CHARACTERIZING ADIPOKINES, VISFATIN AND RESISTIN, AND

DETERMINING THE CONTRIBUTION OF KINASE SIGNALING IN

AN OBESITY-INDUCED LIVER CANCER PHENOTYPE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. REVIEW OF THE LITERATURE

Liver Cancer

Liver Cancer is the sixth most common cancer and the leading cause of cancer deaths worldwide.[1] More than 700,000 people are diagnosed with liver cancer each year throughout the world with 600,000 deaths each year.[1] Liver cancer is more common in sub-Saharan Africa and Southeast Asia with liver cancer being the most common type of cancer in these countries.[1] In the United States, the American Cancer Society estimates that approximately 40,710 new cases of liver cancer were diagnosed in 2017.[1] Of these new cases, 29,200 were male and 11,510 were female.[1] Also, approximately 28,290 of these newly diagnosed patients will die due to liver cancer.[1] Since 1980, liver cancer incidence has more than tripled and deaths have increased by almost 3% per year since 2000.[1] Liver cancer is one of the most lethal cancers with a five year survival rate of 4-8%.[2] Unfortunately, signs and symptoms related to liver cancer do not appear until later stages leading to a high mortality rate.[1] Risk factors for liver cancer include gender (more common in males than females), race/ethnicity (more common in Asian Americans and Pacific Islanders), chronic viral hepatitis, cirrhosis, aflatoxins, non-alcoholic fatty liver disease, heavy alcohol use and obesity.[1] The link between obesity and liver cancer is likely to be mediated by a state of chronic inflammation.[2]

Liver Cancer and Obesity Link

Obesity has become a public health concern with more than one third of adults being obese.[2,3] Obesity can lead to liver diseases such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).[2] NAFLD affects approximately

30% of the general population and 90% of morbidly obese individuals.[4] NASH can lead to fibrosis, cirrhosis, and liver cancer if left untreated.[2] Low grade inflammation that is associated with obesity contributes to the progression from NAFLD to NASH and finally liver cancer.[2] Also, obesity in the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can lead to DNA damage and ultimately liver cancer.[2] In a large prospective mortality study, the relative risk of mortality from liver cancer was 1.68 times higher in women and 4.52 times higher in men with a BMI greater than 35 kg/m^2 compared to patients with normal BMI.[4] Obesity along with low grade inflammation results in elevated plasma concentrations of insulin and insulin-like growth factor 1 (IGF-1), increased secretion of adipokines such as visfatin and resistin along with inflammatory cells including macrophages.[2]

Adipocytes in obese individuals go through hypertrophy due to excess fat accumulation which leads to the secretion of free fatty acids along with pro-inflammatory cytokines including IL-6.[2] Obesity may increase cell proliferation, ROS production, lipogenesis, and promote the invasive capacity of liver cancer cells along with matrix-metalloproteinase-9.[5] Also, according to the Warburg Effect, cancer cells choose aerobic glycolysis for glucose metabolism instead of oxidative phosphorylation.[6] Also, certain cancer cells shift from using mitochondrial oxidative phosphorylation to pyruvate reduced to lactate for energy. Cells get glucose from their microenvironment and secrete lactic acid to meet their energy needs.[6] Further, an increase in lactate dehydrogenase, an enzyme that synthesizes lactate, is correlated with tumor burden and is thought to reflect the invasive potential of cancer cells.[7] Activating invasion and metastasis processes along with evading growth suppressors are key hallmarks of cancer.[8] Studies have shown

that cancer cells in the presence of obesity are able to evade these growth suppressors and sustain proliferation.[5,8] HepG2 liver cancer cells exposed to obese sera had a 50% increase in proliferation compared to cells exposed to normal weight sera.[5] Therefore, there is a need to understand obesity-associated hallmarks of cancers including sustained proliferative signaling, invasion and metastasis, enabling inflammation, and cellular energetics.

ROS causes DNA damage and destruction of liver cells.[5,9] The production of reactive oxygen species can activate hepatic stellate cells which are characterized by increased production of the extracellular matrix and accelerated proliferation.[10] ROS production has been shown to be 50% higher in liver cancer cells exposed to obese sera compared to normal weight sera.[5] ROS production is a byproduct of cellular metabolism. Changes in lipid metabolism may lead to the progression of liver cancer.[5] FASN enzyme catalyzes the de novo synthesis of long chain fatty acids and an increase in FAS has been shown in multiple tumor types.[11,12] Increased de novo lipogenesis contributes to the initiation and progression of tumors.[11,12] De novo lipogenesis may promote cancer progression due to an increase in cancer cell proliferation requiring the synthesis of lipids for generation of biological membranes.[13] Lipid biosynthesis is induced as part of the anabolic metabolism of cancer cells.[13] The production of these lipids may provide energy to cancer cells during times of nutrient depletion.[13] Studies have shown that obese sera increases lipogenesis 45% more compared to normal weight sera.[5] The link between obesity and invasive liver cancer may be partially due to the increase in expression of adipokines such as visfatin and resistin, which leads to a cascade of physiological changes in the liver. Visfatin and resistin are found in higher levels in obese individuals.

These data show the importance of further investigating the role of visfatin and resistin in inducing an invasive liver cancer phenotype.

Visfatin

Visfatin is found in the visceral adipose tissue and is commonly referred to as nicotinamide phosphoribosyltransferase (Nampt) and pre-B-cell colony-enhancing factor (PBEF).[14] Macrophages of visceral fat in obese individuals are a major source of visfatin production.[14] Visfatin serves as a cytokine, growth factor and an enzyme.[14] It also exhibits anti-apoptotic, proliferative, pro-inflammatory, pro-angiogenic and metastatic properties.[14] Visfatin is also an insulin-mimetic but its function remains unclear.[14] Studies have shown that visfatin is elevated in many cancers including obesity-associated malignancies.[14] Elevated visfatin has been shown to be associated with a bad prognosis along with a higher tumor grade.[14] Circulating visfatin along with adipocyte and macrophage-associated visfatin may impact pathways involved in the development of obesity and cancer by acting on glucose and lipid metabolism, insulin sensitivity, chronic inflammation and oxidative stress response.[14] Visfatin may not only contribute to cancer promotion but also its progression and prognosis.[14] It has been reported that visfatin is a very potent IL-6 inducer both *in vitro* and *in vivo*.[14]

Pathways that visfatin are known to be involved in include nuclear factor-kB, the mitogen-activated protein kinases (MAPKs), ERK½ and p38, phosphatidylinositol 3 kinase (PI3K), and the generation of reactive oxygen species.[14] Visfatin has a role in metabolic, stress response and cellular bioenergetics specifically NAD synthesis.[14] Visfatin is a key enzyme of the NAD pathway.[14] NAD is formed from precursors such as

nicotinic acid, tryptophan, nicotinamide riboside or by the salvage pathway in which visfatin catalyzes the rate limiting step.[14] Cancer cells have an increased demand for NAD due to an increase in proliferation and DNA repair rate.[15] Visfatin is considered a potential target for anti-cancer therapies.[15] During the development of hepatocellular carcinoma, AMP-activated protein kinase (AMPK) becomes dysregulated.[15] In prostate and breast cancer cells, visfatin was shown to increase cellular proliferation but the use of a visfatin inhibitor had antitumor effects by inducing apopotosis.[16] This suggests that visfatin is one of the adipocytokines that links obesity and tumorigenesis and could potentially be an effective target for the inhibition of obesity-related carcinogenesis.[16] To date, there are no detailed studies that examine the relationship between visfatin and hepatocellular carcinomas in the context of obesity. We aim to further characterize visfatin and examine if inhibition of kinase signaling suppresses visfatin-induced liver cancer progression.

Resistin

Resistin was discovered in mice in 2001 and was named so because it resists insulin action.[17] Initially, resistin was said to be a link between obesity, insulin resistance and diabetes.[17] Studies have shown that resistin is an adipokine that represents an important link between obesity, inflammation, and insulin resistance.[18] Also, resistin has been associated with an increased risk of cancer.[18] The expression of resistin was initially defined in adipocytes.[17] Resistin is mainly located in mononuclear leukocytes, macrophages of adipose tissue, spleen, and bone marrow cells.[17,18] Resistin's effects may be mediated through paracrine and endocrine modes.[18]The functional receptor for resistin has been identified as adenylyl cyclase-associated protein 1 (CAP1).[18, 19] Resistin binds to

CAP1 in monocytes and up-regulates intracellular cAMP concentration, PKA activity and NF-kβ.[17] Overexpression of CAP1 in monocytes increases inflammation in adipose tissue and suppression of CAP1 has been shown to significantly reduce inflammation *in vitro* and *in vivo*.[17] Resistin regulates glucose homeostasis, insulin resistance, and inflammation which all play pathogenic roles in obesity, diabetes, and cardiovascular disease.[18] Also, resistin up-regulates the mRNA and protein expression of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2) along with matrix metalloproteinases.[17] This is an indication that resistin may be responsible for promoting angiogenesis.[17]

In vivo studies indicate that infusion of resistin increases glucose production and impairs insulin sensitivity in the liver.[20] Also, phosphorylation of the enzyme AMPK increases the expression of glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK).[20] The human homolog of resistin only shares 59% amino acid homology with mouse resistin and the source of resistin differs between the two.[20] Previous studies have investigated the effects of resistin on the phosphorylation state of AMPK.[20] HepG2 cells were exposed to resistin and they found that resistin decreased Akt phosphorylation through an AMPK-independent mechanism suggesting that biological processes including metabolism, cell cycle regulation, and apoptosis become dysregulated leading to proliferation/anti-apoptosis, tumor progression, cellular differentiation, and other physiological changes promoting invasive liver cancer.[20] Reports of resistin and the phosphorylation of Akt are inconsistent. Lastly, resistin can also activate the ERK pathway by stimulating pro-inflammatory cytokines and causing

oxidative stress.[17] Studies have shown that a specific p38 inhibitor blocked resistin-induced ROS production.[17]

Kinase Signaling Pathways

Although it is known that visfatin and resistin are involved in kinase signaling pathways, the contribution of kinase signaling in visfatin and resistin-induced invasive liver cancer is less understood. Few studies have investigated visfatin and resistin mediated activation of kinase signaling with corresponding changes in cell function which may promote an invasive liver cancer phenotype. Akt and ERK may be two important pathways in visfatin or resistin-induced liver cancer progression.

Previous studies have shown that the ERK signaling pathway is involved in promoting cell proliferation, migration, survival along with tumor progression.[21] For the ERK pathway to be activated, first there is membrane receptor activation.[21] Then, RAS is recruited by adaptor proteins.[21] There are a series of phosphorylation steps to amplify the signal (Raf to MEK and finally ERK).[21] There are over 100 targets downstream of ERK, many of them being transcription factors.[21] Altered levels of these transcription factors following ERK activation can lead to a change in expression levels of genes involved in cell cycle progression.[21] Studies have shown that the ERK pathway stimulates pro-inflammatory cytokines and adipokines, visfatin and resistin, causing oxidative stress[17] By inhibiting the ERK pathway, more will be known about the effect of this inhibition on visfatin along with resistin and overall liver cancer progression. Studies have shown crosstalk between the ERK and Akt pathways.[21]

The Akt pathway is another important pathway involved in liver cancer. Akt is downstream of PI3K and when PI3K is activated, Akt is recruited to the plasma membrane.[21] Phosphoinositide dependent kinase 1 (PDK1) phosphorylates residues in the activation loop of Akt to initiate kinase activity.[21] The Akt pathway is known to be up-regulated in various carcinoma cell lines.[21] In summary, these data suggest that visfatin and resistin contribute significantly in promoting an invasive liver cancer phenotype. Inhibition of the kinase signaling pathways would then potentially suppress visfatin and resistin-induced liver cancer progression.

Objectives

The aim of this research project was two-fold to 1) determine the contribution of visfatin and resistin in an *in vitro* model of obesity-induced liver cancer and 2) determine if the inhibition of kinase signaling suppresses visfatin or resistin-induced liver cancer progression. We hypothesize that adding a neutralizing antibody targeting visfatin and resistin will reduce the physiological impact of obesity on invasive liver cancer. Also, the inhibition of Akt and the ERK pathway will suppress visfatin and resistin induced liver cancer progression.

II. VISFATIN AND RESISTIN IN AN IN VITRO MODEL OF OBESITY-INDUCED INVASIVE LIVER CANCER

Abstract

Obesity is associated with the development and progression of liver disease to hepatocellular carcinoma (HCC). Obesity is characterized not only by low grade inflammation but elevated plasma concentrations of insulin and insulin-like growth factor 1 (IGF-1), visfatin, and resistin. Obesity and liver cancer promote physiological changes related to cellular proliferation, ROS, MMP section, invasion, and lipid accumulation. Adipokines, visfatin and resistin, have been shown to promote liver cancer incidence and progression. Studies have yet to determine the role of visfatin and resistin in an obesity-induced liver cancer phenotype. Using an *in vitro* model, sera from obese (OB) or normal weight (NW) males (based on BMI) were used to determine the efficacy of neutralizing antibodies of visfatin and resistin in reducing an obesity-induced liver cancer phenotype. HepG2 and SNU-449 cells were exposed to OB and NW ± neutralizing antibodies for visfatin or resistin. The neutralizing antibodies suppressed obesity-induced growth, invasion, and MMP secretion. These physiological changes corresponded with a decrease in phosphorylated ERK and Akt along with CAP1 and β-catenin. We describe the effect of neutralizing antibodies on OB and NW sera to determine the contribution of visfatin and resistin in obesity-induced invasive liver cancer phenotype.

Introduction

Liver Cancer is the sixth most common cancer and the leading cause of cancer deaths worldwide.[1,22,23] In 2012, approximately 23,000 people died from liver cancer in the United States, which is a 56% increase in deaths since 2003.[1] New liver cancer cases

9

rose 38% from 2003-2012.[1] This rise in liver cancer incidence coincides with the current

obesity trend with one third of adults being obese.[3,24] An increase in BMI is an important

risk factor in the development of liver cancer.[25] Studies from Denmark and Italy both

showed a 2-fold increase in liver cancer incidence in obese individuals.[13,25] A study

conducted in the United States of more than 900,000 adults, showed that the risk of death

from HCC was 2-fold higher in men with a BMI of 30-34.9 and 4.5 times higher in men

with a BMI greater than 35.[25] Recently, it has been shown that the second most common

cause of HCC is fatty liver disease which is associated with obesity.[25] It is expected in the

future that fatty liver disease will account for over one third of HCC cases.[25] Obese

individuals have been shown to have higher levels of serum visfatin and resistin

compared to normal weight individuals but the role of visfatin and resistin in fatty liver

disease and liver tumorigenesis is largely unknown.[5]

Preliminary studies from our laboratory have demonstrated that obesity promotes

an invasive liver cancer phenotype evidenced by increased physiological and molecular

changes related to viability, invasion, reactive oxygen species (ROS), and matrix

metallopeptidase 9 (MMP-9) secretion.[5] Previous studies have shown that visfatin

strongly induces proliferation in hepatocellular carcinoma (HCC) cells and acts as a

growth factor in HCC cells.[16] Additionally, visfatin is one of the key adipocytokines that

links obesity and the progression of HCCs.[16] Studies have also shown that resistin can act

directly on cancer cells by stimulating proliferation and migration, promoting cells

adhesion, and altering the tumor microenvironment.[18] While previous studies have shown

that obesity and high levels of pro-tumorigenic adipokines, visfatin and resistin, promote

liver cancer incidence and progression, the mechanism through which these adipokines

promote liver cancer progression has not been fully elucidated.[16,18] Therefore, the objective of this study is to determine the contribution of visfatin and resistin in obesity-induced invasive liver cancer phenotype. We hypothesize that adding a neutralizing antibody targeting visfatin and resistin will reduce the physiological impact of obesity on invasive liver cancer.

Materials and Methods

Cell Lines

Human liver cancer cells (HepG2) were purchased from the American Type Tissue Culture Collection (ATCC) (Rockville, MD). Cells were cultured in DMEM containing penicillin and streptomycin plus 10% fetal bovine serum (FBS). SNU-449 liver cancer cells were purchased from ATCC. Cells were cultured in RPMI containing penicillin and streptomycin plus 10% FBS. Cells were maintained at 37°C in a 5% (v/v) CO_2 humidified incubator.

Serum Samples

An *in vitro* model using 5% pooled male human sera from OB (BMI > 30 kg/m^2) and NW (BMI< 25 kg/m^2) individuals (n=5 for each group). Sera was acquired from Equitech Enterprise Inc. (Kerrville, TX), and individuals were screened to ensure they did not have diseases that would affect results including HIV, hepatitis, diabetes, or cancer.

11

Neutralizing Resistin and Visfatin in Human Sera

An ELISA assay was used to measure resistin levels in human sera. The values

obtained from the ELISA assay were taken into consideration when determining the

concentration of resistin antibodies. The obese serum contained 2.46 ng/ml of resistin and

the normal weight BMI category contained 0.8 ng/ml of resistin (data not shown).

Visfatin concentration was determined based on levels of visfatin in obese individuals.

Neutralizing antibodies can be used in concentrations ranging from 1ug/ml-10ug/ml. Ten

ug/ml of neutralizing antibodies was selected to ensure adequate inhibition of visfatin and

resistin in the obese serum which would contain the highest amounts of these

adipocytokines. Neutralizing antibodies were incubated with OB and NW weight sera

for 1 hour prior to *in vitro* experiments.

Cellular Proliferation

Cellular proliferation was assessed by MTT dye conversion to formazan and

analyzed in a plate reader at 540nm. 1 X 10^4 cells were seeded per well in a 96-well plate

overnight. After 24 hours, cells were washed with PBS and treated with one of the

following experimental conditions: SFM, SFM +5% OB sera, SFM + 5% OB sera +

visfatin antibody, SFM + OB sera + resistin antibody, SFM + 5% NW sera, SFM + 5%

NW sera + visfatin antibody, SFM + 5% NW sera + resistin antibody for 72 h. After 72 h,

20 µl of MTT reagent (5 mg/ml) was added to each well. After 2 hours, MTT reagent was

aspirated and 100 µl of DMSO was added to each well and shaken for 10 minutes.

Absorbance was measured using Cytation 5.

Reactive Oxygen Species Assay

Intracellular levels reactive oxygen species (ROS) were assessed using a cell-permeable DCFH-DA probe. HepG2 and SNU-449 cells were seeded in a dark, clear bottom 96-well microplate. On day 2 cells were exposed to the following experimental conditions: SFM, SFM +5% OB sera, SFM + 5% OB sera + visfatin antibody, SFM + OB sera + resistin antibody, SFM + 5% NW sera, SFM + 5% NW sera + visfatin antibody, SFM + 5% NW sera + resistin antibody. On day 3, the media was removed, and cells were washed with 1X buffer, and incubated for 45 minutes in 1X buffer containing 25 µM DCFDA at 37˚C. Fluorescent intensity was measured at excitation wavelength 485 nm and emission wavelength 529 nm with Cytation 5.

Oil Red O Staining

Approximately 30,000 HepG2 and SNU-449 cells were seeded in a 24-well plate. After 24 hours, cells were washed with PBS and treated with one of the following conditions: SFM, SFM +5% OB sera, SFM + 5% OB sera + visfatin antibody, SFM + OB sera + resistin antibody, SFM + 5% NW sera, SFM + 5% NW sera + visfatin antibody, SFM + 5% NW sera + resistin antibody. After 48 hours, the media was removed, washed with PBS, fixed for 10 min with 10% formalin, and washed with 60% isopropanol. Cells were stained with filtered oil red O solution for 10 minutes. Lipid droplets were imaged using Cytation 5 (Winooski, VT) at 20X magnification. Lipid droplets were eluted and quantified using 100% isopropanol. Absorbance was measured at 500 nm with Cytation 5.

Invasion Assay

The Corning BioCoat Matrigel Invasion Chamber assay was utilized to assess if the neutralizing antibodies targeting visfatin and resistin decreased the invasive capacity of HepG2 and SNU-449 cells.[26] The matrigel chamber was hydrated in serum free media for 2 hours prior to seeding. After the chambers were rehydrated, cells were seeded on the upper side of the chamber at 80,000 cells per well in a 24-well plate with one of the following treatments: SFM, SFM +5% OB sera, SFM + 5% OB sera + visfatin antibody, SFM + OB sera + resistin antibody, SFM + 5% NW sera, SFM + 5% NW sera + visfatin antibody, SFM + 5% NW sera + resistin antibody. The chemoattractant, 10% FBS, was added to the well below the chamber. After 48 hours cells were stained with crystal violet, and images were captured using Cytation 5. After imaging, crystal violet was dissolved using a distain solution (methanol, acetic acid, and water), and absorbance was measured with the Cytation 5 microplate reader. Cell counts were conducted after 48 hours and there were no statistical differences observed between treatment groups (data not shown).

ELISA for MMP-9

To determine MMP-9 secretion from cells, HepG2 and SNU-449 cells were exposed to the experimental conditions for 1 hour. After 1 hour, cells were washed with PBS and SFM was added to each well to capture MMP-9 secretion. The ThermoFisher (Waltham, MA) ELISA kit was used to measure MMP-9 secretion.[27]

Western Blot Analysis

HepG2 and SNU-449 cells were plated at 400,000 cells per well in a 6-well plate. After 24 hours, the cells were serum starved for six hours. The cells were then exposed to their respective experimental conditions for 15 minutes. After the treatment, cells were harvested using lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5 ml 10% SDS, 36.86 ml ddH$_2$O) and quantified using Pierce BCA protein assay kit.[28] Fifty µg of protein lysate was electrophoresed through a 10% gel (4 ml H$_2$O, 3.3 ml 30% acrylamide mix, 2.5 ml 1.5 M TRIS, 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulfate (APS), 8 µl TEMED along with the stacking gel (3.4 ml ddH$_2$O, 0.83 ml 30% acrylamide mix, 0.63 ml 1.0 M TRIS, 0.05 ml 10% SDS, 0.05 ml 10% APS and 6 µl TEMED). It was then transferred to a nitrocellulose membrane and blocked with 5% bovine serum albumin for 45 minutes. After measuring phosphorylated proteins, blots were stripped with Restore Plus western blot stripping buffer before measuring total levels of protein. Target proteins for western blot analysis are listed in Table 1.

Table 1. Summary of target proteins for western blot analysis	
Primary Antibody	**Secondary Antibody**
β-catenin Dilution: 1:1000 Incubation time: overnight Manufacturer: Thermo Fisher Scientific, catalogue #71-2700, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Phospho-ERK Dilution: 1:2000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #4370, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:5000 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Total-ERK Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #9102, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Table 1 Continued. Summary of target proteins for western blot analysis	
Phospho-Akt Dilution: 1:1000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #9271, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Total-Akt Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #4691, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
β-actin Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #4967, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Table 1 Continued. Summary of target proteins for western blot analysis

CAP1	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey
Dilution 1:250	Dilution: 1:1250
Incubation time: overnight	Incubation time: 45 minutes
	Manufacturer: GE Healthcare UK Limited
Manufacturer: Thermo Fisher Scientific, catalogue #PA5-56354, Waltham, MA	catalogue #NA934V, Pittsburg, PA

Statistical Analysis

Values are presented as mean ± s.e. of the mean. Experiments were repeated three times. Statistical analyses were performed between treatment groups except as noted. For all tests, GraphPad Prism 7.0 software was used (GraphPad Software Inc., La Jolla, CA, USA), and $P<0.05$ was considered statistically significant. Results were compared using either Mann-Whitney U Test or Student's T Test.

Results

The effect of adipocytokine depletion in serum-induced proliferation

To assess cell viability, HepG2 and SNU-449 cells were exposed to serum with or without a neutralizing antibody for visfatin and resistin for 72 hours. HepG2 cells exposed to OB serum increased proliferation compared to NW but did not reach statistical significance (Figure 1A). SNU-449 cells exposed to OB serum increased proliferation by 24% when compared to NW ($p<0.05$) (Figure 1B). In both cell lines, depleting OB and NW serum of visfatin and resistin did not reduce cell proliferation. These data suggest that in the short-term, visfatin and resistin do not contribute to obesity-induced proliferation.

The effect of visfatin and resistin depletion on lipogenesis

Lipid accumulation in liver cells provides the necessary biomolecules for cell growth. HepG2 and SNU-449 cells were exposed to serum with or without antibodies for visfatin and resistin for 48 hours. In both cell lines, there was a trend for OB to increase lipogenesis compared to NW, but these findings were not statistically significant (Figure 2A/B). Further, depleting OB serum of visfatin and resistin reduced lipogenesis to levels

similar to that of NW but was not statistically significant. These results suggest that other factors in OB serum may be contributing to lipogenesis to a greater degree than visfatin or resistin.

Visfatin and resistin depletion reduces ROS production

ROS induced DNA damage can lead to mutations that promote initiation and progression of liver cancer. HepG2 and SNU-449 cells were exposed to serum with or without neutralizing antibodies for 24 hours. In HepG2 cells, OB serum increased proliferation by 14% compared to control ($p < 0.05$) (Figure 3A). Depleting the serum of visfatin or resistin reduced ROS production to levels similar to that of cells exposed to NW serum, 15% and 12%, respectively ($p < 0.05$). No differences in ROS production were observed in cells exposed to NW serum with or without depletion of visfatin or resistin. OB sera increased ROS production by 10% compared to SNU-449 cells exposed to NW sera. In SNU-449 cells, resistin depletion decreased ROS production by 10% ($p < 0.05$) while no differences were observed in OB plus visfatin antibody (Figure 3B). Further, visfatin or resistin depletion had no effect on cells exposed to NW serum.

The effect of visfatin and resistin on MMP-9 secretion

Matrix metalloproteinase-9 may promote the invasive capacity of liver cancer cells. The ELISA assay was used to determine the effect of neutralizing antibodies on secretion of MMP. The trend for both HepG2 and SNU-449 cells is almost identical (Figure 4A/B). OB sera increased MMP-9 secretion significantly compared to control ($p < 0.05$). The addition of the neutralizing antibodies decreased MMP-9 secretion to

levels similar to that of NW. These data suggest that visfatin and resistin contribute to obesity-induced MMP secretion in liver cancer cells.

Resistin suppresses OB-induced invasion in liver cancer cells

Hormones and adipocytokines may play a role in metastasis of liver cancer cells. Matrigel chambers were used to assess the invasive capacity of liver cancer cells exposed to OB or NW serum with or without visfatin or resistin depletion. In HepG2 cells, OB sera increased invasion 2-fold compared to NW ($p<0.05$) (Figure 5A). Depleting OB sera of resistin decreased invasion 2-fold ($p<0.05$). The invasive capacity of HepG2 and SNU-449 cells were similar across all groups exposed to NW sera with or without visfatin or resistin. Neutralizing antibodies for visfatin or resistin suppressed OB-induced invasion by 40% and 30%, respectively ($p<0.05$) (Figure 5B). These results demonstrate that resistin contributes to OB-induced invasion in HepG2 cells. In addition, visfatin and resistin are important for OB-induced invasion in SNU-449 cells.

Visfatin and resistin signaling

The ERK and Akt signaling pathways undergo mutations in a cancerous environment to promote cancer progression and metastasis. These two proteins were measured to confirm that they are involved and active in liver cancer. The functional receptor for resistin is CAP1 and β-catenin serves a regulator of cell adhesion and gene transcription. HepG2 and SNU-449 cells exposed to OB sera increased phosphorylation of ERK compared to cells exposed to NW sera. Depleting the OB sera with a neutralizing antibody for visfatin did not decrease OB-induced phospho-ERK in HepG2 and SNU-449 cells. The resistin antibody slightly decreased OB-induced phospho-ERK. In HepG2 and

SNU-449 cells, OB sera increased phosphorylation of Akt. Visfatin and resistin inhibition decreased OB-induced activation of Akt in HepG2 cells but not SNU-449 cells. CAP1 and β-catenin protein levels were not differentially affected by any of the experimental conditions (Figure 6).

Discussion

Previous reports have shown that obese serum has high levels of visfatin and resistin compared to normal weight serum.[5] The link between obesity and invasive liver cancer may be partially due to the increase in expression of adipokines such as visfatin and resistin, which leads to a cascade of physiological changes in the liver such as cellular proliferation, ROS, MMP, invasion, and lipogenesis. Other studies have shown that visfatin and resistin are elevated in a variety of cancers and diseases.[29,30,31] To our knowledge, there are no studies that examine the role of visfatin and resistin in obesity-induced liver cancer phenotype. Additional research would aid in understanding the obesity and liver cancer link.

The results from this *in vitro* study establish that visfatin and resistin are important adipocytokines in obesity-induced liver cancer. Cellular proliferation was assessed because it is important in the context of not only cancer development but also cancer progression.[32] We found that both HepG2 and SNU-449 liver cancer cells exposed to OB serum increased proliferation compared to NW. Previous studies have shown that OB sera promotes proliferation compared to NW sera.[5] Also, the depletion of visfatin and resistin in OB and NW serum did not reduce cellular proliferation. We did not observe

differences with the neutralizing antibodies, but this may indicate that there are other factors in the serum responsible for promoting cellular proliferation.

One of the key hallmarks of cancer is an increase in lipogenesis.[33] Tumor cells synthesize approximately 95% of fatty acids de novo despite sufficient lipid supply.[33] The activation of fatty acid synthesis may be required for tumor survival.[33] We found that OB serum increased lipogenesis compared to NW serum in both HepG2 and SNU-449 cells. Also, depleting the OB serum of visfatin and resistin reduced lipogenesis to levels similar to that of NW. Other studies have found that obesity induces lipogenesis.[34] Although we saw a trend for OB to increase lipogenesis compared to NW it was not statistically significant. There may be a shift from lipogenesis, lipid accumulation, to lipolysis, lipid breakdown. It has been suggested that tumors may promote intracellular lipolysis in adipose tissue but the exact mechanism remains unclear.[33] However, lipolysis was not measured in this study.

Cancer cells have an increase in the production of reactive oxygen species due to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction.[35] This increase in ROS leads to DNA damage and promotes genetic instability.[35] This study shows that in HepG2 cells OB serum increased ROS production and the addition of the neutralizing antibodies reduced ROS production similar to that of cells exposed to NW serum. In the SNU-449 cells, OB sera increased ROS production and the addition of only the resistin antibody to the OB sera reduced ROS production.

Tumor cells secrete matrix metalloproteinases (MMPs) which are extracellular matrix-degrading enzymes.[36,37,38] Studies have shown that an increase in MMP-9

secretion leads to an increase in invasion and metastasis.[39,40] HepG2 and SNU-449 cells

exposed to OB sera had a significant increase in MMP-9 secretion compared to control

and the addition of neutralizing antibodies decreased secretion similar to that of NW.

MMP-9 and obesity promoted the invasive capacity of liver cancer cells. HepG2 cells

exposed to OB sera had a robust increase in invasive capacity compared to control. Also,

the addition of the neutralizing antibodies led to a significant decrease in invasive

capacity. This same trend continues with the SNU-449 cells. Based on these results, we

can conclude that visfatin and resistin play an important role in obesity-induced invasion.

As expected, OB sera increased phosphorylation of Akt and ERK compared to

NW sera in HepG2 and SNU-449 cells. Other studies have also shown increased levels of

ERK in the presence of obesity.[5] The neutralizing antibody for visfatin did not decrease

OB-induced phospho-ERK and there was only a slight reduction in the presence of the

resistin antibody. CAP1 and β-catenin protein levels were not affected by any of the

experimental conditions. This indicates that CAP1 may need a longer time point exposed

to experimental conditions. To properly assess β-catenin, one may need to visualize

localization and not total levels of protein. In the future, β-catenin should be assessed by

subcellular fractionation.

Collectively, visfatin and resistin play an important role in promoting an obesity-

induced liver cancer phenotype by mediating proliferation, lipogenesis, ROS, MMP, and

invasion. The limitations of this study include the study design being correlative but

necessary to warrant future mechanistic research. Also, there are other growth factors

present in the serum that may be contributing to the physiological parameters assessed

other than visfatin or resistin. Using serum is also a strength of the study because specific

targets can be analyzed, and other factors can be eliminated. Another strength of this study is that we are addressing a timely issue with high prevalence of obesity and increasing incidence of liver cancer. Future studies aim to investigate the mechanism through which visfatin and resistin are promoting an invasive phenotype. Two potential targets include STAT3 and NF-kB. Studies have suggested that activation of NF-kB or STAT3 can promote invasion and overall metastasis. Therefore, future studies can determine if visfatin or resistin mediate their effects through STAT3 or NF-kB activation.
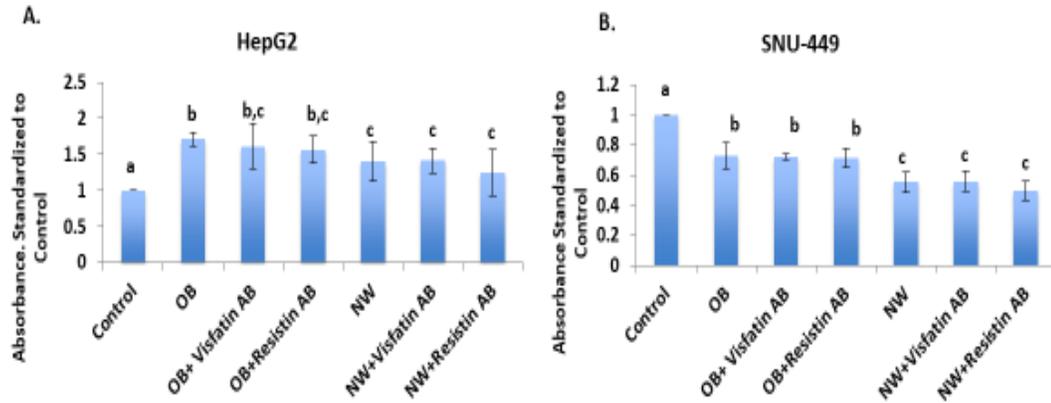
Figure 1. The role of visfatin and resistin in cell viability. MTT assay was used to assess cell viability. Growth was assessed by MTT dye conversion. A. HepG2 cells were exposed to the treatments for 96 hours. B. SNU-449 cells were exposed to the treatments for 96 hours. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
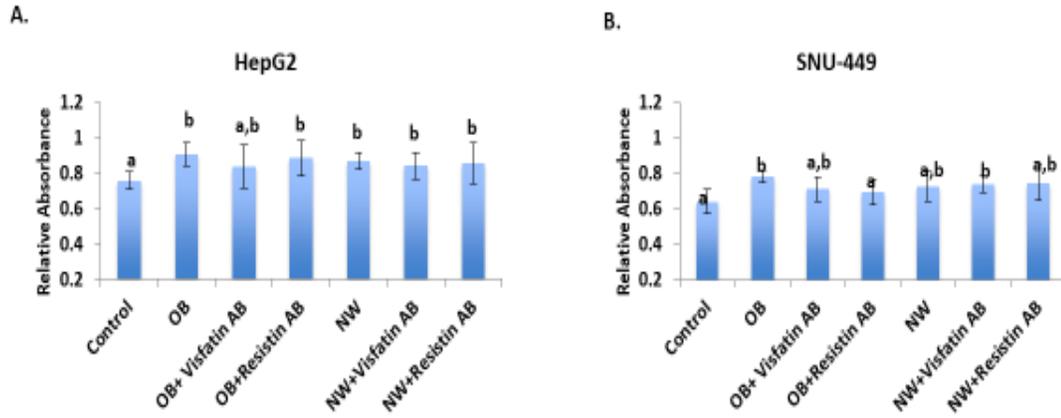
Figure 2. Visfatin, resistin and lipogenesis. A. HepG2 cells were cultured with treatments for 24 hours. B. SNU-449 cells were exposed to treatments for 24 hours. Oil Red O staining was used to visualize lipid accumulation in liver cancer cells. Cells were distained, and absorbance was used to measure lipid accumulation in liver cancer cells. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
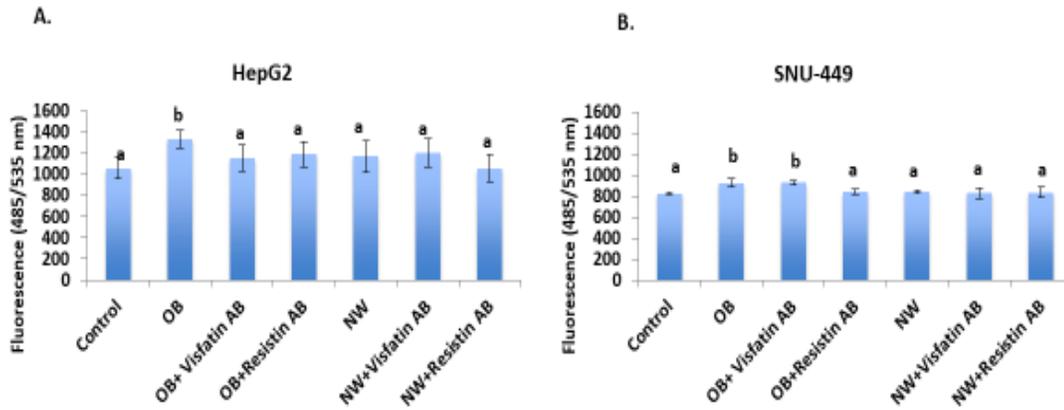
Figure 3. Obesity promotes ROS production. A. HepG2 and B. SNU-449 cells were cultured with visfatin or resistin. After 24 hours, cells were labeled with DCFDA (20uM) and then analyzed on a fluorescent plate reader. Different letters indicate significant differences between experimental conditions, P < 0.05.
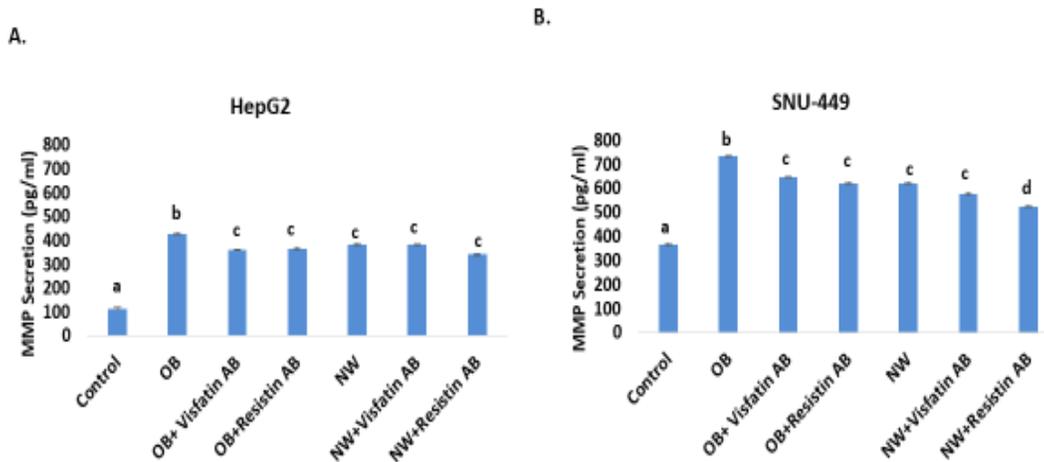
Figure 4. Visfatin and resistin blockade suppresses obesity-induced MMP secretion. A. HepG2 and B. SNU-449 cells were conditioned for 1 hour with treatments. After an hour, cells were washed and replaced with serum-free media (SFM) for 24 hours. This conditioned media was analyzed by ELISA assay. Different letters indicate significant differences between experimental conditions, P < 0.05.
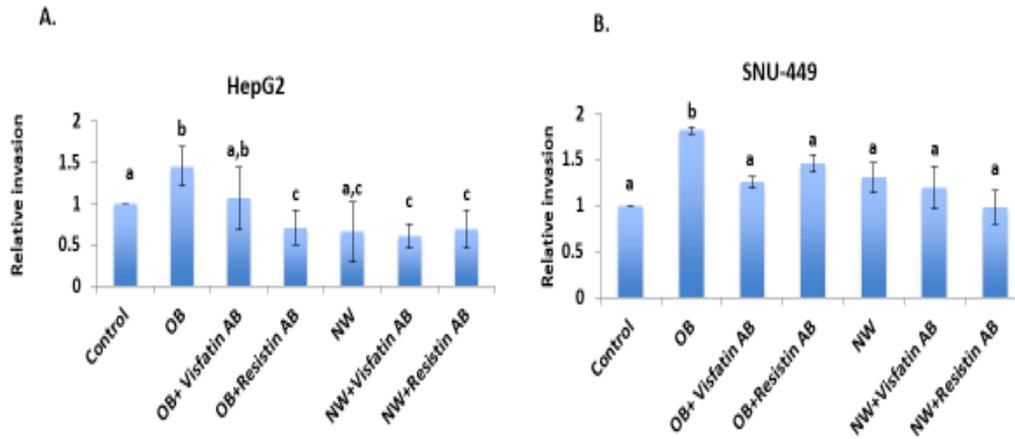
Figure 5. The differential effects of visfatin and resistin inhibition in obesity-induced invasion. A. HepG2 and B. SNU-449 cells were seeded in BD Biocoat Matrigel Chambers with treatments and FBS was used as a chemoattractant (in the lower chamber). After 24 hours, cells were fixed, stained, and dissolved. 100ul of dissolution was measured using optical densitometry. Representative images of cells that migrated through the membrane (lower panel) were captured using Cytation 5. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
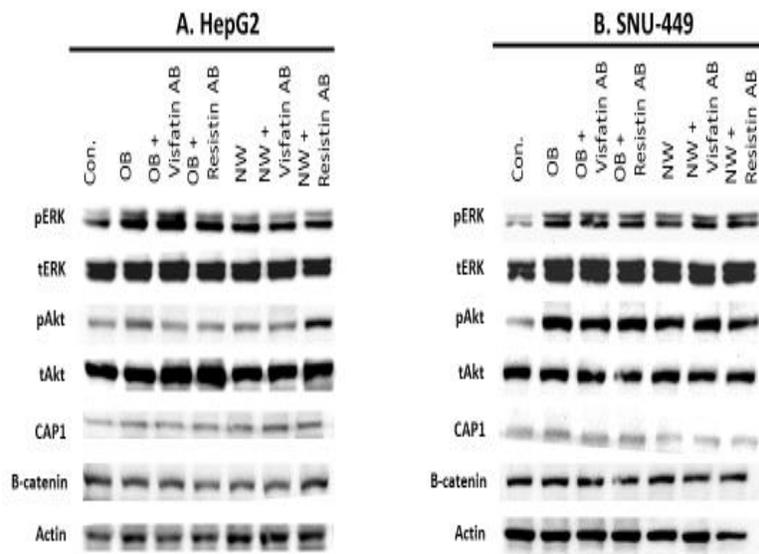
Figure 6. The effects of neutralizing antibodies on protein expression. HepG2 and SNU-449 cells were cultured in experimental conditions for 15 minutes. Western blot analysis was carried out with antiphospho-ERK, anti-ERK, antiphospho-Akt, anti-Akt, anti-CAP1, anti-β-catenin, and anti-actin.

III. INHIBITION OF KINASE SIGNALING IN VISFATIN-INDUCED LIVER CANCER

Abstract

Visfatin is found in the visceral adipose tissue and is commonly referred to as nicotinamide phosphoribosyltransferase (Nampt).[16] Visfatin has anti-apoptotic, proliferative, pro-inflammatory, pro-angiogenic, and metastatic properities.[16] Pathways that visfatin are known to be involved include but are not limited to mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERK).[16] The ERK pathway promotes cellular proliferation, migration, survival of cancer cells, and tumor progression.[41] The Akt pathway is known to be up-regulated in many cancers including liver cancer.[41] Studies have yet to determine whether inhibition of kinase signaling will suppress visfatin-induced liver cancer progression. Using an *in vitro* model, HepG2 and SNU-449 liver cancer cells were exposed to visfatin ± ERK, PI3K/Akt, or both inhibitors. The following physiological parameters were assessed: cellular proliferation, ROS, lipogenesis, invasion, and MMP secretion. The inhibition of kinase signaling suppressed most of these parameters. These physiological changes corresponded with a decrease in phosphorylated Akt and ERK, β-catenin, and FASN. We describe the effect of Akt and ERK inhibitors to determine if blocking these pathways suppress visfatin-induced liver cancer progression.

Introduction

Over the past two decades the death rate from cancer has decreased by 23%.[1] Although there is an overall reduction in cancer incidence and death, some cancers are

seeing an increase.[1] Liver cancer incidence has more than tripled in the past 35 years and deaths have increased by almost 3% per year since 2000.[1] The increase in liver cancer incidence coincidences with the current U.S. obesity trend.[3,24] Obese individuals have excessive fat stores in which adipocytokines are located. Macrophages of visceral fat are a major source of the production of the adipocytokine visfatin.[16] Visfatin functions as a cytokine, growth factor, and an enzyme along with exhibiting anti-apoptotic, proliferative, pro-inflammatory, pro-angiogenic, and metastatic properties.[16] This adipocytokine has been shown to be essential in NAD production.[42] Visfatin also acts on glucose metabolism, lipid metabolism and insulin sensitivity which may lead to the promotion and progression of liver cancer.[16]

Previous research has shown that phosphorylation of Akt and ERK were elevated by treatment with visfatin.[43] The Akt pathway is activated by phosphorylation and induces cell proliferation and survival of cancer cells.[43] The ERK pathway is also activated through phosphorylation in response to numerous growth factors.[43] Additionally, visfatin has been shown to induce proliferation in cancer cells but this effect was repressed when Akt and ERK inhibitors were added.[20,43] The effect of inhibiting ERK and Akt signaling on visfatin-induced liver cancer progression will be assessed. These kinase signaling pathways have been shown to promote cell proliferation, migration, survival along with tumor progression, ROS production and invasion.[5,21] Studies have shown that these kinase signaling pathways are involved in various cancers including liver cancer.[5,14,15,16,21] We expect that the inhibition of kinase signaling pathways will suppress adipocytokine-induced liver cancer progression which will be

seen by a decrease in physiological parameters associated with cellular proliferation, ROS, lipogenesis and invasion.

Materials and Methods

Cell Culture and Reagents

Human liver cancer cells (HepG2) were purchased from the American Type Tissue Culture Collection (ATCC) (Rockville, MD). Cells were cultured in DMEM containing penicillin and streptomycin plus 10% fetal bovine serum (FBS). SNU-449 liver cancer cells were purchased from ATCC. Cells were cultured in RPMI containing penicillin and streptomycin plus 10% FBS. Cells were maintained at 37˚C in a 5% (v/v) CO_2 humidified incubator. Cells were treated with 10 μM PI3K inhibitor ly290004 (LY) (Sigma Aldrich) and 10 μM ERK inhibitor PD98059 (PD) (Cell Signaling) 1 hour prior to the addition of 50 ng/ml visfatin. The concentrations of the PI3K and ERK inhibitors were selected based on the previous literature with the use of these inhibitors in human cancer cells.[32,44,45] The concentration of visfatin was selected based on previous studies that measured visfatin levels in obese or diabetic individuals.[46,47,48] The intention of using these concentrations is to closely recapitulate the physiological levels observed in obese individuals.

Cellular Proliferation

Cellular proliferation was assessed by MTT dye conversion to formazan and analyzed in a plate reader at 540nm. 1 X 10^4 cells were seeded per well in a 96-well plate overnight. After 24 hours, cells were washed with PBS and treated with one of the following experimental conditions: 2.5% charcoal stripped media + DMSO (vehicle

control), 2.5 % charcoal stripped media + visfatin, 2.5% charcoal stripped media +

visfatin + PI3K inhibitor (LY), 2.5% charcoal stripped media + visfatin + ERK inhibitor

(PD) , and 2.5% charcoal stripped media + visfatin + LY + PD for72 h. Charcoal stripped

media was selected because liver cancer cells grow too quickly in complete media. After

72 h, 20 µl of MTT reagent (5 mg/ml) was added to each well. After 2 hours, MTT

reagent was aspirated and 100 µl of DMSO was added to each well and shaken for 10

minutes. Absorbance was measured using Cytation 5.

Reactive Oxygen Species Assay

Intracellular levels reactive oxygen species (ROS) were assessed using a cell-

permeable DCFH-DA probe. HepG2 and SNU-449 cells were seeded in a dark, clear

bottom 96-well microplate. On day 2 cells were exposed to the following experimental

conditions: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS +visfatin, SFM

+ 5% FBS + visfatin + LY, SFM + 5% FBS + visfatin + PD, SFM+5% FBS + visfatin +

LY + PD. On day 3, the media was removed, and cells were washed with 1X buffer, and

incubated for 45 minutes in 1X buffer containing 25 µM DCFDA at 37˚C. Fluorescent

intensity was measured at excitation wavelength 485 nm and emission wavelength 529

nm with Cytation 5.

Oil Red O Staining

Approximately 30,000 HepG2 and SNU-449 cells were seeded in a 24-well plate.

After 24 hours, cells were washed with PBS and treated with one of the following

conditions: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS +visfatin, SFM

+ 5% FBS + visfatin + LY, SFM + 5% FBS + visfatin + PD, SFM+5% FBS + visfatin +

LY + PD. After 48 hours, the media was removed, washed with PBS, fixed for 10 min with 10% formalin, and washed with 60% isopropanol. Cells were stained with filtered oil red O solution for 10 minutes. Lipid droplets were imaged using Cytation 5 (Winooski, VT) at 20X magnification. Lipid droplets were eluted and quantified using 100% isopropanol. Absorbance was measured at 500 nm with Cytation 5.

Invasion Assay

The Corning BioCoat Matrigel Invasion Chamber assay was utilized to assess if the neutralizing antibodies targeting visfatin and resistin decrease the invasive capacity of the HepG2 and SNU-449 cells.[26] The matrigel chamber was hydrated in serum free media for 2 hours prior to seeding. After the chambers were rehydrated, cells were seeded on the upper side of the chamber at 80,000 cells per well in a 24-well plate with one of the following treatments: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS +visfatin, SFM + 5% FBS + visfatin + LY, SFM + 5% FBS + visfatin + PD, SFM+5% FBS + visfatin + LY + PD. The chemoattractant, 10% FBS, was added to the well below the chamber. After 48 hours cells were stained with crystal violet, and images were captured using Cytation 5. After imaging, crystal violet was dissolved using a distain solution (methanol, acetic acid, and water), and absorbance was measured with the Cytation 5 microplate reader. Cell counts were conducted after 48 hours and there were no statistical differences observed between treatment groups (data not shown).

ELISA for MMP-9

To determine MMP-9 secretion from cells, HepG2 and SNU-449 cells were exposed to the experimental conditions for 1 hour. After 1 hour, cells were washed with

PBS and SFM was added to each well to capture MMP-9 secretion. The ThermoFisher

(Waltham, MA) ELISA kit was used to measure MMP-9 secretion.[27]

Western Blot Analysis

HepG2 and SNU-449 cells were plated at 400,000 cells per well in a 6-well plate.

After 24 hours, the cells were serum starved for six hours. The cells were then exposed to

their respective experimental conditions for 15 minutes. After the treatment, cells were

harvested using lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5 ml 10% SDS,

36.86 ml ddH$_2$O) and quantified using Pierce BCA protein assay kit.[28] Fifty µg of protein

lysate was electrophoresed through a 10% gel (4 ml H$_2$O, 3.3 ml 30% acrylamide mix,

2.5 ml 1.5 M TRIS, 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulfate (APS), 8 µl

TEMED along with the stacking gel (3.4 ml ddH$_2$O, 0.83 ml 30% acrylamide mix, 0.63

ml 1.0 M TRIS, 0.05 ml 10% SDS, 0.05 ml 10% APS and 6 µl TEMED). It was then

transferred to a nitrocellulose membrane and blocked with 5% bovine serum albumin for

45 minutes. After measuring phosphorylated proteins, blots were stripped with Restore

Plus western blot stripping buffer before measuring total levels of protein. Target proteins

for western blot analysis are listed in Table 2.

Table 2. Summary of target proteins for western blot analysis

Primary Antibody	Secondary Antibody
FASN (C.60.10) Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Thermo Fisher Scientific, catalogue #MA5-14887, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
β-catenin Dilution: 1:1000 Incubation time: overnight Manufacturer: Thermo Fisher Scientific, catalogue #71-2700, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Phospho-ERK Dilution: 1:2000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #4370, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:5000 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Total-ERK Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #9102, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Phospho-Akt Dilution: 1:1000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #9271, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Total-Akt Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #4691, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Table 2 Continued. Summary of target proteins for western blot analysis

β-actin	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey
Dilution: 1:1000	Dilution: 1:2500
Incubation time: 1 hour	Incubation time: 45 minutes
Manufacturer: Cell Signaling Technology, catalogue #4967, Danvers, MA	Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Statistical Analysis

Values are presented as mean ± s.e. of the mean. Experiments were repeated three times. Statistical analyses were performed between treatment groups except as noted. For all tests, GraphPad Prism 7.0 software was used (GraphPad Software Inc., La Jolla, CA, USA), and P<0.05 was considered statistically significant. Results were compared using either a Mann-Whitney U Test or Student's T Test.

Results

The effect of PI3K/Akt and ERK inhibition on proliferation

Cellular proliferation was assessed in HepG2 and SNU-449 liver cancer cells exposed to visfatin with or without an LY, PD, or both inhibitors for 96 hours. HepG2 cells exposed to visfatin promoted cellular proliferation by 42% when compared to control (p<0.05) (Figure 7A). The addition of the LY and PD suppressed cellular proliferation by 2-fold and almost 3-fold respectively when compared to visfatin (p<0.05). When both inhibitors when added, proliferation was suppressed to levels similar to that of LY as a single agent. SNU-449 cells exposed to visfatin increased cellular proliferation by 52% (p<0.05) (Figure 7B). The addition of the PI3K inhibitor suppressed proliferation by 2-fold (p<0.05). Further, the ERK inhibitor did not suppresses cellular differentiation but had a value similar to that of visfatin. When both inhibitors were used the effect was similar to that of visfatin with LY.

The effect of PI3K and ERK inhibition on lipogenesis

One of the key hallmarks of cancer is the activation of de novo lipogenesis. This process can occur even in the presence of an adequate lipid supply in the body. HepG2 and SNU-449 liver cancer cells exposed to visfatin with or without an Akt inhibitor, ERK inhibitor, or both inhibitors showed no overall difference (Figure 8A/B). These data suggest that the liver cancer cells are receiving energy from another source.

The effect of PI3Kand ERK inhibitors on ROS production

The production of reactive oxygen species can lead to abnormalities in the structure and function of the liver.[49] HepG2 and SNU-449 liver cancer cells were exposed to visfatin with or without LY, PD, or both inhibitors. In the HepG2 cell line there was no difference between the control and the conditions exposed to the inhibitors (Figure 9A). In SNU-449 cells, the addition of visfatin increased ROS production by 13% compared to control ($p<0.05$) (Figure 9B). The addition of LY decreased ROS production by 29% compared to SNU-449 cells exposed to visfatin ($p<0.05$) (Figure 9B). When the ERK inhibitor was added the level of ROS production was similar to that of visfatin. Further, the addition of both inhibitors reduced ROS production to a level similar to LY.

The effect of PI3K/Akt and ERK inhibition on MMP-9 secretion

To determine MMP-9 secretion, cells were exposed to visfatin ±PD, LY, or both inhibitors. The trend for both HepG2 and SNU-449 cells is almost identical (Figure 10 A/B). Visfatin increased MMP-9 secretion significantly compared to control ($p<0.05$). The addition of Akt, ERK, or both inhibitors decreased MMP-9 secretion to levels similar to that of control.

42

PI3K and ERK inhibitors suppress liver cancer cell invasion

Cancerous cells can invade into local tissues and eventually metastasize to other parts of the body. HepG2 cells exposed to visfatin increased invasive capacity by 36% compared to control ($p<0.05$) (Figure 11A). The addition of the LY, PD, or both inhibitors reduced the invasive capacity by 38%, 58%, and 53% respectively. SNU-449 cells exposed to visfatin increased the invasive capacity by 33% compared to control (Figure 11B). The addition of the inhibitors had the same trend with a decrease in invasive capacity compared to visfatin. These data suggest that visfatin contributes to the invasive capacity of liver cancer cells and the addition of LY and PD are useful in decreasing the invasiveness of liver cancer.

Visfatin and resistin signaling

Protein levels of phospho-ERK and phospho-Akt were measured to determine that these pathways are involved visfatin-induced liver cancer. FASN was also assessed due to its role in lipid synthesis and β-catenin has a role in cell-cell adhesion. SNU-449 cells treated with visfatin increased phospho-ERK and LY did not inhibit these results, but this was not seen with HepG2 cells. As anticipated, resistin +PD decreased phospho-ERK in HepG2 and SNU-449 cells. Both cells lines treated with visfatin increased the phosphorylation of Akt and as expected visfatin + LY decreased active Akt. in HepG2 and SNU-449. Surprisingly, the combination of resistin + PD + LY did not decrease the phosphorylation of Akt. In SNU-449 but not HepG2, visfatin increased b-catenin protein levels and LY decreased visfatin-induced β-catenin. However, visfatin + PD was more effective in suppressing the effect of visfatin on β-catenin protein levels in SNU-449.

Interestingly, HepG2 cells treated with visfatin + LY + PD did not result in lower protein levels of β-catenin. Visfatin increased FASN and the combination of LY + PD was more effective in inhibiting visfatin-induced FASN expression (Figure 6).

Discussion

Previous research has shown that phosphorylation of Akt and ERK were elevated by treatment with visfatin.[43] The Akt pathway is activated by phosphorylation and induces cell proliferation and survival of cancer cells.[43] The ERK pathway is also activated through phosphorylation in response to numerous growth factors.[43] Studies have shown that these kinase signaling pathways are involved in various cancers including liver cancer.[5,14,15,16,21] Additional research would determine if inhibition of kinase signaling would suppresses visfatin-induced liver cancer progression.

The results from this *in vitro* study suggest that Akt and ERK signaling pathways are involved in visfatin-associated liver cancer. Cancer metabolism centers around oncogenes being able to rewire cellular metabolism to go through increased growth and division.[50] HepG2 cells exposed to visfatin significantly increased cellular growth compared to the control and the addition of the inhibitors greatly suppressed proliferation. SNU-449 cells demonstrated the same trend with the exception of the ERK inhibitor. The addition of the ERK inhibitor had similar levels of cellular proliferation as visfatin without any inhibitors. This may be due to inherent higher levels ERK due to KRAs mutation in SNU-449 cells. Studies have shown that some ERK mutations might be resistant to MEK inhibitors.[51]

Excessive accumulation of lipids can cause lipotoxicity, cell dysfunction, and changes in metabolic pathways.[52] This is a major risk factor for the development of fatty liver disease and hepatocellular carcinomas.[52] HepG2 and SNU-449 liver cancer cells exposed to visfatin with or without an PI3K/Akt inhibitor, ERK inhibitor, or both inhibitors showed no overall difference in lipogenesis. This suggests that if cells are not accumulating lipids they must be breaking them down. Lipolysis promotes the mobilization of fuel from the adipose tissue to peripheral tisses.[52] This trend continues in the case of ROS. Oxidative stress is associated with the development of hepatocellular carcinoma because the body is being stimulated by free radicals from ROS.[53] This process leads to DNA damage and abnormal protein expression.[53] Studies have shown that patients with hepatocellular carcinoma have an increase in oxidative DNA damage.[53] HepG2 cells exposed to visfatin along with ERK and PI3K/Akt inhibitors did not demonstrate difference in ROS production. SNU-449 cells exposed to visfatin had a modest increase in ROS production compared to control. The addition of the PI3K/Akt inhibitor reduced ROS levels similar to the control but the addition of the ERK inhibitor had no effect on reducing ROS levels. This may also indicate that ERK is constitutively active in this cell line.

Extracellular matrix metalloproteases are critical for the invasive capacity of tumors.[54] MMP-9 secretion in HepG2 and SNU-449 cells were significantly increased when cells were exposed to visfatin compared to control and MMP-9 secretion decreased with addition of the inhibitors. HepG2 and SNU-449 cells significantly increased in invasive capacity when exposed to visfatin. The addition of the inhibitors reduced

invasiveness to that of the control in both cell lines. Based on our results we can conclude that Akt and ERK are critical in visfatin-induced liver cancer progression.

SNU-449 cells treated with visfatin increased phospho-ERK, but the addition of LY did not inhibit these results, but this trend was not observed with HepG2. Resistin + PD decreased phospho-ERK in HepG2 and SNU-449 cells. Both cells lines treated with visfatin increased the phosphorylation of Akt and as expected visfatin + LY decreased active Akt. Surprisingly, the combination of resistin + PD + LY did not decrease the phosphorylation of Akt. There may be a compensatory mechanism in place that allows PD to be effective even in the presence of LY. In SNU-449 cells, visfatin increased β-catenin protein levels and LY decreased visfatin-induced β-catenin but the addition of PD was more effective in suppressing the effect of visfatin on β-catenin protein levels. HepG2 cells treated with visfatin + LY + PD did not result in lower protein levels of β-catenin. There may be crosstalk between the two inhibitors allowing for the expression of β-catenin.

Collectively, we propose that Akt and ERK pathways are key in the development of visfatin-induced liver cancer. The limitation of this study is that it was correlative. Future *in vitro* studies aim to look at the mechanism in which Akt and ERK inhibitors are modifying physiological processes that were assessed.

A.
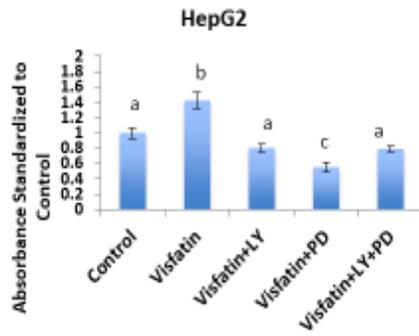
B.
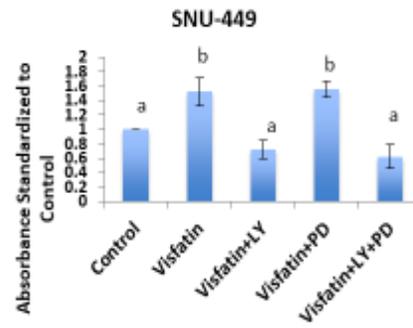
Figure 7. The role of visfatin in cell viability. MTT assay was used to assess cell viability. Growth was assessed by MTT dye conversion. A. HepG2 cells were exposed to the treatments for 72 hours. B. SNU-449 cells were exposed to the treatments for 72 hours. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
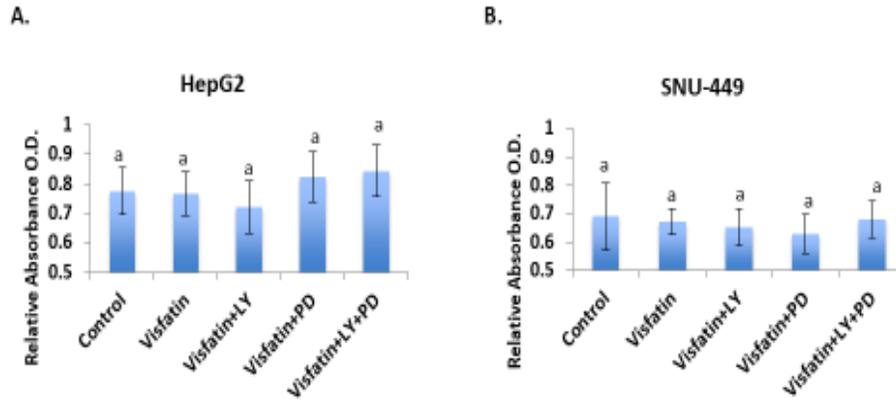
Figure 8. The role of visfatin in lipogenesis in liver cancer cells. A. HepG2 cells were cultured with treatments for 24 hours. B. SNU-449 cells were exposed to treatments for 24 hours. Oil Red O staining was used to visualize lipid accumulation in liver cancer cells. Cells were distained and absorbance was used to measure lipid accumulation in liver cancer cells. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
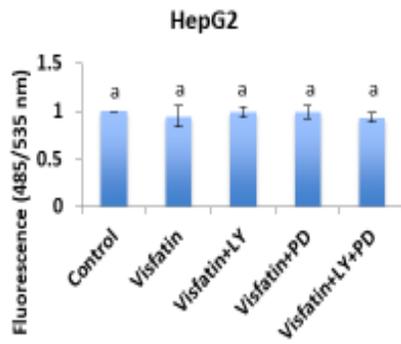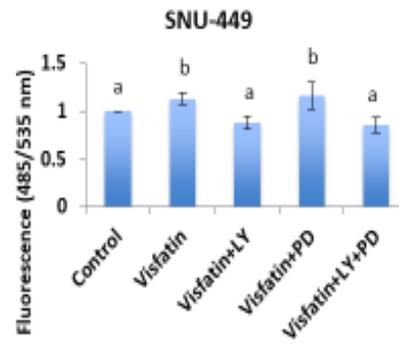
Figure 9. The differential effects of visfatin on ROS production. A. HepG2 and B. SNU-449 cells were cultured with visfatin or resistin. After 24 hours, cells were labeled with DCFDA (20uM) and then analyzed on a fluorescent plate reader. Different letters indicate significant differences between experimental conditions, P < 0.05.

A.

B.
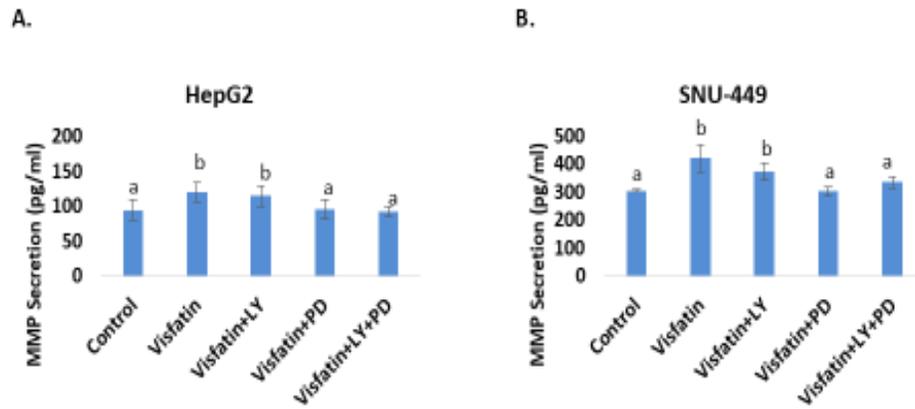
Figure 10. ERK inhibition suppresses visfatin-induced MMP secretion. A. HepG2 and B. SNU-449 cells were conditioned for 1 hour with treatments. After an hour, cells were washed and replaced with serum-free media (SFM) for 24 hours. This conditioned media was analyzed by ELISA assay. Different letters indicate significant differences between experimental conditions, P < 0.05
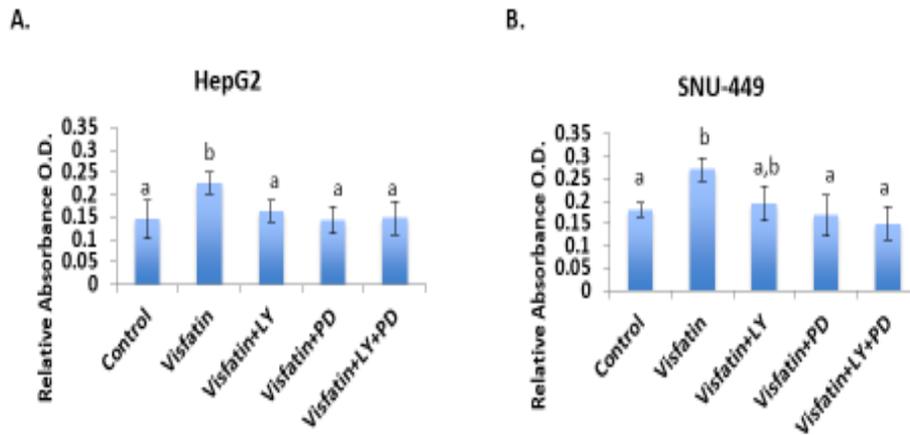
Figure 11. The differential effects of PI3K/Akt and ERK inhibition in visfatin-induced invasion. A. HepG2 and B. SNU-449 cells were seeded in BD Biocoat Matrigel Chambers with treatments and FBS was used as a chemoattractant (in the lower chamber). After 24 hours, cells were fixed, stained, and dissolved. 100ul of dissolution was measured using optical densitometry. Representative images of cells that migrated through the membrane (lower panel) were captured using Cytation 5. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, P < 0.05.
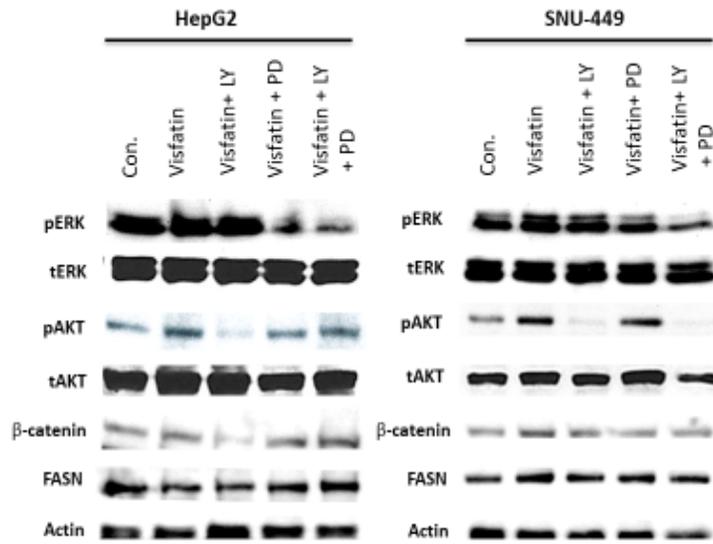
**Figure 12. The effects of PD and LY on protein expression.** HepG2 and SNU-449 cells were cultured in experimental conditions for 15 minutes. Western blot analysis was carried out with antiphospho-ERK, anti-ERK, antiphospho-Akt, anti-Akt, anti-β-catenin, anti-FASN, and anti-actin.

IV: INHIBITION OF KINASE SIGNALING IN RESISTIN-INDUCED LIVER CANCER

Abstract

Resistin is located in monocytes and macrophages of adipose tissue.[20] This adipocytokine is an important link between obesity, inflammation, insulin resistance, and cancer risk.[20] Pathways that resistin are known to be involved include but are not limited to mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERK).[16] The ERK pathway promotes cellular proliferation, migration, survival of cancer cells, and tumor progression.[41] The Akt pathway is known to be up-regulated in many cancers including liver cancer.[41] Studies have yet to determine whether inhibition of kinase signaling will suppress resistin-induced liver cancer progression. Using an *in vitro* model, HepG2 and SNU-449 liver cancer cells were exposed to resistin ± ERK, Akt, or both inhibitors. The following physiological parameters were assessed: cellular proliferation, ROS, lipogenesis, invasion, MMP, and lactate dehydrogenase activity. The inhibition of kinase signaling suppressed most of these parameters. These physiological changes corresponded with decreased phosphorylated Akt and ERK, CAP1, pyruvate dehydrogenase, pyruvate kinase, and FASN. We describe the effect of Akt and ERK inhibitors to determine if inhibition suppresses resistin-induced liver cancer progression.

Introduction

The American Cancer Society estimates that there were 40,710 new cases of liver cancer in 2017 and 28,920 of those cases resulted in death.[1] Liver cancer is considered one of the most lethal cancers with a relatively low survival rate.[8] Also, liver cancer is

one of the few cancers that have had an increase in incidence and mortality within the last two decades.[1] This trend is comparable to our current obesity epidemic with more than one third of adults being obese.[3,8] Studies have shown through an adipokine array that there is an increase in the expression of resistin in an obesity-induced liver cancer phenotype.[5]

Resistin is an adipocytokine that is involved not only in insulin resistance but glucose homeostasis and inflammation which tie into diabetes, cardiovascular disease, and obesity.[20] Also, there is an association between resistin and an increased risk of cancer.[20] Resistin may mediate its effects through the Akt and ERK signaling pathways.[43] The Akt pathway is activated by phosphorylation and induces cell proliferation and survival of cancer cells.[43] The ERK pathway is also activated through phosphorylation in response to numerous growth factors.[43] Additionally, resistin has been shown to induce proliferation in cancer cells but this effect was repressed when Akt and ERK inhibitors were added.[20,43] We hypothesize that the inhibition of Akt and the ERK pathways will suppress resistin induced liver cancer progression. The effect of inhibiting ERK and Akt signaling on resistin-induced liver cancer progression will be assessed. These kinase signaling pathways have been shown to promote cell proliferation, migration, survival along with tumor progression, ROS production, and invasion.[5,21] Studies have shown that Akt and ERK kinase signaling pathways are involved in various cancers including liver cancer.[5,19,21]

Materials and Methods

Cell Culture and Reagents

Human liver cancer cells (HepG2) were purchased from the American Type

Tissue Culture Collection (ATCC) (Rockville, MD). Cells were cultured in DMEM

containing penicillin and streptomycin plus 10% fetal bovine serum (FBS). SNU-449

liver cancer cells were purchased from ATCC. Cells were cultured in RPMI containing

penicillin and streptomycin plus 10% FBS. Cells were maintained at 37°C in a 5% (v/v)

CO_2 humidified incubator. Cells were treated with 10 μM Akt inhibitor ly290004 (Sigma

Aldrich) and 10 μM ERK inhibitor PD98059 (Cell Signaling) 1 hour prior to the addition

of 50 ng/ml visfatin. The concentrations of the PI3K/Akt and ERK inhibitors were

selected based on the previous literature with the use of these inhibitors in human cancer

cells.[32,44,45] The concentration of resistin was selected based on previous studies that

measured resistin levels in obese or diabetic individuals.[55,56,57] The intention of using

these concentrations is to closely recapitulate the physiological levels observed in obese

individuals.

Cellular Proliferation

Cellular proliferation was assessed by MTT dye conversion to formazan and

analyzed in a plate reader at 540nm. 1 X 10^4 cells were seeded per well in a 96-well plate

overnight. After 24 hours, cells were washed with PBS and treated with one of the

following experimental conditions: 2.5% charcoal stripped media + DMSO (vehicle

control), 2.5 % charcoal stripped media + resistin, 2.5% charcoal stripped media +

resistin + PI3K/Akt inhibitor (LY), 2.5% charcoal stripped media + resistin + ERK

inhibitor (PD), and 2.5% charcoal stripped media + resistin + LY + PD for 72 h. Charcoal stripped media was selected because liver cancer cells grow too quickly in complete media. After 72 h, 20 µl of MTT reagent (5 mg/ml) was added to each well. After 2 hours, MTT reagent was aspirated and 100 µl of DMSO was added to each well and shaken for 10 minutes. Absorbance was measured using Cytation 5.

Reactive Oxygen Species Assay

Intracellular levels reactive oxygen species (ROS) were assessed using a cell-permeable DCFH-DA probe. HepG2 and SNU-449 cells were seeded in a dark, clear bottom 96-well microplate. On day 2 cells were exposed to the following experimental conditions: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS + resistin, SFM + 5% FBS + resistin + LY, SFM + 5% FBS + resistin + PD, SFM+5% FBS + resistin + LY + PD. On day 3, the media was removed, and cells were washed with 1X buffer, and incubated for 45 minutes in 1X buffer containing 25 µM DCFDA at 37°C. Fluorescent intensity was measured at excitation wavelength 485 nm and emission wavelength 529 nm with Cytation 5.

Oil Red O Staining

Approximately 30,000 HepG2 and SNU-449 cells were seeded in a 24-well plate. After 24 hours, cells were washed with PBS and treated with one of the following conditions: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS + resistin, SFM + 5% FBS + resistin + LY, SFM + 5% FBS + resistin + PD, SFM+5% FBS + resistin + LY + PD. After 48 hours, the media was removed, washed with PBS, fixed for 10 min with 10% formalin, and washed with 60% isopropanol. Cells were stained with filtered

oil red O solution for 10 minutes. Lipid droplets were imaged using Cytation 5 (Winooski, VT) at 20X magnification. Lipid droplets were eluted and quantified using 100% isopropanol. Absorbance was measured at 500 nm with Cytation 5.

Invasion Assay

The Corning BioCoat Matrigel Invasion Chamber assay was utilized to assess if the neutralizing antibodies targeting visfatin and resistin decreased the invasive capacity of the HepG2 and SNU-449 cells.[26] The matrigel chamber were hydrated in serum free media for 2 hours prior to seeding. After the chambers were rehydrated, cells were seeded on the upper side of the chamber at 80,000 cells per well in a 24-well plate with one of the following treatments: SFM + 5% FBS + DMSO (vehicle control), SFM + 5% FBS + resistin, SFM + 5% FBS + resistin + LY, SFM + 5% FBS + resistin + PD, SFM+5% FBS + resistin + LY + PD. The chemoattractant, 10% FBS, was added to the well below the chamber. After 48 hours cells were stained with crystal violet, and images were captured using Cytation 5. After imaging, crystal violet was dissolved using a distain solution (methanol, acetic acid, and water), and absorbance was measured with the Cytation 5 microplate reader. Cell counts were conducted after 48 hours and there were no statistical differences observed between treatment groups (data not shown).

ELISA for MMP-9

To determine MMP-9 secretion from cells, HepG2 and SNU-449 cells were exposed to the experimental conditions for 1 hour. After 1 hour, cells were washed with PBS and SFM was added to each well to capture MMP-9 secretion. The Thermo Fisher (Waltham, MA) ELISA kit was used to measure MMP-9 secretion.[27]

Lactate Dehydrogenase Activity

To determine lactate dehydrogenase activity, HepG2 and SNU-449 cells were plated at 10,000 cells per well in a 96-well plate. The Sigma-Aldrich Lactate Dehydrogenase Activity Assay Kit was used, according to the manufacturer's protocol to measure the activity of lactate dehydrogenase.[58]

Western Blot Analysis

HepG2 and SNU-449 cells were plated at 400,000 cells per well in a 6-well plate. After 24 hours, the cells were serum starved for six hours. The cells were then exposed to their respective experimental conditions for 15 minutes. After the treatment, cells were harvested using lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5 ml 10% SDS, 36.86 ml ddH$_2$O) and quantified using Pierce BCA protein assay kit.[28] Fifty µg of protein lysate was electrophoresed through a 10% gel (4 ml H$_2$O, 3.3 ml 30% acrylamide mix, 2.5 ml 1.5 M TRIS, 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulfate (APS), 8 µl TEMED along with the stacking gel (3.4 ml ddH$_2$O, 0.83 ml 30% acrylamide mix, 0.63 ml 1.0 M TRIS, 0.05 ml 10% SDS, 0.05 ml 10% APS and 6 µl TEMED). It was then transferred to a nitrocellulose membrane and blocked with 5% bovine serum albumin for 45 minutes. After measuring phosphorylated proteins, blots were stripped with Restore Plus western blot stripping buffer before measuring total levels of protein. Target proteins for western blot analysis are listed in Table 3.

Table 3. Summary of target proteins for western blot analysis

Primary Antibody	Secondary Antibody
FASN (C.60.10) Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Thermo Fisher Scientific, catalogue #MA5-14887, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Phospho-ERK Dilution: 1:2000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #4370, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:5000 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Total-ERK Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #9102, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Table 3 Continued. Summary of target proteins for western blot analysis

Phospho-Akt Dilution: 1:1000 Incubation time: overnight Manufacturer: Cell Signaling Technology, catalogue #9271, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Total-Akt Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #4691, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
β-actin Dilution: 1:1000 Incubation time: 1 hour Manufacturer: Cell Signaling Technology, catalogue #4967, Danvers, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Table 3 Continued. Summary of target proteins for western blot analysis

CAP1 Dilution 1:250 Incubation time: overnight Manufacturer: Thermo Fisher Scientific, catalogue #PA5-56354, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:1250 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Pyruvate Dehydrogenase Dilution 1:1000 Incubation time: overnight Manufacturer: Thermo Fisher Scientific, catalogue #PA5-17190, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA
Pyruvate Kinase Dilution 1:1000 Incubation time: overnight Manufacturer: Thermo Fisher Scientific, catalogue #PA5-13790, Waltham, MA	ECL Anti-Rabbit IgG, horseradish peroxidase linked whole antibody from donkey Dilution: 1:2500 Incubation time: 45 minutes Manufacturer: GE Healthcare UK Limited catalogue #NA934V, Pittsburg, PA

Statistical Analysis

Values are presented as mean ± s.e. of the mean. Experiments were repeated three times. Statistical analyses were performed between treatment groups except as noted. For all tests, GraphPad Prism 7.0 software was used (GraphPad Software Inc., La Jolla, CA, USA), and P<0.05 was considered statistically significant. Results were compared using either a Mann-Whitney U test or student's *t* test.

Results

The effect of PI3K/Akt and ERK inhibition on proliferation

To assess for cellular proliferation, HepG2 and SNU-449 liver cancer cells were exposed to resistin with or without an LY, PD, or both for 72 hours. HepG2 cells exposed to resistin exhibited similar levels of cellular proliferation as control. The addition of PI3K/Akt and ERK inhibitors resulted in a 72% decrease and a 2-fold decrease in cellular proliferation respectively (p<0.05) (Figure 13A). SNU-449 cells expose to resistin increased proliferation by 9% compared to control (p<0.05) (Figure 13B). The addition of the LY, PD, or both inhibitors decreased cellular proliferation by 55%,18%, and 44% respectively.

The effect of PI3K/Akt and ERK inhibitors on lipogenesis

The process of lipogenesis provides energy to cancer cells thereby leading to an increase in cell division and overall survival enhancing tumor growth.[59] HepG2 and SNU-449 cells were exposed to resistin with or without LY or PD for 48 hours. In HepG2 cells, the addition of resistin decreased lipogenesis by 19% (p<0.05) (Figure 14A). When

the inhibitors were added there was a slight increase in lipogenesis compared to control, but it was not statistically significant. In SNU-449 cells, the addition of resistin also decreased lipogenesis (Figure 14B). Further, the ERK inhibitor was the only condition to statistically decrease lipogenesis.

The effect of PI3K/Akt and ERK inhibitors on ROS production

Almost all cancers have been found to have an increase in ROS production which promotes tumor development and progression. Although there is an increase in ROS, there is also an increase in the levels of antioxidant proteins expressed by the tumor cells to detoxify from ROS production.[60] HepG2 and SNU-449 cells were exposed to resistin with or without LY or PD for 24 hours. In both cell lines, there was a trend for resistin to increase ROS production compared to control, but these findings were not statistically significant (Figure 15A/B). Further, the addition of PI3K/Akt, ERK, or both inhibitors decreased ROS production, but these results were not statistically significant.

PI3K/Akt and ERK inhibitors suppress the invasive capacity of liver cancer cells

Matrigel invasion chambers were used to assess the invasive capacity of liver cancer cells exposed to resistin with or without LY, PD, or both inhibitors. In HepG2 cells, resistin increased invasion by 36% compared to control (p<0.05) (Figure 16A). The addition LY, PD, or both inhibitors suppressed the invasive capacity by 39%, 57%, and 66% respectively. SNU-449 cells exposed to resistin increased invasion by 35% compared to control (p<0.05) (Figure 16B). The SNU-449 cells had the same trend as the HepG2 cells with a decrease in invasive capacity in the conditions exposed to LY, PD, or both inhibitors (p<0.05).

The effect of resistin on LDH

Certain cancer cells shift from using mitochondrial oxidative phosphorylation to pyruvate reduced to lactate for energy. Lactate dehydrogenase (LDH) is an enzyme that catalyzes interconversion of pyruvate and lactate.[58] In HepG2 cells, resistin significantly increased LDH activity compared to control ($p<0.05$) (Figure 17A). The addition of LY, PD, and both inhibitors were all statistically significant compared to control ($p<0.05$). The trend is almost identical in the SNU-449 cells. Resistin significantly increased LDH activity compared to control ($p<0.05$) (Figure 17B). The addition of LY reduced LDH activity but was not found to be statistically significant ($p<0.05$). The addition of PD and combination of inhibitors significantly decreased LDH activity compared to control ($p<0.05$).

The effect of PI3K/Akt and ERK inhibition on MMP-9 secretion

To determine MMP-9 secretion, cells were exposed to resistin $\pm$ ERK, Akt, or both inhibitors. In HepG2 cells, there were no differences in the experimental groups (Figure 18A). In SNU-449 cells, resistin promoted MMP secretion compared to control ($p<0.05$) (Figure 18B). Resistin + LY reduced MMP secretion to levels similar to control. Resistin + PD and Resistin plus both inhibitors significantly reduced MMP secretion compared to resistin ($p<0.05$)

Resistin signaling

Protein levels of phospho-ERK and phospho-Akt were measured to ensure that these pathways are in fact involved in resistin-induced liver cancer progression. Fatty acid synthase catalyzes the final steps of de novo lipogenesis and CAP1 was measured

because it is a functional receptor for resistin. Pyruvate is the end product of glycolysis and drives many pathways in the TCA cycle. The enzyme pyruvate dehydrogenase goes through an irreversible reaction in which pyruvate and NAD+ are converted into acetyl-CoA and NADH. From there the acetyl-CoA can enter the TCA cycle. Pyruvate kinase is involved in the last step of glycolysis in which phosphoenolpyruvate is converted to pyruvate. In HepG2 cells but not SNU-449, resistin increased phospho-ERK. Resistin + LY did not decrease phospho-ERK but as expected, resistin + PD did decrease phospho-ERK. Further, the combination of resistin + LY + PD decreased resistin-induced phospho-ERK in HepG2 cells and SNU-449. Both HepG2 and SNU-449 cells exposed to resistin increased the phosphorylation of Akt. As anticipated, resistin + LY suppressed the phosphorylation of Akt while PD had no effect. Only HepG2 cells exposed to resistin increased CAP1 and FASN while the addition of LY or PD did not change protein levels of either CAP1 or FASN. HepG2 cells treated with resistin increased PDH but this difference was not observed in SNU-449. Resistin + LY was the most effective in decreasing PDH compared to treatment with PD in HepG2 and SNU-449. The combination of PD and LY also decreased PDH which may be due to LY rather than PD. In HepG2 cells, resistin increased PK and the addition of LY or PD did not inhibit this increase. Lastly, differences in PK protein levels were not observed in SNU-449 cells (Figure 7 A/B).

Discussion

The Akt pathway is activated by phosphorylation and induces cell proliferation and survival of cancer cells.[43] The ERK pathway is also activated through phosphorylation in response to numerous growth factors.[43] These kinase signaling

pathways have been shown to promote cell proliferation, migration, survival along with tumor progression, ROS production, and invasion.[5,21] Additionally, resistin has been shown to induce proliferation in cancer cells but this effect was repressed when PI3K/Akt and ERK inhibitors were added.[20,43] Additional research would aid in our understanding of the effectiveness of PI3K/Akt and ERK inhibitors in a resistin-induced liver cancer phenotype.

The results from this *in vitro* study suggest that Akt and ERK signaling pathways are involved in resistin-induced liver cancer. The ability of cancer cells to sustain proliferation is a fundamental trait.[13] In HepG2 cells, the addition of the PI3K/Akt and ERK inhibitors reduced cellular proliferation and the addition of both inhibitors was most effective. In the SNU-449 cells, resistin increased proliferation more so than control and the addition of the PI3K/Akt inhibitor significantly reduced cellular proliferation. The addition of the ERK inhibitor was not as effective and the combination treatment was similar to the cells treated with the PI3K/Akt inhibitor alone. This may be due to KRAs mutation in SNU-449 cells which express higher levels of ERK.

Lipogenesis and fatty acid oxidation are important mechanisms in the context of liver damage since the liver is the central organ in lipogenesis.[61] In HepG2 cells the addition of resistin decreases lipogenesis compared to control. The addition of the inhibitors slightly increased lipogenesis compared to resistin. In SNU-449 cells, the trend continues with resistin not promoting lipogenesis as much as control and inhibitors slightly increasing lipogenesis except the ERK inhibitor which significantly reduced lipogenesis. Other studies have shown that resistin promotes lipid accumulation.[62] Although we did not observe these effects, it may be due to a switch from lipogenesis to

lipolysis. However, this study did not measure lipolysis but should be integrated in future studies. The production of reactive oxygen species modulates a multitude of cell signaling pathways which are mediated through a number of transcription factors, cytokines, and enzymes that are involved in inflammation, tumor survival, proliferation, invasion, angiogenesis, and cancer metastasis.[63] In both HepG2 and SNU-449 cells there was a trend for an increase in resistin and a slight decrease in cells exposed to ERK and PI3K/Akt inhibitors but the results were not significant.

High levels of MMP may correlate with an increase in invasive capacity. There was no difference in MMP secretion in HepG2 cells. In SNU-449 cells, resistin significantly promoted invasive capacity compared to control and the addition of PI3K/Akt and ERK inhibitors significantly decreased the invasive capacity of the cells. To our knowledge, there is no research thus far that investigates the effect of PI3K/Akt and ERK inhibitors on MMP secretion in resistin.

In HepG2 cells, resistin increased phospho-ERK, LY did not decrease phospho-ERK, but as expected, resistin + PD did decrease phospho-ERK. The combination of resistin + LY + PD decreased resistin-induced phospho-ERK in HepG2 cells and SNU-449. Resistin + LY suppressed the phosphorylation of Akt while PD had no effect in either cell line. Only HepG2 cells exposed to resistin increased CAP1 and FASN while the addition of LY or PD did not change protein levels. HepG2 cells treated with resistin increased PDH but this trend was not observed in SNU-449. Resistin + LY was the most effective in decreasing PDH compared to treatment with PD in HepG2 and SNU-449. The combination of PD and LY also decreased PDH which may be due to LY rather than PD. In HepG2 cells, resistin increased PK and the addition of LY or PD did not inhibit

this increase. Lastly, differences in PK protein levels were not observed in SNU-449

cells. Future studies should do a dose response with resistin at various time points to

determine if resistin will eventually increase PK. Two glycolytic enzymes, PK and PDH

were assessed, along with Lactate dehydrogenase activity to determine if there is an

increase in glucose uptake and fermentation of glucose to lactate.[6] Resistin significantly

increased LDH activity compared to control. This suggests a switch from using

mitochondrial oxidative phosphorylation to pyruvate reduced to lactate for energy, The

Warburg Effect.[6] Protein levels of PK and PDH also increased in the presence of resistin.

This indicates that more acetyl-CoA is being made which is needed to supply the cell

with NADH. NADH is necessary for the conversion of pyruvate to lactate.

The results of this *in vitro* study identify PI3K/Akt and ERK as being critical

pathways for resistin-induced liver cancer. Overall, resistin promotes cellular

proliferation, ROS, MMP, invasion and LDH activity. The limitation of this study is that

it is correlative but necessary to determine the role of Akt and ERK inhibition on resistin-

induced liver cancer. Future *in vitro* studies aim to look at the mechanism in which

PI3K/Akt and ERK inhibitors are modifying physiological processes that were assessed.
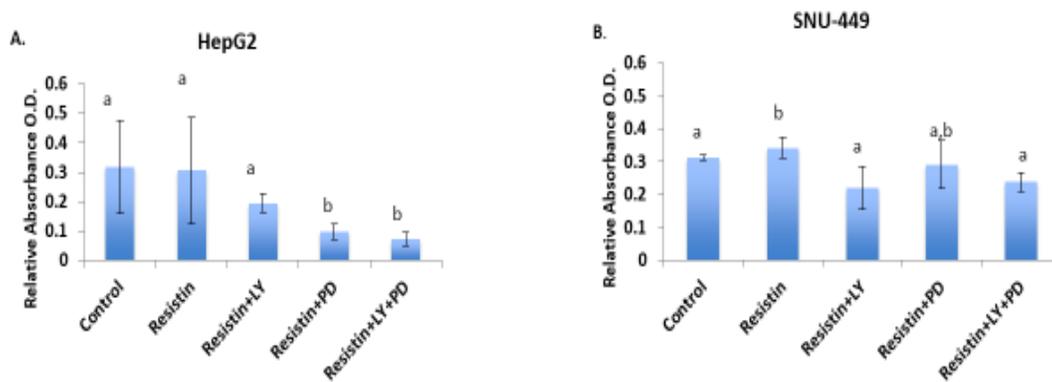
Figure 13. Resistin promotes cell viability in SNU-449 cells. MTT assay was used to assess cell viability. Growth was assessed by MTT dye conversion. A. HepG2 cells were exposed to the treatments for 72 hours. B. SNU-449 cells were exposed to the treatments for 72 hours. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
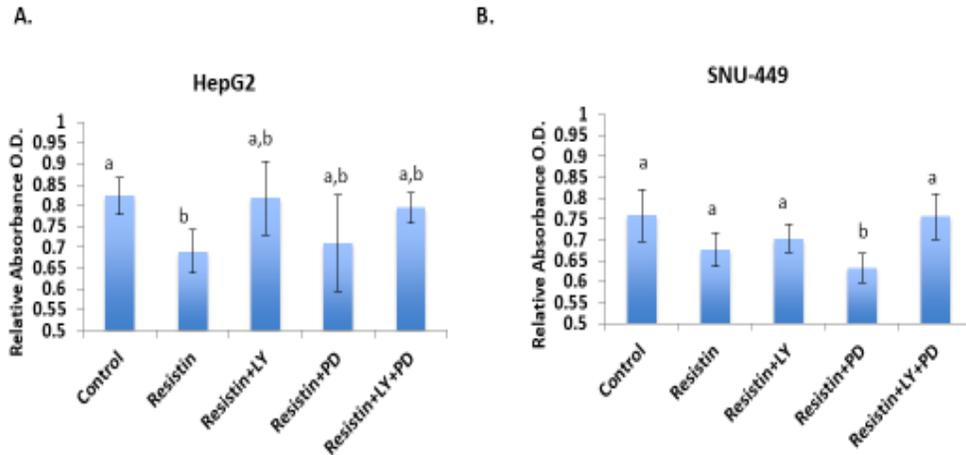
Figure 14. The role of resistin in lipogenesis in liver cancer cells. A. HepG2 cells were cultured with treatments for 24 hours. B. SNU-449 cells were exposed to treatments for 24 hours. Oil Red O staining was used to visualize lipid accumulation in liver cancer cells. Cells were distained and absorbance was used to measure lipid accumulation in liver cancer cells. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
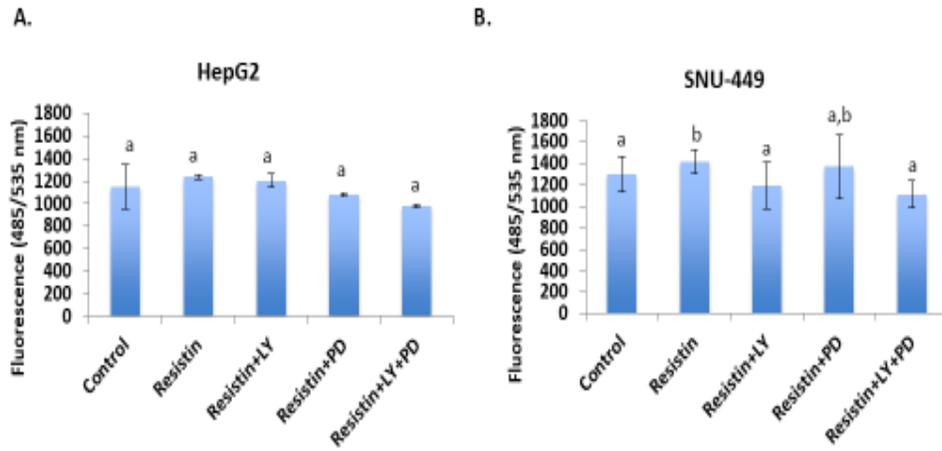
Figure 15. The role of Akt and ERK in resistin-associated ROS production. A. HepG2 and B. SNU-449 cells were cultured with visfatin or resistin. After 24 hours, cells were labeled with DCFDA (20uM) and then analyzed on a fluorescent plate reader. Different letters indicate significant differences between experimental conditions, $P < 0.05$.
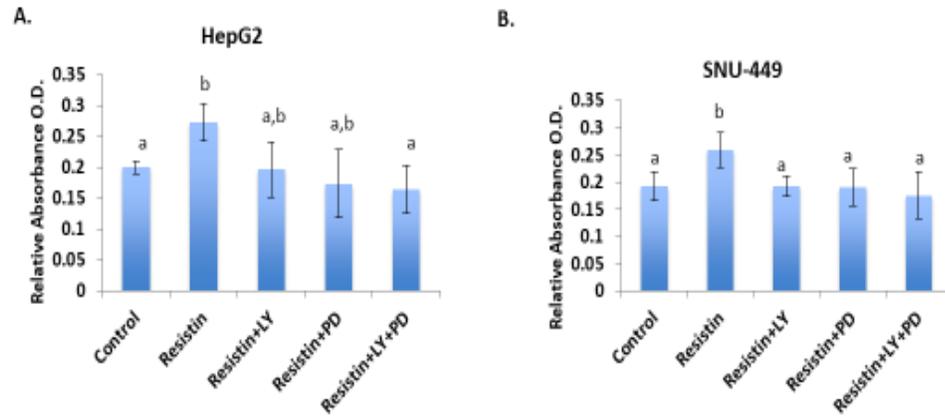
Figure 16. The differential effects of PI3K/Akt and ERK inhibition in resistin-induced invasion. A. HepG2 and B. SNU-449 cells were seeded in BD Biocoat Matrigel Chambers with treatments and FBS was used as a chemoattractant (in the lower chamber). After 24 hours, cells were fixed, stained, and dissolved. 100ul of dissolution was measured using optical densitometry. Representative images of cells that migrated through the membrane (lower panel) were captured using Cytation 5. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, P < 0.05.
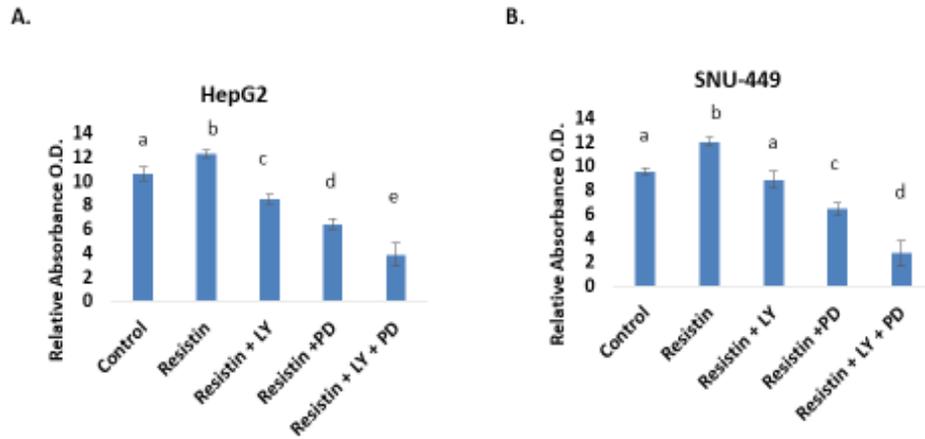
Figure 17. Changes in lactate dehydrogenase activity in liver cancer cells. A. HepG2 and B. SNU-449 liver cancer cells were exposed to treatments for 24 hours followed by LDH detection.
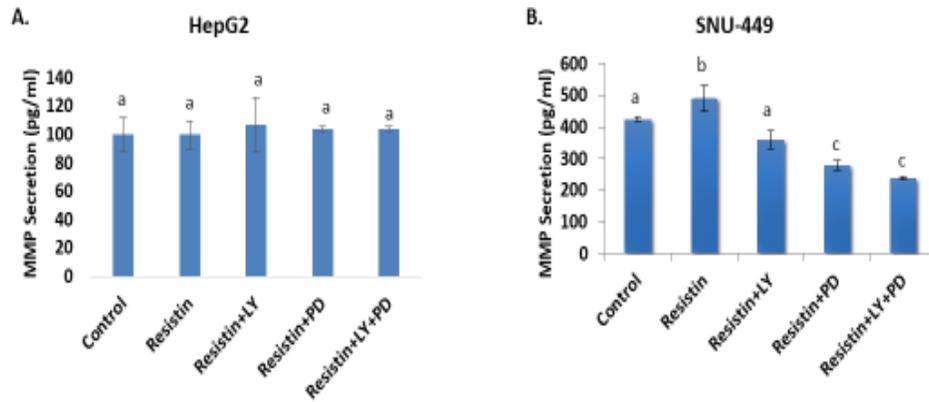
Figure 18. The differential effects of PI3K/Akt and ERK suppression on MMP secretion. A. HepG2 and B. SNU-449 cells were conditioned for 1 hour with treatments. After an hour, cells were washed and replaced with serum-free media (SFM) for 24 hours. This conditioned media was analyzed by ELISA assay. Different letters indicate significant differences between experimental conditions, P < 0.05.
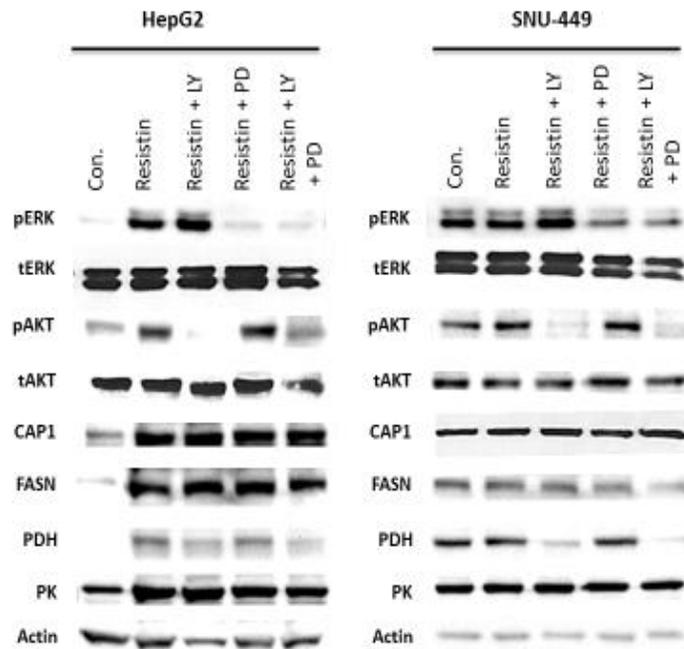
Figure 19: The effects of PD and LY on protein expression. HepG2 and SNU-449 cells were cultured in experimental conditions for 15 minutes. Western blot analysis was carried out with antiphospho-ERK, anti-ERK, antiphospho-Akt, anti-Akt, anti-CAP1, anti-FASN, anti-PDH, anti-PK, and anti-actin.

V. IMPLICATIONS FOR FUTURE STUDIES

In the study outlined in Chapter 2, obesity increased ROS, MMP, invasion, ERK, and Akt compared to normal weight. Visfatin contributed to an increase in MMP, ROS, and invasion in HepG2 cells. Visfatin inhibition was effective in decreasing the phosphorylation of Akt in a less aggressive cell line. This suggests that an intervention with a visfatin antibody would be more effective in the earlier stages of liver tumorigenesis. Resistin was the most effective in inhibiting phosphorylation of Akt and ERK along with ROS, MMP, and invasion. This may suggest that resistin inhibition may be important in multiple stages of liver cancer progression. Visfatin and resistin play an important role in promoting an invasive liver cancer phenotype. Studies have suggested that the activation of NF-kB or STAT3 can promote invasion and overall metastasis. Future studies can determine if visfatin or resistin mediate their effects through STAT3 or NF-kB activation.

In the study outlined in Chapter 3, visfatin treatment increased cell viability, MMP, invasion, and phosphorylation of Akt. Inhibition of PI3k/Akt was effective in decreasing cell viability, ROS, invasion, phospho-Akt, phospho-ERK, and β-catenin. However, there were differential effects which were cell line specific. This suggests that visfatin has multiple effects on physiological parameters and protein expression which are mediated through either Akt or ERK inhibition. In summary, these results suggest that inhibition of the visfatin protein itself may be more effective than targeting its downstream signaling. Future research will expand, mechanistically, on how visfatin mediates invasion. Visfatin may bind to IR or IGF-1R which can activate Akt/ERK along

with NF-kB and initiate gene transcription for genes associated with invasion and progression in liver cancer cells.

In Chapter 4, resistin treatment increased cell viability, invasion, LDH activity, PDH, PK, ERK, Akt, FAS, and CAP1. These results were not consistent in both cell lines which suggests that differences in mutations that contribute to the development of liver cancer might determine the importance of resistin and its downstream targets. In general, inhibiting LY in the aggressive cell line, SNU-449, was more effective than PD in cells exposed to resistin. In summary, this suggests that resistin and Akt signaling is important in liver cancer progression evidenced by a decrease in invasion, cell viability, and changes in cellular energetics. Resistin was shown to be a classic example of the Warburg Effect. Future studies aim to look at the underlying mechanism in the glycolytic pathway leading to an increase in lactate production.

Collectively, in an *in vitro* model of obesity and adipocytokine depletion, visfatin and resistin did not contribute to proliferation and lipogenesis. However, as single agents visfatin and resistin increased proliferation and lipogenesis. These results suggest that there are other obesity-related hormones or cytokines mediating these effects. When visfatin and resistin were used as single agents they did not promote ROS or MMP. Although, when obese serum was depleted of visfatin or resistin, ROS and MMP decreased. Therefore, there are other ROS and MMP promoting factors in the serum that work synergistically to promote ROS production and MMP secretion. Among the three studies, visfatin and resistin induced invasion and promoted phosphorylation of Akt and ERK. Future studies should target visfatin and resistin rather than their downstream targets because the inhibition of these adipocytokines was more effective than inhibiting

77

Akt or ERK. Targeting visfatin and resistin may slow down liver cancer progression because suppressing adipocytokine signaling was found to decrease both Akt and ERK. These findings are important because visfatin and resistin inhibitors could be used in future clinical studies to reduce the impact of obesity on liver cancer progression.

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79

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