EFFECT OF METFORMIN ON INSULIN MEDIATED PROLIFERATION OF LUNG CANCER CELL LINES

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In

Biosciences

BY

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ABSTRACT

EFFECT OF METFORMIN ON INSULIN MEDIATED PROLIFERATION OF LUNG CANCER CELL LINES

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Key words: Non small cell lung carcinoma, Diabetes mellitus, Insulin resistance, Metformin, Insulin

Background: The incidence of cancer in diabetes mellitus (DM) patients is a matter of concern. Lung cancer is the most commonly diagnosed cancer and leading cause of death in males. Smoking is the main risk factor contributing to lung cancer progression. The occurrence of cancer is more with the type 2 DM. Besides, hyperglycemia and endogenous insulinaemia exist together for a long duration as a result of insulin resistance. As a consequence of this, the mitogenic activity of insulin is amplified. Moreover, other growth factors, and hormones are activated under the influence of insulin that further enhances this effect. It is also related to obesity, central fat accumulation, physical inactivity and smoking. The nicotine of smoke induces oxidative stress and endothelial malfunction creating metabolic abnormalities in lung cancer. In this perspective, role of insulin sensitizing drug, metformin in inhibiting the growth proliferation of lung cancer cells is hereby explored.

Objective: The present study was aimed to evaluate the growth proliferation effect of insulin on non small cell lung carcinoma cell lines. It also proposed to evaluate role of metformin in preventing insulin mediated proliferation in p53 and liver kinase B1 (LKB1) mutant and wild type cell lines.

Materials and methods: Two non small cell lung carcinoma cell lines, A549 and H1299 (p53 and LKB1 wild type and mutant) were used to analyze the mitogenic role of insulin by incubating for 24 hours with human recombinant insulin at a range of concentrations from 1nM to 10μM. This was followed by the metformin (concentrations from 1μM to 50mM) treatment for 24 hours along with insulin (500μM for A549 and 1mM for H1299). The proliferations were assessed by MTT dye reduction test and the percentage of the survival of the treated cells was compared with the control. One way ANOVA was used for the data analysis and the proliferation between cell lines were evaluated by student's t-test and two way analysis of variance (Two way ANOV).

Results: Both the cell lines exhibited a significant proliferation ($p<0.001$) with the concentrations of insulin. Insulin stimulated the proliferation approximately by two fold and 1.78 times for A549 cells and H1299 cell line respectively compared to control cells. The growth of two lung carcinoma cell lines were significantly ($p<0.001$) inhibited by metformin treatment for 24 hours. The maximum reduction in growth was 73% and 67% for A549 and H1299 respectively for a concentration of 50mM of metformin compared to the control. The results followed a dose dependant response pattern for insulin as well as metformin treatment. Concentration at which 50% inhibition of growth observed (IC_{50}) was comparable for both the cell lines.

Conclusions: Insulin in high circulating concentrations can augment the growth proliferation of lung cancer cells. Metformin can inhibit this insulin mediated proliferation of lung cancer cells in a multifaceted way. The mechanism of action is independent of p53 and liver kinase B1.

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviation
1	Advanced glycation end products	AGE
2	Body mass index	BMI
3	Diabetes mellitus	DM
4	Human leukocyte antigen	HLA
5	Hybrid receptors	IGF-IR/IR-A & IGF-IR/IR-B
6	Insulin growth factor I &II	IGF-I & II
7	Insulin growth factor -I Receptor	IGF-IR
8	Insulin receptor	IR
9	Insulin receptor isoforms A & B	IR-A & IR-B
10	Insulin receptor substrates 1-4	IRS1-4
11	Liver kinase B1	LKB1
12	Mammalian Target of rapamycin	mTOR
13	Major histocompatibility complex	MHC
14	Mitogen activated protein kinase	MAPK
15	Non small cell lung carcinoma	NSLC
16	Tuberous sclerosis complex	TSC
17	Transcription factor 7-like 2	TCF7L2
18	Vascular endothelial growth factor	VEGF

Student Approval Form

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Declaration

I declare that all the changes suggested by the external examiner in the dissertation entitled " E**ffect of metformin on insulin mediated proliferation of lung cancer cell lines"** submitted by me for the award of degree of Master of Philosophy in the Centre for Biosciences has been incorporated in the dissertation.

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Chapter one

Introduction

1.1 Diabetes Mellitus: Definition and Epidemiology

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia. It affects majority of the organ systems and is associated with partial or complete deficit of pancreatic insulin that leads to metabolic disturbances. Based on aetiology, diabetes mellitus is classified into two types: type 1and type 2. Type 1 diabetes is a T- cell mediated autoimmune disease in which β cells in the pancreatic islets undergo destructive process causing a severe insulin deficiency. Type 2 is linked with resistance of insulin to its biological responses in liver and muscle cells along with the impaired pancreatic β cell function (Colledge, 2010).

The enormity of this disease has been aptly brought out by the World Health Organization reports which highlights the major brunt borne by developing countries(Diabetes fact sheets, 2011). As per their statistics, number of death cases attributed to diabetes mellitus, worldwide, is expected to double in the time period from 2005-30. It is therefore a major public health problem due to the numerous sufferings it causes to patients and the serious economic impact it poses to health care giver systems.

1.2 Genetics and Pathophysiology of DM

The interaction of genetic and environmental factors is seen in both types of diabetes. The key genetic locus of type 1 is on human leucocyte antigen (HLA) within the major histocompatibility complex (MHC) on the short arm of chromosome 6 (Kantarova & Buc, 2007). An important genetic determinant of type 2 DM is transcription factor-7 like 2 gene (TCF7L2) (Rees et al., 2011). Environmental factors like, viral and microbial infections, dietary factors, and various nitrosamines are found to accelerate the disease (van Belle, Coppieters, & von Herrath, 2011). Diet, tobacco smoking and obesity are other contributing reasons of DM, which shows a close predilection to middle aged population (Hamilton, Hamilton, & Zderic, 2007).

At the cellular level, type 1 Diabetes Mellitus (DM) is caused by specific antibodies acting on the pancreatic β cell causing its destruction. This irregularity of auto immune system may be due to genetic changes which are elicited with other environmental factors. Inflammation and damage of cells are mediated by macrophages and lymphocytes. Pathophysiology is complex in type 2 compared to type 1. Type 2 diabetes mellitus is primarily related to insulin resistance. There is an inappropriate signaling system from the insulin receptors for producing the desired metabolic effect that leads to a state of hyperglycemia. This in turn stimulates pancreatic insulin secretion that results in secondary hyperinsulinaemia. Both hyperglycemia and hyperinsulinaemia occurs in early stages (Zeitler, 2008). Towards final stages, dysfunction of β cells occurs which leads to the decrease in plasma insulin. Many aspects have been suggested for the development of insulin resistance. Abnormalities in the lipid metabolism and accumulation of free fatty acid (FFA) seen with obesity, impaired nutrition and physical inactivity play crucial roles in insulin resistance syndrome (Lewis, Carpentier, Adeli, & Giacca, 2002).

1.3 Antidiabetic Drugs

Glycaemic control can be maintained in the early stages by suitable changes in dietary habits and lifestyle measures. Oral anti hyperglycemics like Biguanides (Metformin), Thiazolidinediones, Sulphonylureas, Meglitinides and α-glucosidase inhibitors are drugs commonly used for type 2 DM (Tripathi, 2008). These are having different mechanisms of action: insulin sensitizing mechanism which helps uptake of glucose into the cells (metformin), delaying glucose absorption (α-glucosidase inhibitors) and by stimulation of insulin production (sulphonylureas). A combination of oral anti-diabetic agents and insulin is used in chronic cases. The later stages most often require insulin replacement therapy due to the reduction in the β cell mass of pancreas (Tripathi, 2008).

1.4 Complications of DM

Diabetes mellitus is mostly present along with hypertension and dyslipidemia. Further, patients develop various secondary complications as a result of poor glycaemic control for a long time period. These complications can be micro vascular

or macro vascular. Retinopathy, cataract, nephropathy, peripheral neuropathy, microangiopathy and ulceration of foot follow in such chronic cases. Moreover, it increases the risk of coronary heart diseases (Kim, 2002; Plutzky, 2011; Schalkwijk & Stehouwer, 2005). Development of cancer in people with type 2 diabetes mellitus is another dangerous finding (Barone et al., 2008; Vigneri, Frasca, Sciacca, Pandini, & Vigneri, 2009). This is proved by the fact that 10% of mortality of diabetes is due to cancers of different organs coexisting with renal complications (Colledge, 2010).

1.5 Diabetes and Cancer

The influence of diabetes mellitus (more with type 2) in cancer progression is more critical as cancer too carries a higher rate of mortality. According to 2008 world estimates, about, 12.7 million cancer cases and 7.6 million deaths are accounted and out of which 56% of cases and 64% of deaths occurred in developing world (Jemal et al., 2011). Carcinogenesis is a multi step process initiated as an alteration in the gene. Deviation from the normal genetic process of cell division and differentiation lead to a stage of transformation from cellular normality to neoplasm. When the cells invade or disrupt the surrounding tissue, it is considered as malignant. Metastasis is the stage of spreading the tumor to other organs and forms neoplasm in new locations. Activation of proto oncogenes and suppression of tumor suppressor genes are believed to be main factors for initiating carcinogenesis. Many other genetic variations and dysregulation of signaling and metabolic pathways are also involved in its progression. Characteristics that contribute to a high morbidity for cancer are its heterogeneity, variations in clinical presentation, late diagnosis, nature of metastasis and recurrence (Colledge, 2010). The origin of cancer can be from all the organ systems and the present study is focusing on the lung cancer.

1.6 Lung cancer: Epidemiology and Classification

Among all types of cancers, lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer death in males. It is accounted for 13% of total cases and 18% of deaths in 2008 (Jemal et al., 2011). In US, estimated cancer deaths were approximately 28% irrespective of the sex in 2011 (Siegel, Ward, Brawley, & Jemal, 2011). This cancer is formed from the tissues of the lung which are

lining the air passages. Histologicaly they are of two types: Small Cell Lung Carcinoma and Non Small Cell Carcinoma (NSLC). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma fall under NSLC which constitute the major group (Gamez-Pozo et al., 2009). Squamous cell carcinoma is the common type that originates from the epithelial lining of bronchial tubes. Adenocarcinoma shows mostly involve mucus secreting glandular cells but rarely affects alveolar sacs. The large cell carcinoma is seen in the periphery of lung. This classification is mainly meant for treatment decisions (Rueth et al., 2012).

1.7 Lung cancer: Aetiology and Symptoms

Lung diseases like bronchitis and pneumonia with coughing are the initial symptoms which undiagnosed and not treated can aggravate. Diagnosis is by histological evaluation of sputum cytology, bronchoscopy, lymph node biopsy, thoracocentesis and imaging techniques like computerized tomography and positron emission tomography (Matsuoka, Miyoshi, Morimoto, Hino, & Tsuyuguchi, 2011). Survival rates decrease with late diagnosis and is generally seen in people at 45-50 years. Development of secondary tumors in the upper aero digestive tract is another complication. Delay in timely diagnosis and treatment has been found to decrease patient survival time for the lung cancer (Indovina, Marcelli, Maranta, & Tarro, 2011).

The most important cause of lung cancer is cigarette smoking. People working with asbestos, silica, beryllium, cadmium and chromium are also in the high risk for developing lung cancer. In addition, high levels of air pollution, also have a contributory role (Hueper, 1956). For all these reasons, prevalence of lung cancer is high in India (Behera & Balamugesh, 2004). Though environmental factors do play a role in lung cancer progression, genetic aspects are also involved in it (Roth, 1995).

1.8 Diabetes and Lung Cancer

There are diverging evidences for the association of lung cancer and diabetes type 2. The increased association of diabetes in lung cancer is noticed in many studies (Hemminki, Li, Sundquist, & Sundquist, 2010; Lee, Lin, Hsiao, & Shin, 2012; Swerdlow et al., 2005). Diabetes is considered as a predictive factor for recurrence and patient survival. Moreover, the prime defect of diabetes, insulin resistance has

been included as a potential factor for the survival of lung cancer patients (Park, Lim, Shin, & Yun, 2006). The rate of incidence varies with intensity of smoking, or time of onset of the disease. However, the inverse correlation of diabetes and lung cancer is also documented. It is owing to the high body mass index (BMI) seen in diabetes patients which is negatively related to lung cancer (Atchison, Gridley, Carreon, Leitzmann, & McGlynn, 2011; Ehrlich, Quesenberry, Van Den Eeden, Shan, & Ferrara, 2010). The increased metabolic rate due to smoking results in weight loss (Koh, Yuan, Wang, Lee, & Yu, 2010). Another reason relays on the age of incidence of lung cancer at which the patient may succumb to other diabetic complications (Hall, Roberts, Boulis, Mo, & MacRae, 2005).

1.9 Diabetes and Cancer: Molecular Links

Type 2 DM and cancer have common risk factors like obesity, tobacco use and sedentary life style (Hemminki et al., 2010). The long term hyperinsulinaemia secondary to the insulin resistance may enhance proliferative capacities of the cells. Figure1 represents the factors of diabetes mellitus contributing to cancer progression. They stimulate the synthesis of insulin growth factors (IGF-I & IGF-II). Insulin acts on receptors IR-A and IR-B (isoforms of insulin receptors), in which the signaling through IR-A is more mitogenic than metabolic. IGF-I mainly signals through IGF-IR and cause growth promoting effects. IGF-II can act through IR-A for the proliferative effect. Many hybrid receptors are also noticed in tumor cells (Gallagher & LeRoith, 2010; Tsugane & Inoue, 2010). The molecular mitogenic mechanisms of insulin/IGF signaling are illustrated in Figure 2. The metabolic disturbances seen in diabetes may add on to this factor. High glucose concentrations activate reactive oxygen species which induces oxidative stress. The nicotine of smoke generate imbalance of free radical axis which also causes oxidative stress in the cells. This results in hemodynamic changes and DNA alterations (Reuter, Gupta, & Aggarwal, 2010).

1.10 Metformin: Anti Proliferative Role in Cancer

The increased incidence of cancer affecting various organs in type 2 varies with the therapeutic drugs used in diabetes (Swerdlow et al., 2005; Vigneri et al.,

2009). The risk is more with patient on sulphonylurea and insulin (Bowker, Majumdar, Veugelers, & Johnson, 2006) and less with metformin usage (Landman et al., 2010). The antiproliferative effects of metformin are reported in lung cancer attributed to tobacco induced tumorigenesis (Ben Sahra, Le Marchand-Brustel, Tanti, & Bost, 2010; Memmott et al., 2010). Metformin hydrochloride (*N, N*-dimethylimidodicarbonimidic diamide hydrochloride) had been used as a first line treatment for diabetes since long time. They are used mainly in patients with insulin resistance in type 2 diabetes and along with insulin in type 1 obese cases.

Metformin is extracted from the herb, *Galega officinalis*. This was first used along with tea for symptoms of repeated urination. The practice of it as a medicine for hyperglycemia started in Britain in 1958, and later to US and all part of the world (Dowling, Goodwin, & Stambolic, 2011).

Figure 1. Mechanisms contributing to cancer progression in diabetes mellitus (Hemminki et al., 2010; Lewis et al., 2002)

Metformin increases the insulin sensitivity of the cells thereby helping utilization of peripheral glucose. They inhibit the hepatic gluconeogenesis. It will not

cause hypoglycemia in non diabetic (Tripathi, 2008). At molecular level, metformin activates AMP regulated protein kinase. This kinase is activated when the adenosine monophosphate (AMP): ATP ratio increases. This leads to decreasing of protein synthesis and proliferation of cells by inhibiting the mTOR pathway (Landman et al., 2010). Direct targeting of mammalian Target of Rapamycin (mTOR) pathway independent of AMPK is also reported. These molecular mechanisms may explain the beneficial effect of metformin in cancer (Figure 3).

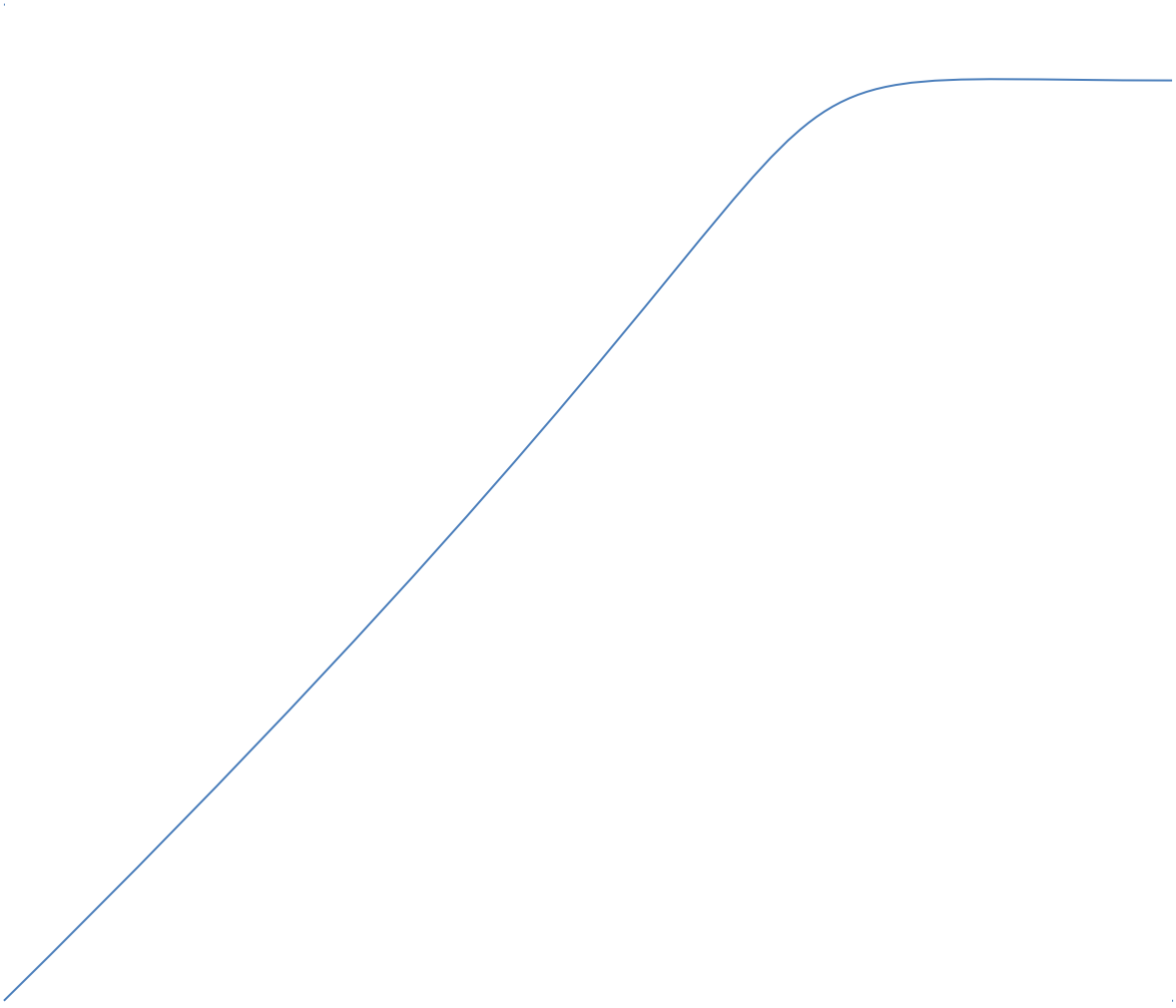

Figure 2. Molecular mitogenic mechanisms of insulin/IGF axis (Ben Sahra, Laurent et al., 2010; Gallagher & LeRoith, 2010; Pollak, 2008)

IGF-I&II: Insulin growth factors I&II. IR-A&IR-B: Insulin receptor isoforms, IGF-IR: Insulin growth factor -I Receptor, IGF-IR/IR-A & IGF-IR/IR-B: Hybrid receptors, IRS1-4: Insulin

receptor substrates1-4, mTOR: mammalian target of rapamycin, TSC- Tuberous sclerosis complex

Figure 3. Anti mitogenic roles of metformin (Ben Sahra, Laurent et al., 2010; Gallagher & LeRoith, 2010; Pollak, 2008)

AMPK: Adenosine monophosphate kinase, IGF-I&II: Insulin growth factors I&II, LKB1: Liver kinase B1, mTOR: mammalian target of rapamycin, TSC: Tuberous sclerosis complex

However, there are reports that shows the necessity of Liver Kinase B1 (LKB1), a tumor suppressor functions for activation of AMPK pathway, and the selectivity of mutated p53 towards expressing the anti tumor effect of metformin (Ben Sahra, Laurent et al., 2010; Buzzai et al., 2007; Memmott & Dennis, 2009).

From the foregoing, it is prudent to study the role of insulin on proliferation and the effect of metformin on lung cancer. This *in vitro* study focuses on the mitogenic or proliferative role of insulin and the anticancer effect of metformin by using non small

cell lung cancer cell lines. The cell lines selected are, A549 and H1299, which is p53 and LKB1 wild type and mutant so as to evaluate the specificity of p53 and LKB1 mutation in metformin action as well.

Chapter Two

Review of Literature

2.1 Diabetes Mellitus: Characteristics

The incidence of clinical symptoms like polyurea of diabetes mellitus is narrated 3000 years ago by the ancient Egyptians. Later many developments in extracting the cause and reasons of the disease took place, the most important being the discovery of excess sugar in blood and urine, role of liver, pancreas and isolation of hormone, insulin (Ahmed, 2002). Now, it is a common disorder that needs lifelong treatment. The literature is replete with description of aetiology and pathology of the disease (DeFronzo, 2004; van Belle et al., 2011). The characteristic feature of the disease is the high levels of blood glucose (hyperglycemia) due to the anomaly of insulin mediated utilization of it by liver, muscle and adipose tissue. The impairment in muscle glycogen synthesis, hexokinase II activity and muscle glucose transportation is studied by using the Nuclear magnetic resonance (NMR) spectroscopy (Shulman, 2000).

2.1.1 DM Complications: Effects of Hyperglycemia

Guillausseau and Laloi (2003) depicts hyperglycemia and secondary hyperinsulinaemia form the basis of complications of diabetes. The uncontrolled diabetes mellitus is highly degenerative. According to Polak et al. (1997) at molecular level, glycation of proteins and lipids due to excessive circulating glucose level forms glycotoxins and advanced glycation end products (AGE). Harmful effects of which include increase in extracellular matrix production, neuropathy, and vascular complications. Excessive production of growth factors is suggested to cause retinopathy and renal complications. Involvement of vascular endothelial growth

factor (VEGF) and protein kinase C are also described. Smoking adds to the deleterious effects of cardiovascular complications (Mancini et al., 2012). Many metabolic pathways are interrupted or defective in this disease and hence poorly controlled diabetes mellitus (both the types) influence harshly on the general health of the affected individual. Continuous and prolonged inconsistencies in type 2 DM make it a complicated and multifarious one.

2.1.2 DM Complications: Effects of Hyperinsulinaemia

In addition to hyperglycemia, compensatory hyperinsulinaemia seen in early stages is a distinctive feature of type 2 DM. As insulin is having various metabolic functions like, glucogenesis, glucogenolysis, gluconeogenesis and protein synthesis, the impairment of insulin secretion and inactivity affects almost all organ systems. Defronzo (2004) documented that the loss of glucose homeostasis owing to the derangement of insulin secretion and nonresposiveness leads to aberrant metabolic pathways. Deprivation of energy from glucose source subsequently leads to the utilization of stored triglycerides. This fat metabolism releases free fatty acids to the circulation which amplifies the difficulty due to the insulin resistance. Moreover, the circulating levels of glucose stimulate the pancreas and consequently increasing the plasma insulin concentration (Lewis et al., 2002). Concentration of plasma insulin increases as that of glucose in type 2 DM.

2.1.3 Insulin Resistance: Causes and Implications

Recent advancements in technology have been added onto the know-how of this metabolic impairment. Obesity, physical inactivity, extra energy diet, hyperlipidaemia are some of the reasons predicted to cause this non receptiveness of insulin by the cells (Guilherme, Virbasius, Puri, & Czech, 2008; Kahn & Flier, 2000; Savage, Petersen, & Shulman, 2005). Higher body weight due to physical inactivity leads to the central fat accumulation which disturbs insulin system. Smoking is considered as a causative factor of insulin resistance (Attvall, Fowelin, Lager, Von Schenck, & Smith, 1993). Various studies explain the mechanisms involved in the smoking and metabolic impairment. Chiolero et al. (2008) relates the body weight of smokers to the impairment. They mentioned the hormonal imbalance also as a

cause. Another mechanism proposed is due to the stimulative effect of nicotine, carbon monoxide and other agents in the smoke on the growth hormones and adrenal gland hormones. In addition, nicotine increases the oxidative stress which impairs the endothelial dysfunction that can lead to the resistance to insulin directed glucose uptake (Attvall et al., 1993; Axelsen et al., 1995; Schalkwijk & Stehouwer, 2005; Zeiher, Schachinger, & Minners, 1995). The insulin resistance and compensatory insulinaemia is associated with many other disorders including, hypertension, cardiovascular difficulties, kidney abnormalities and defects in the nervous system (Brands & Hall, 1992). Jee, Kim &Lee (2005) pointed out insulin resistance as one of the risk factors for cancer in diabetes patients. The authors also discuss the interactive roles of inflammation, insulin resistance, diabetes, atherosclerosis and cancer.

2.2 Diabetes Mellitus and Cancer: Epidemiology

The risk of incidence of diabetes has been a topic of research among scientists from long back. In a multisite case controlled study conducted at Roswell Park Memorial Institute between 1957 and 1965 showed the risk of endometrial cancer in diabetes (O'Mara, Byers, & Schoenfeld, 1985). Different types of epidemiological and group studies have been reported during these years to analyze the risk of cancer occurrence in diabetes mellitus. The relation has been observed for hepatic, pancreas and endometrial cancer (La Vecchia, Negri, Franceschi, D'Avanzo, & Boyle, 1994). Friberg, Mantzoros & Wolk (2007) noticed the risk with endometrial cancer and assessed the possible ways of management in a cohort study. The rate is found to be more in colorectal cancer cases that use insulin as a treatment of diabetes in two works done at US. The involvement of other site of origin of cancer like, breast, liver, pancreas and bladder have also been specified (Coughlin, Calle, Teras, Petrelli, & Thun, 2004; Vinikoor et al., 2009). Some inverse or inconclusive relations are also reported in the case of prostate cancer. The protective effects of prostate cancer may be explained due to the effect of leptin and testosterone (Kasper & Giovannucci, 2006). However, the contribution of insulin resistance has been proved in the development of benign prostate hyperplasia (Vikram, Jena, & Ramarao,

2010). The time of onset of the disease, stage of diagnosis, the study group and severity of the disease are some of which may influence the variations in the reports.

2.3 Diabetes Mellitus and Lung Cancer: Epidemiology

Conflicting reports exist for the risk of lung cancer in diabetes. In a study conducted in Japan, the increased risk of lung cancer is detected irrespective of the sex in diabetes patients (Kuriki, Hirose, & Tajima, 2007). Independency of lung cancer incidence with hypertension, hyperlipidaemia and gout is cited by (Lee et al., 2012). The initial age-specific increase of the occurrence of lung cancer in diabetes at the age of 30-49 years is found to be declining in the long term follow-up (Swerdlow et al., 2005). Similar information is documented at University of Alberta (Johnson, Bowker, Richardson, & Marra, 2011). The diabetes is considered to play a major part in predicting the survival rate as well as the local recurrence of common lung cancer. The irregularity in metabolism of biomolecules in DM may augment the complications of cancer (Varlotto et al., 2012; Win et al., 2008).

However, some epidemiological studies do not support the correlations between diabetes and lung cancer. Asthma, chronic obstructive pulmonary disease and pneumonia were found to be associated with the difficulties of diabetes, but lung cancer is not categorized in this group. This is connected with the loss of lung function as diabetes advances (Ehrlich et al., 2010). The low body mass index or decreased survival of diabetes patients are cited as the explanations for the negative correlations (Atchison et al., 2011; Ehrlich et al., 2010). Varying degree of smoking habits, severity and complication of diabetes and time of onset and diagnosis of disease are highly influential in predicting the risk in lung cancer. The stage of diabetes mellitus is the prime factor to decide the possibility of episode of cancer as the pathophysiology differs in early and progressive stages.

2.4 Diabetes Mellitus and Cancer: Cellular Biology

Many researches explored the cellular events of diabetes destining the cancer progression. Vingeri et al. (2009) evaluated the position of elevated levels of glucose and insulin in blood which can contribute the transformation of normal cells. They pointed out the severity of occurrence associated with type 2 DM as the difference in

distribution of endogenous insulin and that used in the treatment. Unlike the insulin secreted from pancreas, the injected insulin reaches to the liver and peripheral tissue with the same concentration and which is not remaining in circulation for long period. The prevalence and the age of onset are the favoring aspects for type 2. Moreover, the hyperinsulinaemia secondary to the reduced response of insulin in type 2, as discussed in the pathophysiology of diabetes, lasts for decades before reaching to the stage of lack of insulin production by pancreatic β cells (Vigneri et al., 2009). Severe complications results for type 2 and are basically due to the reductive response of metabolic hormone. Nevertheless, the hyperglycemia and exogenous insulin of type 1 may also have some deleterious effects in proliferation.

2.4.1 Role of Insulin

Multiple and complex systems operate at receptor level for the enhanced mitogenic activity of insulin. Insulin acts through insulin receptor (IR) which has two isoforms IR-A and IR-B. These are produced as an effect the alternative splicing. Signaling through IR-A is mitogenic (Gallagher & LeRoith, 2010). Moreover, Insulin stimulates the growth hormone signaling through the growth hormone receptors (GRH receptors) and the synthesis of insulin like growth factor-1(IGF-1). Supportively more expression of IGF-I and IGF-II is observed in most of the neoplasm. As shown in Figure 1, IGF-1 binds to the IGF-IR and mostly is mitogenic. IGF–II binds to IR-A as well as IGF-1R, creating mitogenic changes (Vigneri et al., 2009). Quite often the hybrid receptors are also noted on the tumor cell surface. These are formed by the by alpha and beta subunits of IR-A and IR-B. IGF can bind to either of the receptors whereas IGF-II can bind only to IGF-IR/IRA hybrid. Insulin has negligible effects on this. Besides these alterations, decreased levels of IGF-1 Binding proteins, (IGFBP-1 &IGFBP-2) and make available these factors in the biologically active form which is more interactive (Pollak, 2008). A decrease in circulating levels of sex-hormone binding globulin and increases the bioavailability of estradiol and testosterone has been noticed in the case of breast cancer. This intensifies the IGF signaling.

2.4.2 Role of Growth Factors

In addition to the direct pathways, function of interleukin 6, plasminogen activator inhibitor-1, adiponectin leptin and tumor necrosis factor in the growth promoting effect is proposed (van Kruijsdijk, van der Wall, & Visseren, 2009). Besides this growth factor mechanism, oxidative stress formation in cells due to the imbalance between reactive oxygen species and antioxidants unfavorably modulate and modify certain intercellular reactions. This directly or indirectly have profound outcome in various stages of cancer progression, cell survival, radio resistance, chemo resistance and angiogenesis. Implications and relations of oxidative stress, chronic inflammation, and carcinogenesis were reviewed (Reuter et al., 2010). The continuous elevated glucose concentration in blood directly plays a crucial role of induction of oxidative stress which deteriorates the cell function.

There is a positive correlation already cited for FFA and insulin resistance (Lewis et al., 2002). Several metabolic pathways are elucidated to explain the oxidative stress, inflammation and tumor growth. Investigations on the effect of hyperglycemia in advanced glycation end products AGE, protein kinase C, polyol pathway has intensely carried out. The nuclear factor-B9 NF-B, p38 mitogen activated kinases (MAP) and hexosamine pathways are also added to this function (Evans, Goldfine, Maddux, & Grodsky, 2003). Many deviations from the normal metabolism which happen in the pathology of diabetes mellitus outline the origin of mitogenic activities. These researches signify the glycaemic control of diabetes mellitus for preventing the undesirable complications including cancer.

2.4.3 Diabetes Mellitus and Lung Cancer: Specific Mechanisms

The oxidative stress induced by nicotine of tobacco and smoke cause insulin resistance can lead to the hyperinsulinaemia and metabolic syndrome in lung cancer. This is emphasized by the study of Petridou et al. (2011) in finding of the risk of insulin resistance in developing lung cancer. The proliferative effects of circulating insulin in lung cancer are supported by the increase insulin and IGF receptors in tumor cells of lung. The up regulation of vascular endothelial growth factors by insulin is proved in NSLC (Gray et al., 2008). IGF and insulin receptors were increased in

number in lung cancer cells which supports the role of insulin (Spiliotaki et al., 2011; Vigneri et al., 2009). The amplification of IGF signaling through the estrogen β receptors in lung cancer is reported (Tang et al., 2012). Smoking increases the metabolic rate and obesity is seldom observed in chronic smokers, and hence BMI (Atchison et al., 2011). However, the other harmful effects of smoke can lead to derangements in metabolic pathways.

2.5 Diabetes Mellitus and Cancer: Role of Anti-Diabetic Drugs

The cancer rate in diabetes- affected individuals depends to a certain level on the therapy they are undergoing. Mostly, this focuses on the mechanism of action of the particular drug in reducing the blood sugar level. The insulin secretogenic drugs like sulphonylurea activate the ATP dependant channel on pancreatic β cells. These kinds of drugs become less effective in late stages when the degeneration of β cells of pancreas occurs. The patients who are on the exogenous insulin falls in the same high risk category as that of patients on sulphonylurea (Bowker et al., 2006). Hemkens et al. (2009) compared the effects of insulin analogues also. Accordingly, gargline had more potent effect than insulin in its proliferative effects, the reason being the high affinity of insulin analogues for IGF receptors. On the other hand, insulin sensitizing drugs like, metformin and thiazolidinedions favor the prevention of proliferation. Very few clinical studies have been reported for thiazolidinedions (Giovannucci et al., 2010). Numerous investigations are going on to confirm the effect of metformin in cancer irrespective of the origin (Andujar-Plata, Pi-Sunyer, & Laferrere, 2012; Chong & Chabner, 2009; Libby et al., 2009). *In vivo* and *in vitro* studies have performed to find out the effect of metformin in tobacco induced tumor of lung (Memmott et al., 2010).

2.6 Metformin and Cancer

2.6.1 Antiproliferative Effects of Metformin

Antiproliferative action of metformin is answered by understanding its mechanism of action at molecular level. In our body it stimulates the glucose storage pathways and preventing the gluconeogenesis. Different mechanisms are studied at post receptor level (Ben Sahra, Le Marchand-Brustel et al., 2010; Dowling et al.,

2011). Intracellularly it activates adenosine monophosphate kinase (AMPK) pathway. The AMPK is turned on in cells whenever there is a less production of ATP. This is followed by the suppression of energy requiring processes of the cell. Subsequent activation of many downstream complexes reduces or inhibits the cholesterol metabolism, protein and fatty acid synthesis etc. Pollak (2008) describes the inhibition of protein synthesis through the mammalian target of rapamycin (mTOR) pathway by activating the tumor suppressor tuberous sclerosis complex 2 (TSC 2). Dowling et al. (2011) has documented the other routes of metformin effect, by directly inhibiting the mTOR. In the case of lung cancer, in an experiment worked out by Memmott & Dennis (2009) pointed out the antitumorigenic effect of metformin is through the direct inhibition of mTOR pathway without activating the AMPK.

Effect of metformin on cell cycle arrest at G_0/G_1 phase is independent of the AMPK activation in preventing cell proliferation in breast, colon and prostate have been described (Ben Sahra et al., 2008). In the same study it is noted that metformin could reduce the tumor size by the continuous use of the drug. This implied the cyclin D1 and retinoblastoma protein (pRb) may be the targets of metformin. The metformin individually and in combination was effective in its anti tumor activity in lung and breast cancer cell lines (Rocha et al., 2011). There are different pathways by which metformin can contribute to the anti proliferative effect. Illustrations are given in Figure 2.

2.6.2 Role of p53 and LKB1 in Metformin Effect

Inconsistent opinions are presented for the requirement of LKB1 gene for the action of the biguanides. Zakikhani et al. (2008) in their *in vitro* study using cell lines of prostate, colon and cervical adenoma verified the LKB1 role in repressing the protein synthesis. HeLa (cervical adenoma cells) with non functional LKB1 allele was not responsive to metformin at all concentrations they used. Ben Sahra et al. (2010) reviewed the association of downstream elements in the serial pathway of antineoplastic role of metformin. In an *in vitro* experiment to verify the combined effect of metformin with chemotherapeutic drug by using LKB1 wild type and mutant cell line, no significant difference in the effect has been noticed (Rocha et al., 2011).

Metformin can add its antiproliferative effect not only through AMPK pathway, but indirect inhibitory pathways can also be adopted (Dowling et al., 2011). This implies the biguanides interacting directly and indirectly to the growth inhibitory function.

Moreover, another relevant issue regarding the targets of metformin is the mutation of the p53 gene. This is significant in the case of cancers as this tumor suppressor is altered in many of the origins of cancer. The selectivity of metformin in cancer preventive action in p53 deficient cells is evaluated in colon cancer and proved the specificity of metformin action by observing fatty acid oxidation, lactate production and glucose consumption (Buzzai et al., 2007). However, metformin could cause a cell cycle arrest independent of p53 as was evidenced in the study of Ben Sahra (2010). The group discusses various studies regarding the p53 altered and wild type in the drug effect and concludes the ultimate inhibition of mTOR pathway and the cell cycle arrest leading to the anticancer activities of the drug (Ben Sahra, Le Marchand-Brustel et al., 2010).

The diabetes pathophysiology can lead to insert cancers of different origin as one of its complications. Cancer which is multifactorial in its aetiology will be more degenerative if associated with diabetes. Lung cancer, the major risk is linked with tobacco use, can create a worsened situation in diabetes mellitus patients. Individual and group variations are seen particularly in this form of cancer due to the different kinds of risk factors associated with the disease. As insulin resistance and oxidative stress are found to be chief reasons in this incidence, the insulin sensitizing mechanism of metformin may help to prevent this risk factor, but warrants more information.

Chapter Three

Aim and Objectives

The increasing incidence of lung cancer in diabetes type 2 patients may be attributed to the mitogenic role of insulin which is seen in high circulating levels in the early phases of the disease. In lung cancer patients, smoking or related risk factors often leads to insulin resistance and subsequent diabetes. It is now understood that this effect can be reduced by insulin sensitizing drugs like, metformin. So, the present study is based on the hypothesis that hyperinsulinaemia can have proliferative /mitogenic role in lung cancer progression by signaling through insulin-IGF axis and an oral hypoglycemic drug like metformin can inhibit or suppress this growth.

The aims and objectives of this *in vitro* study were:

1. To find the proliferation of non small cell lung carcinoma cell lines (A549 and H1299) by insulin
2. To find the effect of metformin on the insulin mediated proliferation of non small cell lung cancer cell lines
3. To compare the effect of metformin in p53 and LKB1 mutant and wild type cells

Chapter Four

Materials and Methods

4.1 Lung Cancer Cell Lines

Human non small cell lung cancer cell lines used for the experiment were A549 and H1299. A549 is an alveolar adenocarcinoma cell line. It was first established in 1972 from 58 year old Caucasian male by D J Giard (Giard et al., 1973). They are basal epithelial cells of the alveoli and are squamous in nature. This cell line is hypo triploid with a modal chromosome number of 66. Modal number 64 and 67 also are seen in high frequencies. They are p53 wild type and LKB1 (liver kinase B1) mutated (Rocha et al., 2011). In addition, more deletions are reported in cytogenetic analysis. These squamous epithelial cells are keratin positive and have the ability to synthesize lecithin. In culture, they form adherent monolayer with doubling time of approximately 22 hrs (American Type Culture Collection, 2011).

H1299 is non small cell lung carcinoma cell line derived from the metastatic site of lymph node. This is deposited from a 43 year old male Caucasian male by Dr. A. Gazdar and Dr. J. MInna (American Type Culture Collection, 2011). In culture, they grow adherent to form monolayer with a doubling time of approximately 15-16 hours. They have a homozygous deletion of p53 gene and hence no expression of p53 protein (Tsai et al., 1996). Studies revealed the capability of synthesis of peptide neuromedin by this cell line. This cell line is LKB1 wild type (Zhang et al., 2008).

4.2 Maintenance of Cell Lines

Both the non small cell lung cancer cell lines, A549 and H1299 were obtained from National Centre for cell Sciences, Pune. The cell lines were transported in nutrient medium with 5% FBS. As soon as cells were received in the laboratory, it was observed under inverted microscope (Magnus Olympus) for any focus of

contamination and for understanding the general state of the cells. The half of the transport media was removed and FBS was added to make a final concentration of 10%. The culture flask was kept in incubator for aeration of the cells at 37^{0}C and 5% CO_2.The removed media was centrifuged to obtain the detached cells during transfer. The pellet formed is seeded in cell culture flask and incubated.

The cell lines were maintained in Dulbecco's modified eagle's medium, high glucose, 4.5gm/l (DMEM) (Gibco, invitrogen) supplemented with 10% heat inactivated FBS, 1% solution of penicillin (5000units/ml) and streptomycin (500µg/ml). The incubation was done at 37^{0}C and 5% CO_2 and saturating humidity. This was made possible in a special type of incubator with a constant supply of CO_2 (New Brunswick Scientific, Galaxy 170R, eppendorf, Germany). The purpose of using CO_2 was to maintain the pH as CO_2 dissolves in the culture medium containing bicarbonate ions which was in equilibrium with water and carbon dioxide thereby maintaining the pH (Freshney, 2005)**.** For getting a proper supply of CO_2, vented flasks were used. The experiments were performed at complete sterilized air by removing particles greater than 0.3 micrometers through High Efficiency Particulate Air (HEPA) filter (Freshney, 2005).

The cells were observed microscopically every day. The medium was renewed at every 2-3 days for both the cell lines. When growth attained 60-70% confluent, the sub culturing was done. The medium from the flask which was to be sub cultured was discarded. The cells were washed with phosphate buffered saline. Pre warmed trypsin (0.25%) – EDTA (0.02%) solution was added, kept in incubator for 1-2 minutes and observed for detachment of cells from monolayer. The clumps of the cells were disrupted gently by pipeting and centrifuged at 1200 rpm for 5 min. The cells were resuspended in prewarmed growth medium and sub cultured into 2-3 flasks. According to the cell density, the split ratio was varied. The cells were incubated at 37^{0}C and 5% CO_2. The sub culturing flasks and morphology of the cell lines are shown in Figure 4. For preservation, healthy non contaminated cells were harvested by trypsinization and centrifuged at 1200rpm for 5min. The cells were then suspended in cell freezing medium. Complete growth medium with 10% dimethyl sulphoxide (DMSO) was used as the cell freezing medium. I ml of the (approximately

2-3x10^4 cells) cell suspension was transferred to cryovials and labeled with date, passage number and cell name. The vials were placed at -80^{o}C overnight and then transferred to liquid nitrogen.

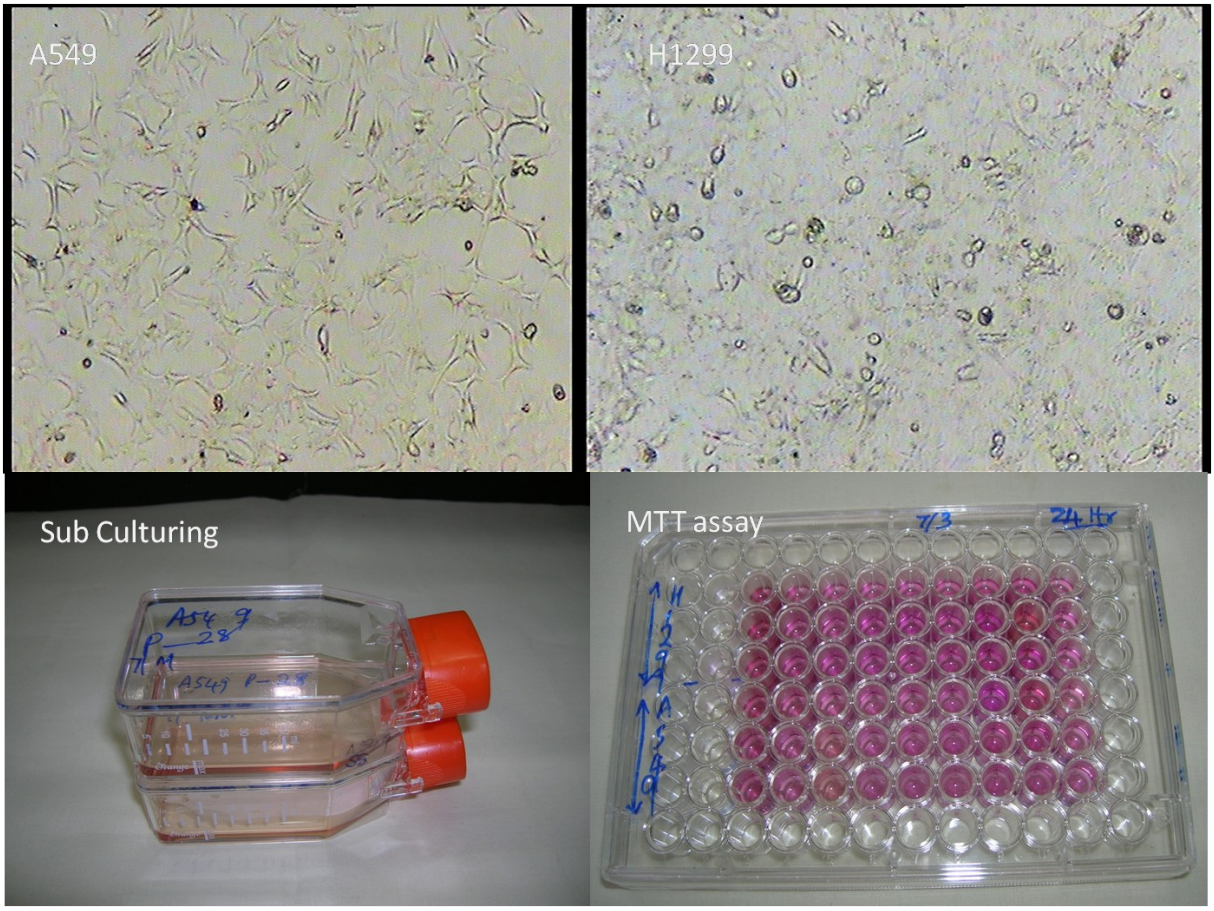

Figure 4. Subculturing and morphology of non small cell lung cancer cell lines, A549 (A) and H1299 (B). 96 well plate with cells after MTT assay is shown. Photographs taken at Tissue Culture Laboratory, Central University of Punjab

4.3 Cell Counting Using Hemocytometer

The cells were harvested by trypsinization and resuspended in prewarmed growth medium. The counting was done with improved neubauer counting chamber. 70% ethanol was used to clean the surface of the counting chamber and cover slip. By using a micropipette, the counting area was charged with cell suspension. The cells were drawn into the area by capillary action. The four corner squares of volume 0.4mm^3 was selected for counting. The cells per ml were calculated by multiplying the

total number of cells counted in four squares by 2500. According to required cell density the cell suspension was diluted.

4.4 Insulin and Metformin Treatment

The cell count was performed by hemocytometry. Viability was assessed by trypan blue staining test. Approximately, 10,000 cells of A549 and H1299 were seeded with complete growth medium in the wells of 96 well plate and incubated at 37^0C and 5% CO_2 for 24 hours for adherence of the cells on to the surface. The viability was checked with trypan blue dye test. Next day, the medium was removed and discarded. Growth medium without FBS was added to each well to provide starvation to serum for the cells to synchronize the cell cycle dividing phase. Human recombinant insulin (10mg/ml, High media, Mumbai, India) is diluted with the foetal bovine serum free growth media to various doses like 1nM, 5 nM, 10nM, 50nM, 100nM, 500nM, 1000nM, 5000nM and 10,000 nM. Each concentration of insulin was added to the cells along with growth media free from FBS. Cells without insulin were treated as the negative control. The wells with media only have been considered as blank. The experiment was repeated in triplicates. After incubation of 24 hours, the reagents were discarded from the wells and the cells were subjected to MTT assay for detecting the proliferation.

Similarly approximately, 10,000 cells were seeded on to the 96 well plate (Tarsson, India) for finding the inhibitory effect of metformin. Viability is checked by trypan blue dye test. Metformin hydrochloride (Ranbaxy India) is prepared at various concentrations by dissolving in sterile water. The concentrations used were, 1µM, 5µM, 10µM, 50µM, 100µM, 500 µM, 1mM, 5mM, 10mM, and 50mM. Each cell line was given treatment with this varying concentration of metformin along with sub maximal concentration of insulin showing proliferation. The cells were incubated for 24 hours and on the next day, the reagents were removed and MTT assay was performed the to find the inhibitory effect of metformin on these lung cancer cell lines. The experiment was conducted in triplicates.

4.5 Viability Staining

Viable cells are defined as the cells which are capable of growth. The staining technique which is based on the dye exclusion method is utilized to check the viability of the cells. The cells with intact membrane cannot uptake the dye and are considered as viable. Whereas, non viable cells will be able to take up the dye and stain the colour of the dye. The dye used here was 0.5% trypan blue prepared in phosphate buffered saline. Equal volume of the dye and the cell suspension were mixed and allowed to stand for 1-2 minutes. This mixture was charged to the counting chamber and the cells which stained blue were counted as nonviable and non stained were considered as viable (Freshney, 2005).

Percentage of viability was calculated as, % of viability = $\frac{\text{Number of viable cells x 100}}{\text{Total number of cells}}$

4.6 MTT Dye Reduction Test

This assay is a colorimetric quantitative method used to detect cell proliferation and uses the yellow tetrazolium salt MTT (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Reduction of MTT takes place in the mitochondria of the living cells by succinic dehydrogenase and purple formazan are formed. The formazan conversion is directly proportional to the number of viable cells. The formazan crystals formed are insoluble in aqueous solutions. Solvents like DMSO are added to make it soluble. At pH 7.0, it has two peaks of absorbance, at 500nm and 570nm (Plumb, 1999). MTT (50mg % w/v) was prepared in prewarmed phosphate buffered saline and stored in amber coloured bottle as the solution is sensitive to light. After the insulin and metformin treatment, the cells are added with 200µl of MTT solution and incubated for 3-4 hours (Figure 4). Then the solvent, 200µl of DMSO was added and optical density is measured at 492nm with a reference filter at 620nm in micro plate reader (LMR 9602 G, Indian Scientific, India). The percentage of survival was calculated by

$$\% \text{ of survival} = \frac{\text{Absorbance of test- Absorbance of the blank X 100}}{\text{Absorbance of the control- Absorbance of the blank}}$$

4.7 Data Analysis

The percentage survival of were analyzed for significance by one way ANOVA. Duncan's multiple range was used for multiple comparisons. The effect between cell lines at various concentrations of insulin and metformin were compared by student's t- test and Two Way ANOVA. The level of significance accepted was at $p<0.05$.

Chapter Five

Results

5.1 Mitogenic Effect of Insulin on A549

The concentrations ranging from 1nM to 10μM were used to evaluate the growth promoting effects of insulin on serum starved lung adenocarcinoma cell line, A549. Statistically significant growth proliferation (One way ANOVA, $p<0.001$) was noticed for various concentrations of insulin as shown in Table 1. The mean percentage of survival of cells in each concentration with standard deviation is shown in Figure 5A. The proliferation of cells followed a dose-dependent manner. There was no significant proliferation for lower concentrations, 1nM and 5nM compared with the control cells where there was no treatment with insulin. Maximum proliferation was obtained with I μM of insulin, approximately 2 fold increase compared with control cells. Further increase in insulin concentration did not enhance the growth; instead it reached a plateau (Figure 5B).

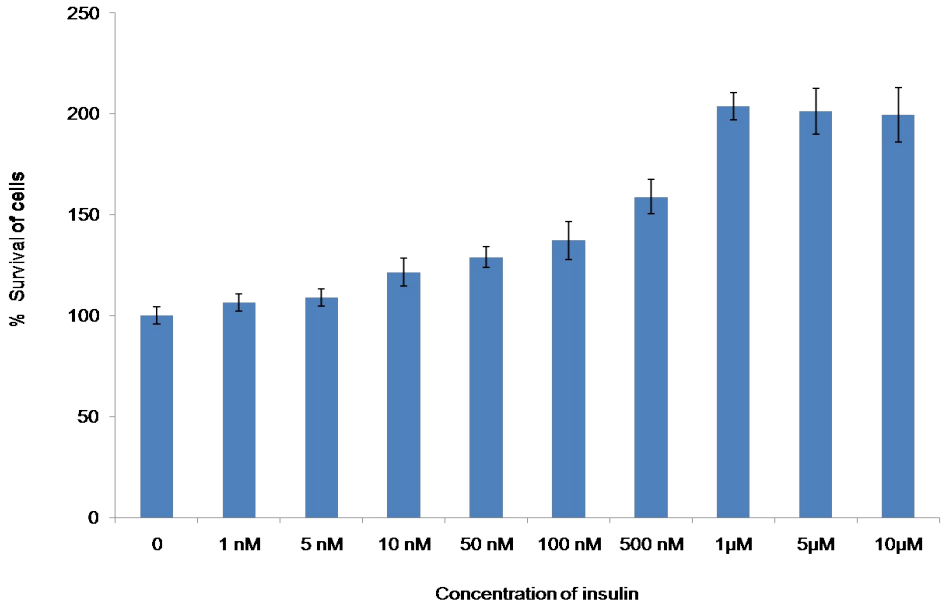

Figure 5A. Proliferative effect of insulin on lung cancer cell line (A549). The data shown here is the percentage proliferation with ± SD after insulin treatment for 24 hours

Table 1. Analysis of variance (One way ANOVA) of percentage survival of A549 cells following treatment with different concentrations of Insulin

Parameter	Concentrations of insulin	Mean percentage Survival	± SD	F value	P value
Percentage Survival for A 549 Cell Line	Negative Control	100.25[a]	4.27	201.93	< 0.001
	1 nM	106.55[a]	4.31		
	5 nM	108.92[a]	4.32		
	10 nM	121.55[b]	6.88		
	50 nM	129.07[bc]	5.16		
	100 nM	137.34[c]	9.41		
	500 nM	158.90[d]	8.51		
	1 µM	203.76[e]	6.89		
	5 µM	201.25[e]	11.4		
	10 µM	199.50[e]	13.3		

a, b, c, d, e, f - Means with same superscript within each cell line do not differ each other (Duncan's Multiple Range Test)

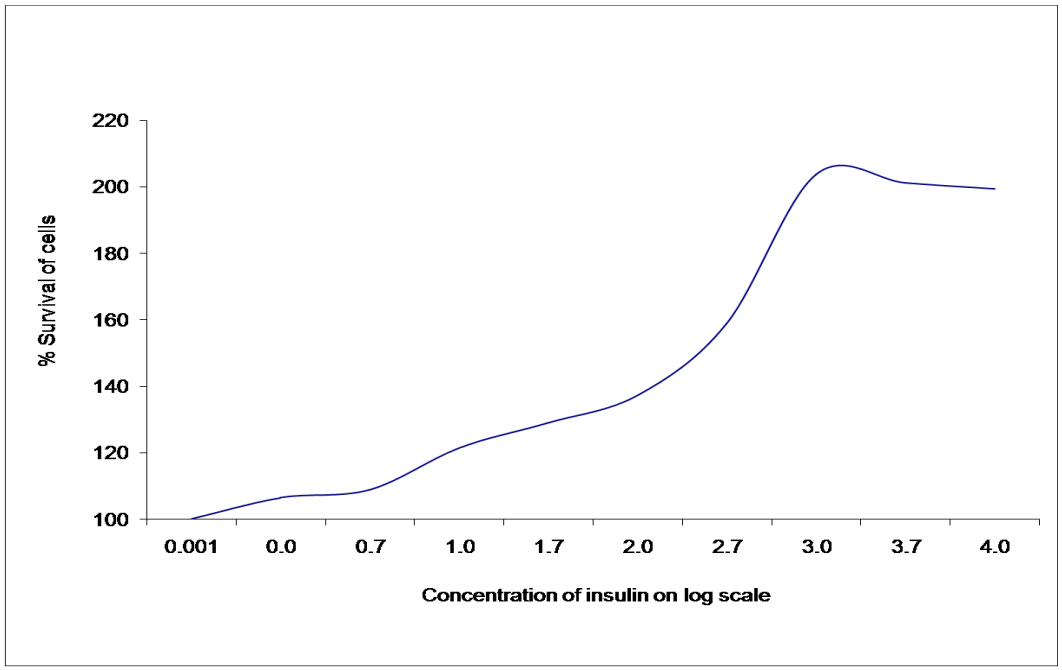

Figure 5B. Dose dependant effect of insulin on proliferation of A549

5.2 Mitogenic Effect of Insulin on H1299

Insulin stimulated the proliferation of HI299, the non small cell lung carcinoma cell line, for the concentrations used, 1nM to 10µM. Results showed a significant proliferation in all concentrations compared to control (One way ANOVA, $p<0.001$) as shown in Table 2. No differences in percentage of proliferation were obtained for the concentrations between 10nM and 50nM as well as between 5µM and 10µM according to Duncan's multiple range test. Maximum proliferation obtained at a dose of 1µM which is approximately, 1.78 fold compared to negative control where no treatment of insulin was provided (Figure 6A). Further increase in concentration of insulin showed no increase in proliferation. The proliferation of cells on treatment with insulin followed a dose dependant pattern as shown in Figure 6B.

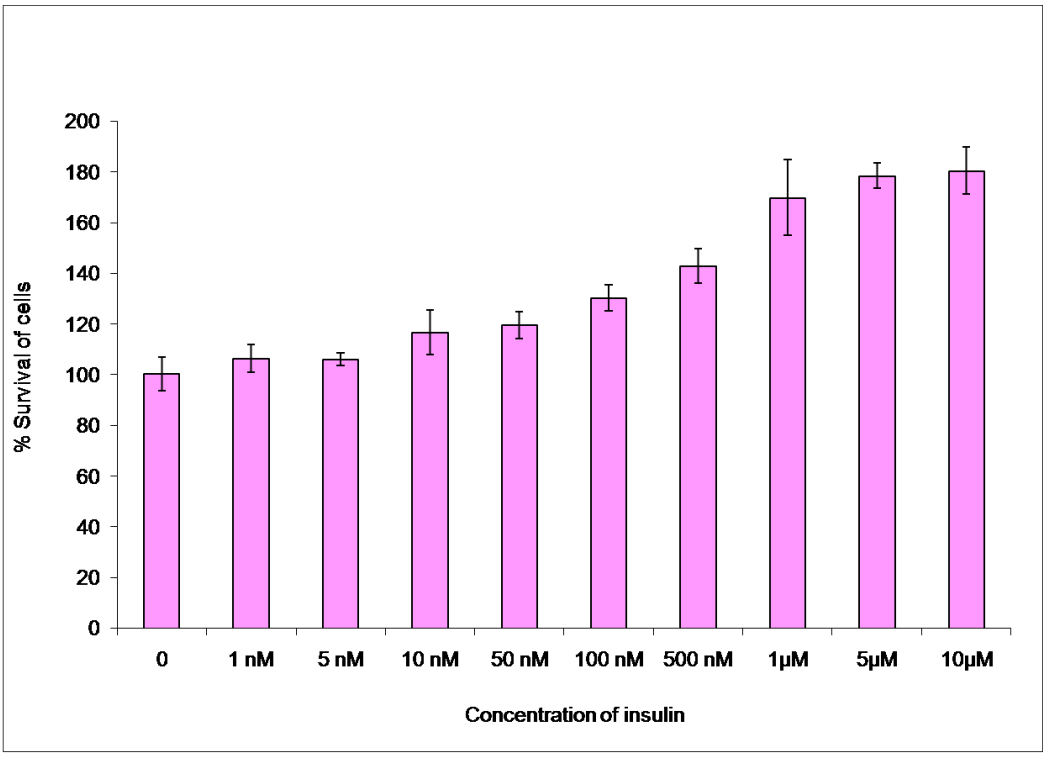

Figure 6A. Effect of insulin on H1299, lung cancer cell line. The data shown here is the percentage proliferation with ± SD after the treatment with insulin for 24 hours

Table 2. Analysis of variance (One way ANOVA) of percentage survival of H1299 cells following treatment with different concentrations of Insulin

Parameter	Concentrations of insulin	Mean percentage Survival	± SD	F value	P value
Percentage Survival for H 1299 Cell Line	Negative Control	100.34[a]	6.69	125.437	< 0.001
	1 nM	106.39[a]	5.46		
	5 nM	106.17[a]	2.51		
	10 nM	116.75[b]	8.75		
	50 nM	119.60[b]	5.32		
	100 nM	130.32[c]	5.06		
	500 nM	143.05[d]	6.76		
	1 µM	169.85[e]	14.90		
	5 µM	178.56[f]	4.96		

	10 µM	180.57[f]	9.25

a, b, c, d, e, f - Means with same superscript within each cell line do not differ each other (Duncan's Multiple Range Test)

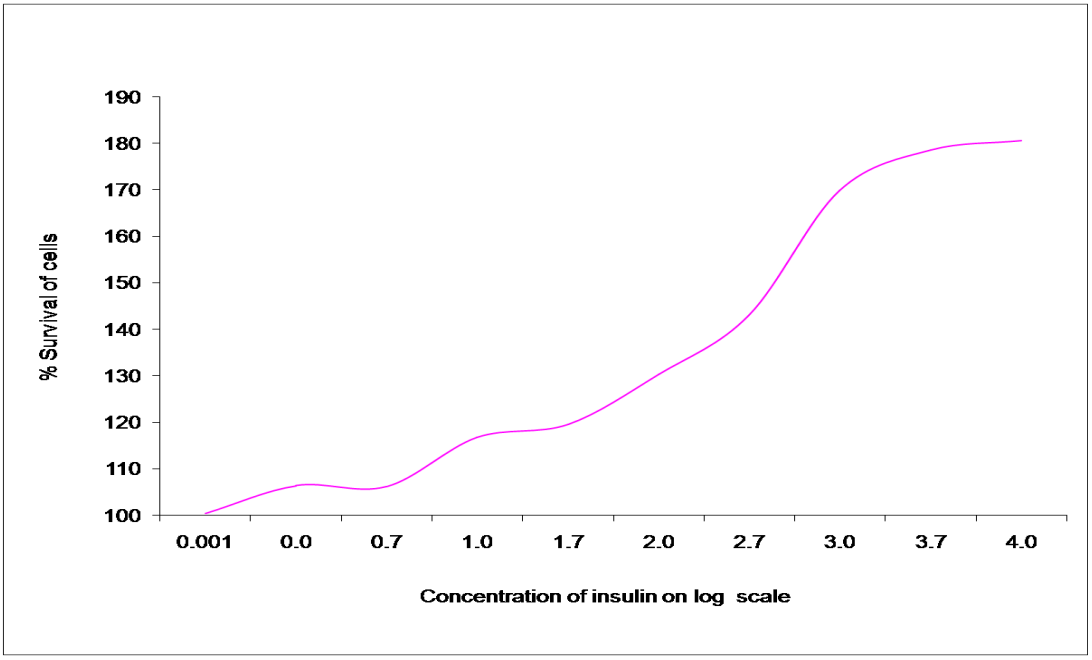

Figure 6B. Dose dependant effect of insulin on proliferation of H1299

5.3 Comparison of Effect of Insulin between Two Cell Lines

Effect of insulin on the growth proliferation is compared at all concentrations using student's t- test and Two Way ANOVA (Table 3&4). Significant differences were not noticed for lower concentrations, but were found to be significant in higher doses of insulin. Proliferation percentage was on the higher side for A549 compared to H1299 as shown in Figure 7. The dose response pattern obtained was similar for both the cell lines.

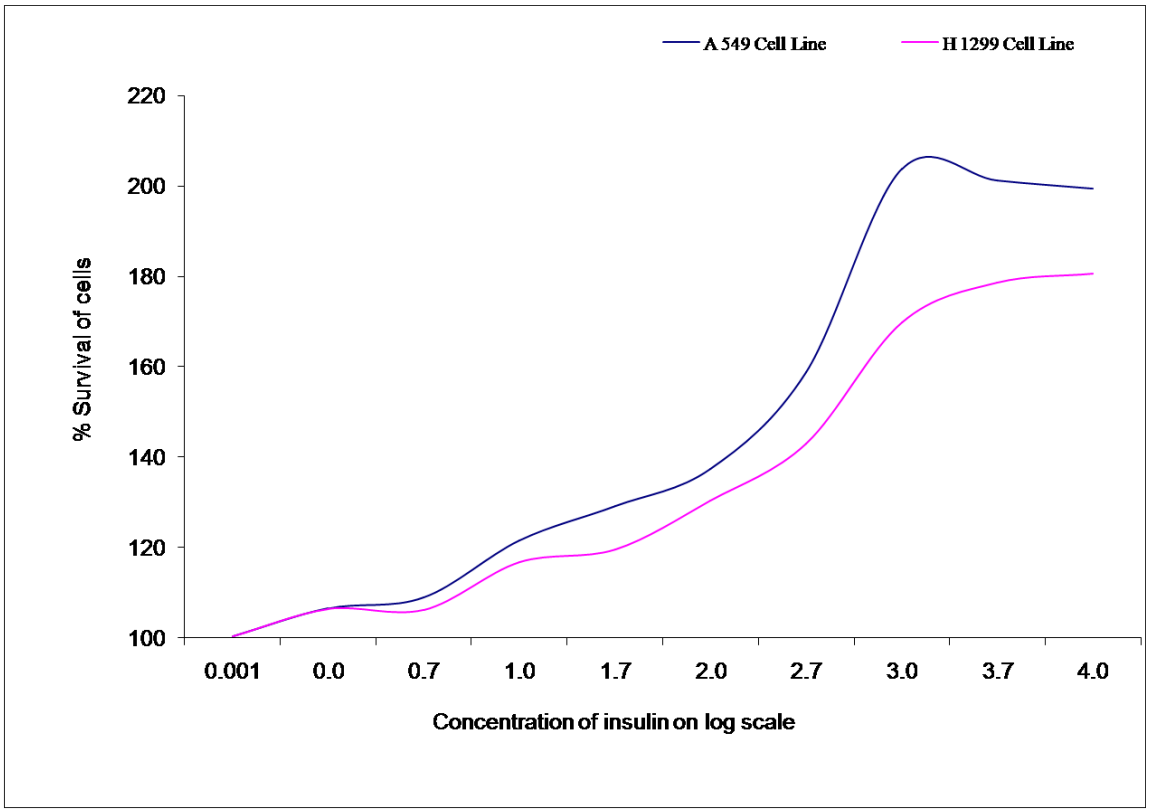

Figure 7. Comparison of effect of insulin on growth proliferation on A549 and H 1299

Table 3. Comparison of percentage survival on treatment with insulin between two cell lines by student's t-test

Concentrations of insulin	Cell Line	Mean Percentage survival	± SD	t value	P value
Negative Control	A 549	100.25	4.27	- 0.093	> 0.05
	H 1299	100.34	6.69		
1 nM	A 549	106.55	4.31	0.044	> 0.05

	H 1299	106.39	5.46		
5 nM	A 549	108.92	4.32	1.594	> 0.05
	H 1299	106.17	2.51		
10 nM	A 549	121.55	6.88	0.877	> 0.05
	H 1299	116.75	8.75		
50 nM	A 549	129.07	5.16	2.774	< 0.05
	H 1299	119.60	5.32		
100 nM	A 549	137.34	9.41	1.394	> 0.05
	H 1299	130.32	5.06		
500 nM	A 549	158.90	8.51	12.587	< 0.001
	H 1299	143.05	6.76		
1 µM	A 549	203.76	6.89	4.521	< 0.01
	H 1299	169.85	14.90		
5 µM	A 549	201.25	11.43	9.906	< 0.001
	H 1299	178.56	4.96		
10 µM	A 549	199.50	13.36	2.873	< 0.05
	H 1299	180.57	9.25		

Table 4. Comparison of percentage survival between two cell lines on treatment with insulin by Two Way ANOVA

Source	Type III Sum of Squares	df	Mean Square	F value	P value
Corrected Model	193373.64	19	10177.56	161.81	< 0.001
Intercept	3076780.0	1	3076780.0	48916.99	< 0.001

	2		2		
Group	5202.64	1	5202.64	82.72	< 0.001
Treatment	183597.19	9	20399.69	324.33	< 0.001
Group X Treatment	4337.39	9	481.93	7.66	< 0.001
Error	8554.13	136	62.90		
Total	3338847.3	156			
Corrected Total	9 201927.77	155			

5.4 Effect of Metformin on Insulin Mediated Proliferation of A549 Cell Line.

A549 cell line was treated with different concentrations of metformin from 1µM to 50mM for 24 hours. The sub maximal concentration of insulin proliferation, 500 µM was used for proliferation along with metformin. The negative control was treated with insulin only. Significant inhibition (One way ANOVA, $p<0.001$) of proliferation was observed for cellular growth as shown in Table 3. Lower concentrations didn't inhibit the growth proliferation of cells. Maximum inhibition of 73% was observed with metformin concentration 50mM. No differences in inhibition have been noticed between lower concentrations of (1µM to 500µM) metformin, according to Duncan's Multiple Range test. Figure 8A shows the mean percentage survival of cells with ±SD after the treatment with various concentrations of metformin for 24 hours. The concentration at which 50% inhibition (IC_{50}) is found to be 9.98 mM by log transformed probit analysis. The dose response curve is showed in Figure 8B.

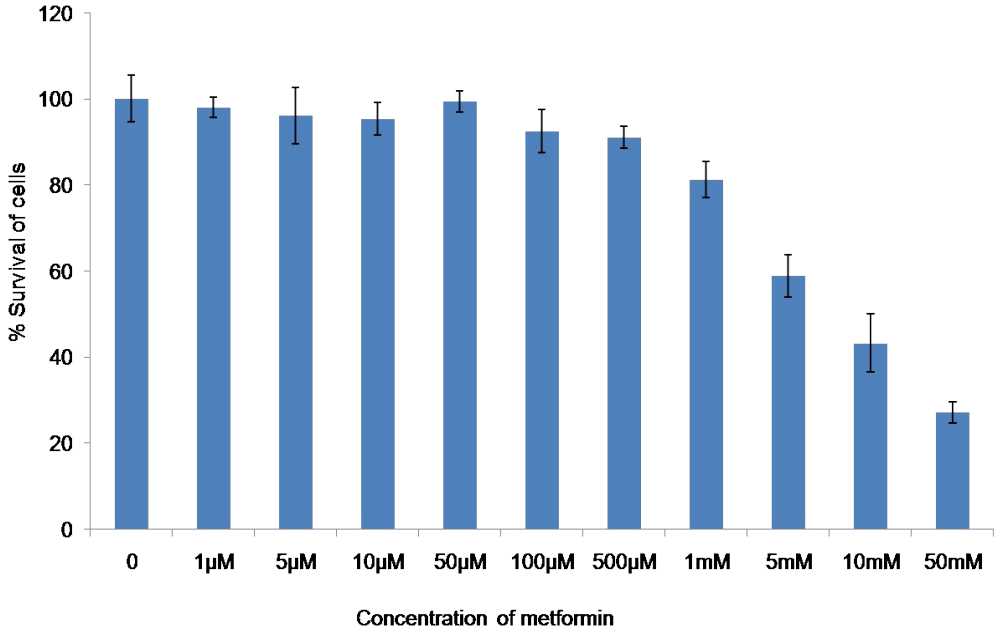

Figure 8A. Inhibitory effect of metformin on A549 cell line. The mean percentage of survival ± SD after treatment with metformin for 24 hours is shown.

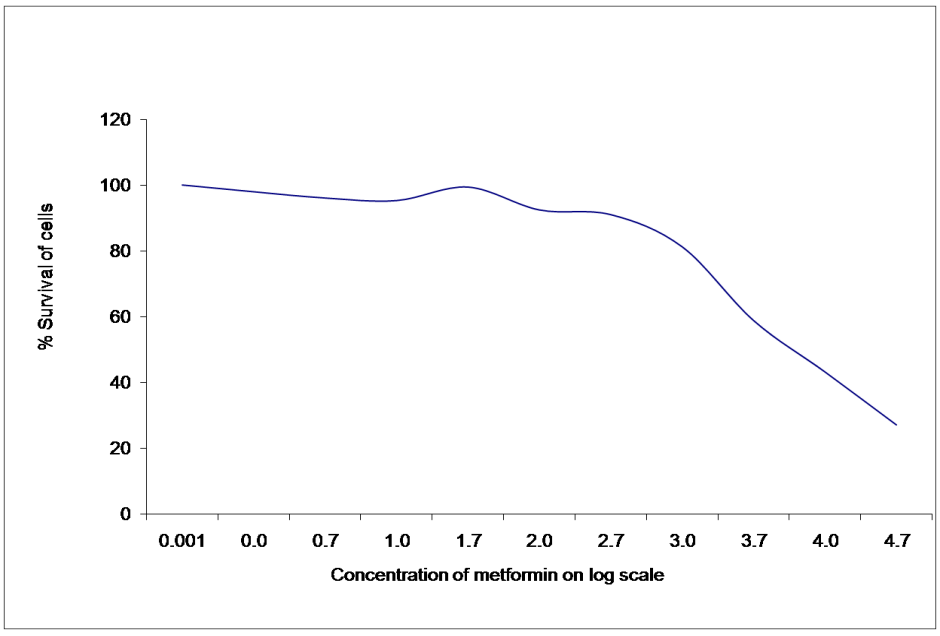

Figure 8B. Dose dependant curve of inhibitory effect of metformin on A549

Table 5. Analysis of variance (One way ANOVA) of percentage survival of A 549 cells following treatment with different concentrations of metformin

Parameter	Concentrations of Metformin	Mean percentage ±SD Survival		F value	P value
Percentage Survival - A 549	Negative Control	100.158[g]	5.366	255.89	<0.001
	1 µM	98.104[g]	2.349		
	5 µM	96.209[fg]	6.567		
	10 µM	95.419[ef]	3.797		
	50 µM	99.526[g]	2.508		
	100 µM	92.575[ef]	5.020		
	500 µM	91.153[e]	2.516		
	1 mM	81.359[d]	4.121		
	5 mM	58.926[c]	4.969		
	10 mM	43.286[b]	6.772		
	50 mM	27.172[a]	2.385		

a, b, c, d, e, f – Means with same superscript within each cell line do not differ each other (Duncan's Multiple Range Test)

5.5 Effect of metformin on Insulin Mediated Proliferation of H1299 Cell line

The inhibitory effect of metformin on H1299 was assessed with concentrations ranging from 1µM to 50mM along with 1µM insulin. One way ANOVA showed a significant inhibition of growth of H1299, non small cell lung carcinoma cell line ($p<0.001$) as shown in Table 5. For lower concentrations, no inhibitory effect is detected Duncan's multiple range test showed no significant differences in inhibition between lower concentrations of metformin with the control, which was not treated

with metformin. The mean percentage of survival of cells and SD after 24 hours of incubation with metformin is shown in Figure 9A. 67% of inhibition of proliferation is observed for 50mM concentration of metformin. The concentration at which 50% inhibition (IC_{50}) is found to be 17.2mM by log transformed probit analysis. The inhibitions at various concentration of metformin showed a dose dependant response 9B.

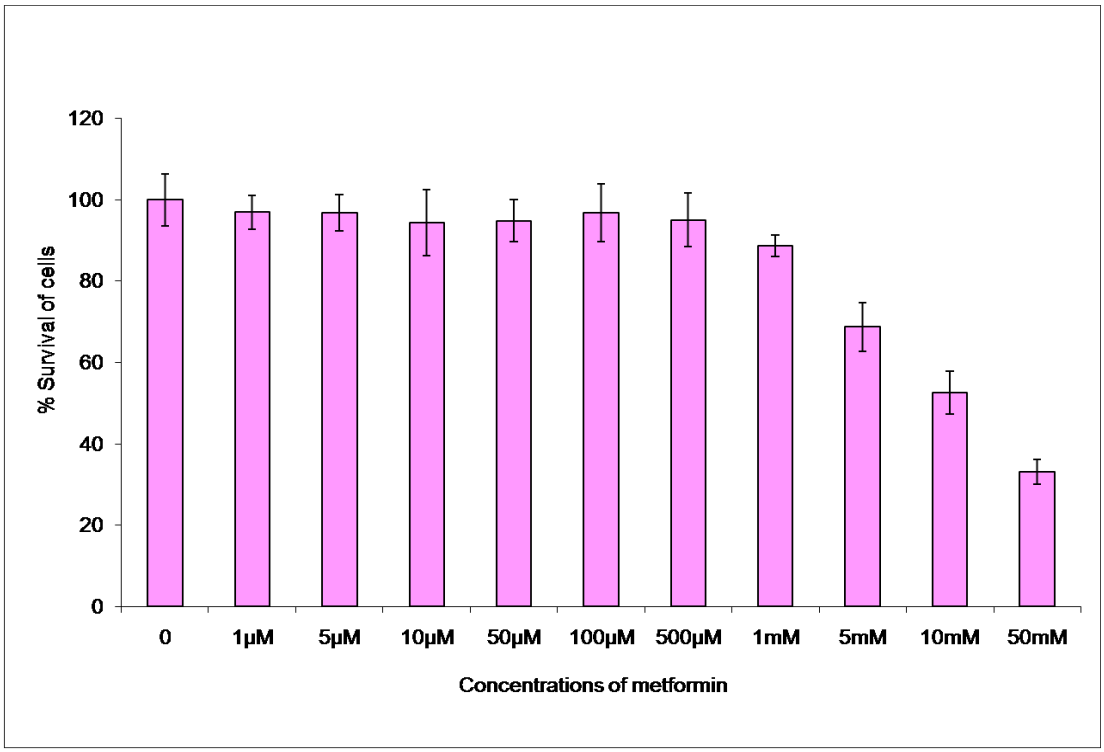

Figure 9A. Effect of metformin on the inhibition of H1299 cell line. The mean percentage of survival ± SD at different concentrations of metformin is displayed.

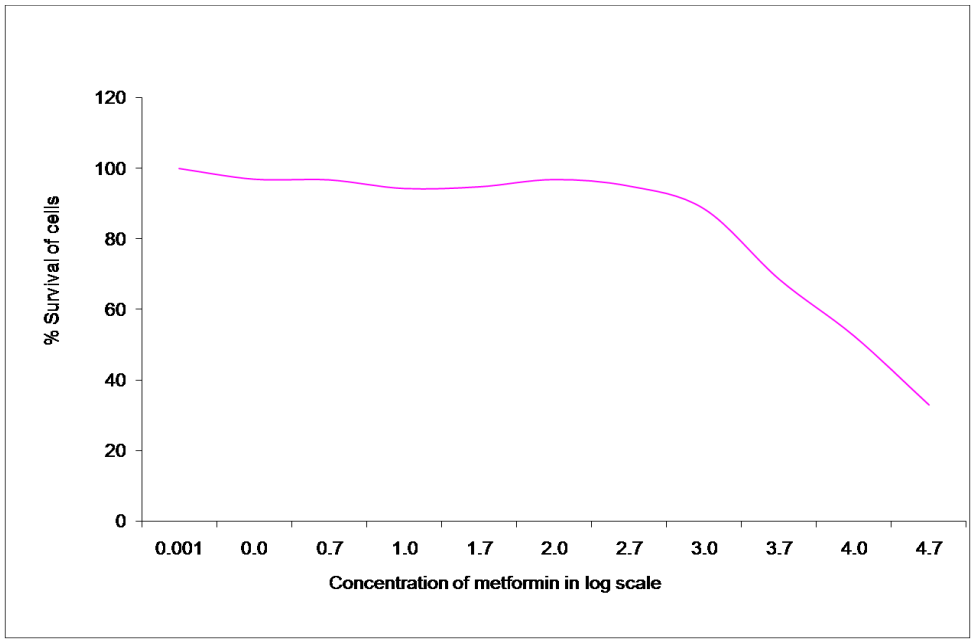

Figure 9B. Inhibitory effect of metformin in a dose dependant pattern on H1299

Table 6. Analysis of variance (One Way ANOVA) of percentage survival of H1299 cells following treatment with different concentrations of metformin

Parameter	Concentrations of metformin	Mean percentage survival	± SD	F value	P value
Percentage Survival - H 1299	Negative Control	99.903^{e}	6.407	125.927	<0.001
	1 µM	96.881^{e}	4.137		
	5 µM	96.686^{e}	4.423		
	10 µM	94.249^{e}	8.080		
	50 µM	94.737^{e}	5.226		
	100 µM	96.784^{e}	7.083		
	500 µM	94.932^{e}	6.558		
	1 mM	88.499^{d}	2.637		
	5 mM	68.616^{c}	5.982		
	10 mM	52.534^{b}	5.306		
	50 mM	33.041^{a}	3.094		

a, b, c, d, e, f – Means with same superscript within each cell line do not differ each other (Duncan's Multiple Range Test)

5.6 Comparison of Effect of Metformin between Two Cell Lines

The effect of metformin on the insulin mediated proliferation of two lung cancer cell lines were compared by student's t- test and by Two Way ANOVA at the various concentrations. The results are shown in Table 7 and 8. For high concentrations of metformin, 50nM, differences were not significant ($p>0.005$). Albeit, individually metformin showed significant inhibitory effect on both the cell lines, the effect was more pronounced for A549 cell lines. Maximum of 73% inhibition was seen with A549, whereas a maximum of 67% was only observed for H1299 for a concentration after 24 hours of treatment with metformin. A549 could inhibit 50 % of cells at a lower concentration than H1299.The dose dependant response was observed for both the cell line upwards from concentration 1mM. This is presented in Figure 10.

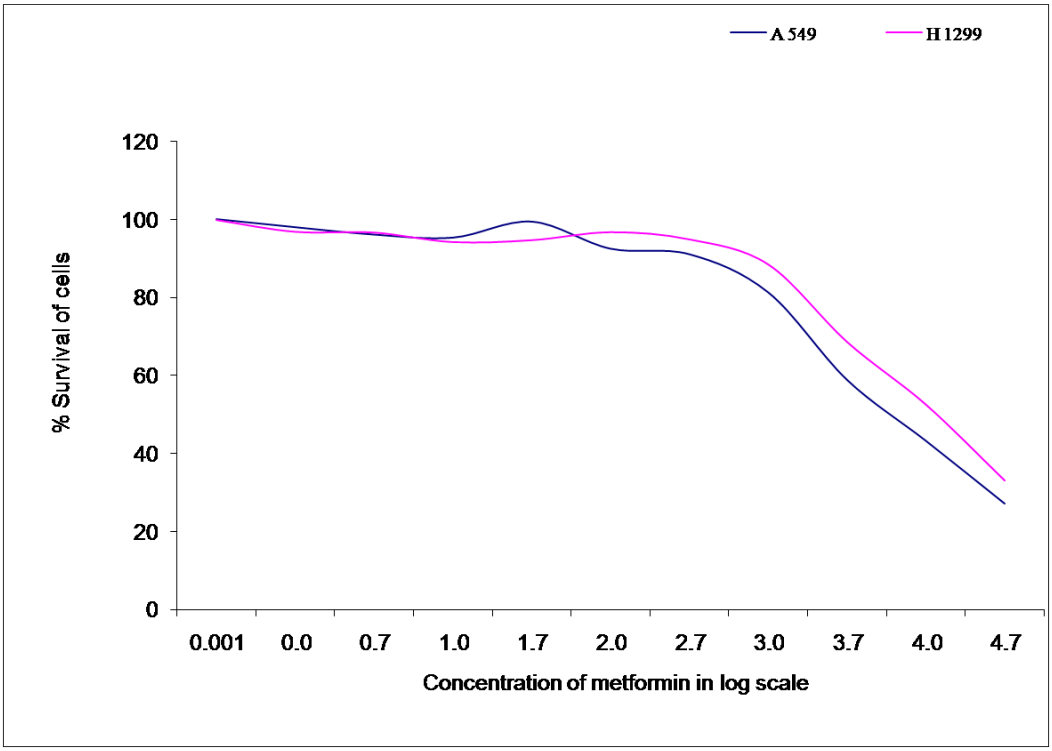

Figure 10. Comparison of inhibitory effects of metformin on insulin mediated proliferation of two lung cancer cell lines, A549 and H1299

Table 7. Comparison of percentage survival between two cell lines at different concentrations of metformin by Two Way ANOVA

Source	Type III Sum of Squares	df	Mean Square	F value	P value
Corrected Model	91626.32	21	4363.16	169.33	< 0.001
Intercept	1179160.98	1	1179160.98	45762.44	< 0.001
Group	395.42	1	395.42	15.35	< 0.001
Treatment	90334.55	10	9033.46	350.58	< 0.001
Group X Treatment	896.36	10	89.64	3.48	< 0.001
Error	3968.12	154	25.77		
Total	1274755.43	176			
Corrected Total	95594.44	175			

Table 8. Comparison of percentage survival between two cell lines at different concentrations of metformin by student's t-test

Concentration	Cell Line	Mean Percentage survival	± SD	t value	P value
Negative Control	A 549	100.158	5.366	0.063	> 0.05
	H 1299	99.903	6.407		
1 µM	A 549	98.104	2.349	0.577	> 0.05
	H 1299	96.881	4.137		
5 µM	A 549	96.209	6.567	- 0.135	> 0.05
	H 1299	96.686	4.423		
10 µM	A 549	95.419	3.797	0.296	> 0.05
	H 1299	94.249	8.080		
50 µM	A 549	99.526	2.508	1.933	> 0.05
	H 1299	94.737	5.226		
100 µM	A 549	92.575	5.020	- 1.102	> 0.05
	H 1299	96.784	7.083		
500 µM	A 549	91.153	2.516	- 2.598	< 0.05
	H 1299	94.932	6.558		
1 mM	A 549	81.359	4.121	- 4.331	< 0.01
	H 1299	88.499	2.637		

5 mM	A 549	58.926	4.969	- 3.689	< 0.01
	H 1299	68.616	5.982		
10 mM	A 549	43.286	6.772	- 6.247	< 0.001
	H 1299	52.534	5.306		
50 mM	A 549	27.172	2.385	- 5.044	< 0.01
	H 1299	33.041	3.094		

Discussion

The increased incidence of cancer in diabetes mellitus type 2 patients is primarily due to the impairment of insulin resistance. This is explained on the basis of failure of expression of insulin dependent glucose transporters on the surface of the cells (Morino, Petersen, & Shulman, 2006). This causes increasing circulating levels of glucose and insulin for a long duration. The glycotoxins formed as a result of hyperglycemia have effect on the extracellular matrix and vascular system. Insulin is a metabolic hormone and hence the fault in glucose transportation in muscle, liver and adipose tissue, adversely harm the regulatory measures of normal blood glucose concentration which imbalances the energy production pathways. High insulin level in extracellular fluid exerts the mitogenic effects on cells and accelerates the growth proliferation of cancer cells. Further, insulin stimulates the synthesis of IGF-I and IGF-II and reduces the level of IGF binding proteins (Pollak, 2008). All these mechanisms together favor the mitogenic effect of insulin. The receptor ligand interactions mediate the activation of tyrosine kinase by autophosphorylation and subsequent activation of P13 kinase and mitogen activated protein kinase (MAPK) signaling pathways (Richardson, Hamilton, Davis, Brito, & De Leon, 2011). As shown in the Figure 1, the causes and reasons of the metabolic syndrome are mainly owed to the consequences of the sedentary life style and deleterious habits like smoking.

According to this study, insulin stimulated the growth proliferation of serum starved non small cell lung cancer (NSLC) cell lines, A549 and H1299. The results

strongly support the mitogenic/proliferative effect of insulin on non small cell lung carcinoma cells. These findings corroborates earlier investigation on effect of insulin on A549 (Li, Wang, Yang, & Jiao, 2009). Moreover, it correlates the molecular link between diabetes and cancer. There are reports about the correlation of lung cancer incidence in diabetes (Hemminki et al., 2010; Lee et al., 2012; Swerdlow et al., 2005). This *in vitro* experiment suggests that the hyperinsulinaemia in diabetes can augment the carcinogenesis of lung. A549, cell line of adenoma of alveoli showed a maximum of 2 fold increases in growth, whereas the H1299 cells responded to a peak of 1.78 fold compared to the control. Variation noticed in the proliferation percentage between two cell lines may be attributed to the differences in expression of insulin and IGF receptors on cell surfaces. However, 4 to 7 fold increase in proliferation for breast cancer cell lines (MCF-7 and T47D) and a 20-30% proliferation for prostate cancer cell line (PC-3) with insulin treatment for 48 hours in two similar previous studies were done in the same laboratory (Sharma, 2011; Singh, 2011). This shows the less sensitivity of lung cancer cell lines to insulin compared with the breast cancer cells and more with prostate cancer cells which is in agreement with study of insulin receptor analysis (Spiliotaki et al., 2011; Vigneri et al., 2009). In an *in vivo* experiment, risk of lung cancer with inhaled insulin was reported (Mitri & Pittas, 2009). In a preclinical study, the adverse effect of reduced survival of diabetic patients on insulin therapy was described in NSLC patients (Farhan, 2009). Reports of our *in vitro* study are well in conformity with the above mentioned reports which concluded that high concentrations of circulating insulin can be a risk factor for carcinogenesis.

Notwithstanding this correlation, decreased incidence of lung cancer in diabetes patients has also been documented. Atchison et al (2011) in a study done in US, including both the types of DM, couldn’t correlate the lung cancer frequency in diabetic cases. They suggested high BMI and less smoking habit of diabetes patients as the causes. However, a dose response relation has been established between smoking and diabetes. Hall et al. (2005) proposed the decrease in life expectancy as the reason for this negative relationship. According to them, cardiovascular complications adversely affect the physical condition of patients prior to

carcinogenesis. On the other hand, present findings imply that hyperinsulinaemia or hyperglycemia due to insulin resistance can accelerate the progression of tumor of lung. The reason may not be the obesity, high BMI and central fat accumulation, but the metabolic impairment originated due to smoking, one of the major risk factor for lung cancer. This is in agreement to our findings which underlines the role of insulin resistance in the survival of lung cancer cases (Attvall et al., 1993; Park et al., 2006). Effects of smoking can hence be the reason for the proliferation of NSLC cells in diabetes. Therefore, this study points that hyperinsulinaemia/insulin resistance as a result of oxidative stress, abnormality in endothelial function; hemodynamic changes can enhance the lung tumorigenesis.

There are many epidemiological evidences to explain the higher cancer incidence in diabetes mellitus with insulin or insulin stimulating drugs. On the contrary, improvement in clinical condition has been observed for diabetes patients with cancer who are on metformin, a hypoglycemic drug so widely used. Metformin increases the insulin sensitivity thereby favoring the uptake and utilization of glucose. Intra-cellularly, metformin sensitizes the cells for insulin and restricts the anabolism of fat. Many molecular mechanisms have been suggested for anti cancer activity of metformin. Most commonly cited mechanism is the activation AMPK, the indicator of cellular stress. The signaling of growth factors is mainly regulated by the p13 kinase (Desbois-Mouthon et al., 2000). Dowling et al. (2011) suggested indirect activation of metformin on AMPK due to disruption in the mitochondrial respiratory chain that decreases the level of ATP. Adenosine mono phosphate binds to AMPK and produces a conformational change which phosphorylates its catalytic subunit which requires the involvement of Liver Kinase B1 (LKB1). The binding of AMP to AMPK protects it from the action of phosphatase. It holds back the protein synthesis by inactivation of pathway (Dowling et al., 2011). Also, metformin can directly suppress the mTOR and reduce proliferative events. In addition, metformin repress the mitotic elements like, kinesins, tubulins and histones. It induces cell cycle arrest at G_0/G_1 phase and inhibits the proliferation of cells (Ben Sahra et al., 2008).

In this study, both non small cell lung carcinoma cell lines showed a significant growth inhibitory effect on the insulin mediated proliferation. A549, at a concentration

of 50mM of metformin exhibited a 73% inhibition compared with the control cells which were treated with only insulin. H1299 reduced 67% of the growth proliferation of cells. A dose dependant pattern is followed by both the cell lines. These results are in accordance with the *in vitro* study conducted for A549 and the tobacco induced carcinogenesis (Memmott et al., 2010; Rocha et al., 2011). Activation of mTOR has already been discussed in lung tumorigenesis. Reduction of glucose 6 phosphatase enzyme activity is accompanied by the decrease of ATP production and AMPK activation (Ota et al., 2009). The effect of metformin is investigated along with the chemotherapeutic agent, paclitaxel by Rocha et al. (2011) by using non small cell lung carcinoma cell lines, A549. In addition, Memmott et al. (2010) specifies metformin that could inhibit the IGF and hybrid receptor phosphorylation, protein kinase B and mTOR in lung tissue without activating the AMPK pathway. These data substantiate our results of inhibition of growth proliferation of lung cancer cells.

In the current study, we compared cell lines of p53 mutant and wild type. A549 is the p53 wild type and H1299, not expressing p53 protein due to the homozygous deletion. p53, a tumor suppressor gene, has a wide range of functions like, apoptosis, DNA repair and cell cycle arrest to prevent cancer progression (Vousden & Ryan, 2009). In glucose deprived cancer cells, p53 helps to arrest the cell proliferation until the energy source is restored. Hence p53 is required to make use of the available nutrients of the cell for its survival. Buzzai et al. (2007) suggested that metformin could stimulate metabolic processes which helps the cell survival in p53 wild type cells. These effects were not observed in p53 mutant cells and hence could exert its inhibitory effects. Hence, metformin could only inhibit the growth of p53 mutated cells (Buzzai et al., 2007). This is not in accordance with our findings that both the p53 mutant and wild type lung cancer cell lines were sensitive to metformin in a highly significant manner. Though A549 could inhibit 50% of cells at a lower concentration (9.98mM) than H1299 (17.2mM), the increase is only 1.7 fold. This report is similar to the observation of Ben Sahra et al. (2008) which couldn't find any dissimilarity in metformin effect to p53 mutant and wild type breast and prostate cell lines. They interpret the inhibitory effects of metformin could be by arresting the cell cycle at G_0/G_1 phase independently of p53. This implies metformin effectively block

the molecular energy utilization mechanisms and prevents the mitogenic effect caused by insulin.

The specificity of metformin action in Liver kinase B1 (LKBI) mutated cells is another important area. Mainly lung cancer cell lines are LKB1 mutated. This tumor suppressor is involved in maintaining cellular integrity. The main mechanism of action of metformin is by activating AMPK pathway and suppressing the protein synthesis which prevent the cell growth. Various reports suggest the requirement of LKBI for an effective growth reduction by metformin. The inhibition of cell growth was not detected in HeLa cells which have no allele for the LKBI gene (Zakikhani et al., 2008). However, the cell line selected in this study, LKBI mutant (A549) and wild type (H1299) showed a significant inhibitory effect even though variations exist in the percentage of inhibition. This is similar to the reports of the study conducted by Rocha et al, 2011. As demonstrated in Figure 3, many pathways have been suggested for the effect of metformin. It may take the indirect or direct pathway for producing the effect. It was ample clear in this study, as both the cell lines followed the similar pattern of linearity at any particular concentration.

The present study therefore confirms that, hyper insulinaemia can contribute to the lung carcinogenesis and metformin could effectively prevent this process of proliferation. The anti proliferative or anti mitotic activity of metformin, on lung cancer is independent of p53 and LKBI mutation. More receptor based studies are necessary to elucidate the specific mechanism involved in the metformin activity.

Summary and Conclusion

The hyperglycemia, a clinical state that occurs in diabetes mellitus type 2, results from the metabolic disturbances related to the insulin resistance. This results in increasing levels of circulating glucose and insulin in blood for a long period of time that exerts a mitogenic effect through IGF/IR axis. The first part of the study evaluating growth enhancing effect of serum starved non small cell lung carcinoma cells reaffirmed the mitogenic or proliferative effect of insulin. Though the basis of development of insulin resistance is attributed in various cancers as obesity, physical inactivity, disturbances in fat metabolism and oxidative stress, in lung cancer, smoking associated parameters contributes more to it. These effects may increase with the therapy of insulin or insulin stimulating drugs leading to poor prognosis of carcinoma patients. Hyperinsulinaemia accelerate this effect. In this context, insulin sensitizing mechanism of metformin, an oral anti hypoglycemic from the biguanides family, assumes significance as a therapeutic measure for cancer. A number of epidemiological, molecular, clinical, and therapeutic evidences are supporting this hypothesis of less risk of cancer progression in diabetic cases that are on metformin therapy. More so, several *in vitro* and in *vivo* data justifies the use of metformin in a prophylactic manner.

The present study attempted to evaluate the effects of using metformin in lung cancer. The reports confirmed the anticancer role of metformin. More over, it compared the metformin effects in two non small cell lung carcinoma cell lines which

are mutant and wild type of tumor suppressor genes p53 and liver kinase b1(LKB1). Though diverse reports regarding the involvement of these genes in metformin mechanism exist, this *in vitro* study demonstrated significant inhibition of insulin mediated proliferation for both the cell lines. That implied the different roles played by metformin in causing anti-mitotic effect. This report demands for receptor based analysis to elucidate the mechanism of action of metformin in non small cell lung carcinoma.

The study proved the mitogenic effect of insulin, in high concentrations, that can augment cell growth in lung cancer. On contrary insulin lowering therapy with metformin can prevent or restrict the growth by multiple mechanisms. The usefulness of metformin in the treatment for premalignant stages of cancer progression, either individually or in combination with other modalities of cancer therapeutics needs to be studied in detail.

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