensitizer that can promote reactive oxygen species (ROS)/free radicals production, essential for the induction of radiation-induced DNA damage (Karar and Maity, 2009). As tumors grow, the microenvironment lacks an adequate blood supply, leading to regions that are underperfused and poorly oxygenated or hypoxic (Yeom et al., 2011). This can lead to radiation resistance as a tumor microenvironment in oxygen deficit can not facilitate radiation-induced DNA damage. Hypoxic tumor cells are particularly known to up-regulate hypoxia-inducing factor 1α (HIF-1α), a key transcription factor which increases the expression of VEGF (Kung et al., 2000). After radiation exposure, the induction of a variety of transcription factors can activate transcription of growth factors, cytokines and cell cycle-related genes involved in multiple pathways and affect tumor cell survival or alter tumor cell proliferation. As for angiogenesis, radiation exposure can result in activation of EGFR which can activate PI3K/AKT and STAT3 pathways, and upregulate VEGF production (Bowers et al., 2001). The release of angiogenic growth factors like VEGF and FGF have been recognized as part of the radiogenic response of epithelial tumors (Gorski et al., 1999). Protection of tumor vessels by high VEGF levels could

thereby contribute to the radio-resistance of tumors (Brieger et al., 2007). It has also been shown that Hsp90, EGFR, VEGF and AKT are known to play a role in radiation resistance (Sheridan et al., 1997; Tanno et al., 2004). Radiation therapy itself contributes to radioresistance by upregulating angiogenic and pro-survival factors, like Bcl-2, Bcl-xL and Survivin (Ho et al., 2010; Khan et al., 2010). The increased tumor cell proliferation that is often seen after radiation may be the result of up-regulated angiogenic pathways (Horsman and Siemann, 2006; Timke et al., 2008). This may lead to factors contributing to radiation resistance such as increased interstitial fluid pressure and vascular permeability, decreased tumor perfusion, increased oxygen consumption, increased hypoxic microenvironment, and up-regulated survival pathways, which makes radiation less effective (Jain, 2005).

Antiangiogenic Interactions and Radiation

Antiangiogenic agents with radiation have been tested in experimental conditions with various tumor models, tumor host strains, starting tumor size, final tumor volume measured, and dosing and scheduling (Hendry, 1999). Tumor size can affect oxygen tension, nutrient supply, and pH, which are all factors in determining radiation response (Horsman and Siemann, 2006). As tumor size increases, oxygen tension and pH decrease

because of a greater demand for oxygen and nutrients, and glycolysis dominates, leading to acidosis (Vaupel, 2004). A previous study also showed that radiation dose required to achieve the same biologic effect is around 3 times higher in the absence of oxygen than in its presence, the so-called "oxygen enhancement effect" (Gray et al., 1953). Antiangiogenic therapy produces a specific "vascular normalization window", a break when function, structure of tumor blood vessels and microenvironment temporarily become normalized (Jain, 2005). Since tumor growth and angiogenesis are part of a codependent cycle and antivascular treatments can break this cycle and prevent revascularization after radiation (Wachsberger et al., 2003), the potential function behind this, is to decrease interstitial fluid pressure (IFP) in tumor tissues and increase blood perfusion, so that antitumor drugs can easily penetrate into the tumors. Additionally, it will temporarily overcome hypoxia, improve oxygenation to produce more free radicals, result in more DNA damage, apoptotic cell death and increase the sensitivity to radiotherapy (Tong et al., 2004). Therefore, the alternation of radiotherapy and short term antiangiogenic therapy is what produces this seemingly paradoxical effect of antiangiogenic therapy via vascular normalization. The concurrent administration of radiotherapy and contiguous antiangiogenic therapy will not produce a decrease in IFP and increased blood perfusion.

Central Hypothesis

The central hypothesis of our project is that, Linifanib (ABT-869), a VEGFR/PDGFR multi-receptor tyrosine kinase inhibitor, can induce an antitumor effect and radiosensitize HNSCC cells via inhibition of STAT3 signaling pathway and augmenting DNA double strand break in tumor cells.

Significance

Angiogenesis plays an important role in the pathogenesis of HNSCC. VEGF and its receptors are expressed in most cases of HNSCC, and multiple preclinical studies have shown that these markers are associated with tumor progression, changes in microvessel density and development of lymph node metastasis (Hicklin and Ellis, 2005). Previous animal studies demonstrated that inhibition of VEGF markedly decreases angiogenesis and tumor growth (Kim, 1993). STAT3 is a potential modulator of VEGF expression and regulates cell differentiation, cell-cycle progression, angiogenesis, metastasis and apoptosis (Garg et al., 2005). The mechanism of Linifanib (ABT-869), a VEGF/PDGF receptor tyrosine kinase inhibitor, combined with radiation therapy as a radiosensitizer on STAT3 signaling pathway and DNA damage response has not yet been identified in HNSCC. Our study's contribution is significant; because, for the first time, it defines the

mechanism for the role of ABT-869 in the regulation of STAT3 pathway in HNSCC, it will be expected to lead to further research on STAT3 signaling mechanism in pharmacology. In addition, the positive effects of ABT-869 have been observed in the treatment of leukemia and other solid tumors, such as breast, liver, lung and colorectal cancers. Identification of mechanisms underlying ABT-869 combined with radiation suppression of STAT3 signaling pathway could provide an effective therapeutic for HNSCC treatment in the future.

CHAPTER TWO

LINIFANIB (ABT-869) ENHANCES RADIOSENSITIVITY OF HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS

By

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This paper has been published by Oral Oncology. 49:591-597, 2013.

Abstract

Novel targeted therapeutic strategies to overcome radio-resistance of cancer cells traditionally treated with radiation may improve patient survival with the added benefit of reduced systemic toxicity. Herein, we tested the feasibility of Linifanib (ABT-869), a multi-receptor tyrosine kinase inhibitor of members of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) receptor families, on radio-sensitization of head and neck squamous cell carcinoma (HNSCC). UMSCC-22A and UMSCC-22B cells were treated with Linifanib and γ-radiation response was determined as well. Cell viability, cytotoxicity, apoptosis induction and cell cycle distribution were examined by MTT assay, colony formation assay and flow cytometry. In addition, expression of STAT3 and downstream signaling proteins were assessed using western immunoblotting. To evaluate DNA double strand break γH2AX was used as a marker. Treatment with Linifanib resulted in cell growth inhibition, G2/M cell cycle arrest, induction of cell death via apoptosis, reduced phosphorylation of STAT3, which has been linked to radio-resistance, lower expression of cyclin D1, survivin, increased PARP cleavage and γH2AX expression. In addition, Linifanib overcame the radio-resistance of the cell lines and significantly enhanced radiation-induced cytotoxicity ($p < 0.05$). These data suggest the possibility of combining targeted therapeutic such as Linifanib with

radiation to enhance inhibition of cell growth and apoptosis in HNSCC cells. Thus, it may provide a novel therapeutic strategy and improve efficacy of radiation against HNSCC in the future.

Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the most common epithelial malignancy arising in the upper aerodigestive tract, which includes cancers of the oral cavity, oropharynx, hypopharynx, pharynx and larynx. It is the sixth most common cancer worldwide, with approximately 600,000 new cases diagnosed each year (Jemal et al., 2009). Despite advancements in therapeutic regimens, up to 50% of HNSCC patients will experience treatment failure, patients who have frequent recurrence, the median survival rate will limit less than 1 year (Cooper et al., 2004). The standard treatment for loco-regional disease involves surgery and/or radiotherapy in either the neo- or adjuvant setting. Concurrent chemoradiation is frequently used as primary treatment for patients with advance-stage disease, but only a portion of patients have durable responses to cisplatin-based chemoradiation. In addition, cisplatin has a number of side-effects that can limit its use (Bernier et al., 2004; de Castro et al., 2007).

Targeted biological therapies that selectively interfere with cancer cell growth signals may improve patients' survival by enhancing the effects of radiation, with the added benefit of reduced systemic toxicity (Yin et al., 2011). Based on retrospective cohort study, overexpression of epidermal growth factor receptor (EGFR) correlates with worse clinical outcome, making it a logical therapeutic target (Agra and Carvalho, 2008). However, the majority of these tumors fail to respond to EGFR inhibitors. Presence of EGFR variant III, overactivation of the Ras/MAPK, STAT3 and PI3-K/mTOR pathways independent from EGFR by other stimuli such as hypoxia-inducible factor-1α (HIF-1α), which upregulates vascular endothelial growth factor (VEGF) expression, are potential reasons for response failure (Matta and Ralhan, 2009).

Signal transducer and activator of transcription 3 (STAT3), an oncogenic transcription factor, is present in a number of different cancer cells including head and neck cancers (Buettner et al., 2002; Leong et al., 2003; Yin et al., 2010). It is activated by tyrosine phosphorylation via upstream receptor that binds to growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and interleukin-6 (Garg et al., 2005). It is also a potential modulator of VEGF expression and regulates a variety of critical functions, including cell differentiation, cell-cycle progression, angiogenesis, metastasis and apoptosis (Sternberg

and Licht, 2005). Approximately 80% of HNSCC exhibit up-regulation of STAT3 expression, which theoretically mediates radio-resistance and chemo-resistance as demonstrated in pancreatic and breast cancer studies (Greten et al., 2002; Real et al., 2002). Therefore, inhibition of STAT3 may render tumor cells growth arrest and/or apoptosis. In addition, it has been shown that STAT3 blockade in tumor cells resulting to increased expression of proinflammatory chemokines and cytokines, which led to subsequent activation of innate and adaptive anti-tumor immunity (Wang et al., 2004).

Linifanib (ABT-869) is a novel ATP-competitive receptor tyrosine kinase inhibitor in the vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) receptor families. It is under active clinical development primarily in solid tumors. Previous studies had shown that Linifanib can inhibit PI3K/AKT, RAS/MAPK and STAT pathway in acute myeloid leukemia (AML) (Wong et al., 2009; zhou et al., 2009), and in combination with mTOR inhibitor can inhibit VEGF expression in several types of cancers (Jasinghe et al., 2008; Semenza, 2003).

In search for novel targeted therapeutic strategies to overcome radio-resistance of cancer cells, we investigated the role of ABT-869 on radio-sensitization in HNSCC. To the best of our knowledge, the effect of ABT-869 on radio-sensitization of head and neck cancer cells has not yet been reported. Furthermore, this study aimed to examine whether

STAT3 signaling pathway could be inhibited by ABT-869, as a new therapeutic strategy to reduce radio-resistance of HNSCC. We found that ABT-869 enhances the radiation-induced inhibition of proliferation and apoptosis in two HNSCC cell lines. In addition, Linifanib reduces phosphorylation of STAT3, which has been linked to radio-resistance. Therefore, Linifanib may offer a new therapeutic strategy to reduce radio-resistance of HNSCC.

Materials and Methods

Cell Culture and Reagents

Radio-resistant HNSCC cell lines were used for this study. UMSCC-22A (SCC-22A) and UMSCC-22B (SCC-22B) originated from the same patient's hypopharynx, but were derived from primary tumor and metastatic cervical lymph node, respectively. The original tumor grade for SCC-22A was T2N1M0, for SCC-22B was T2N1M0 as well (Zhao et al., 2011). Linifanib (ABT-869) was kindly provided by Abbott Laboratories, Abbott Park, IL.

Cell Viability Assay

The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and streptomycin and 1% nonessential amino acids. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 ^oC. Both cells were seeded in triplicate at 8000 cells/well in 96-well plates. After growth overnight, the cells were then treated for 48 h and 72h at 37°C with varying doses (0 [control], 5, 10, 20 and 40 μM) of ABT-869. Cell viability was assessed with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent, Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The plates were read on a microplate reader (Bio-Rad Model 3550). The similar outcomes were observed, in a dose- and time-dependent manner, the shown result was for 48 h. The IC50 values (50% growth inhibition) were determined for each cell line and displayed as mean ± SEM from at least 3 experiments.

Clonogenic Survival Assay

Cells were exposed to IC25 & IC50 of ABT-869 for 12 hours before-irradiation with a dose of 2, 4 or 8 gray (Gy) at a dose rate of 1.678 Gy/min, using a ^{60}Co source (Eldorado machine, Atomic Energy of Canada Ltd, Ottawa, Canada). Culture media was replaced with fresh media the next day. Colonies were stained with crystal violet after 12-

14 days, and the number of colonies containing at least 50 cells was counted. Each experiment was done in triplicate.

Cell Cycle Analysis

Cells ($5x10^5$) were exposed to ABT-869 (20 μM) or radiation (4 Gy). After 24 & 48 hrs cells were collected, fixed with 75% ethanol, then treated with propidium iodide (PI) and ribonuclease staining buffer (BD Pharmingen) according to the manufacturer's protocol. Samples were analyzed by flow cytometry (FACSCalibur™; Becton Dickinson, Franklin Lakes, NJ). For radio-sensitization experiments, cells were treated with ABT-869, irradiated (4 Gy) and analyzed after 24 & 48 hrs.

Analysis of Apoptosis

Cell death by apoptosis was evaluated by trypan blue dye exclusion using light microscopy (Olympus IX70, Olympus America Inc., PA) and Annexin-V & PI apoptosis detection kit (BD Biosciences, San Jose, CA). Briefly, cells were treated the same as for the cell cycle analysis. After 24 hrs cells were stained with trypan blue (Thermo Scientific) for 2 hrs and then tested under the light microscope (100X & 400X). Also

treated cells were stained with FITC-conjugated Annexin-V in the presence of PI analyzed by flow cytometry. Annexin V^+ cells were scored as apoptotic cells.

Western Immunoblotting

The treatment protocol used was the same as for the cell cycle analysis. Twenty-four hours after radiation treatment the cells were harvested, washed and resuspended in NP-40 lysis buffer. Whole cell lysates (40 μg) were separated through 10-12% sodium dodecyl sulfate (SDS) polyacrylamide gels under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). The membranes were blocked and incubated with the following antibodies; Phosphor-STAT3, STAT3 and cPARP (Cell Signaling Technologies, Beverly, MA), cyclin D1, Bcl-2, Bcl-xL, Mcl-1, AIF (Santa Cruz Biotechnology, Santa Cruz, CA), Survivin (Novus, Littleton, CO), γH2AX (EMD Millipore, Billerica, MA) and HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technologies, Beverly, MA). Data were normalized to corresponding values of GAPDH densitometry.

Statistical Analysis

Each assay was performed at least three times as independent experiments.

Statistical analyses were done with two-tailed Student's t-test and performed with Prism 5.01 software (GraphPad Software, San Diego, CA). A *p*-value of <0.05 was considered as statistically significant.

Results

Effect of ABT-869 on Cell Growth Inhibition

To evaluate the cytotoxic effect of ABT-869 on SCC-22A and SCC-22B cell lines, MTT assay was used. ABT-869 induced a significant growth inhibition in a dose-dependent manner (Figure 2). IC50 for these cell lines were 21.2 and 19.4 μM, respectively.

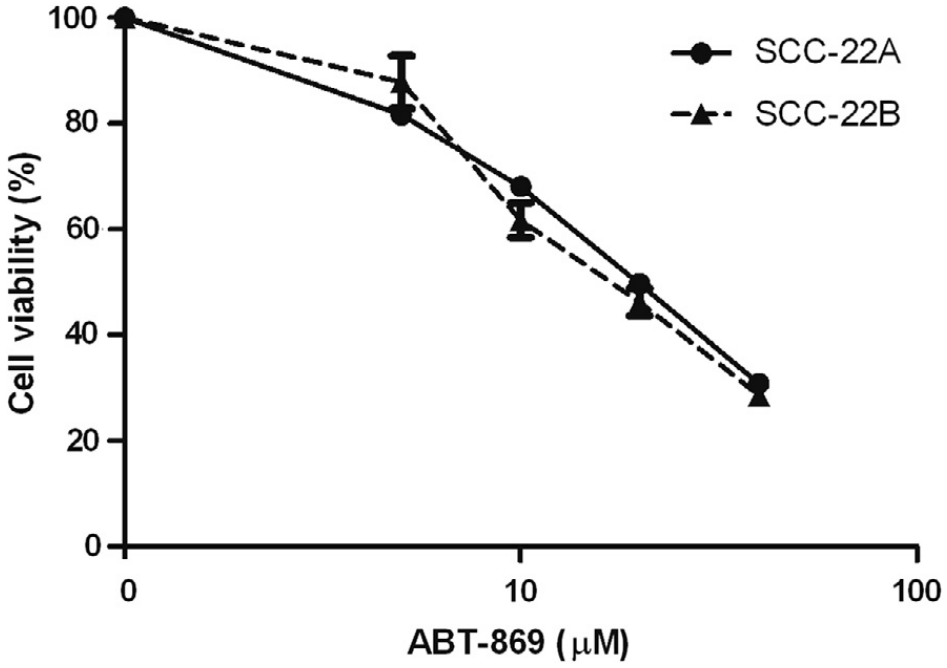

Figure 2. Growth inhibition curve of HNSCC cell lines after ABT-869 treatment. Cells were treated with increasing concentration of ABT-869 for 48 hr. Live cells were quantitated by MTT assay. Data are displayed as mean ± SEM from at least 3 experiments.

ABT-869 Enhances the Antitumor Growth Effect of Radiation

To determine whether ABT-869 enhances radiation-induced cell death in HNSCC cells, the relatively radiation–insensitive (a 50% killing dose is approximately 8 Gy) SCC-22A&B cells were exposed to ABT-869 for 12 hr followed by radiation (2, 4, or 8 Gy). The impact of the single and combination treatments on cell proliferation was then measured by clonogenic cell survival assay. Figure 3 shows that the surviving fraction at 4 and 8 Gy for ABT-869 treated cells was significantly lower than that of untreated cells ($p < 0.05$). This observation suggests that the growth inhibition effect of ABT-869 could overcome radio-resistance and significantly enhance the effect of radiation.

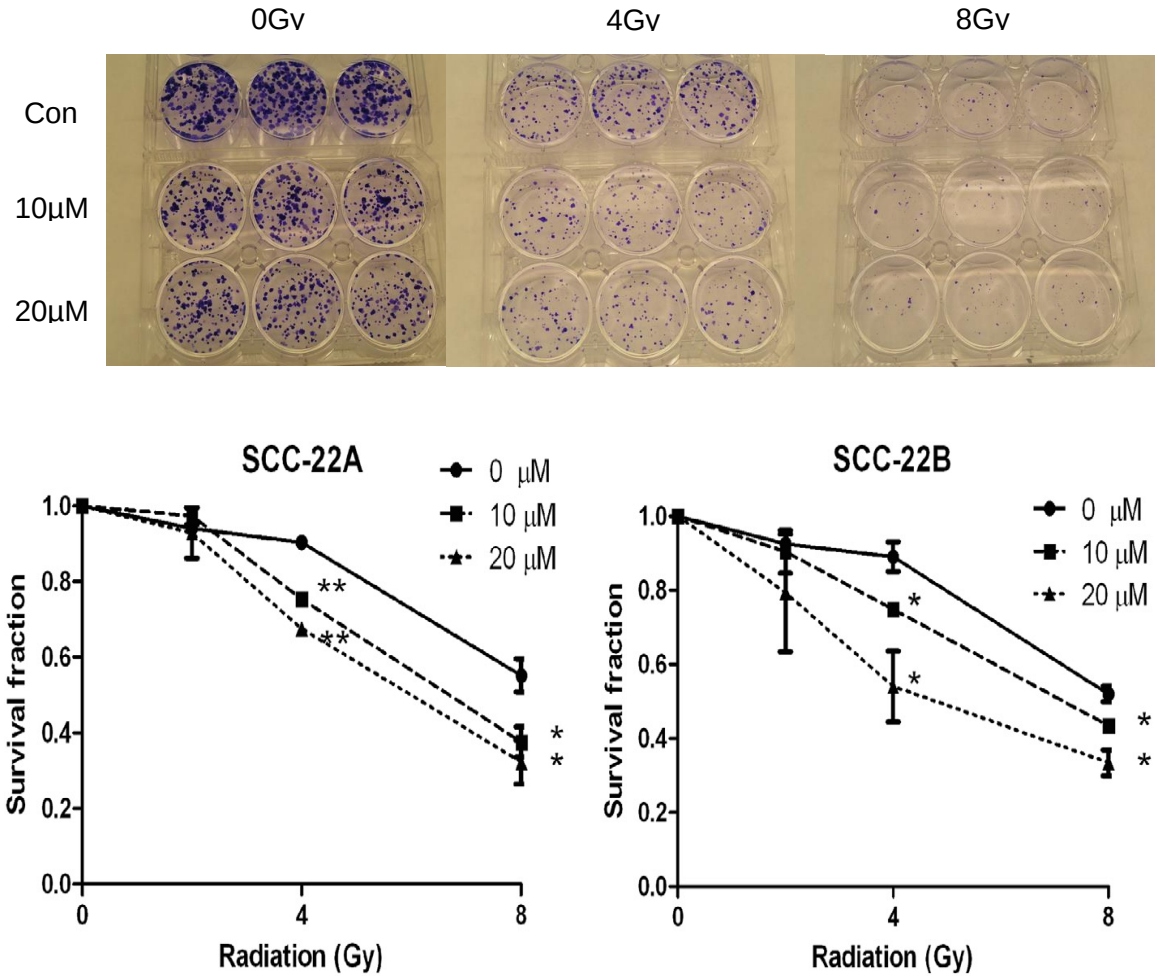

Figure 3. Radio-sensitization effect of ABT-869 on HNSCC cells. SCC-22A & B cells were plated and exposed to ABT-869 for 12 hr followed by single radiation dose of 2, 4, or 8 Gy. Colony formation picture was shown above. Survival fraction was assessed at 12-14 days after irradiation. Data are the mean ± SEM of 3 independent experiments. Asterisks represent significant difference as compared to untreated control group (*p < 0.05, **p < 0.01).

ABT-869 Induces G2/M Cell Cycle Arrest and Increases Sub-G0 Population Alone and Enhances when Combined with Radiation

The observed inhibition of cell growth by ABT-869 could be the result of the induction of cell cycle arrest and/or apoptosis. To examine this, SCC-22A&B cells were treated with ABT-869 for 24 or 48 hr. The percentages of cells were then examined by flow cytometry after PI staining (Figure 4A&B). Compared to control group, we observed significant accumulation in G2/M phase in SCC-22A (42.2% *versus* 23.4% in control) and in SCC-22B cells (24.6% *versus* 17% in control), after ABT-869 treatment, indicating that a higher number of cells were blocked in a more radiosensitive phase of the cell cycle. In addition, ABT-869 treatment increased sub-G0 population in SCC-22A (14.3% *versus* 4.4% and 22.6% *versus* 5.2% in control) and SCC-22B (21.6% *versus* 8.6% and 31.4% *versus* 12% in control) after 24-48 hr, respectively. Interestingly, radiation alone induced only a transient arrest at G2/M phase at 24 hr. In contrast, the combination treatment blocked recovery from radiation-induced cell cycle arrest in SCC-22A and caused higher accumulation of sub-G0 population in SCC-22B cells, compared to radiation alone (48 hr). Next we tested whether ABT-869 enhances the effect of radiation on sub-G0 population. Cells were treated (12 hr) with ABT-869 prior to radiation (4Gy) and collected 24 hr later. As shown in Figure 4C, ABT-869 in

combination with radiation increased sub-G0 population by around 2 fold in both cell lines, compared to radiation alone, confirming that ABT-869 sensitized the cells to irradiation, hence the synergistic effect of the two treatments.

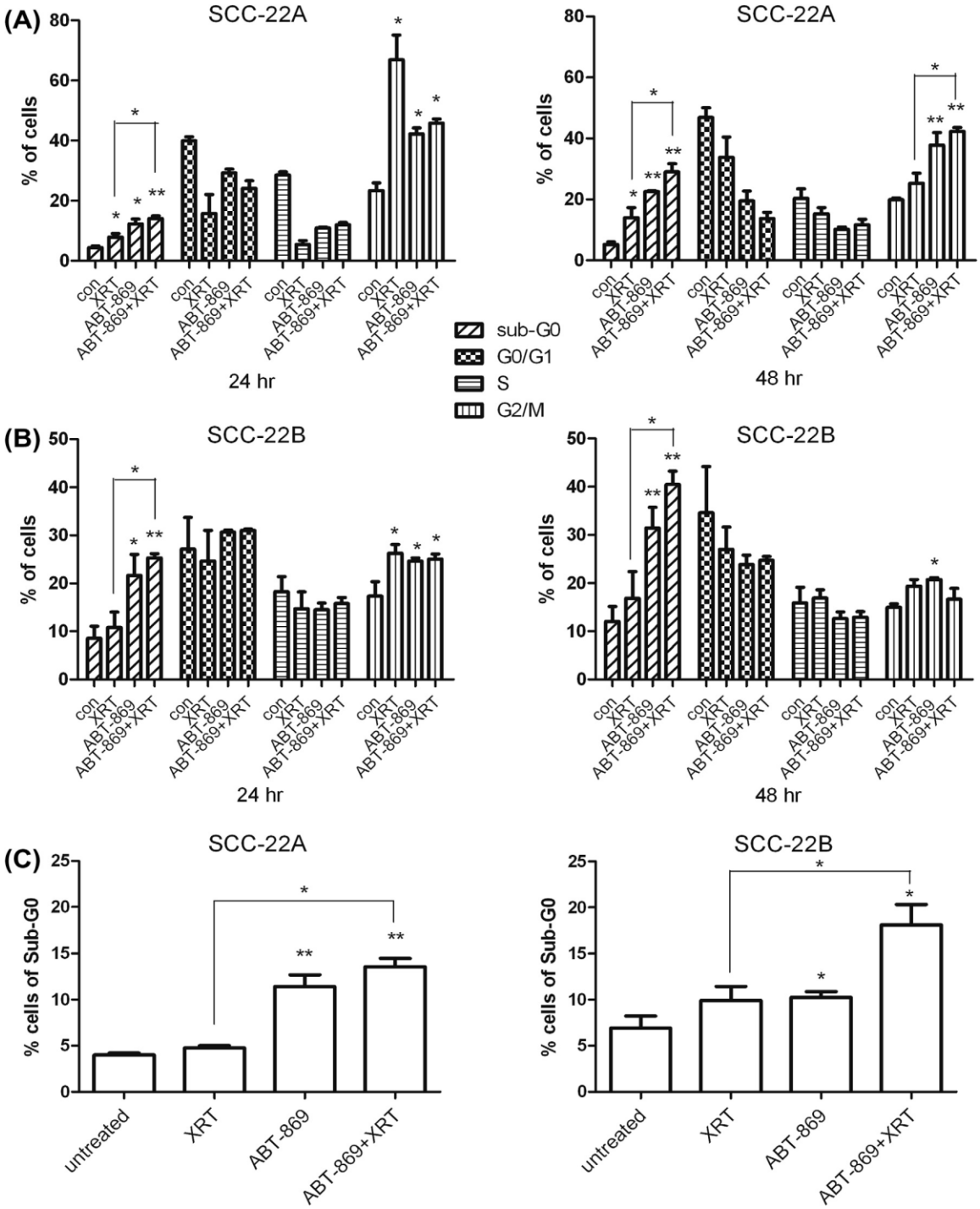

Figure 4. The effects of ABT-869 on cell cycle distribution. (A) SCC-22A and (B) SCC-22B were treated with ABT-869 20 μM combined with radiation and analyzed 24 or 48 hrs later. (C) SCC-22A & B cells were pre-treated with ABT-869 20 μM for 12 hrs, then irradiated at 4 Gy. Cells were harvested 24 hrs later. The percentages of cells were determined by flow cytometry after PI staining. Data are the mean ± SEM of 3 independent experiments. (*$p < 0.05$, **$p < 0.01$)

ABT-869 Induces Cell Death via Apoptosis

To confirm that the observed ABT-869-induced cell growth inhibition is by apoptotic death, cells were treated with either ABT-869, radiation, the combination and stained with trypan blue dye exclusion or Annexin-V and PI. We clearly observed increased trypan blue dye uptake by cells (dead cells) and morphological changes considered dead by apoptosis (cell shrinkage, cytoplasmic blebbing, cytoplasmic condensation and irregular shape) in the combination group compared with untreated, radiation and ABT-869 alone groups (Figure 5A). We then performed Annexin-V and PI staining to confirm and determine apoptotic population changes. ABT-869 treatment increased the apoptotic population by 4.61 and 3.11-fold and by 9.15 and 5.33-fold increase in combination in SCC-22A & B cells, as compared to untreated and radiation alone groups respectively (Figure 5B). Apoptotic cell death after combination treatment was significantly higher ($p< 0.05$-0.01) than that caused by either of the agents alone. This was also consistent with increased sub-G0 population (Figure 4C). These data suggest that apoptosis could be a major contributor in the ABT-869-caused cell growth inhibition and synergistically enhanced the antitumor growth effect of radiation in both cell lines.

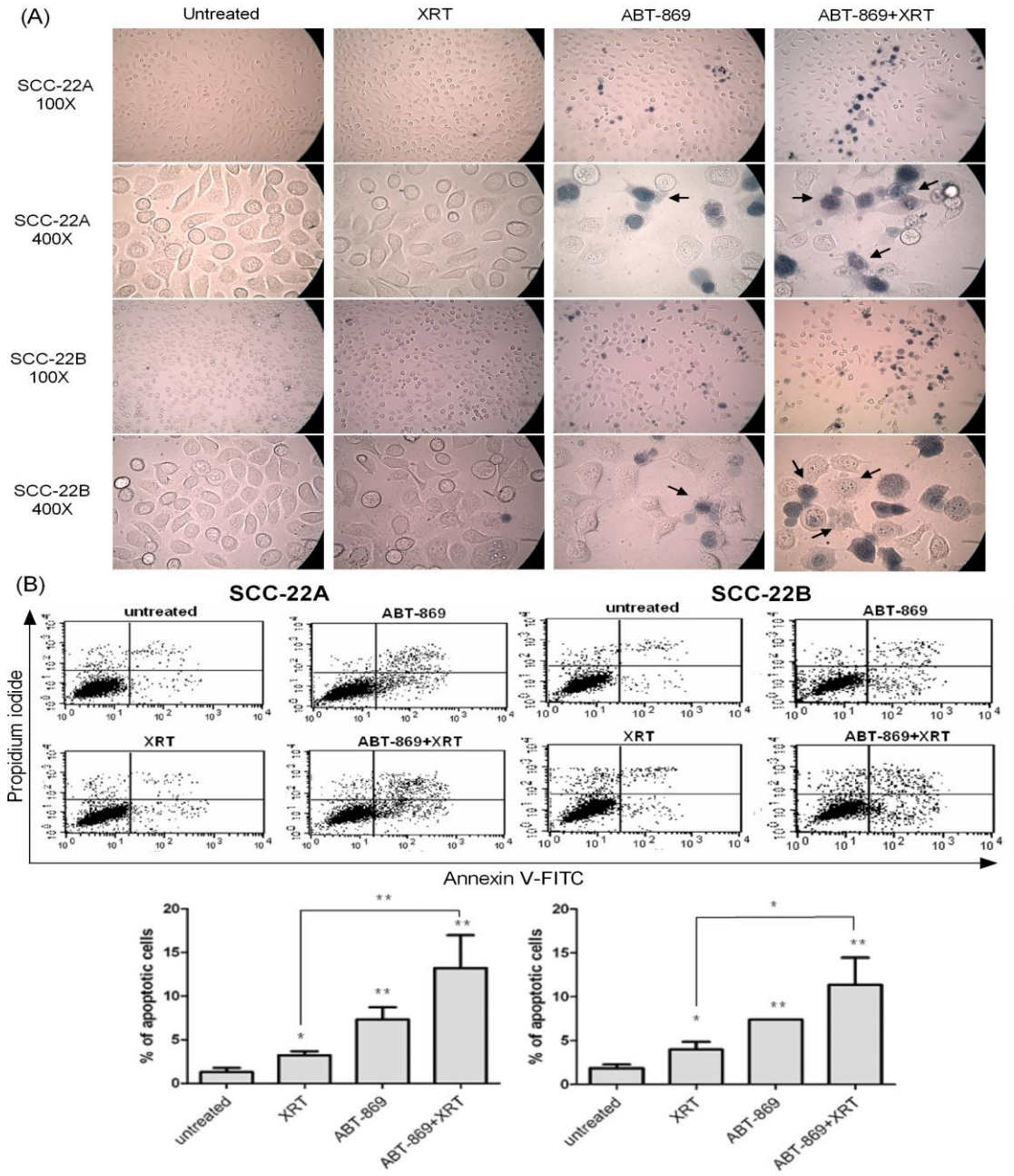

Figure 5. ABT-869 can induce cells to undergo apoptosis. SCC-22A & B cells were pre-treated with ABT-869 20 μM for 12 hrs, or in combination with 4 Gy radiation. Cells were harvested 24 hrs after radiation. (A) Light microscopy (100X & 400X) showed that ABT-869 treated and combination group resulted in morphological changes, decreased cell numbers and more cell death. Trypan blue stain positive cells were considered as dead cells. (B) Annexin V and PI staining were used and Annexin V positive cells were counted as apoptotic cells. Data are the mean ± SEM of 3 independent experiments (*$p < 0.05$, **$p < 0.01$).

Using Stattic as Positive Control to Compare the Apoptotic Effects Induced by ABT-869

Stattic is a well known STAT1 and STAT3 inhibitor in previous literature (Bill et al., 2010), we further used it as positive control to evaluate whether the apoptotic effects caused by Stattic is similar to those by ABT-869. We first determined its IC50 after 48 hrs treatment for both SCC-22A&B was around 6 μM. Cells were treated with either ABT-869 or Stattic IC50 concentration for 24 hrs. Flow cytometry was performed to determine apoptotic population changes like previously described. The data showed that ABT-869 can induce similar amount of cell apoptosis like Stattic. (Figure 6)

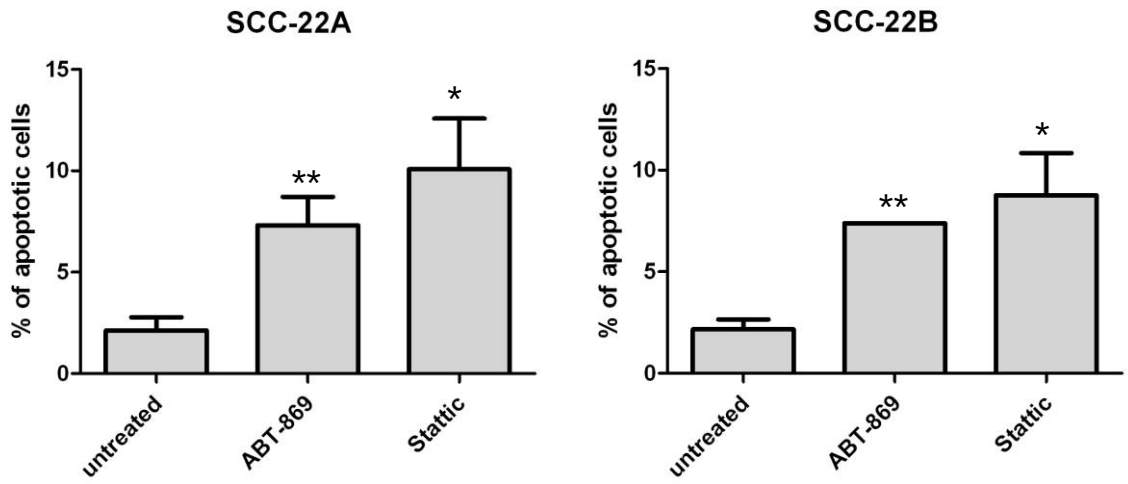

Figure 6. ABT-869 can induce similar apoptotic population changes like Stattic. Cells were treated for 24 hrs. Annexin V and PI staining were used and Annexin V positive cells were counted as apoptotic cells. Data are the mean ± SEM of 3 independent experiments (*$p < 0.05$, **$p < 0.01$).

Combination of ABT-869 and Radiation Inhibits Activation of the STAT3 and Downstream Signaling Pathways

Because STAT3 is critical in regulating the expression of downstream genes involved in apoptosis (Bcl-2, Bcl-xL, Mcl-1, survivin) and proliferation (cyclin D1), which has also been associated with both chemo- and radio-resistance in HNSCC, we examined the phosphorylation level of STAT3 after cells were treated with either ABT-869, radiation alone or in combination by western blot analyses. STAT3 is constitutively activated at high level in these two cell lines. Densitometry analysis demonstrated that combination treatment significantly reduced the level of STAT3 phosphorylation (Figure 7). We next investigated the effect of ABT-869 on STAT3-regulated proteins. A concomitant reduction of expression level of cyclin D1, Bcl-xL, Bcl-2, Mcl-1, survivin and increased level of poly (ADP-ribose) polymerase cleavage (cPARP), a hallmark of apoptotic cell death (Yoo et al., 2004), were observed in both cell lines.

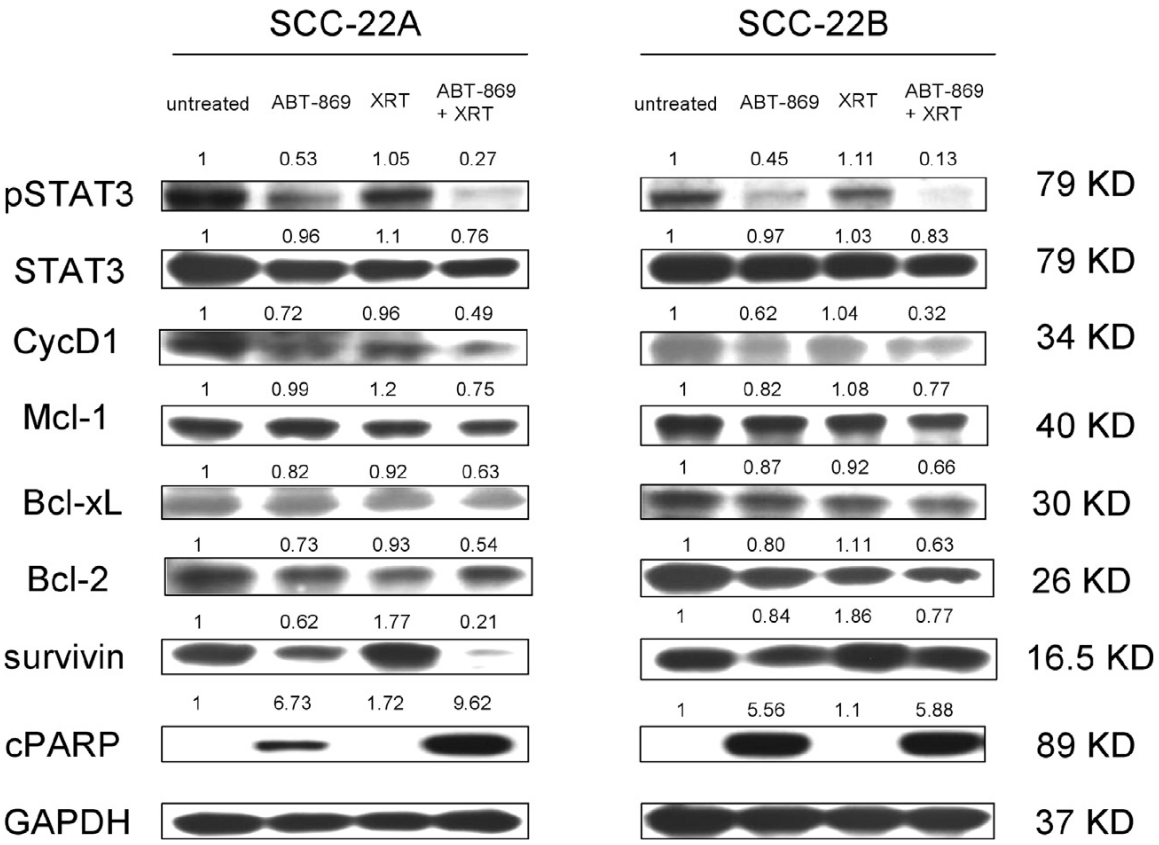

Figure 7. The effects of ABT-869 and radiation on STAT3 and downstream effectors in HNSCC cells. Cells were either treated with 20 μM ABT-869 or 4 Gy for 24 hrs or pre-treated for 12 hrs subsequent radiation. Protein expressions were determined by western blot. GAPDH was used as loading control. Data are the mean ± SEM of 3 independent experiments.

Combination of ABT-869 and Radiation Also Induces Caspase-Independent AIF-mediated Cell Death

To identify if ABT-869 could induce caspase-independent cell death, we further detect the expression of apoptosis inducing factor (AIF) (Figure 8). While AIF was released to cytosol from mitochondria, later on it would translocate to the nucleus and cause DNA fragmentation and cell death. Therefore, increased nuclear AIF expression implies more cell death. We observed that combination of ABT-869 and radiation has higher nuclear AIF and lower cytosolic AIF expression compared to ABT-869 alone on both cell lines. In SCC-22B metastatic cell line, an even more obvious trend was observed compared to primary SCC-22A cell line. Thus, combination of ABT-869 and radiation can also induce caspase-independent AIF-mediated cell death in HNSCC.

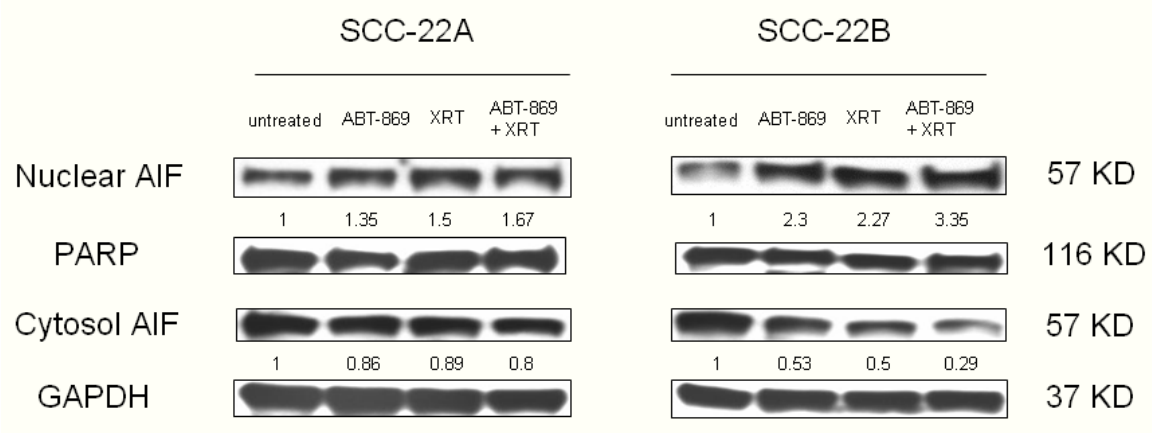

Figure 8. The effects of ABT-869 and radiation on cytosol & nuclear AIF expression in HNSCC cells. Cells were either treated with 20 μM ABT-869 or 4 Gy or combination for 24 hrs. Protein expressions were determined by western blot. PARP was used as nuclear loading control for nuclear AIF. GAPDH was used as cytoplasm loading control for cytosol AIF. Data are the mean ± SEM of 3 independent experiments.

Combination of ABT-869 and Radiation Increases DNA Damage - Double Strand Breaks (DSBs)

We further investigate the effect of ABT-869 on DNA damage response. To assess DNA DSBs, we examined the well documented marker γH2AX expression for DNA double strand breaks (Bonner et al., 2008). We found that ABT-869 alone could induce DNA damage, when combining with radiation resulted in more DNA damage. (Figure 9) These results indicated that, inhibition of STAT3 signaling by ABT-869 could increase DNA double strand breaks and sensitize the HNSCC cells to radiation in a synergistic manner.

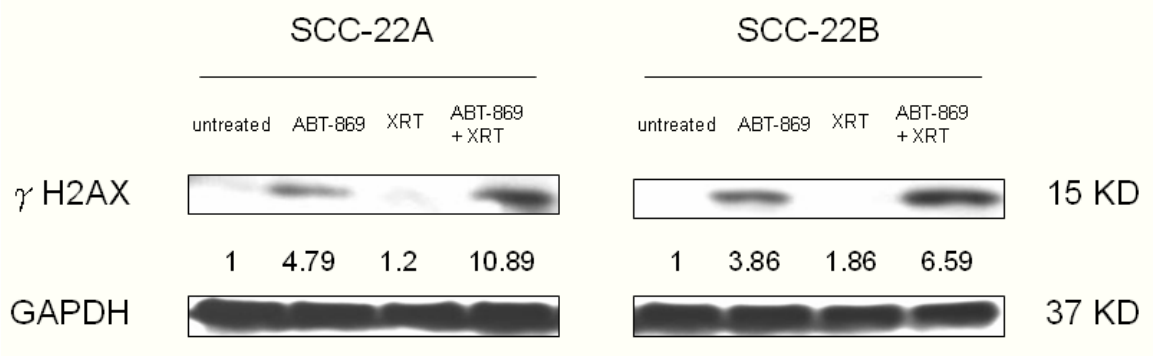

Figure 9. The effects of ABT-869 and radiation on DNA double strand breaks in HNSCC cells. Cells were either treated with 20 μM ABT-869 or 4 Gy or combination for 24 hrs. Protein expressions were determined by western blot. DNA double strand break marker γH2AX was detected. GAPDH was used as loading control. Data are the mean ± SEM of 3 independent experiments.

Discussion

Current radiation and chemotherapy protocols can control HNSCC but many tumors do not respond well. In addition, both chemotherapy and radiotherapy have dose limiting toxicity. Recent studies have focused on the use of novel molecular-targeted agents with limited side effects in an attempt to improve existed treatments of HNSCC. Targeting EGFR becomes a rational approach for HNSCC treatment since higher expression of EGFR has been associated with resistance to radio- and/or chemo-therapy (Bonner et al., 2006; Vermoken et al., 2008). However, such improvement on disease control by EGFR targeting was incremental and novel targeting strategies are needed.

VEGF and its receptors are potential targets for cancer therapy and both are expressed in increased numbers primarily during periods of tumor growth (Brekken et al., 2000). Protection of tumor vessels by VEGF could thereby contribute to the radio-resistance of tumors and high VEGF levels may additionally contribute to blood vessel and tumor cell protection as a cause of radio-resistance (Brieger et al., 2007). Considering the regulatory role of VEGF/PDGF as modulators of tumor growth and response to radiation (Timke et al., 2007), we hypothesized that Linifanib (ABT-869) would overcome radio-resistance of HNSCC cell lines. We demonstrated that ABT-869 augments head and neck cancer cells' susceptibility to the radiation and that the cell

growth inhibition could be achieved at lower radiation dose in combination with ABT-869 in both cell lines compared to either ABT-869 or radiation alone (Figure 3), which may prevent undesired radiation damage. To the best of our knowledge, this work shows for the first time the synergistic effect of ABT-869 and radiation in HNSCC *in vitro*. The mechanism of enhanced cell growth inhibition involves ABT-869-mediated cell cycle arrest in G_2/M phase and apoptosis.

Recent reports also showed that STAT3 can activate downstream molecules (e.g., c-myc, cyclin D1, Bcl family proteins, IAPs and VEGF) in HNSCC, therefore, promote tumor cells proliferation and survival. Constitutive activation of STAT3 suppresses apoptosis, and also has a positive correlation with cyclin D1 expression in laryngeal carcinoma (Masuda et al., 2010). In addition, upregulation of cyclin D1, which is involved in G1 and G2 cell cycle arrest (Michalides et al., 2002; Zhang et al., 2011), has been specifically associated with resistance to anti-EGFR treatment and poor prognosis of HNSCC patients. Therefore, STAT3 and cyclin D1 can be effective targets to control the growth of cancer cells and facilitate their apoptotic death. The activity of STAT3 and cyclin D1 expression were down-regulated after ABT-869 treatment alone and to a greater extent in combination with radiation, which is consistent with observed G2/M cell cycle arrest and capability to enhance the cytotoxicity of radiation. It also has been shown

that radiation enhances STAT3 phosphorylation and increases anti-apoptotic protein expression in several cancers (Ho et al., 2010; Lee et al., 2008). After combination treatment of ABT-869 and radiation we detected an altered/reduced expression of STAT3 downstream effectors, Mcl-1, Bcl-2 and Bcl-xL, which have been shown to influence radio-sensitivity (Masuda et al., 2010; Nix et al., 2005).

Several studies have documented a positive correlation between survivin, a member of the inhibitor of apoptosis protein, tumor aggressiveness and radio-resistance in head and neck cancer cells (Farnebo et al., 2011; Khan et al., 2010; 2012). Zhou et al. showed that survivin is a direct target of STAT3 pathway in an AML cell line (Zhou et al., 2009). Moreover, down-regulation of survivin can arrest cancer cells at G2/M phase and increase caspase-dependent apoptosis (Liu et al., 2010). Our results indicated that radiation-induced survivin expression was significantly down-regulated and the inhibition of cell growth was correlated with significantly increased expression of cleaved PARP, a hallmark of apoptosis, after treatment with ABT-869 alone and in combination with radiation in SCC-22A and to a lesser extent in SCC-22B cells.

In summary, we demonstrated that ABT-869 significantly radio-sensitizes primary and metastatic HNSCC cells (Figure 3) by inducing cell cycle arrest and cell death. However, some differences were observed in ABT-869-induced effects between

primary versus metastatic cell lines such as a) prolonged G2/M cell cycle arrest, b) higher level of survivin down-regulation and c-PARP expression in primary compared to the metastatic cell lines. This can be explained by reports of significantly higher survivin expression in cervical lymph node metastases than in primary HNSCCs, and its negative regulation of G2/M and apoptosis (Marioni et al., 2006; Mehlen and Puisieux, 2006). These results suggest that the combination treatment of ABT-869 and radiation may affect multiple pathways to induce cell death in metastatic cells, such as apoptosis inducing factor (AIF) and endonuclease G (Endo G) mediated caspase-independent apoptosis (Cao et al., 2012; Huerta et al., 2009). AIF can translocate from cytosol to nucleus and cleave DNA, so we further detected the expression of nuclear AIF. The combination of ABT-869 and radiation resulted in higher nuclear AIF and lower cytosolic AIF expression. It implies the combination treatment can also induce caspase-independent cell death; in SCC-22B metastatic cell line, the trend is even more obvious than SCC-22A primary cell line. Our results are also consistent to previous studies, showing that some reagents/compounds can affect on caspase dependent or AIF-mediated capspase independent cell death or both pathways in various cancer types (Artus et al., 2006; Croci et al., 2008; Jeong et al., 2011; Liu et al., 2004; Rashimi et al., 2005; Yu et al., 2012).

Since approximately 80% of HNSCC exhibits up-regulation of STAT3 expression, inhibition of STAT3 may cause tumor cells growth arrest and/or apoptosis. Recent studies showed that, in colon and lung cancer cell lines, DNA damage could induce the expression of IL-6, resulting in the activation of STAT3 signaling pathway. Therefore, by inhibiting STAT3, one can also inhibit DNA damage repair and induce apoptosis in tumor cells (Barry et al., 2010; Yun et al., 2012). We confirmed this relationship by examining the expression of DNA double strand break marker γH2AX. Inhibition of STAT3 signaling by a VEGFR/PDGFR inhibitor, ABT-869, could increase DNA double strand breaks and synergistically sensitize HNSCC cells to radiation.

Taken together, our results serve as proof of principle that a multi-receptor tyrosine kinase inhibitor, such as ABT-869 can be a promising radio-sensitizer and deserve further clinical development in the treatment of HNSCC.

Acknowledgments

We thank Abbott Laboratories for providing Linifanib, Mr. Celso Perez for the excellent technical assistance with radiation experiments and Dr. Lubo Zhang's lab members. This project was funded by Loma Linda University Cancer Center.

CHAPTER THREE

GENERAL DISCUSSION

Targeting Angiogenesis Agents Combined with Radiation on Head and Neck Cancer

Our study demonstrates a VEGFR/PDGFR multi-receptor tyrosine kinase inhibitor, ABT-869, that inhibits angiogenesis can radiosensitize HNSCC cells. An overview of current antiangiogenic agents combined with radiation on HNSCC *in vitro* and *in vivo* studies is given in Table 1. A schematic diagram of antiangiogenic agents is also shown in Figure 10. These studies showed that the combination of antiangiogenic agents with radiation or chemotherapy may improve clinical responses to treat HNSCC patients.

Postulated Mechanisms of Antiangiogenic Therapy and Radiation

The precise mechanism by which angiogenesis inhibition improves clinical outcome is not fully understood yet. On one hand, antiangiogenic agents traditionally are presumed to inhibit tumor vasculature formation, depriving the tumor of necessary

nutrients and oxygen. Studies showed that the excess of EGF, VEGF and PDGF cause poor blood flow in disorganized and leaky tumor vessels, resulting in increased IFP, and poor drug delivery and hypoxia (Wchsberger et al., 2005). Data for head and neck cancer suggests that bevacizumab (VEGF-A monoclonal antibody) as a single agent is not effective *in vitro*, but did have *in vivo* activity in preclinical models. It is therefore believed that the physiological vascular microenvironment is critical for tumor angiogenesis (Hoang et al., 2012; Wachsberger et al., 2003). Since VEGF is a survival factor for endothelial cells via the induction of AKT and other proteins, anti-VEGF agents have been shown to enhance the apoptosis of endothelial cells *in vitro* (Gorski et al., 1999). Given these observations, it has been proposed that anti-VEGF therapy can enhance the ability of radiotherapy to induce destruction of tumor vasculature. There are several possible advantages for the combination of antiangiogenic drugs with radiation to improve cancer treatment. (1) Antiangiogenic agents are directly cytotoxic to endothelial cells and can target VEGF and its receptors, instead of having to access the tumor masses. (2) Decreases in the proportion of hypoxic cells and increased oxygen content enhances oxygen-induced free radical formation resulting more DNA damage to tumor cells. (3) Angiogenesis occurs in certain limited circumstances, like wound healing and ovulation, therefore, antiangiogenic therapies targeting specific receptors on proliferating

tumor endothelium will be safer and reduce normal tissue toxicities (Scappaticci et al., 2002). Since oxygen is a potent radiosensitizer, the combination of ionizing radiation and antiangiogenic agents would be a favorable approach. A recent study also indicated that oxygen levels may actually increase after treatment with antiangiogenic agents and ionizing radiation. Combination of antiangiogenesis and radiation can cause apoptosis of both endothelial cells and tumor cells (Griffin et al., 2002; Horsman and Siemann, 2006). (Figure 11)

Prognostic Factors / Biomarkers

Hypoxia is a characteristic pathophysiological property of locally advanced solid tumors and such areas have been found in a wide range of human malignancies including cancers of the prostate, pancreas, rectum, breast, uterine cervix, brain tumors, malignant melanomas and head & neck cancers. Molecular studies investigating the tissue distribution of HIF-1α and of its target proteins CA-9 and GLUT-1 showed worse outcomes in cases exhibiting an overexpression of these endogenous markers (Evans et al., 2003; Vaupel et al., 2002; 2004). Lactate accumulation is another factor proportional to malignant levels and increased risk of metastases in head and neck cancer, cervical cancer, and colorectal cancer (Brizel et al., 2001; Walenta et al., 2000; 2003).

Overexpression of EGFR is detected in 90 % of all HNSCC tumors, and high levels of this protein expression is associated with decreased survival, radio-resistance, increased rates of distant metastases and recurrence (Dorsey and Agulnik, 2013). In a meta-analysis of 12 studies, including 1002 patients of oral cavity, pharyngeal and laryngeal cancers, higher VEGF expression showed positive association with a 2-fold higher risk of death within 2 years (Kyzas et al., 2005). VEGF plasma levels have been described as potential prognostic and predictive biomarkers as well (Allen et al., 2005; Druzgal et al., 2005). Other factors like VEGFR/KDR, pKDR/KDR, Bax, BcL-xL, BcL-2, cyclooxygenase-2 (COX-2) and survivin, all correlated to clinical outcomes in retrospective clinical studies (Cohen et al., 2009; Farnebo et al., 2011; Seiwert and Cohen, 2008). A recent study also showed that vascular normalization induced by antiangiogenic therapies can stimulate tumor microenvironmental immune response, thus, enhance cancer immunotherapy to kill cancer cells (Huang et al., 2012). An increase in tumor-infiltrating $CD8^+$ T cells could be a potential biomarker for vascular normalization (Huang et al., 2013). Thus, it is critical to validate these potential biomarkers in perspective randomized studies.

Conclusions

Despite recent advances in therapy for head and neck squamous cell carcinoma, chemotherapeutics cytotoxicity is of major concern. Novel therapy with targeted agents is a promising direction. There are some practical points needed to be considered. These agents have a cytostatic function but not curative potential, theoretically should be used in combination with radiation or other chemo-therapies instead of single therapy to achieve appreciable impact on patient survival (Loges et al., 2009). Increasing the dose of antiangiogenic agent or double antiangiogenic agent combination treatment might do harm to normal tissues and destroy vasculature due to rapid/excessive pruning of tumor vessels, leading to hypoxia and poor drug delivery in the tumor (Jain, 2005). Thus, optimal doses and schedules of these reagents tailored to the angiogenic profile of tumors can normalize tumor vasculature and its microenvironment without harming normal tissues. Combining ABT-869 with other chemo-drugs/DNA damaging agents like cisplatin and 5-Fluorouracil or PARP inhibitor to result in more persistent DNA double strand breaks might mimic the results of this study. Here, we have shown VEGFR/PDGFR multi-receptor tyrosine kinase inhibitor, ABT-869, is an attractive option to overcome radioresistance. Antiangiogenic therapy in combination with

radiotherapy is a promising strategy, which could lead to less morbidity and increased efficacy in the treatment of HNSCC.

Table 1. Antiangiogenic agents in combination with radiation for head and neck cancers

Agent	Type of agent and target	Treatment	Developmental stage	Results	Ref.
Bevacizumab	Humanized monoclonal antibody VEGF-A	Bevacizumab+RT	Preclinical	Enhanced inhibition of tumor growth and blood vessel formation	Hoang, 2012
		Bevacizumab+erlotinib+RT	Preclinical	Enhanced anti-tumor growth	Bozec, 2008
		Bevacizumab+FHX	Phase I	Enhanced anti-tumor activity	Seiwert, 2008
		Bevacizumab+FHX	Phase II	2-year survival 68%	Salama, 2011
		Bevacizumab+erlotinib +chemoradiotherapy	Phase II	3-year PFR 71%, OSR 82%	Hainsworth, 2011
		Bevacizumab+cisplatin+IMRT	Phase II	2-year PFR 75.9%, OSR 88%	Fury, 2012
		Bevacizumab+erlotinib+CCRT	Phase II	3-year PFR 82%, OSR 86%	Yoo, 2012
		Bevacizumab+cetuximab +pemetrexed+RT	Phase II	Ongoing	
Vandetanib	TKI VEGFR/EGFR	Vandetanib + RT	Preclinical	Enhanced anti-tumor activity	Sano, 2011
		Vandetanib+ cisplatin+RT	Phase II	Ongoing	
Sunitinib	TKI VEGFR, PDGFR, Kit	Sunitinib+cetuximab+RT	Preclinical	Reduced tumor growth vessel formation	Bozec, 2009
		Sunitinib+IGRT	Phase II	18-month PFR 56%, OSR 71%	Tong, 2012
		Sunitinib+cetuximab+RT	Phase I	Ongoing	
Sorafenib	TKI VEGFR, PDGFR, Kit B-Raf, C-Raf, Flt3	Sorafenib+RT	Preclinical	Enhanced anti-proliferation effects	Yadav, 2011
Motesanib	TKI VEGFR, PDGFR, Kit	Motesanib+RT	Preclinical	Decreased tumor growth	Kruser, 2010
Linifanib	TKI VEGFR, PDGFR, Kit	Linifanib+RT	Preclinical	Arrested cell cycle in G2/M and augmented the cell killing and apoptosis effect of radiation	Hsu, 2013

Abbreviaition: RT, radiation; FHX, fluorouracil, hydroxyurea, radiation; IMRT, intensity-modulated radiation therapy; TKI, tyrosine kinase inhibitor; CCRT, concurrent chemoradiation; PFR, progression free rate; OSR, overall survival rate; IGRT, hypofractionated image-guided radiotherapy

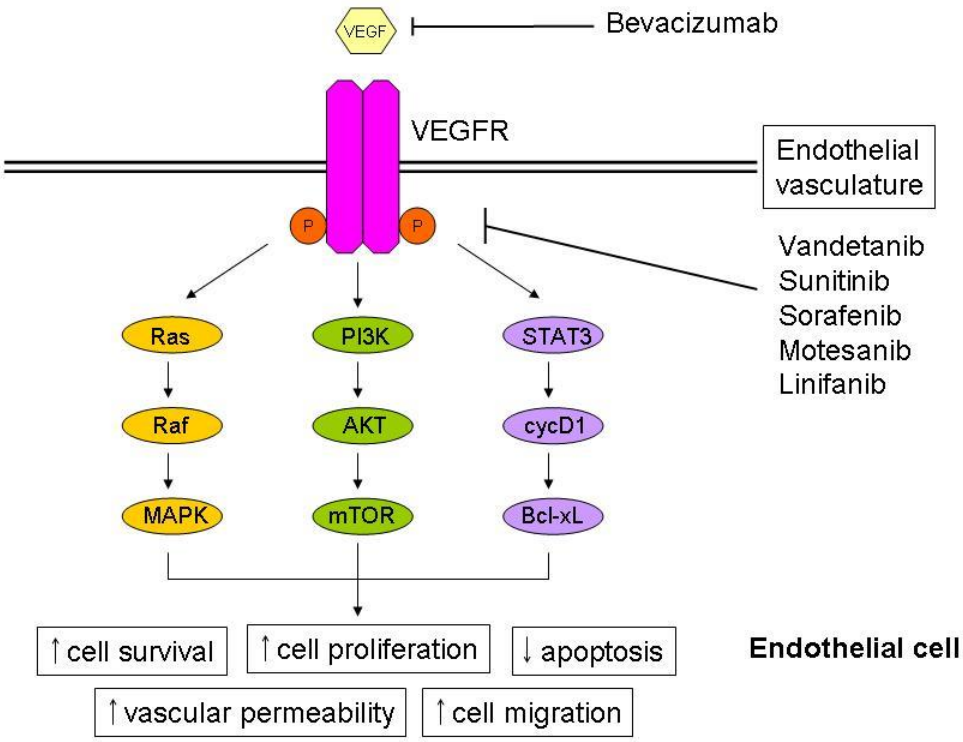

Figure 10. The effects of antiangiogenic agents on cell signaling pathways that lead to enhanced radiosensitivity.

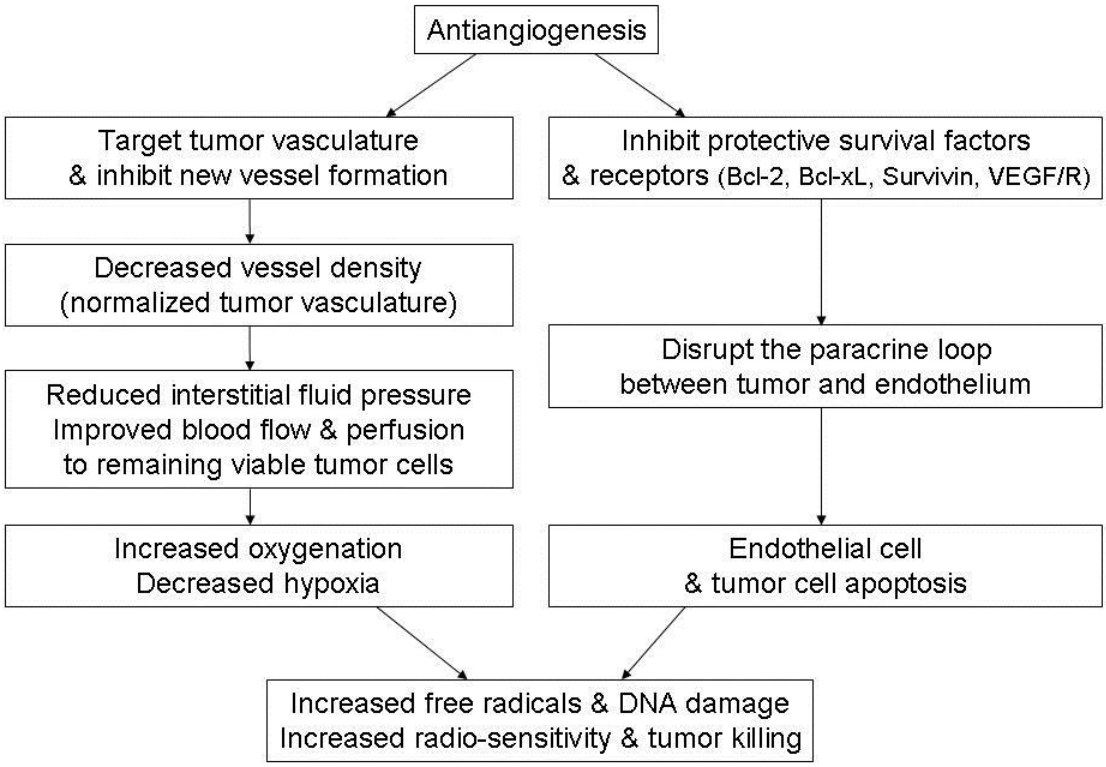

Figure 11. Possible advantages and mechanisms of using antiangiogenic therapies to enhance tumor response to radiation. These agents can improve tumor oxygenation via targeting tumor vasculature and inhibiting new vessel formation, also disrupt the interaction between tumor cells and tumor endothelial cells. When combining with ionizing radiation, it results in both tumor and endothelial cell apoptosis, work synergistically to improve radiosensitization effects.

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