Glucose-deprivation increases thyroid cancer cells sensitivity to metformin

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Abstract

Metformin inhibits thyroid cancer cell growth. We sought to determine if variable glucose concentrations in medium alter the anti-cancer efficacy of metformin. Thyroid cancer cells (FTC133 and BCPAP) were cultured in high-glucose (20 mM) and low-glucose (5 mM) medium before treatment with metformin. Cell viability and apoptosis assays were performed. Expression of glycolytic genes was examined by real-time PCR, western blot, and immunostaining. Metformin inhibited cellular proliferation in high-glucose medium and induced cell death in low-glucose medium. In low-, but not in high-glucose medium, metformin induced endoplasmic reticulum stress, autophagy, and oncosis. At micromolar concentrations, metformin induced phosphorylation of AMP-activated protein kinase and blocked p-pS6 in low-glucose medium. Metformin increased the rate of glucose consumption from the medium and prompted medium acidification. Medium supplementation with glucose reversed metformin-inducible morphological changes. Treatment with an inhibitor of glycolysis (2-deoxy-D-glucose (2-DG)) increased thyroid cancer cell sensitivity to metformin. The combination of 2-DG with metformin led to cell death. Thyroid cancer cell lines were characterized by over-expression of glycolytic genes, and metformin decreased the protein level of pyruvate kinase muscle 2 (PKM2). PKM2 expression

was detected in recurrent thyroid cancer tissue samples. In conclusion, we have demonstrated that the glucose concentration in the cellular milieu is a factor modulating metformin's anti-cancer activity. These data suggest that the combination of metformin with inhibitors of glycolysis could represent a new strategy for the treatment of thyroid cancer.

Keywords

metformin; glucose; oncosis; thyroid cancer; cell signaling

Introduction

Targeting cancer cell metabolism has emerged as a novel approach to prevent or treat cancers (Pollak 2012). Recent epidemiological and laboratory studies have demonstrated that the anti-diabetic drug metformin has anti-cancer activity (Pollak 2012, Margel *et al.* 2013, Yin *et al.* 2013, Thompson 2014). Currently, there are more than 200 ongoing clinical trials in patients with breast, ovarian, lung, colorectal, and pancreatic cancers using metformin alone or in combination with other therapies (ClinicalTrials.gov). Recent meta-analyses, however, have indicated that metformin-inducible reduction of cancer incidence and mortality does not affect all populations equally (Gandini *et al.* 2014). Clarification of the molecular mechanisms underlying cancer cell sensitivity to metformin could provide critical information for selective use of metformin in clinical trials.

Experimental studies have demonstrated that metformin may influence tumorigenesis, both indirectly, through the systemic reduction of insulin levels, and directly, via the induction of energetic stress (Pernicova & Korbonits 2014). Metformin has been shown to have a strong anti-proliferative effect in many cancer cell lines due to activation of AMP-activated protein kinase (AMPK), inhibition of mammalian target of rapamycin (mTOR) activity, and cell cycle arrest (Ben Sahra *et al.* 2010, Choi & Park 2013). Metformin can also inhibit mTOR signaling in an AMPK-independent manner through induction of REDD1, a known negative regulator of mTOR (Ben Sahra *et al.* 2011). Induction of autophagy was demonstrated in cancer cells after treatment with metformin, and inhibition of autophagy by Beclin1 knockdown was sufficient for prevention of the anti-proliferative effects of metfor-min on endometrial cancer cells (Takahashi *et al.* 2014). Metformin is considered as a mitotoxic drug and studies in human cancer cells have demonstrated its inhibitory effect on mitochondrial complex I activity (Wheaton *et al.* 2014).

In cell lines derived from follicular and papillary thyroid cancers, metformin inhibited cell growth, activated AMPK, down-regulated p70S6K/pS6 signaling, antagonized the growth-stimulatory effects of insulin, inhibited clonal cell growth, and reduced thyroid cancer sphere formation (Chen *et al.* 2012, Klubo-Gwiezdzinska *et al.* 2013). Metformin treatment also significantly delayed tumor growth in animal models of thyroid cancer (Cho *et al.* 2014). Diabetic patients treated with metformin who were subsequently diagnosed with thyroid cancer, and were treated with thyroidectomy and ^{131}I, had an increased likelihood of complete response and higher remission rates as compared to matched thyroid cancer diabetic patients not receiving metformin (Klubo-Gwiezdzinska *et al.* 2013). Together these

data suggested the potential utility of metformin as an adjunct treatment for non-diabetic patients with thyroid cancer.

Numerous factors may influence the anti-cancer effectiveness of treatment with metformin. Recent studies have indicated that the anti-proliferative effects of metformin in cancer cells are highly dependent on the glucose concentration in the extracellular milieu (Wahdan-Alaswad *et al.* 2013, Zordoky *et al.* 2014). It has been demonstrated that metformin inhibited cellular proliferation in the presence of glucose, but induced cell death upon glucose deprivation (Birsoy *et al.* 2012, Zordoky *et al.* 2014). Experimental *in vitro* and *in vivo* studies have demonstrated that use of the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) in combination with metformin leads to significant cell death associated with a decrease in cellular ATP, prolonged activation of AMPK, and sustained autophagy (Cheong *et al.* 2011). In a xenograft model of human breast cancer, combined treatment with the mitotoxic drug Mito-CP and 2-DG led to significant tumor regression (Cheng *et al.* 2012).

Cancer cells exhibit diverse responses to glucose limitation. Over-expression of glycolytic genes (pyruvate kinase muscle (PKM), ENO1, and ALDOA) was demonstrated in cancer cells with high sensitivity to glucose deprivation (Birsoy *et al.* 2014). The glucose-utilization gene signature has been proposed as a biomarker for identifying tumors that are sensitive to treatment with biguanides.

There is limited information regarding the effects of micro-environmental factors on the efficacy of treatment with metformin in differentiated thyroid cancer. In this study, we examined the effects of glucose in the extracellular milieu on thyroid cancer cell response to treatment with metformin. We also determined how pharmacological blockade of glycolysis with 2-DG affected sensitivity of thyroid cancer cells to treatment with metformin.

Materials and methods

Human thyroid tissue samples

The protocol was approved by the Institutional Review Boards at the Medstar Washington Hospital Center and the Human Use Committee at the Uniformed Services University of the Health Sciences. Paraffin embedded thyroid tissue samples were obtained from 16 patients that developed progressive disease after initial treatment and underwent a second surgery for metastatic lesions. Informed consent from individual patients was not necessary because all data were rendered anonymous.

Thyroid cancer cell lines and reagents

Human thyroid cancer cell lines derived from follicular thyroid cancer 133 (FTC133) and papillary thyroid cancer (BCPAP) were obtained from Dr Motoyasu Saji (The Ohio State University) with permission from the researchers who originally established the cell lines. These cell lines had been tested and authenticated by DNA analysis to be of thyroid origin (Schweppe *et al.* 2008). In our laboratory, we confirmed the presence of BRAF mutation in BCPAP cells, and loss of PTEN expression in FTC133 cells.

Cancer cells were propagated in conventional DMEM supplemented with 10% of fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator. The cells were sub-cultured with 0.5% trypsin and 0.02% EDTA (Sigma–Aldrich) when the cell confluency reached 80%. All experiments were performed using thyroid cancer cell lines which had been passaged <25 times. For glucose deprivation experiments, cells were washed with PBS twice, and then medium was replaced with non-glucose containing DMEM (Invitrogen). The glucose concentration was maintained at 0 mM or adjusted to either 5 mM (low-glucose) or 20 mM (high-glucose). Cells were incubated with either control medium or medium containing metformin (Sigma Chemical Co.). The pharmacological inhibitor of glycolysis, 2-DG was obtained from Sigma Chemical Co.

Cell proliferation, viability, and caspase activity assays

Cell proliferation rate was determined by cell counting using Vi-CELL Cell Viability Analyzer from Beckman Coulter (Fullerton, CA, USA). Cell viability was assessed by alamarBlue Assay (Invitrogen) according to manufacturer instructions. Cell viability also was determined by evaluation of mitochondrial membrane potential with the fluorogenic lipophilic cation JC-1 (Cayman Chemical Company, Ann Arbor, MI, USA). Detection of mitochondrial membrane potential was performed according to the manufacturer's instructions, followed by fluorescent microscopy. Cytotoxicity was examined using the LIVE/DEAD Cell Imaging Kit (Invitrogen). FAM-FLICA Caspase 3/7 Assay for apoptosis detection via activated caspases 3 and 7 was obtained from ImmunoChemistry Technologies, LLC (Bloomington, MN, USA). Dead cells were detected using propidium iodine staining. All experiments were repeated at least three times and the average values of representative experiments are reported.

Evaluation of glucose concentration and pH in the medium

The glucose concentration was measured using the glucose oxidase method (GlucCell, CescoBioproducts, Atlanta, GA, USA).

Protein extraction and western blot analysis

Thyroid cancer cells were incubated with ice-cold cell lysis buffer; 25 μg of total protein lysates were subjected to SDS–PAGE (7%). The separated proteins were transferred to nitrocellulose membranes (Invitrogen) by electrophoretic blotting. Membranes were incubated overnight with the primary antibody against phospho-p70S6K (Thr389), total-p70S6K, phospho-pS6 (Ser235/236), phos-pho-AMPK (Thr172), total AMPK, phospho-AKT (Ser473), poly (ADP-ribose) polymerase (PARP), cleaved-caspase 3, LC3B, p62, BIP, PKM2 (Cell Signaling, Boston, MA, USA), cyclin D1, perforin, and b-actin (Santa Cruz Biotechnologies). Membranes were then incubated with the secondary antibody and, after washing protein bands on the blots were visualized using the Li-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Image Studio Lite version 3.1 (LI-COR) was used for band densitometry measurement and quantification of protein levels.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from thyroid cancer cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. SYBR Green-based qPCR master mixes were obtained from Qiagen. Expression of genes with an established role in regulation of glycolysis was examined using 'glycolysis' real-time PCR arrays (Qiagen). Expression of *PKM* (with specific isoforms being *PKM1* and *PKM2*), glucose-6-phosphate dehydrogenase (*G6PD*), and fructose-1,6-biphosphatase 1 (*F1*) were examined using specific primers.

Immunostaining

Immunostaining was performed on paraffin-embedded tissue sections and on cancer cell lines that were cultured on eight-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA, USA). Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Sections and cancer cell lines were incubated overnight with primary anti-PKM2 and immunostaining was performed using the DAKO Universal LSAB Kit (DAKO, Carpinteria, CA, USA). Negative controls were performed as described earlier except that the primary antisera were not included. We adopted the semi-quantitative scoring system in considering the staining intensity and area extent. Every tumor was given a score according to the intensity of the staining (no staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3) and the extent of stained tumor areas (0%, 0; 1–10%, 1; 11–50%, 2; 51–80%, 3; and 81–100%, 4). The final immunoreactive score was determined by multiplying the intensity scores with the extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12.

Confocal microscopy

Cells were grown on coverslips and fixed with 4% PFA in PBS followed by treatment with 0.2% Triton X-100 in PBS. Perforin primary antibody (1:100) was incubated for 1 h at room temperature and visualized by means of AlexaFluor-488 (1: 300, Invitrogen). Coverslips were mounted in antifade (SlowFade; Invitrogen) and examined under a confocal microscope (Zeiss 700).

Time-lapse microscopy

Live cell imaging was performed using Leica AF6000 Time-Lapse Imaging System.

Results

Metformin inhibits thyroid cancer cell viability in low-glucose medium

We first examined the effect of low (5 mM) and high (20 mM) glucose on growth of thyroid cancer cells. During the first 48 h, no difference was noted in the proliferation rate of cells growing in either high- or low-glucose conditions (Fig. 1A). Starting from 72 h, the number of cells was higher in cells grown in high-glucose medium, and the difference was more prominent in BCPAP cells. In glucose-free DMEM medium supplemented only with 10% FBS, the rate of growth was decreased in both cells lines.

To determine the effects of glucose on thyroid cancer cell response to metformin, we performed treatment with metformin in thyroid cancer cells growing in high- and in low-

glucose medium. The AlamarBlue Assay showed that in high-glucose medium, treatment with metformin at concentration 5 mM for 48 h was required for inhibition of cell viability (Fig. 1B). In low-glucose medium, thyroid cancer cell viability was significantly reduced after exposure to metformin at concentrations ranging from 0.1 to 0.5 mM. ViCell analysis demonstrated that metfor-min had no significant effect on the number of dead cells in high-glucose medium, but induced cell death in low-glucose medium.

Western blot analysis demonstrated that metformin decreased expression of cyclin A, and its effects were more prominent in low-glucose medium (Fig. 1C). Treatment with metformin was not associated with induction of caspase-3 and PARP cleavage in cancer cells growing in high- or low-glucose medium. Caspase 3/7 activity assay showed no caspase activation in thyroid cancer cells treated with metformin for 48 h in low-glucose medium (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Analysis of autophagy-related molecules showed that treatment with metformin resulted in cell type specific responses. Loss of p62 was observed in FTC133 cells treated with metformin in low-glucose condition. In BCPAP cells, switch from high- to low-glucose medium was associated with loss of p62 (Fig. 1D). The lipidated form of LC3B (LC3B-II) was detected after exposure to metformin in FTC133 cells cultured in low-glucose but not in high-glucose medium. In BCPAP cells, LC3B expression was detected in cells cultured in high- and low-medium conditions, and was not significantly affected by metformin.

These results showed that thyroid cancer cells were more sensitive to treatment with metformin in low-glucose compared to high-glucose medium metformin.

Metformin induces oncosis in thyroid cancer cells in low-glucose medium

To clarify the effects of metformin on thyroid cancer cells, we performed real-time imaging of thyroid cancer cells treated with metformin. In high-glucose medium, met-formin (5 mM) had no significant effect on cellular morphology. In low-glucose medium, time-dependent morphological changes suggesting oncosis were observed in metformin treated thyroid cancer cells (Supplementary Videos 1 and 2). These morphological changes occurred gradually, with time points corresponding to the following stages: non-reactive stage (24 h), cell swelling (24–36 h), cell shrinkage (36–40 h), 'anchored' detachment (40–48 h), and membranous blebbing with cytoplasmic ballooning (48–72 h). Representative images of morphological changes in thyroid cancer cells under treatment with metformin are presented in Fig. 2A.

Analysis of mitochondrial membrane potential by JC-1 staining showed cytoplasmic localization of JC-1 aggregates in non-treated thyroid cancer cells (Fig. 2B). The perinuclear accumulation of JC-1 aggregates was noted in cells that were treated with metformin for 48 h, suggesting that metformin affected localization of mitochondria in thyroid cancer cells. Loss of mitochondrial membrane potential in cells that detached from the plates after treatment with metformin for 72 h was associated with propidium iodine nuclear staining, indicating cell death.

Since increased permeability of the endoplasmic reticulum (ER) membrane is considered an early event of oncosis, we examined expression of BiP, a well-characterized regulator of transport across the ER membrane. Treatment with metformin for 48 h was associated with overexpression of BiP in cells cultured in low-, but not in high-glucose medium (Fig. 2C). Expression of a molecular marker of oncosis (perforin) was also induced in thyroid cancer cells treated by metformin (Fig. 2C). Confocal microscopy demonstrated membranous localization of perforin in metformin treated thyroid cancer cells (Fig. 2D).

Together these results suggest that oncosis may represent a mechanism of metformin-inducible thyroid cancer cell death.

AMPK is activated in thyroid cancer cells cultured in low-glucose medium

Oncotic cell death is triggered by ATP depletion, and AMPK is a known sensor of decreased ATP/AMP ratio. Therefore, we examined the effects of metformin on phosphorylation of AMPK and activation of pS6 in thyroid cancer cells growing in high- and low-glucose medium.

Time-course experiments demonstrated that culturing of thyroid cancer cells in high-glucose medium for up to 48 h had no significant effects on p-AMPK (Fig. 3A). In contrast, in low-glucose medium a progressive increase in p-AMPK over time was documented, especially in BCPAP cells. At 48 h, the level of p-AMPK was increased by more than twofold when compared to control cells.

In high-glucose medium, treatment with metformin for 48 h was associated with a dose-dependent induction of p-AMPK and inhibition of p-pS6 (Fig. 3B). In low-glucose medium, metformin increased the level of p-AMPK with subsequent down-regulation of p-pS6 at 24 and 36 h (data not shown). At 48 h of incubation in low-glucose medium, however, the level of p-AMPK was not significantly different between metformin-treated and non-treated cells (Fig. 3C). Interestingly, despite the negligible effects of metformin on p-AMPK at 48 h, there was significant down-regulation of p-pS6.

These data show that thyroid cancer cell response to metformin includes AMPK-dependent as well as by AMPK-independent mechanisms.

Glucose depletion increases cytotoxic effects of metformin on thyroid cancer cells

Since growth inhibitory effects of metformin were prominent in thyroid cancer cells cultured in low-glucose conditions, we performed experiments using glucose-free medium supplemented with FBS. As demonstrated in Fig. 4A, metformin significantly decreased the number of viable cells within 24 h. BCPAP cells were more sensitive to metformin treatment in these conditions.

Measurement of glucose in the medium during treatment with metformin showed that in both high-and low-glucose media, metformin accelerated the decline in glucose concentration. Data from measurements in high-glucose medium are presented in Fig. 4B, with an identical trend noted in low-glucose medium. The increased consumption of glucose from the medium with metformin treatment was prominent in FTC133 cells but not in

BCPAP cells. Treatment with metformin also prompted acidification of medium consistent with induction of glycolysis. In FTC133 and BCPAP cells treated with metformin (1 mM for 48 h), the levels of pH decreased from 7.35 ± 0.02 to 6.8 ± 0.12 (P=0.01) and from 7.25 ± 0.1 to 6.8 ± 0.2 (P=0.01) respectively.

To determine if glucose concentration in the medium is a factor influencing metformin action, we adjusted the level of glucose to 20 mM after cell incubation with metformin in low-glucose medium for 48 h. Supplementation of glucose reversed metformin-inducible morphological changes (Fig. 4C), and prevented metformin-inducible death in thyroid cancer cells. In contrast, supplementation of medium with FBS was not sufficient to rescue thyroid cancer cell from metformin-inducible cell death.

These data demonstrate that glucose concentration in the medium is a factor influencing metformin activity against thyroid cancer cells.

Treatment with 2-DG potentiates the effects of metformin on thyroid cancer cells

To confirm that activation of glycolysis is a critical mechanism underlying thyroid cancer cell survival when treated with metformin, we used the anti-glycolytic agent 2-DG. In high-glucose medium, treatment with 2-DG (5 mM) for 48 h had no significant effects on cell viability. In FTC133 cells, treatment with 2-DG increased p-AMPK in high- as well as in low-glucose medium, and down-regulated p-pS6, especially in low-glucose medium. In BCPAP cells, the level of p-AMPK was increased in low-glucose medium and was not significantly affected by 2-DG. However, treatment with 2-DG (5 mM) down-regulated p-pS6 in BCPAP cells in low-glucose medium (Fig. 4D). Treatment with 2-DG (5 mM) also resulted in induction of BiP expression in FTC133 and BCPAP cells. Analysis of apoptotic markers showed that as a single agent, 2-DG had no significant effect on caspase-3 and PARP cleavage at 24 h. Treatment with 2-DG (5 mM) for 36 and 48 h was associated with slight induction of caspase-3 cleavage.

In a low-glucose environment, the combination of 2-DG with metformin resulted in robust activation of AMPK and down-regulation of p-pS6 at 6 h of treatment. The combination of 2-DG (5 mM) with metformin (1 mM) for 24 h resulted in activation of apoptosis as demonstrated by western blot with anti-caspase-3 and anti-cleaved PARP (Fig. 4E). Dose–response experiments demonstrated that pretreatment of thyroid cancer cells with 2-DG for 24 h sensitized them to clinically achievable concentrations of metformin. Under these conditions, treatment with metformin at micromolar concentrations (25 μM) induced caspase-3 and PARP cleavage at 24 h (Fig. 4F) and led to massive cell death at 48 h in the examined cell lines.

These data indicate that the inhibition of glycolytic activity by 2-DG increases thyroid cancer cells sensitivity to metformin, and suggest that anti-cancer effects of metformin can be mediated via regulation of genes controlling glycolysis.

Metformin inhibits expression of PKM2 in thyroid cancer cells cultured in low-glucose medium

We performed a RT-PCR screening of 84 glycolytic genes with well-established role in regulation of glycolysis using commercially available RT-PCR arrays. The mRNA extracted from normal thyroid tissue was used as control. The set of genes that were commonly up- or down-regulated in thyroid cancer cell lines comparing to the normal thyroid is presented in Table 1. Both FTC133 and BCPAP cell lines were characterized by over-expression of genes that were described in 'glucose-deprivation sensitive' cancer cells (Birsoy *et al.* 2014). We also performed qRT-PCR analysis of PKM2, G6PD, and F1 using RNA from 15 human thyroid papillary cancers and normal thyroid tissue. Compared to a normal thyroid, the mRNA levels of PKM2 and G6PD were higher in 14/15 PTCs (mean 2.59 ± 1.3, P=0.003) and 13/15 PTCs (mean 3.6 ± 2.0; P=0.007) respectively. The mRNA level of F1 was decreased in 10/15 PTCs (mean 0.72 ± 0.4), however the difference was not statistically significant (P=0.08).

Since PKM expression was significantly up-regulated in human PTCs, we focused our study on this regulator of glycolysis. The switch in a splice isoforms of the PKM is sufficient for the shift in cellular metabolism to aerobic glycolysis (Christofk *et al.* 2008). Therefore, we next examined the effects of metformin on expression of PKM1 and PKM2 isoforms in FTC133 and BCPAP cells. RT-PCR analysis showed that a switch from high- to low-glucose medium did not affect expression of PKM isoforms. Treatment with metformin was not associated with significant changes in PKM isoforms mRNA levels in FTC133 cells and in BCPAP cells (Fig. 5A). Western blot analysis demonstrated that metformin had no significant effects on protein expression of PKM2 in high-glucose medium, but significantly inhibited PKM2 in thyroid cancer cells cultured in low-glucose medium (Fig. 5B). The inhibitory effects of metformin on PKM2 protein expression were confirmed by immunostaining (Fig. 5C).

These data show that metformin decreases the level PKM2 protein in thyroid cancer cells, and suggest

Expression of PKM2 is increased in human thyroid cancers

Our *in vitro* experiments suggested that detection of PKM2 in human thyroid cancer could serve as a biomarker underlying the efficacy of treatment with metformin. Therefore, we assessed expression of PKM2 by immunostaining in a series of metastatic thyroid cancers.

As demonstrated in Fig. 6A, the level of PKM2 was low in normal follicular cells, but was increased in all examined primary thyroid cancers. However, the level of PKM2 expression significantly varied between examined tumors. Strong homogenous immunostaining was detected in 7/16 primary tumors, whereas a different degree of heterogeneity was observed in the remaining nine PTCs. Tumors with mixed follicular and papillary patterns of growth frequently displayed heterogeneous staining with anti-PKM2, and the intensity of immunostaining was higher in the area formed by papillary structures. Interestingly, the levels of PKM2 were also different between neighboring cancer cells forming the same papilla.

We next examined PKM2 in metastatic lesions that developed after initial therapy. Strong homogenous immunostaining was detected in 12/16 cases. The seven metastatic lesions that originated from tumors with strong homogenous PKM2 expression maintained the same pattern of PKM2 staining. Metastases that derived from primary tumors with heterogeneous PKM2 expression were characterized by loss of PKM2 immunoreactivity in one case, persistent heterogeneity in three cases, and increased PKM2 immunostaining in five cases (Fig. 6B).

The average immunohostichemical scores in primary PTCs (mean score 8.4) and metastases (mean score 11) were higher than in normal thyroid tissue mean score (mean score 0.9). As demonstrated in Fig. 6C, PKM2 immunoreactivity in metastatic lesions was significantly higher than in primary tumors (P=0.018).

These results show that most metastatic lesions from patients with progressive thyroid cancers are characterized by high level of PKM2 expression.

Discussion

To survive in an adverse tumor microenvironment with limited nutrients, cancer cells reprogram canonical biochemical pathways to provide the necessary precursors of proteins, nucleic acids, and membrane lipids via shifts from anaerobic to aerobic glycolysis (Vander Heiden *et al.* 2009). We have explored molecular mechanisms implicated in the thyroid cancer cell response to glucose deprivation, and to treatment with metformin. We demonstrate that: i) the level of glucose in medium significantly affects the efficacy of metformin as an anti-tumor agent; ii) in low-glucose medium, metformin induces autophagy and oncosis, and these effects are mediated via AMPK-dependent and AMPK-independent mechanisms; iii) metformin induces glycolytic activity in thyroid cancer cell lines, increasing their dependency on glucose in the extracellular milieu, and sensitizing them to a pharmacological inhibitor of glycolysis; iv) metformin inhibits expression of the master regulator of aerobic glycolysis PKM2 in cell lines; and v) expression of PKM2 was elevated in tissue samples from thyroid cancer recurrences.

In our prior report (Klubo-Gwiezdzinska *et al.* 2013), we demonstrated that supra-physiological concentrations of metformin were required to inhibit thyroid cancer cell proliferation in medium containing 20 mM of glucose. Similar results were found in the current study when treatment with metformin was performed in DMEM medium with 20 mM glucose. The efficacy and sensitivity of treatment with metformin was increased when cells were cultured in 5 mM glucose medium, with micromolar concentrations of metformin being sufficient to inhibit viability of thyroid cancer cells.

Our results corroborate previously reported findings in breast cancer cells, demonstrating pro-apoptotic activity of metformin in low-, but not in high-glucose medium (Wahdan-Alaswad *et al.* 2013). It also has been shown that metformin led to massive cell death in a glucose-deprived microenvironment (Menendez *et al.* 2012).

To clarify the mechanisms of metformin-inducible cell death in low-glucose medium, we initially examined a caspase-dependent pro-apoptotic cascade. Treatment of thyroid cancer

cells with metformin in low-glucose medium was not associated with induction of caspase-3 or PARP cleavage, suggesting a role for non-apoptotic mechanisms. In low-glucose medium, metformin activated autophagy, a process that permits recycling of cellular constituents as internal fuel sources when external nutrient supplies are limited. We observed differences in regard to expression of LC3B-lipidated form between FTC133 and BCPAP cells at baseline and after exposure to metformin. BCPAP cells harbor the BRAFV600E mutation resulting in constitutive activation of MAPK/ERK signaling. In melanomas, oncogenic BRAF has been associated with inhibition of mTOR and upregulation of basal autophagy (Maddodi *et al.* 2010). It is possible that autophagic activity is constitutively activated in thyroid cancer cells harboring BRAF mutations.

Morphological changes in thyroid cancer cells that were treated with metformin in low-glucose evoked oncosis. Oncosis is considered as a multi-step process and a recent study (Balvan *et al.* 2015) has demonstrated that oncotic and apoptotic types of cell death can be distinguished using time-lapse microscopy. The real-time imaging demonstrating that metformin induces cellular swelling, followed by cell shrinkage, membranous blebbing, cell detachment, and finally death, suggested that oncosis is a mechanism of metformin-inducible thyroid cancer cell death.

Oncosis is a specific type of cellular damage that was described in cardiomyocytes after profound myocardial ischemia following coronary occlusion (Majno & Joris 1995). *In vitro*, oncosis was demonstrated in oxygen–glucose deprivation models used to mimic the effects of stroke. It has been postulated that oncosis may be induced by ATP depletion, leading to progressive membrane injury with resultant ion and water leakage and cell swelling. Consequently, the cell membrane becomes leaky to propidium iodide, indicating the development of a non-selective increase in membrane permeability, with eventual physical disruption of the cell membrane and necrosis (Weerasinghe & Buja 2012). The demonstration of ER stress in combination with cellular swelling, followed shortly thereafter by cell detachment and death, suggests that oncosis is a mechanism of metformin-inducible thyroid cancer cell death. There is limited information on molecular mechanisms underlying the crosstalk between specific types of cancer cell death. A recent study (Del Nagro *et al.* 2014) demonstrated that cancer cell lines with a slower rate of ATP depletion (average $t\frac{1}{2}$of 45 h) activate caspase-3 and show evidence of apoptosis and autophagy, whereas cell lines with rapid depletion of ATP (average $t\frac{1}{2}$of 32 h) do not activate caspase-3 or show signs of apoptosis or autophagy. Oncosis was identified as a predominant form of cell death once the ATP level was depleted by >20-fold. Results of our study suggest that treatment with metformin in a low-glucose environment decreases the ability of thyroid cancer cells to regenerate ATP, leading to induction of oncosis. At the present time, there are limited data on molecular markers of oncosis in cancer cells. Gene expression profiling of oncotic and apoptotic cardiomyocytes revealed that over-expression of perforin was associated with oncosis (Weerasinghe *et al.* 2013). In our study, treatment with metformin was associated with induction of perforin in FTC133 as well as in BCPAP cells cultured in low-glucose medium.

Together these data support the statement that induction of oncosis can be one of the mechanisms implicated in the anti-cancer effects of metformin.

Since ATP depletion is considered as a triggering event in oncosis, we extended our study by analysis of AMPK, a well-characterized sensor of intracellular ATP deficiency.

We found that culturing thyroid cancer cells in low-glucose for 48 h resulted in AMPK activation. Interestingly, in low-glucose conditions, treatment with metformin for 48 h did not further increase the level of phospho-AMPK but diminished the phosphorylation of pS6, suggesting a role for AMPK-independent mechanisms of action. It has been demonstrated previously that metformin directly inhibits mTOR by enhancing the association of PRAS40 with RAPTOR in an AMPK-independent manner (Liu *et al.* 2014). It also has been shown that metformin can inhibit mTORC1 signaling via Rag GTPases in the absence of AMPK (Kalender *et al.* 2010).

In our study, the level of AMPK activation inversely correlated with the concentration of glucose in the extracellular milieu, and metformin increased the rate of glucose uptake from the medium. These data suggested that metformin induced a metabolic switch toward glycolysis and increased the dependency of thyroid cancer cells on glucose. Rescue experiment at the early stage of metformin-inducible oncosis showed that supplementation with glucose was sufficient to reverse morphological changes and prevent cell death. Our results were consistent with previously reported findings in leukemia cells, demonstrating that metformin increases glycolysis, while decreasing mitochondrial electron transport chain complex I activity (Scotland *et al.* 2013).

We demonstrate that the glucose concentration in the extracellular milieu modulates metformin's anti-cancer activity, suggesting a possible clinical correlate: reduced systemic glucose levels in patients with thyroid cancer could increase the effect of treatment with metformin. Serum glucose levels in non-diabetic individuals is consistently maintained between 4 and 5.5 mM (70–100 mg/dl) and in conditions of prolonged nutrient deprivation, serum glucose level may drop to 3.0–3.5 mM (55–65 mg/dl) (Haymond *et al.* 1982). Blood glucose levels fall by nearly 25% early in a ketogenic diet and are sustained at ~15% below baseline over time (Phinney *et al.* 1983). It also has been shown that the rates of glucose uptake, as well as rates of flux into the glycolytic pathway, are reduced in rats fed a ketogenic diet. *In vivo* experiments demonstrated that metformin inhibition of breast tumor growth was enhanced when the serum glucose level was reduced via a low carbohydrate ketogenic diet (Zhuang *et al.* 2014).

In the current study, the 'low' glucose level used (5 mM) corresponds to 90 mg/dl which lies inside the normal range of glucose levels seen in non-diabetic individuals. On the other hand, the 'high' glucose level of 20 mM used corresponds to the highly supraphysiologic level of 360 mg/dl that can only be encountered in the blood of diabetic individuals. As a result, and by extrapolating our results to humans, the current study does not only apply to diabetic patients, but also to non-diabetic individuals.

To further target substrate utilization, we employed a 'tumor cell hyper-sensitization' strategy combining a mitochondrial inhibitor with an inhibitor of glycolysis. We examined the combination of metformin with 2-DG, which is phosphorylated by hexokinase and subsequently inhibits ATP generation via the glycolytic pathway. Treatment with 2-DG

dramatically increases thyroid cancer cell sensitivity to metformin. The combination of 2-DG (5 mM) with low micromolar concentrations of metformin (25 μM) led to massive cell death in all examined cell lines. Results of our study are consistent with previously reported findings in experimental breast cancer models. In addition, it has been shown that the combination of a mitochondria-targeted drug (Mito-CP) with 2-DG can induce significant tumor regression in the absence of significant morphological changes in kidney, liver and heart (Cheng *et al.* 2012). Our data suggested a potential utility of combination of metformin with 2-DG for the treatment of thyroid cancer, and additional animal studies are needed to confirm these findings.

The identification of molecular determinants underlying cancer cells sensitivity to the treatment with metabolic agents is important for development of personalized treatment of patients with cancer. A recent study has identified that PKM2 is one of the critical enzymes underlying cancer cell sensitivity to glucose deprivation and to treatment with biguanides (Birsoy *et al.* 2014). We demonstrated that thyroid cancer cell lines were characterized by overexpression of genes controlling glycolysis, and that metformin decreased the protein level of PKM2 in both FTC133 and BCPAP cells. Increased expression of PKM2 was demonstrated in human papillary thyroid cancers, and its overexpression was associated with advanced tumor stages and lymph node metastasis (Feng *et al.* 2013). Our data demonstrating a high level of PKM2 in recurrent lesions corroborate previously reported findings, and suggest potential utility of metformin for patients with progressive PTCs.

Clinical trials for glioma have demonstrated that a combination of 2-DG (65 mg/kg per day) with radio-therapy is well tolerated without any acute toxicity or late radiation damage to the normal brain tissue (Dwarakanath *et al.* 2009). Currently, 2-DG treatment is undergoing clinical trials for hormone-refractory prostate cancer and advanced solid tumors (lung, breast, pancreatic, gastric, head, and neck cancers). Reducing glucose availability also has been attempted as a cancer treatment. At this time, there are 12 clinical trials (ClinicalTrials.gov) examining the potential utility of a ketogenic diet in combination with chemotherapy or radiation for the treatment of various types of cancer.

In summary, we have demonstrated that glucose concentration in the cellular milieu is a factor modulating the anti-cancer activity of metformin. Results of our *in vitro* experiments suggest that the combination of metformin with inhibitors of glycolysis could represent a new strategy for the treatment of thyroid cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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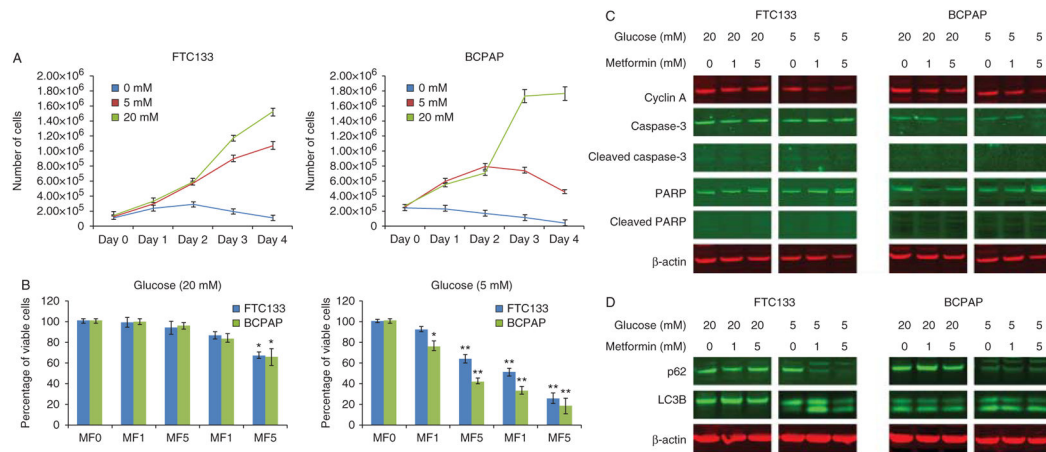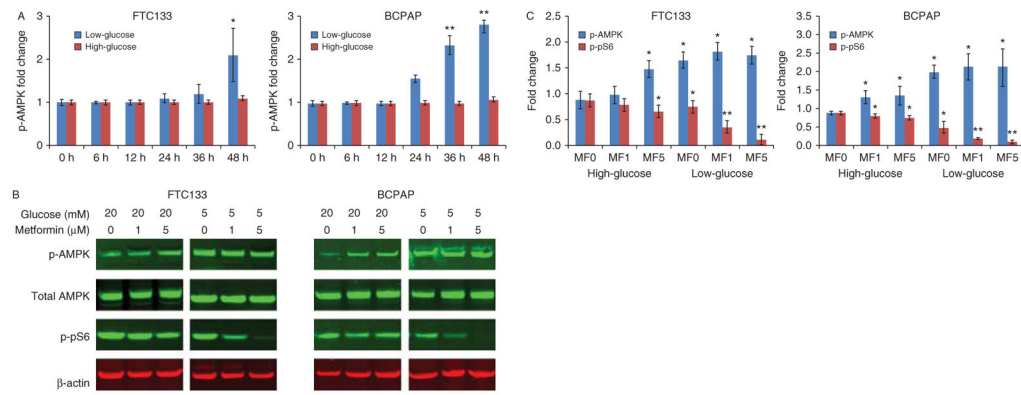
Figure 1.
The effects of metformin on the viability of thyroid cancer cells. (A) Growth curves of FTC133 and BCPAP cells in 10% FBS media supplemented with 0, 5, or 20 mM glucose. Experiments were performed at least three times, and data are presented as mean±S.D. (B) Dose-dependent effect of metformin (MF) on FTC133 and BCPAP cell's growth in high- and low-glucose medium. Data generated from AlamarBlue Assay demonstrated that the decrement in number of viable cells was dependent upon the concentration of metformin and was more prominent in low-glucose medium. (*P value <0.05 and **P value <0.01). (C) Western blot analysis demonstrating metformin-inducible down-regulation of cyclin A expression in dose-dependent manner, with no significant effect on PARP and caspase-3 cleavage. (D) Western blot demonstrating the effects of metformin on expression of p62 and LC3B in thyroid cancer cells cultured in high- and low-glucose medium. Loss of p62 with concomitant increase in LC3BII indicates induction of autophagy.
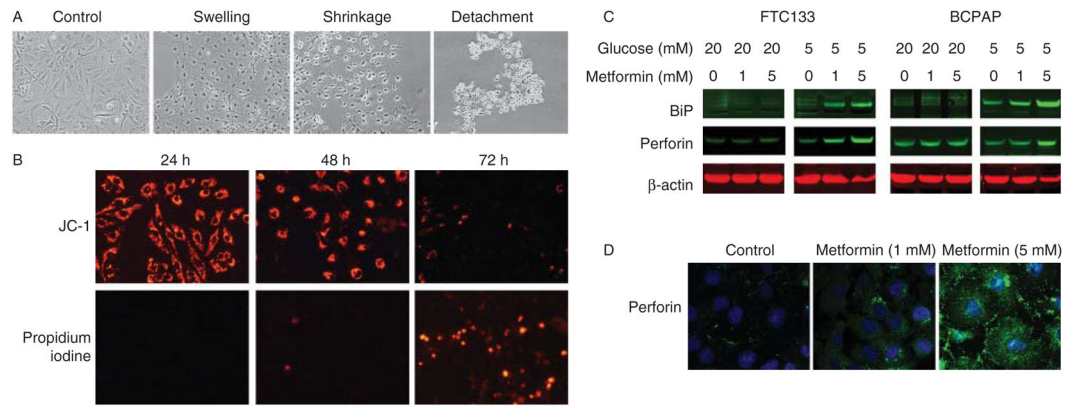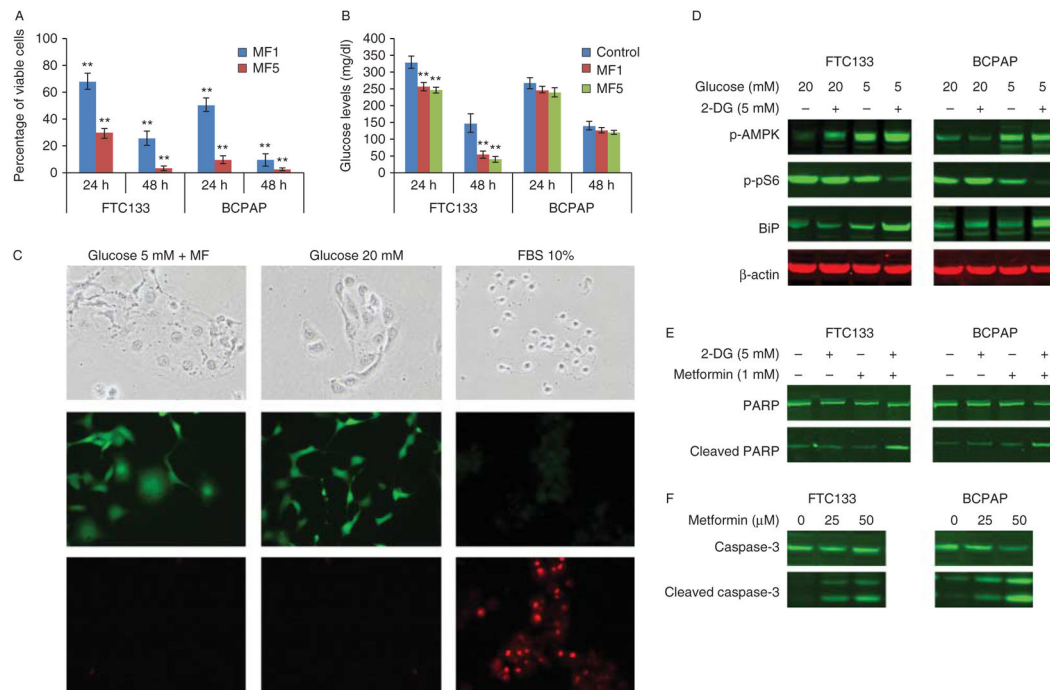
Figure 2.
Metformin induces oncosis in thyroid cancer cells. (A) Metformin induces morphological changes suggesting oncosis in FTC133 cells cultured in low-glucose medium for 48 h. (B) Metformin in low-glucose conditions: affected mitochondrial membrane potential and induced mitochondrial accumulation at 48 h (top panel) and led to cell death and nuclear accumulation of propidium iodine (bottom panel). (C) Western blot analysis showing that metformin in low-glucose medium induced expression of BiP and perforin in FTC133 and in BCPAP cells. (D) Confocal microscopy showing induction and membranous localization of perforin under treatment with metformin in low-glucose conditions for 48 h.
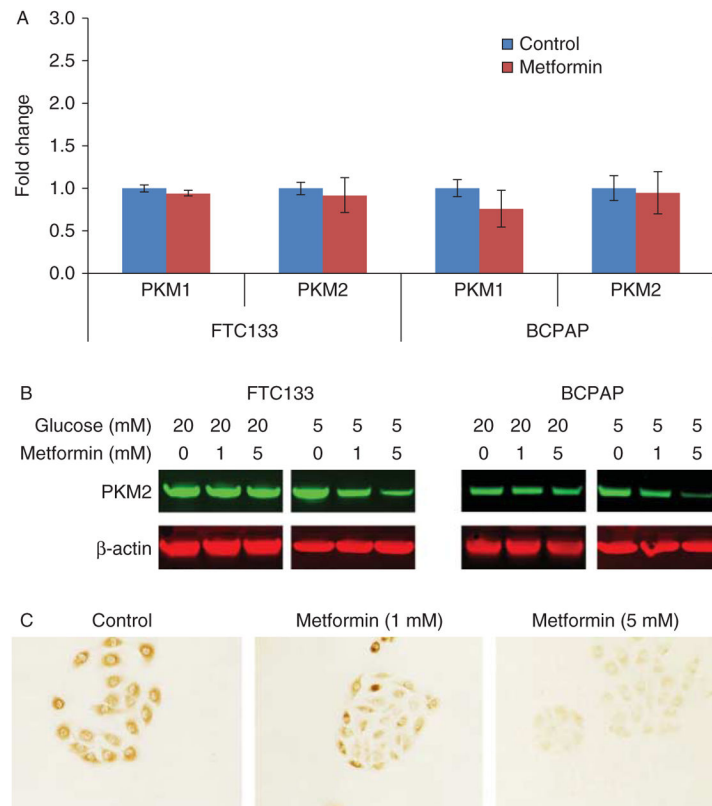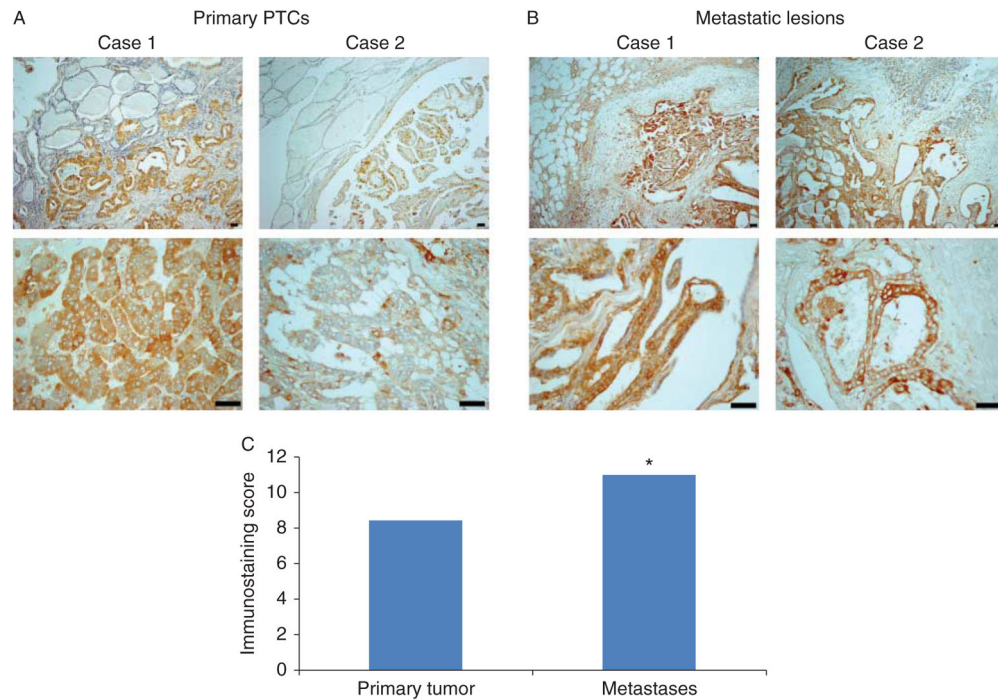
Figure 3.

The effect of metformin on AMPK signaling. (A) Graphical representation of the time-course experiment showing the effects of high- and low-glucose medium on AMPK activation in FTC133 and BCPAP cells. After normalization to the total AMPK, the level of p-AMPK increased over the time in thyroid cancer cells incubated in low-glucose medium (*P value <0.05 and **P value <0.01). (B) Western blot analysis showing that metformin had minimal effects on p-AMPK in thyroid cancer cells incubated in low-glucose medium for 48 h. Dose-dependent inhibition of p-pS6 was noted in FTC133 and in BCPAP cells. (C) Graphical representation of the western blot results demonstrating that the effects of metformin on p-AMPK and p-pS6 in high- and low-glucose medium (*P value <0.05 and **P value <0.01).

Figure 4.
The effects of glucose deprivation and inhibitor of glycolysis (2-DG) on thyroid cancer cells. (A) In glucose-free medium (supplemented only with 10% FBS), treatment with metformin (MF) resulted in significant decrease of number of viable cells, with the effect being more prominent in BCPAP cell line. The viability of thyroid cancer cells is presented as a percentage to that of control cells grown in glucose-free medium. MF1, metformin 1 mM; MF5, metformin 5 mM; h, hours (**P value <0.01). (B) Measurements of glucose concentration in the medium at 24 and 48 h. After MF treatment, an increased consumption of glucose from the medium was noted, especially in FTC133 cells. MF1, metformin 1 mM; MF5, metformin 5 mM; h, hours (**P value <0.01). (C) Top row: morphological changes indicative of oncosis were observed in BCPAP cells after culturing in low-glucose medium containing 1 mM of MF for 48 h. Culture medium then was supplemented with glucose (20 mM) or FBS (10%) and morphological changes were monitored by microscopy after 24 h. Medium supplementation with glucose, but not with FBS, reversed morphological changes and prevented cell death. Medium and bottom row: cells staining with calcein indicating alive cells (medium row), and ethidium bromide indicating dead cells (bottom row). (D) Western blot showing the effects of 2-DG on activation of AMPK/pS6K signaling and expression of BiP in FTC133 and BCPAP cells cultured in high- and low-glucose medium. (E) Western blot with anti-PARP demonstrating that combination of 2-DG (5 mM) with MF (1 mM) induced apoptosis in FTC133 and BCPAP cells cultured in low-glucose conditions. (F) Dose–response experiments using thyroid cancer cells pre-treated with 2-DG (5 mM) showed that treatment with MF (25 μM) was sufficient for induction of caspase-3 cleavages in FTC133 and BCPAP cells cultured in low-glucose conditions.

Figure 5.
PKM2 expression in thyroid cancer cells. (A) The mRNA levels of PKM1 and PKM2 isoforms was determined by qRT-PCR. The fold changes were determined by calculation of Cq values in control and metformin treated cells after normalization to the level of the housekeeping gene (18S). (B) Western blot with anti-PKM2 demonstrating that metformin inhibits PKM2 protein level in FTC133 and BCPAP cells. (C) Immunostaining with anti-PKM2 in FTC133 cells demonstrating inhibitory effects of metformin on PKM2 expression.

Figure 6.
PKM2 expression in human thyroid cancer tissue. (A) Immunostaining with anti-PKM2 showing expression of PKM2 in papillary thyroid cancers. Note the strong, homogeneous anti-PKM2 staining in case 1, and heterogeneous pattern of anti-PKM2 staining in case 2. Magnification for top panel images: objective ×10, scale bar: 50 μM. Magnification for lower panel images: objective ×40; scale bar: 50 μM. (B) Immunostaining with anti-PKM2 showed positive homogenous staining in cervical lymph node metastases in recurrent thyroid cancers corresponding to cases 1 and 2. Magnification for top panel images: objective ×10, scale bar: 50 μM. Magnification for lower panel images: objective ×40; scale bar: 50 μM. (C) Quantification of the immunostaining with anti-PKM2. The graphical representation of the mean immunohistochemical scores for primary and metastatic lesions (*P value <0.05).

Table 1

Glycolytic genes identified by real-time PCR screening as differentially expressed in FTC133 and BCPAP cells as compared to normal thyroid

Target	Description	FTC133 vs normal		BCPAP vs NT	
		Fold change	P value	Fold change	P value
G6PD	Glucose-6-phosphate dehydrogenase	6.8685	0.0000	7.4024	0.0001
PKM2	Pyruvate kinase muscle 2	5.5404	0.0000	4.2029	0.0001
G6PC3	Glucose-6-phosphatase, catalytic, 3	4.7404	0.0000	5.2162	0.0001
PGM1	Phosphoglucomutase 1	4.1554	0.0045	20.5776	0.0001
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4.1125	0.0010	9.3050	0.0001
ENO2	Enolase 2 (gamma, neuronal)	3.9041	0.0031	5.5906	0.0001
PYGM	Phosphorylase, glycogen, muscle	−5.6765	0.0000	−35.1877	0.0000
SUCLG2	Succinate-CoA ligase, GDP-forming, beta subunit	−5.7358	0.0000	−9.2663	0.0001
ALDOC	Aldolase C, fructose-bisphosphate	−7.2350	0.0000	−26.3002	0.0000
GALM	Galactose mutarotase (aldose 1-epimerase)	−64.8934	0.0000	−7.8462	0.0001
F1	Fructose-1,6-bisphosphatase 1	−65.1187	0.0000	−99.5259	0.0000