Metformin reduces glycometabolism of papillary thyroid carcinoma *in vitro* and *in vivo*

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Abstract

More aggressive thyroid cancer cells show a higher activity of glycometabolism. Targeting cancer cell metabolism has emerged as a novel approach to prevent or treat malignant tumors. Glucose metabolism regulation effect of metformin in papillary thyroid cancer was investigated in the current study. Human papillary thyroid carcinoma (PTC) cell lines BCPAP and KTC1 were used. Cell viability was detected by CCK8 assay. Glucose uptake and relative gene expression were measured in metformin (0–10 mM for 48 h)-treated cells by 18F-FDG uptake assay and western blotting analysis, respectively. MicroPET/CT imaging was performed to detect 18F-FDG uptake *in vivo*. After treatment with metformin at 0, 2.5, 5 and 10 mM for 48 h, the ratio of p-AMPK to total AMPK showed significant rising in a dose-dependent manner in both BCPAP and KTC1, whereas p-AKT and p-mTOR expression level were downregulated. 18F-FDG uptake reduced after metformin treatment in a dose-dependent manner, corresponding to the reduced expression level of HK2 and GLUT1 *in vitro*. Xenograft model of PTC using BCPAP cells was achieved successfully. MicroPET/CT imaging showed that *in vivo* 18F-FDG uptake decreased after treatment with metformin. Immunohistochemistry staining further confirmed the reduction of HK2 and GLUT1 expression in the tumor tissue of metformin-treated PTC xenograft model. In conclusion, metformin could reduce glucose metabolism of PTC *in vitro* and *in vivo*. Metformin, by targeting glycometabolism of cancer cells, could be a promising adjuvant therapy alternative in the treatment modality of advanced thyroid carcinoma.

Introduction

Thyroid cancer is increasing globally, especially in young women and differentiated thyroid cancer (DTC) is the most predominant type (Chen *et al*. 2016, Siegel *et al*. 2016). Clinically, it has been widely accepted that 18F-fluoro-2-deoxy-D-glucose (18F-FDG) uptake in metastatic lesions of DTC could provide valuable prognostic information. Metastatic lesions with high 18F-FDG uptake have more dedifferentiated, aggressive and metabolically active tumor cells, thereby indicating a poor prognosis. Conversely, a negative 18F-FDG PET/CT imaging predicts
a more favorable prognosis even in patients with positive radiiodine whole body scan (Masson-Deshayes et al. 2015, Pryma et al. 2006, Salvatori et al. 2015). In molecular level, overexpression of glucose transporter-1 (GLUT1) on the cell membrane of thyroid cancer could contribute to this phenomenon (Schonberger et al. 2002).

Metformin (N,N-dimethylbiguanide), an oral biguanide drug with well-established efficacy and safety profiles, is commonly prescribed as the first-line treatment for patients with type 2 diabetes mellitus (T2DM). Generally, this drug improves blood glucose control and insulin sensitivity by reducing hepatic glucose production, gluconeogenesis and intestinal glucose absorption as well as increasing peripheral glucose uptake (Crandall et al. 2008). Recently, its anti-tumor activity has been widely investigated. There are more than 250 registered clinical trials in patients with breast, ovarian, lung, colorectal and pancreatic cancers using metformin alone or in combination with other therapies (ClinicalTrials.gov). Although popularly studied, the exact effect of metformin on thyroid cancer is still controversial.

Experimental studies have demonstrated that metformin could inhibit cell proliferation and promote apoptosis in multiple thyroid cancer cell lines through adenosine monophosphate-activated protein kinase (AMPK) activation, mammalian target of rapamycin (mTOR) signaling inhibition and cell cycle arrest (Chen et al. 2012, Cho et al. 2014a,b, Hanly et al. 2015). Epidemiological studies have showed that the use of metformin was linked to a decreased risk of thyroid cancer in T2DM patients and a higher remission rate in T2DM patients with thyroid cancer (Klubo-Gwiezdzinska et al. 2013, Tseng 2014). However, meta-analyses have indicated that metformin-induced reduction of cancer incidence and mortality seemed to be of modest magnitude and not affecting all populations equally (Gandini et al. 2014). And more recently, an observational study demonstrated that neither use of metformin nor any other antidiabetic drugs was associated with a decreased risk of thyroid cancer (Becker et al. 2015). However, the total number of metformin-used thyroid cancer patients in this study was much small.

Targeting cancer cell metabolism has emerged as a novel approach to prevent or treat cancers and one of the primary metabolic changes observed in malignant cell transformation is an increased catabolic glucose metabolism (Pollak 2012, Vander Heiden 2011). Anticancer effects of metformin in proliferation and apoptosis of thyroid cancer have been frequently studied. However, few studies have reported the effect of metformin on the glucose metabolism in thyroid cancer. Based on this, we focused on the glycometabolism regulation effect of metformin in thyroid cancer in vitro and in vivo in the current study.

**Materials and methods**

**Cell culture**

Human papillary thyroid carcinoma cell lines BCPAP (DSMZ, German Collection of Microorganisms and Cell Cultures) and KTC1 (kindly provided by Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100U/mL penicillin, 100μg/mL streptomycin and 1% nonessential amino acids (100×) (Gibco). All the cells were incubated at 37°C in a humidified chamber supplemented with 5% CO₂.

**Cell viability assay**

Cells (1 × 10⁴ cells/well) were seeded in 96-well plates. After culturing for 24 h, cells were rinsed with phosphate-buffered saline (PBS) and treated with metformin (MP Biomedicals, Santa Ana, CA, USA) at various concentrations (0–50μM) for 48 h. Cell viability was then evaluated using a CCK8 (Cell Counting kit-8) kit according to the manufacturer’s protocol. Briefly, 10μL CCK8 solution (YEASEN, Shanghai, China) was added to each well, and the samples were incubated at 37°C for 2 h before the absorbance was measured at 450 nm wave length. Each experimental condition, including blank wells, control wells and wells treated with metformin, was assayed in duplicate, and all experiments were performed at least three times.

**In vitro ¹⁸F-FDG uptake assay**

For ¹⁸F-FDG uptake measurement, cells were seeded in 6-well plates (5 × 10⁴ cells per well) overnight, and then subsequently treated with metformin at 0, 2.5, 5 and 10μM for 48 h. Later, the cell culture medium was changed by sugar-free RPMI-1640 culture medium. After 6 h, cells were incubated with ¹⁸F-FDG (Atomic firm Sinovac Pharmaceutical Co., LTD., Shanghai, China) at a final concentration of 37kBq/mL. After 60-min incubation, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with 0.1 M NaOH. Radioactivity was measured using a gamma counter (Capintec, Inc., USA) and expressed as % uptake per well relative to controls. Experiments were repeated for three times.
Western blotting analysis

Cells were seeded in 6-well plates at a density of 1 x 10^6 cells/well. After relevant treatment, cells were harvested in RIPA lysis buffer containing proteinase and phosphatase inhibitors (Beyotime, Nantong, Jiangsu, China). Protein was quantified using a BCA protein assay kit (Beyotime). Equal amounts of cell lysates were separated by 6–10% SDS-PAGE and electrophoretically transferred to PVDF membrane. The membrane was then blocked and probed with primary antibodies (anti-AMPK, anti-phospho-AMPK, anti-GLUT1, anti-HK2, anti-AKT, anti-phospho-AKT, anti-mTOR, anti-phospho-mTOR and anti-β-actin; 1:1000) followed by HRP (horseradish peroxidase)-labeled goat anti-rabbit IgG or HRP-labeled goat anti-rabbit IgG (1:5000). Chemiluminescence was used to analyze protein levels, and β-actin was used as a protein loading control. All antibodies used in this study were purchased from Proteintech Group, Inc., Rosemont, IL, USA. Semi-quantitative analysis was conducted using ImageJ 1.49v (NIH, Bethesda, MD, USA). Experiments were repeated for three times.

Immunohistochemistry (IHC)

Paraffin-embedded tissues from xenograft tumor were cut into 3μm sections. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 15 min. Antigen retrieval was performed by boiling the sections for 10 min in citrate buffer (pH 6.0) and cooling at room temperature, followed by blocking with 10% normal goat serum for 1 h. After incubation with primary antibody (anti-GLUT1 and anti-HK2; 1:100) overnight at 4°C, the sections were incubated with horseradish peroxidase- (HRP-) conjugated anti-mouse/rat secondary antibodies. After washing with PBS three times for 5.0 min each time, peroxidase substrate DAB kit was used for visualization according to the manufacturer's instructions. Semi-quantitative analysis was performed by digital image analysis with the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Xenograft model

Animal studies were carried out in compliance with the guideline on Administration of Lab Animals (Ministry of Science and Technology of China) and approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital. For PTC xenograft implantation, a total of 2 x 10^6 cells were subcutaneously injected into the left forelimb of 6-week-old female BALB/c-nu mice. The animals were kept in individual ventilated cages in compliance with institutional guidelines.

MicroPET/CT imaging and analysis

MicroPET/CT scans and image analysis were performed using a Super Nova MicroPET/CT (Pingseng Healthcare (KunShan), Inc., Kunshan, Jiangsu, China). Water and food were not supplied until 18F-FDG injection at least for 8 h. Each tumor-bearing mouse was injected with 4.959–4.958 MBq (107–134 μCi) of 18F-FDG (Atomic firm Sinovac Pharmaceutical Co., LTD., Shanghai) in 200 μL of saline via tail vein. Twelve-minute static scans were acquired at 1 h after injection, and animals were maintained under 2% isoflurane anesthesia during scanning period. Body temperature was maintained using a heating pad provided with the small-animal PET/CT system. For semi-quantitative analysis, 3-dimensional regions of interest (ROIs) were carefully delineated over the borders of the tumor (T) and a part of the liver as a non-target (NT) reference. 18F-FDG uptake was acquired as SUVmax automatically after delineating the ROIs. The SUVmax ratio (T/NT) was calculated and compared between groups.

Statistics analysis

Analyses were performed using the Statistical Package for the Social Sciences, version 20.0 (SPSS) and GraphPad prism version 5.0 (GraphPad Software). All group data are expressed as means ± standard deviation (S.D.). Student’s t-test was performed to compare means of different groups. All P values are two-tailed. P < 0.05 was considered as a statistically significant difference.

Results

Cell viability inhibition in vitro

To determine the effect of metformin on the viability of thyroid cancer cell, we performed CCK8 assay. Two PTC cell lines, KTC1 and BCPAP, were treated with metformin at various concentrations (0, 10, 20, 30, 40 and 50 mM) for 48 h. The results demonstrated that after treatment with metformin, all thyroid cancer cell lines showed dose-dependent decreases in cell viabilities (Fig. 1).

AMPK activation

To confirm metformin-mediated AMPK activation through AMPK phosphorylation in PTC cell lines, proteins from
metformin-treated cells (KTC1 and BCPAP) at 0, 2.5, 5 and 10 mM for 48 h were harvested and the expression of AMPK and p-AMPK was measured by western blotting. As can be seen in Fig. 2, the ratio of p-AMPK to total AMPK showed a significant rise in a dose-dependent manner in both KTC1 and BCPAP.

18F-FDG uptake in vitro

18F-FDG enters cell via the same facilitative transporters of glucose. It is then phosphorylated and trapped in the cytoplasm. So glucose consumption can be detected and visualized non-invasively by using 18F-FDG. As showed in Fig. 3(A and B), after treatment with metformin at 0, 2.5, 5 and 10 mM for 48 h, PTC cells showed a dose-dependent reduction of 18F-FDG uptake.

Decreased expression of GLUT1 and HK2 in vitro

Glucose transporter-1 (GLUT1) and hexokinase-2 (HK2) are among the major proteins regulating transportation and transformation of glucose, respectively. Their expression level and function status are of great significance in the glucose metabolism in cancer cells (Colell et al. 2007, Mondal et al. 2015). Proteins from metformin-treated cells (KTC1 and BCPAP) at 0, 2.5, 5 and 10 mM for 48 h were harvested, and the expression of HK2 and GLUT1 was measured by western blotting. The results demonstrated that HK2 and GLUT1 in the two PTC cell lines showed dose-dependent decreases (Fig. 3C, D, E and F).

AKT/mTOR signaling suppression

AKT/mTOR pathway is an essential signaling cascade that interacts with glucose metabolism in cancer cells (Robey & Hay 2009). Proteins from metformin (0, 2.5, 5 and 10 mM for 48 h)-treated PTC cells (KTC1 and BCPAP) were harvested, and the expression level of AKT, p-AKT, mTOR and p-mTOR was measured by western blotting. The results demonstrated that after treatment with metformin, p-AKT and p-mTOR were significantly decreased in PTC cell lines (Fig. 4).

In vivo 18F-FDG microPET/CT imaging

Based on the above evidence, 18F-FDG microPET/CT imaging was performed to further confirm the effects of metformin on glucose metabolism of PTC in vivo. Xenograft model of PTC using BCPAP cells was successfully achieved. After 2 weeks, two groups (A and B) were randomly divided with 6 mice per group. Metformin (300 mg/kg, IP, QD) treatment was done for one week in group A (metformin/+), whereas same volume of saline was intraperitoneally injected as control in group B (metformin/−). After three weeks of cells injection, in vivo 18F-FDG microPET/CT imaging was performed. The results showed that, in accordance with the results of in vitro 18F-FDG uptake assay, after treatment with metformin, the SUVmax ratio of T/NT was much lower in group A compared to that in group B (Figs 5 and 6A).

Decreased expression of GLUT1 and HK2 in vivo

After microPET/CT scan, mice were killed, and the expression of HK2 and GLUT1 was detected by
immunohistochemistry. Brown staining was located in the cytoplasm and membrane of BCPAP cell. The intensity of staining was much lower in metformin-treated group compared to that in non-metformin-treated group. These results indicated that the expression of HK2 and GLUT1 was decreased after metformin treatment \textit{in vivo} (Figs 7 and 6B).

**Discussion**

In the current study, we found that metformin, as the most widely prescribed oral hypoglycemic agent in T2DM patients, could downregulate the expression of GLUT1 and HK2, thus decreasing the rate of glucose metabolism in papillary thyroid carcinoma \textit{in vitro} and \textit{in vivo}.

The anticancer effects of metformin have been widely studied recently, whereas the underlying mechanism is still not clearly illuminated. Mainly, the potential mechanisms involved are divided into two ways: ‘direct effect’ and ‘indirect effect’ (Koritzinsky 2015, Luengo et al. 2014). The direct anticancer effects of metformin are found to be mainly mediated by AMPK-dependent way as well as AMPK-independent process. For AMPK-dependent way, studies have demonstrated that metformin’s primary cellular targets are the mitochondrial complex I (Owen et al. 2000, Wheaton et al. 2014) and the mitochondrial glycerophosphate dehydrogenase (mGPD) enzyme (Madiraju et al. 2014). AMPK is activated due to the decrease of oxygen consumption and adenosine triphosphate (ATP) production, which results from the inhibition of complex I and consequently inhibition of the mitochondrial electron transport. This effect induces cell cycle arrest and inhibits protein synthesis in cancer cells (Hawley et al. 2010, Steinberg & Kemp 2009). Cho and coworkers investigated the therapeutic potential of metformin in PTC, and their results indicated that metformin had anti-tumorigenic effects in PTC by activation of AMPK signaling and inhibition of AKT signaling (Cho et al. 2014a,b). However, other
studies have reported that metformin could also regulate cancer cell biology in an AMPK-independent way through the inhibition of the unfolded protein response with a consequent apoptosis, preventing angiogenesis and exerting toxicity on cancer stem cells (Kourelis & Siegel 2012). In addition, metformin-reduced circulating levels of glucose, insulin and other factors that can stimulate tumor cells’ proliferation is considered as its indirect effect (Algire et al. 2010, Sui et al. 2015).

Metabolic changes, known as the Warburg effect or aerobic glycolysis, have been discovered in the context of cancer diseases with the phenomenon that under aerobic conditions, proliferating cancer cells metabolize approximately tenfold more glucose to lactate in a given time than normal surrounding ones (Cairns et al. 2011,
Koppenol et al. 2011). Recently, AMPK activation is found to have the ability to negatively regulate aerobic glycolysis (the Warburg effect) in cancer cells and suppress tumor growth (Faubert et al. 2013). Therefore, based on the fact that metformin is an activator of AMPK, another important role of metformin that could be involved in its anticancer activities is its capability to modulate the tumor cell metabolism.

Studies have showed that metformin could directly inhibit the enzymatic function of HK2 in different cancer models comprising Calu-1 cells as a model of non-small-cell lung cancer and in MDA-MB231 as a model of triple-negative breast cancer (Marini et al. 2013, Salani et al. 2013). In all these cells, metformin determined a dose- and time-dependent reduction in $^{18}$F-FDG uptake. However, Habibollahi et al. (2013) demonstrated that metformin, through activation of the AMPK pathway, exerted a dose-dependent increase in tumor glucose uptake in colon cancer cell lines HT29 (human) and MC26 (murine). In the current study, we examined the effect of metformin on the glucose metabolism in PTC cell lines, and the results demonstrated that metformin could decrease the $^{18}$F-FDG uptake in BCPAP and KTC1 cells. To the best of our knowledge, this is the first study that tried to elucidate the anticancer effect of metformin under a cellular metabolism way in thyroid carcinoma. Although reduction of GLUT1 and HK2, activation of AMPK and inhibition of AKT/mTOR signaling could be of great importance during this process, the exact underlying mechanism of how activation of AMPK could regulate the expression of key enzymes of glycolytic pathway including glucose transporters and hexokinases in thyroid cancer cells is still not clearly known. This is the major limitation of our current work and intensive studies should be performed to further clarify the potential mechanism.

In conclusion, our results demonstrated that after treatment with metformin, PTC cells showed a dose-dependent reduction of $^{18}$F-FDG uptake both in vitro and in vivo in accordance with the downregulated expression of key enzymes in glycolytic pathway including GLUT1 and HK2. Activation of AMPK and inhibition of AKT/mTOR signaling by metformin might play an essential role in the process of this effect. Based on these findings, metformin by targeting glucose metabolism of cancer cell could be a promising adjuvant therapy alternative in the treatment modality of advanced thyroid carcinoma.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Luo designed the study. Shen and Wei performed the experiments. Shen, Wei, Qiu and Song analyzed and interpreted the data. Shen wrote the manuscript. Luo, Sun and Zhang revised the manuscript. All authors read and approved the final manuscript.

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