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Repurposing Metformin for Lung Cancer Management

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<http://dx.doi.org/10.5772/67160>

Abstract

In this article, we introduced the background knowledge of lung cancer management and considered repurposing old drugs to overcome therapy bottleneck. We chose metformin to prove both its antihyperglycemia and antitumor formation effects. Based on the metformin-related AMPK-dependent pathway, we tried to explore the AMPK-independent pathway in inhibition of lung tumorigenesis by metformin. Using preclinical data mining from clinical settings with a literature review, we attempted to clarify the role of metformin in lung cancer management. Additional objective and strong evidence are needed using randomized control studies to verify the benefit of metformin in clinical practice. Furthermore, we proposed two lung cancer animal models and showed the establishment processes thoroughly. We hope that these two lung cancer animal models provide a useful platform for furthering old drug repurposing as well as new drug investigations in the future.

Keywords: lung cancer, metformin, animal model, AMPK pathway, orthotopic injection

1. Introduction

1.1. Background

Lung cancer is known as a major cause of cancer-related mortality worldwide. Newly discovered drugs focus on the issue of improving survival and need vast time and investment. In Ref. [1], it was estimated that it took 13 years and cost of 1.8 billion dollars for one newly developed drug. Additionally, just only one of the 5000 promising antitumor agents had the potential to pass the U.S. Food and Drug Administration (FDA) regulation and obtain final approval. With respect to currently used drugs, it is convenient to quickly access the “repurposing” or “repositioning” effect of converting them into anticancer management. In recent years, more

and more studies have involved the antidiabetes drug metformin. Initially, Evans et al. [2] observed that patients with type 2 diabetes mellitus (DM) under metformin treatment had a reduction of cancer incidence. It caused a 23% reduction of risk of any cancer for the metformin group. Though it was an observational study, more and more research and experimental designs followed the path. Bo et al. [3] showed that the cancer incidence of type 2 DM patients with metformin was lower than that compared with other oral antidiabetic (OAD) agents. For pancreatic and colon cancer patients, Currie et al. [4] found that the metformin users among the type 2 DM group had lower cancer incidence. For breast cancer patients, some studies found that metformin was beneficial for neoadjuvant chemotherapy groups [5,6]. Regarding animal models, Algire et al. [7] proved the efficacy of metformin in lung cancer. From the preliminary result of above studies, we could understand the utilization of metformin in different types of cancers, including lung cancer.

1.2. Diabetes mellitus and metformin

Diabetes mellitus is a common metabolic disease, and the associated prevalence is approximately 7–10% [8]. Patients with DM have higher risk of cardiovascular disease, nephropathy (renal function impairment), retinopathy and polyneuropathy (numbness of distal part of four limbs). It is known that hyperglycemia is crucial for the development of many cancers, including breast, liver, colorectal, kidney and lung cancer. Among the diverse ODA agents for type 2 DM, metformin was the common first-line choice worldwide. It is estimated that approximately 120 million patients initially took metformin for controlling blood sugar. Moreover, the safety of metformin is confirmed due to a lower incidence of lactic acidosis compared with other OAD.

Metformin (N',N'-dimethylbiguanide) belongs to the biguanide class. It possesses hypoglycemic effect by inhibition of gluconeogenesis. Further it could lower insulin resistance, which is very important for cancer growth. From earlier studies, scientists found metformin could activate the adenosine monophosphate-activated protein kinase (AMPK) pathway to negatively regulate the mammalian target of rapamycin (mTOR) pathway with the aid of liver kinase B1 (LKB1) [9,10]. The mTOR pathway helps proliferation for cell viability [11]. Therefore, metformin could demonstrate an antiproliferative effect in cells, even for cancer cells. Based on this implication, metformin showed the potential for an antitumor effect. In addition to the AMPK-dependent pathway, some studies supposed metformin could exert an AMPK-independent pathway in dealing with tumorigenesis [1]. Later, we will focus on the issue of the antitumor effects of metformin in lung cancer.

2. Mechanism of metformin on antitumorigenesis

2.1. The antitumor effect of metformin

2.1.1. Metformin corrects hyperglycemia

Metformin can accumulate within the matrix of mitochondria, and it could exert the inhibition of the complex I of the mitochondrial electron transport chain. Further, reduction of nicotinamide adenine dinucleotide hydride (NADH) oxidation can also cause the reduction

of synthesis of adenosine triphosphate (ATP). After the activation of AMP-activated protein kinase (AMPK), it enhances catabolic activity instead of the anabolic process. It initiates transcriptional signal transduction, inhibition of gluconeogenesis, and induction of glucose uptake into muscle cells via glucose transporters (GLUT2) [12]. Moreover, metformin can indirectly cause the induction of insulin receptor expression to facilitate insulin sensitivity and reduce insulin resistance, which is associated with tumor growth [13].

2.1.2. *Metformin upregulates AMPK*

Metformin can activate AMPK to initiate the downstream signal transduction to affect the transcription of tumor suppressor liver kinase B1 (LKB1) [14]. Once AMPK is activated, it negatively regulates the mTOR pathway by phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) and inhibition of downstream small GTPase (RHEB). The mTOR pathway is crucial for tumor cell survival because mTOR plays a vital role in cell growth, proliferation and protein synthesis. Moreover, the mTOR pathway could be activated via mitogenic responsive phosphoinositide 3-kinase/protein kinase B/AKT (PI3K/PKB/AKT) pathway. When metformin-related AMPK dependent pathway is affected, the inhibition of mTOR signal transduction and reduction of cancer cell proliferation are achieved [15].

2.1.3. *AMPK and p53 pathways*

It is known that p53 can activate numerous genes to negatively regulate the AKT and mTORC1 pathways, resulting in cancer cell quiescence, senescence and further apoptosis. Thus, once AMPK phosphorylates p53, it could lead to p53-mediated cell cycle arrest in p53-expressing cells and cell apoptosis for cells with mutated p53 [11].

2.1.4. *Inflammatory pathway and metformin*

For tumorigenesis, chronic inflammation is attributed to tumor growth and development. Once the inflammatory process is stimulated, it causes DNA adduct formation and increases the amount of inflammation biomarkers (such as cytokine/chemokines, immune-related effectors, acute phase proteins, reactive oxygen and nitrogen species, prostaglandins, cyclooxygenase-related factors and transcription factors and growth factors) [16]. Like tumor necrosis factor-alpha (TNF- α), nuclear factor-kappa B (NF-kB) and signal transducer and activator of transcription 3 (STAT3) are important components of the inflammation reaction. Arai et al. elucidated that metformin reduces the process and production of TNF- α in human monocytes [17].

Reactive oxygen species (ROS) play a vital role in the formation of advanced glycation end products to enrich oxidative stress. Metformin can reduce the production of endogenous reactive oxygen species via inhibition of mitochondrial complex I [18].

2.1.5. *Cell cycle pathway and metformin*

Sahra et al. found that metformin has an antiproliferation effect, which is mediated by G1 cell cycle arrest. In a study with prostate cancer cells, metformin-induced cell cycle arrests by inhibiting the expression of cyclin D1 and retinoblastoma-protein (pRb) [19].

2.1.6. Angiogenesis and metformin

For tumor cells growth, vast amounts of nutrition and oxygen are needed. As the tumor enlarges and begins to invade and cause distant metastasis, angiogenesis is the cornerstone. Tumor cells easily develop pro-angiogenic agents once exposed to a hypoxic environment [8]. The vascular endothelial growth factor (VEGF) formation is the key step. The VEGF group is consisted of four members (VEGF-A, B, C and D), and VEGF-A is the most potent. VEGF-A has four isoforms: VEGF-A₁₂₁, VEGF-A₁₆₅, VEGF-A₁₈₉ and VEGF-A₂₀₆. VEGF-A₁₆₅ functions in both the angiogenic process and cell growth. Moreover, the angiogenesis process needs a cofactor (neuropilin; NRP-1) to facilitate the VEGF ligand interaction with the VEGF receptor (VEGF-R2) [16,20]. In our previous lung cancer cell line (A549) experiments, the expression of NRP-1 decreased after the addition of metformin.

2.1.7. Models of metformin using different cell lines

In the beginning, anticancer studies were started from cell lines and animal models (commonly using a xenograft model). There are a vast number of antitumor studies with metformin on ovarian cancer, gastric cancer, pancreatic cancer and breast cancer. Some emphasize cell cycle-related proteins (CD1, CDK4 and CDK6) and some focus on microRNA (miR) regulation and signal transduction [21–24]. Moreover, cancer stem cells (CSC) were also found to be involved in the antitumor effect of metformin in both cell lines and xenograft models. The dosage and administration route of metformin were diverse. We provided a brief summary of previous findings of the antitumor effects of metformin on different cell lines in **Table 1** [21, 22, 24, 25].

Cancer cell type	Ovarian cancer	Gastric cancer	Breast cancer, prostate cancer, lung cancer	Pancreatic cancer
Lab material	1. Cell line 2. Xenograft model 3. Live tumor analysis	1. Cell line 2. Xenograft 3. miRNA	1. Cell line 2. Xenograft	1. Human pancreatic cancer cells
Mechanism	1. Inhibit tumor proliferation (IHC: Ki-67↓, Cyclin D1↓) 2. pACC↑: downstream target of AMPK (pmTOR↓)	1. Cell cycle-related protein (CD1↓,CDK4↓, CDK6 ↓) 2. Block G0-G1 phase 3. Cell proliferation assay	1. Cancer stem cells (CD44↑, CD24↓)	1. microRNA analysis 2. RT-PCR
Results	1. Inhibits angiogenesis (IHC stain for VEGF, CD-31) 2. Microvessel density: CD-31 3. Inhibits metastasis of ovarian cancer 4. Enhances cytotoxicity of chemotherapy reagents (Cisplatin: colony formation assay)	1. Reduced Cyclin D1 expression	1. Metformin reduces the dosage of chemotherapy (Doxorubicin) 2. Lung cancer cell line (A549)	1. Metformin up-regulates the expression of miR-26a, miR-192 and let-7c. 2. Cell migration 3. Cell proliferation assay 4. Metformin suppresses the oncogene: HMGA1

Cancer cell type	Ovarian cancer	Gastric cancer	Breast cancer, prostate cancer, lung cancer	Pancreatic cancer
Route	Oral feeding metformin in drinking water 200 ml	i.p. injection (intraperitoneal)	Oral intake in drinking water.	1. i.p. (Xenograft) 2. Cell line
Dosage	Reagan-Shaw formula 1. Human: 480 mg/60 kg 2. Mouse: 100 mg/kg	1. 1 mmol/L, 5 mmol/L, 10 mmol/L 1 or 2 mg/day, i.p., 5 times/week for 4 weeks	200 µg/mL (15 mg/kg)	1. 1. i.p: 250 mg/kg (100 µL/mouse) 2. 2. Cell line: (0~10 mmol/L)
Reference	[24]	[22]	[21]	[25]

Table 1. The associated antitumor mechanism of metformin on different cancer cells.

2.2. Literature review of the present studies on the issue of metformin in lung cancer

First, we searched the articles on PubMed that included with metformin and lung cancer in the title. From basic, preclinical research (including cell lines and animal studies) to observational studies, we attempt to explain the association of the antitumor mechanism by metformin.

2.2.1. Preclinical studies

2.2.1.1. Metformin and lung cancer cell lines

Initially, Ashinuma et al. investigated the effect of metformin on the inhibition of clonogenicity, cell growth and proliferation using four different lung cancer cell lines [26].

2.2.1.2. Synergistic effect of metformin with chemotherapy reagents

Chemotherapy is widely used for the treatment of advanced lung cancer (stage IIIB and IV). Some studies aim to overcome chemoresistance by the combination of metformin and chemotherapy. The report of Tseng et al. [27] showed metformin mediated the downregulation of p38 mitogen-activated protein kinase-dependent excision repair cross-complementing 1 and decreased DNA repair ability. Additionally, it could further sensitize human lung cancer cells to cisplatin and paclitaxel agents [27,28].

2.2.1.3. Synergistic effect of metformin and tyrosine kinase inhibitors (TKIs)

Tyrosine kinase inhibitors (TKIs) such as gefitinib (Iressa), erlotinib (Tarceva) and afatinib (Giotrif) are now validated as the first-line therapy for advanced lung adenocarcinoma bearing mutant epidermal growth factor receptor (EGFR). Studies designed to evaluate the possible synergistic effect of metformin and TKIs were launched. Morgillo et al. [29] showed that metformin with gefitinib had more obvious antiproliferative and proapoptotic effects in both cell line and animal models (xenograft).

2.2.1.4. Synergistic effect of metformin and TKI in EGFR-TKI-resistant lung cancer cell line

First-line TKIs in lung adenocarcinoma encountered the issue of drug resistance due to the development of EGFR-resistant strains (such as T790M). To overcome this problem, Li et al. [30] proved that metformin could reverse epithelial-to-mesenchymal transition (EMT) and interleukin-6 (IL-6) signaling activation in EGFR-TKI-resistant lung cancer cells. Then, they proved metformin could increase sensitivity for EGFR-resistant strains to TKIs (gefitinib and erlotinib) therapy.

2.2.1.5. Effect of metformin in radiation therapy for lung cancer

Radiation therapy is one therapy modality for lung cancer patients. However, the possible effects when adding metformin to radiation therapy are unknown. Storozhuk et al. showed that metformin may enhance the radiation response of nonsmall cell lung cancer through the ataxia-telangiectasia mutated protein kinase (ATM) and AMPK pathway [31].

2.2.2. Observational studies

2.2.2.1. Population-based studies in Taiwan

In Taiwan, the National Health Insurance Registered Database (NHIRD) is popular for further assessment and discovery of medical issues. Experts attempt to find the association between metformin and lung cancer risk among type 2 DM patients. Lai et al. performed an epidemiological study and found that in patients treated with metformin, compared with the non-metformin (other OAD) group in type 2 DM patients, the reduction of lung cancer risk was approximately 39–45% [32].

2.2.2.2. Population-based studies outside Taiwan

From a retrospective study performed by Tan et al., they defined diabetic nonsmall cell lung cancer (NSCLC) patients receiving chemotherapy as the first-line treatment. Patients were divided into three groups: (A) Chemotherapy + metformin; (B) Chemotherapy + insulin, and (C) Chemotherapy + other OAD. They found that group A had superior median overall survival (OS) compared with the other two groups (20 months vs. 13.1 months vs. 13.0 months, respectively, $P = 0.007$) [33]. Like the NHIRD in Taiwan, the USA has a similar system called the SEER (surveillance, epidemiology and end results) database. Lin et al. collected 750 diabetic patients diagnosed with stage IV NSCLC and showed that the metformin group was associated with a benefit in survival. The hazard ratio (HR) was 0.80 and 95%, and the confidence interval (CI) is 0.71–0.89, respectively [33,34]. A study by Zhu et al. [35] showed metformin was significantly associated with a 16% reduction of lung cancer risk in type 2 DM patients. The relative risk (RR) is 0.84 and 95% CI (confidence interval was 0.73–0.97, $P < 0.05$).

3. Animal models applied to lung cancer studies

Animal models are indispensable for transforming *in vitro* studies into an *in vivo* setting. Subcutaneous xenograft models are commonly used as lung cancer animal models. In this

model, lung cancer cell lines are injected subcutaneously to the flank side of nude mice. Different designed reagents are administered, and the response after medical therapy is observed. The tumor is not directly initiated from the original lung tissue. Thus, it is an indirect way to observe the so-called lung tumor formation.

Here, we introduced two lung cancer animal models developed in our laboratory. **Model 1** was transgenic mice with an overexpression of human vascular endothelial growth factor (*hVEGF*)-A₁₆₅. The model emphasized angiogenesis in the lung cancer formation process. **Model 2** was an *in vivo* image model of orthotopic lung adenocarcinoma formation in mice by using dual fluorescence reporting genes (pCAG-iRFP-2A-Venus). We could track the lung tumor formation and response to therapy more directly. We provide a summary for these two animal models and compare them with the subcutaneous xenograft model as shown in **Table 2**.

Animal models for lung cancer study	Model 1	Model 2	Current model
Mechanism	hVEGF-A165 over-expression transgenic mice	Orthotopic lung xenograft (transpleural injected) dual fluorescence reporter	Subcutaneous xenograft
Species of animal	Transgenic mice (FVB)	Nude mice (BALB/cAnN. Cg-Foxnlnu/CrlNARL)	Nude mice
Lung cancer cell line selected	Not applied	A549	A549
Tumor formation site	Lung	Lung	Trunk or flank area
Tumor formation duration	Approximately 12 months	Approximately 4–6 weeks	Approximately 4–6 weeks
IVIS setting	No	Yes	No
Lung tumor formation	Direct	Direct	Indirect
Reference	[37]	[40]	[21]

Table 2. Comparison of our two types of animal models with the current subcutaneous xenograft model for lung tumor formation.

3.1. Animal model 1: overexpression of human vascular endothelial growth factor (*hVEGF*)-A₁₆₅-induced lung tumorigenesis in transgenic mice

3.1.1. Background

When a tumor develops, it demands a vast amount of oxygen and nutrition for tumor growth. When a tumor is small in size, approximately 10⁵ to 10⁶ tumor cells, it depends on the diffusion effect for nutrition transport. As the tumor enlarges, it must overcome hypoxia and develop an angiogenic switch, including proangiogenic and angiogenic factors. Once vasculogenesis and angiogenesis are established, the tumor can invade extensively and cause distant metastasis [36]. Based on this theory, we demonstrate lung tumor formation via the angiogenesis model.

3.1.2. Materials and methods, see Ref. [37]

1. Create transgenic mice carrying the *mccsp-Vegf-A₁₆₅-sv40* transgenic fusion gene (Figure 1A)

- A 1975-bp *mccsp-Vegf-A₁₆₅-sv40* transgene was directly microinjected into pronuclear stage FVB mouse embryos and then transferred into the fallopian tube of the recipient females mice.
- Transgenic mice were mated with littermates or normal FVB mice to produce offspring.
- The resulting 12-month transgenic offspring were the candidates for performing the lung cancer model.

2. Gross picture of homozygous transgenic mice compared with wild-type mice (Figure 1B)

- Illustration of the whole picture of lung tissues from transgenic mice (homozygous) and wild type mice. We can observe the mass with a bulging appearance of lung tissue, with tumor formation in the transgenic mice (Figure 1B, upper panel).

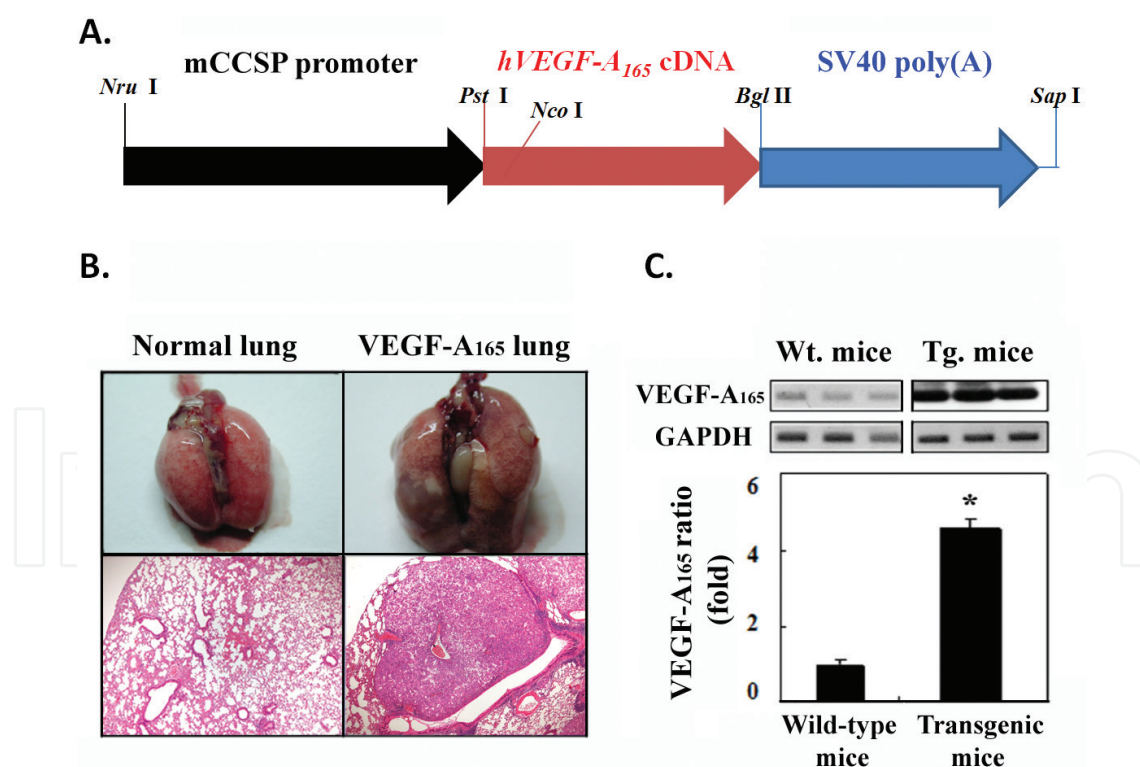


Figure 1. Animal model 1 for overexpressing human vascular endothelial growth factor (*hVEGF*)-A₁₆₅-induced lung tumorigenesis in transgenic mice. (A) Construction map of *hVEGF-A₁₆₅* overexpression, which is controlled by mouse Clara cell-specific protein (*mccsp*) promoter. The structure of the transgene is approximately 1975-base pairs in length. (B) The whole exterior (upper panel) and histopathologic sections of the lung tissues (lower panel) in the transgenic mice (right side) and wild-type mice (left side). (C) Western blot analysis of the *hVEGF-A₁₆₅* protein expression level in the lung tissue of wild type and transgenic mice (upper panel) and the quantification data.

3. Histopathologic analysis of the lung tissue in transgenic mice and wild-type mice (Figure 1B)

- From the lower part of **Figure 1B**, we can easily find the lung tumor formation with bizarre cell shapes and increased nucleus/cytoplasm (N/C) ratios in the lung tissues of transgenic mice. The wild type mice showed no tumor-specific appearance.

4. Validation of *hVEGF-A*₁₆₅ protein expression of transgenic mice was performed by western blot analysis

- In the lung tissue of 12-month transgenic mice, the western blot data proved, there were more than 5-fold higher levels of VEGF expression compared with wild type mice (**Figure 1C**).

3.2. Animal model 2: dual fluorescence reporting genes expressed by an *in vivo* imaging model of orthotopic lung adenocarcinoma in mice

3.2.1. Background

In cancer research, it is important to perform *in vivo* animal experiments to further mimic the effects on human beings. During the study period, it is necessary to record images simultaneously. Real time imaging depends on the *in vivo* image system (IVIS). The creation of good IVIS images is necessary and demands comprehensive consideration. The property of good IVIS images requires an optimal imaging window. For the mammalian tissue study, obtaining deep optical images requires near-infrared (NIR) fluorescent probes. The NIR optical window is around from 650 to 900 nm. Under this optical window, mammalian tissue is considered more transparent to light due to the limited combined absorption of water, melanin and hemoglobin. Moreover, under this spectral region, it could eliminate autofluorescence and have low light scattering [38]. The common used near-infrared fluorescent proteins (iRFP) included iRFP670, iRFP682, iRFP702 and iRFP720 [39]. Based on this concept, we designed a near-infrared fluorescent mice tumor model to further evaluate the IVIS expression during lung tumor formation.

3.2.2. Material and methods, see Ref. [40]

3.2.2.1. Construction of dual fluorescence-expression vector

- The construction map of the pCAG-iRFP-2A-venus transgene is shown (**Figure 2A**).
- Transfection of lung adenocarcinoma cell line (A549) with pCAG-iRFP-2A-venus expression was performed. The iRFP can show red fluorescence. Venus can show green fluorescence. And DAPI (4',6-diamidino-2-phenylindole) emits blue fluorescence in cell nuclei as a background control (**Figure 2B**).

3.2.2.2. Orthotopic lung injection with transfected A549 lung adenocarcinoma cells

- Animal species: Four-week-old male nude mice (BALB/cAnN.Cg-Foxnlnu/CrlNARL) was used.

- A total of 2E+6 iRFP-2A-venus A549 cells were directly injected orthotopically into the transpleural cavity on the left lung side of nude mice.
- IVIS imaging recorded lung tumor formation (**Figure 2C**).

3.2.2.3. Histopathologic analysis confirms the tumor formation of orthotopic lung injected nude mice, which had iRFP-2A-venus A549 expression

- By examining the tissues with an H&E stain, we observe abundant tumor cells formation in the orthotopically injected iRFP-2A-venus A549 cells in mice lung tissue (**Figure 2D**, upper panel).
- Using immunohistochemistry (IHC) and staining with antivenus, lung tumor formation was found (**Figure 2D**, lower panel).

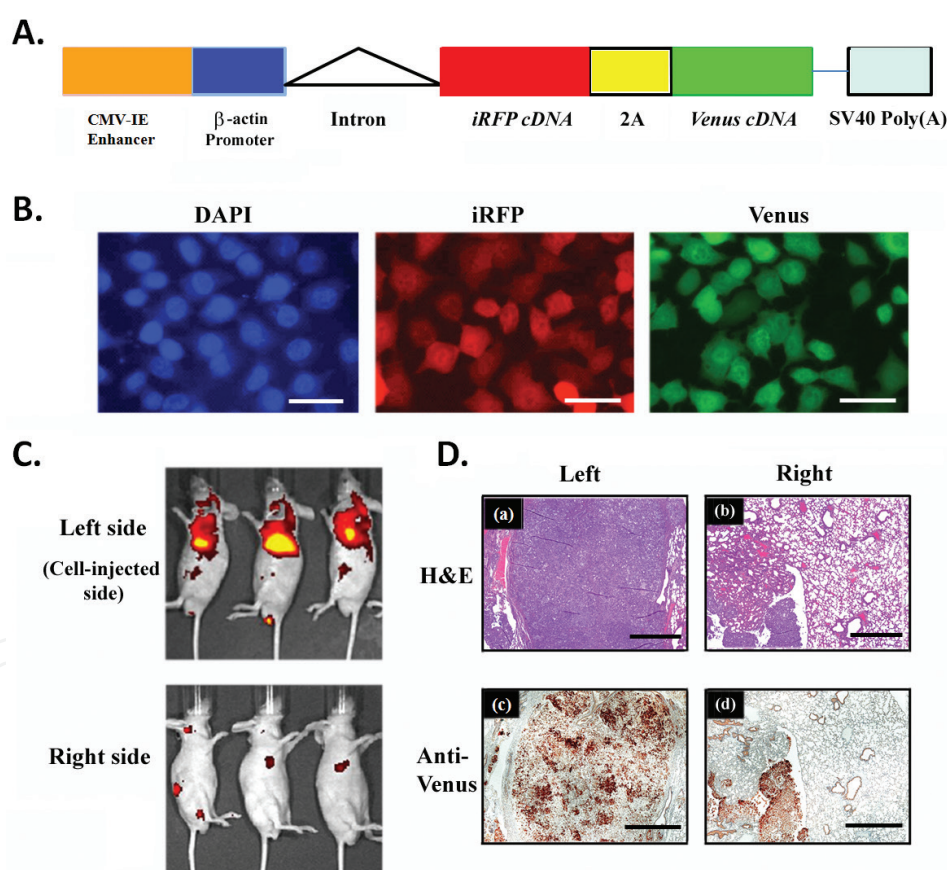


Figure 2. Animal model 2 for dual fluorescence reporting genes expressed by *in vivo* imaging model of orthotopic lung adenocarcinoma in mice. (A) Construction map of dual fluorescence expression vector, pCAG-iRFP-2A-venus transgene. (B) Three different fluorescence signals were used in iRFP-2A-Venus A549 cells expressed under a fluorescent microscope: DAPI (blue), iRFP (red) and venus (green). Scale bar: 50 μ m. (C) The IVIS imaging analysis of nude mice that received iRFP-2A-venus A549 cells by orthotopic injection. Color scale: Max: 4.56e+7, Min: 2.02e+7. (D) Histopathologic study of nude mice lung tumors after receiving iRFP-2A-venus A549 cells by orthotopic injection. L and R represent left and right lungs, respectively. The left side column (a) and (c) represent H&E and IHC expression on the longitudinal sections of lung tumors, respectively. The brown color of antivenus expression in column (c) and (d) indicates lung tumor formation. The scale bar in columns (b) and (d) is 100 μ m.

4. Conclusion

First, we presented a brief introduction of the antihyperglycemic effect of metformin. More and more studies have involved the repurposing of metformin due to its observed antitumor effects. Observational studies found lung cancer patients with type 2 DM under metformin treatment had better outcomes. Through a literature review, we initially sought to examine the potential antitumorigenic effects of metformin in a preclinical setting. Population-based research revealed the survival benefit of type 2 DM patients with metformin under cancer management. Based on the AMPK-dependent pathway, we attempted to discover an AMPK-independent pathway (such as angiogenesis, inflammation, etc.) related to metformin. Later, we illustrated two animal models of lung cancer utilized in our research group. **Model 1** focused on the angiogenesis pathway. Overexpression of human *VEGF-A*₁₆₅ transgenic mice model provided further clues for tumor formation. **Model 2** emphasizes the *in vivo* image of dual fluorescence reporting gene expression created by orthotopic lung injection. IVIS-aided analysis helped track the lung adenocarcinoma formation in real time. This method could shorten the waiting time for lung tumor formation in animal studies. Furthermore, we aim to integrate these two animal models with metformin in a stepwise manner. We look forward to thoroughly elucidating the antitumor effects of metformin for lung cancer management based on current animal model platforms.

Acknowledgements

We thank Dr. Yu-Tang Tung and Dr. Cheng-Wei Lai for their assistance in performing experiments and data collections. This article was financially supported in part by MOST-104-2313-B-005-043-MY3 from the Ministry of Science and Technology, ATU-105-S0508 from the Ministry of Education, TCVGH-NCHU 1027605 and TCVGH-NCHU 1047610 from Rong-Hsing cooperation project, and TCVGH-1033203B, TCVGH-1043204B from VGHTC research project, Taiwan.

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