Khoperia V.G.1, Vasko V.V.2
1 Ukrainian Scientific and Practical Centre for Endocrine Surgery, Transplantation of Endocrine Organs and Tissues of Ministry of Public Health of Ukraine, Kyiv, Ukraine
2 Uniformed Services University of the Health Sciences, Bethesda, MD, USA

EFFECT OF METFORMIN ON THE MEDULLARY THYROID CANCER CELLS

Summary. Background. Medullary thyroid cancer (MTC) is associated with activation of mTOR signaling pathways. Recent studies showed that the anti-diabetic agent metformin decreases proliferation of cancer cells through AMPK-dependent inhibition of mTOR.

The objective of current study — assessment of the effect of metformin on MTC cells.

Materials and Methods. Performed growth, viability, migration and resistance to anoikis assays using two MTC-derived cell lines (TT and MZ-CRC-1). Expressions of molecular targets of metformin were examined in MTC cell lines and in 14 human MTCs tissue samples.

Results. We found that metformin inhibited growth and decreased expression of Cyclin D1 in MTC cells. Treatment with metformin was associated with inhibition of mTOR/p70S6K/pS6 signaling and with down-regulation of pERK in both TT and MZ-CRC-1 cells. Metformin had no significant effects on pAKT in the cell lines examined. Metformin inducible AMPK activation was noted only in TT cells. Treatment with AMPK inhibitor (Compound C) or AMPK silencing did not prevent growth-inhibitory effects of metformin in TT cells. Metformin had no effect on MTC cell migration, but reduced the ability of cells to form multi-cellular spheroids in non adherent conditions. Immunostaining of human MTC showed over-expression of Cyclin D1 in all tumors compared to corresponding normal tissue. Activation of mTOR/p70S6K was detected in 8/14 (57.1 %) of examined tumors.

Conclusions. Together these findings indicate that growth inhibitory effects in MTC cells are associated with down-regulation of both mTOR/6SK and pERK signaling pathways. Expression of metformin’s molecular targets in human MTC cells suggests its potential utility for the treatment of MTC in patients.

Key words: metformin, anoikis, medullary thyroid cancer.

Medullary thyroid carcinoma (MTC) derives from parafollicular C-cells of the thyroid and comprises approximately 4 % of thyroid cancers in United States (Hundahl et al., 1998). MTC occurs in sporadic and hereditary forms. The heritable variants include three different clinical phenotypes: multiple endocrine neoplasia MEN type 2A, MEN type 2B and familial MTC-only syndrome [25]. Prognosis in patients with MTC mainly depends on the stage of tumor progression at the time of diagnosis, with a mean 10-year survival rate of 100, 93, 71, and 21 % for stages I, II, III, and IV, respectively [24].

Dominant-activating mutations in the RET proto-oncogene have been identified as the underlying cause of the development of MTC [7]. The activation of anti-apoptotic PI3K/AKT pathway and mitogenic MEK/ERK cascade are crucial events in cell transformation triggered by oncogenic RET [10, 27]. Recent studies demonstrated that presence of the RET mutation is also associated with activation of the mammalian target of rapamycin (mTOR) signaling in MTC cells [12, 16].

The mTOR protein complex integrates signals from growth factors with protein translation. mTOR regulates cell growth through p70-S6 kinase (p70S6K) which leads to phosphorylation of ribosomal protein S6 [17, 33]. The pathway from growth factor receptor stimulation to mTOR activation proceeds through PI3K/AKT and MAPK/ERK signaling pathways. AKT phosphorylates and inhibits tuberin sclerosis protein complexes (TSC1 and 2) leading to mTOR activation through interactions with the mTOR protein complexes mTORC1 and mTORC2 [1]. In addition to being directly phosphorylated by AKT, TSC2 can be phosphorylated and inactivated by ERK as well as its downstream kinase RSK [29].

Inhibition of signaling pathways activated by oncogenic RET emerged as an alternative strategy for the treatment of patients with MTCs [21]. The kinase inhibitor that blocks activity of RET kinase (Vande-
The anti-diabetic drug metformin has been shown to inhibit mitochondrial complex I, which targets to the plasma membrane for their activation and mitogenic pathway via kinase suppressor of ras 2 (KSR2) [6]. It was suggested that under metabolic stress, binding of AMPK to KSR2 prevents RAF/MEK activation of MEK-ERK and PI3/AKT/mTOR-p70(S6K) pathways selectively target anoikis resistant cancer cells with acceptable effects on normal cells in their proper tissue context [13]. Therefore, pharmacological agents with ability to inhibit both MEK/ERK and PI3/AKT/mTOR signaling pathways could play a role in prevention of metastasis.

Analysis of cell signaling networks interactions reveals that both MEK/ERK and mTOR-p70(S6K) pathways can be inhibited by activated 5′AMP-activated protein kinase (AMPK). AMPK directly phosphorylates the TSC2 tumor suppressor on conserved serine sites distinct from those targeted by other kinases, leading to the inhibition of mTORC1 [22, 28]. Recent studies also demonstrated inter-relationships between AMPK and mitogenic pathway via kinase suppressor of ras 2 (KSR2) [6]. It was suggested that under metabolic stress, binding of AMPK to KSR2 prevents RAF/MEK to be targeted to the plasma membrane for their activation [22].

The anti-diabetic drug metformin has been shown to be a potent AMPK activator. On the intracellular level, metformin inhibits mitochondrial complex I, which leads to an altered AMP/ATP ratio and activation of AMP-activated protein kinase AMPK [11]. Studies in various cancer cell lines and in animal models demonstrated that metformin inhibits growth via activation of AMPK pathway [2, 23, 28]. A recent study demonstrated growth inhibitory effects of metformin on anaplastic thyroid cancer cell lines [5].

In the current study we assessed the in vitro effect of metformin on proliferation and activation of AKT/mTOR/S6K, MAPK/ERK and AMPK signaling pathways in MTC derived cells. We also examined the effects of metformin on MTC cell properties that are required for development of metastases (migration and resistance to anoikis). We also determined expression of metformin molecular targets in human MTC tissue samples.

Materials and Methods

Human thyroid tissue and cell culture. The protocol for study was approved by the Institutional Review Boards at the Washington Hospital Center and the Uniformed Services University of the Health Sciences. The paraffin-embedded thyroid tissue samples from 14 patients with MTC were selected from a thyroid tumor bank maintained at SUHSH. There were 11 samples from patients with sporadic MTC and 3 samples from patients with hereditary MTC (2 MEN2A and 1 familial MTC).

Human medullary thyroid cancer cell lines harboring C634W Ret mutation (TT cells) and M918T Ret mutation (MZ-CRC-1 cells) were obtained from Dr. Motoyasu Saji (The Ohio State University). Cells were maintained in RPMI1640 medium supplemented with heat-inactivated 20% fetal bovine serum (FBS) and 1× nonessential amino acids (1%) at 37 °C and humidified 5% CO₂. For an in vitro model of anoikis, TT and MZ-CRC-1 cells were cultured on poly-HHEMA-treated low-adherent plates. Cells were incubated with either control medium or medium containing metformin (Sigma Chemical Co., St. Louis, MO). The pharmacological inhibitor of αAMPK Compound C was purchased from EMD Biosciences (San Diego, CA).

siRNA transfections. TT cells were transfected with AMPK siRNA-specific to α1 and α2 isoforms of catalytic subunit or with Stealth RNAi siRNA-negative control (Invitrogen, Carlsbad, CA). RNAi duplex–Lipofectamine complexes were prepared using RNAiMAX transfection reagent (Invitrogen Corp., Carlsbad, CA). The efficiency of silencing was assessed by Block-it fluorescent reagent and by detection of αAMPK protein level. Assays were performed 48 h after the beginning of the transfections.

Protein extraction and Western blot analysis. Thyroid cancer cells were incubated with ice-cold cell lysis buffer, scraped, centrifuged and the supernatant was stored at −80 °C. Twenty μg of total protein lysate were suspended in reduced SDS sample buffer and protein lysates were subjected to SDS-PAGE (7%). The separated proteins were transferred to nitrocellulose membranes (0.2-μm pore size; Invitrogen, Carlsbad, CA) by electrophoretic blotting (Invitrogen, Carlsbad, CA). Nonspecific binding was prevented by blocking with 0.1% Tween 20 in PBS (PBS-T) containing 5% nonfat dry milk overnight at 4 °C. Membranes were incubated overnight with the primary antibody against phospho-p70S6K (Thr389), total p70S6K, phospho-pS6 (Ser235/236), total pS6, phospho-AKT (Ser473), total AKT, phospho-ERK (Thr202/Tyr204), total ERK, phospho-AMPKα (Thr172), total AMPKα, PARP and cleaved-caspase 3 (Cell Signaling, Boston, MA), Cyclin D1, E-Cadherin and beta Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with the secondary anti-
Cell viability and proliferation assays. Cell viability was determined by Alamar blue assay (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Cells were plated in 96-well plates and grown until 50% confluence was reached. Cells were treated with metformin at concentrations varying from 0.1 mM to 5 mM for 24, 48 and 72 hours. Ten microliters (10% of sample volume) of Alamar blue solution was added to each well, and cells were subsequently returned to the incubator for 4 h. The absorbance of Alamar blue reagent was monitored at 570 nM and 600 nM by spectrophotometer.

For cell proliferation assay, TT and MZ-CRC-1 cells were plated at a density of 50,000 cells/well in six-well plates. Cells adhered overnight and then were treated with metformin at concentrations varying from 0.1 to 5 mM. After incubation for 24, 48 or 72 hours, cell proliferation rate was determined by cell counting using Vi-CELL™ Cell Viability Analyzer from Beckman Coulter (Fullerton, CA).

In low adherent conditions, cell viability was determined by evaluation of mitochondrial membrane potential with a fluorogenic lipophilic cation (JC-1) (Cayman Chemical Company, Ann Arbor, MI). All experiments were repeated at least three times, and the average values ± standard deviations of representative experiments are reported.

Immunostaining of human thyroid tissue samples. Immunostaining was performed on paraffin embedded tissue sections. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Sections were incubated overnight with Cyclin D1 or phospho-p70S6K antibodies. Immunostaining was performed using Vector kits (Vector Labs, Burlingame, CA) according to the manufacturer’s instruction. For quantification of Cyclin D1 expression, at least 200 cells in three different areas of tumor were examined and percentage of positive nuclei was calculated. For p-p70S6K staining the results of staining were interpreted as: negative — no staining or focal/low intensity of staining in less than 20% of cells and positive staining strong staining in more than 20% of cells. The intensity of immunostaining was scored independently by two investigators (JKG and VV).

The Results and Discussion

Metformin inhibits growth of medullary thyroid cancer cells. To determine the effects of metformin on MTC derived cell lines viability we performed an Alamar blue assay. Treatment with increasing concentration of metformin was associated with inhibition of TT cells growth in dose and time dependent manner. Significant inhibition of TT cells growth was detected after cell exposure for 72 hours with micromolar concentration of metformin. In MZ-CRC-1 cells significant inhibition of growth was observed after exposure to metformin at concentration 1 and 5 mM for 48 or 72 hours. Direct cell counts using Vi-CELL™ Cell Viability Analyzer confirmed data obtained by Alamar blue assay in TT and MZ-CRC-1 cell lines.

The reduction of cell number after treatment with metformin may be the result of either cell cycle arrest or cell death. To address this question we performed Western blot analysis with antibody against proliferation marker (Cyclin D1) and apoptotic markers (cleaved caspase 3 and PARP). Treatment with metformin was associated with inhibition of Cyclin D1 expression in TT and MZ-CRC-1 cell lines without significant changes in cleavage of caspase 3 and PARP. The intensity of immunostaining with antibodies against Cyclin D1 was decreased in metformin treated cells corroborating Western blot data. These data indicate that metformin inhibits MTC cells growth through inhibition of proliferation but not induction of apoptosis.

Metformin inhibits mTOR signaling in MTC-derived cells. Since growth inhibitory effects of metformin in various cancer cell lines were attributed to down-regulation of mTOR signaling, we next examined activation of well established downstream targets of mTOR — p70S6K and its substrate ribosomal protein S6 using specific antibodies. Western blot analysis showed that treatment with metformin was associated with dose dependent inhibition of p-p70-S6K and p-pS6 in MTC-derived cells. Immunostaining also showed that intensity of immunostaining with anti-p-pS6 was decreased in MTC cells after treatment with metformin.

We also examined the effects of metformin on phosphorylation status of Akt, ERK and AMPK in MTC derived cells by Western blot analysis. In TT cells, the level of pAkt was not significantly affected by treatment with increasing concentration of metformin in any of examined cell lines. Metformin inhibited pERK in both TT and MZ-CR-1 cells. Induction of pAMPK was observed in TT cells but not in MZ-CRC-1 cells after treatment with metformin.

Consistent with results of Western blot analysis, the intensity of immunostaining with anti-pAMPK was increased in TT cells treated with metformin but not in MZ-CRC-1 cells.

The role of AMPK in metformin inducible mTOR inhibition. To clarify the role of pAMPK in TT cells response to metformin we performed experiments using pharmacological AMPK inhibitor (Compound C). Treatment with Compound C (2 μM) decreased metformin inducible activation of pAMPK. However, Compound C did not prevent metformin inducible inhibition of p-pS6 and down-regulation of Cyclin D1 in TT cells.

We also performed silencing experiments and examined the effects of metformin on αAMPK deficient TT cells. Combined inhibition of both α1 and α2 isoforms of catalytic subunit prevented metformin inducible AMPK activation. However, loss of AMPK expression did not prevent metformin-inducible down-regulation of p-pS6
and only partially rescued metformin-inducible inhibition of Cyclin D1. These results showed that loss of AMPK activity is not sufficient to completely abrogate inhibitory effects of metformin on mTOR signaling in TT cells.

**The effects of metformin on MTC cells migration and resistance to anoikis.** We next examined the effects of metformin on migration of MTC-derived cells and their resistance to anoikis. TT and MZ-CRC-1 cells intrinsically express E-Cadherin and do not efficiently migrate through 8 μm pore membrane in the Boyden chamber migration assay. Treatment with metformin had no effects on E-Cadherin expression and did not affect migratory ability of TT or MZ-CRC-1 cells (data not shown).

For analysis of anoikis, TT and MZ-CRC-1 cells were maintained in either adherent cell culture plates or low-adherent cell culture conditions. In low-adherent conditions, both MTC-derived cell lines were characterized by establishment of cell-to-cell contacts and formation of multi-cellular spheroids. MTC derived cancer cells that formed spheroids were viable as demonstrated by JC-1 staining.

To determine if metformin can affect spheroid formation, we pre-treated TT and MZ-CRC-1 cells for 24 hours with increasing concentrations of metformin in adherent plates and then transferred these cells into non adherent plates. The number and size of spheroids were decreased in MTC cells pre-treated with metformin. The viability of these cells was significantly reduced as demonstrated by JC-1 staining. We also performed analysis of pro-survival signaling in non adherent cells. Western blot analysis showed that TT and MZ-CRC-1 cells maintained activation of pAKT, pERK and p70S6K/pS6 in low adherent conditions. Pre-treatment with metformin resulted in down-regulation of pAKT, pERK and p70S6K/pS6 in MTC cells growing in non adherent conditions and induction of apoptosis. Together these data demonstrate that metformin impair MTC cells survival after detachment from extra-cellular matrix and sensitizes MTC cells to anoikis.

**Activation of mTOR signaling in human MTCs.** Our in vitro findings demonstrated that metformin inhibits cell growth, decreased Cyclin D1 expression and inhibited mTOR/p70S6K signaling in both MTC derived cell lines. To determine whether these in vitro findings could have relevance in human MTC we assessed expression of Cyclin D1 and activation of mTOR/p70S6K signaling in human MTC tissue samples. The level of immunostaining with anti-Cyclin D1 was increased in all examined MTCs compared to the corresponding normal thyroid tissue. All hereditary MTC and 5/11 sporadic MTC showed strong immunoreactivity with anti-phospho-p70S6K antibody. In two cases of widely invasive MTCs, we detected cancer cells that were located inside the vessels. Positive immunostaining with p-p70S6K was observed in intravascular MTC cells. Together these data showed that human MTC cells are characterized by increased expression of Cyclin D1 and activation of mTOR/p70S6K signaling pathway.

Targeting cancer cell metabolism is a promising strategy to treat cancer. There is increasing evidence of the potential efficacy of a commonly used anti-diabetic drug, metformin, as an anticancer agent. The primary systemic effect of metformin is the lowering of blood glucose levels through reduction of hepatic glucose production, and increased insulin sensitivity enabling appropriate usage of glucose by muscles and adipocytes [3]. Since insulin is a growth-promoting hormone with mitogenic effects, it has been suggested that beneficial effects of metformin in cancer patients are related to its systemic insulin lowering effects. However, numerous studies demonstrated that in addition to its systemic action, metformin has direct growth inhibitory effects on cancer cells through inhibition of mTOR/S6K signaling [3, 17, 22]. In the present study we used MTC derived cells to examine the effects of metformin on medullary thyroid cancer cell growth, migration and resistance to anoikis.

Metformin inhibited MTC cell growth in a dose and time dependent manner. In fact, MTC cells seem to be specifically sensitive to metformin. In contrast to previous studies documenting anti-cancer effects of millimolar concentrations of metformin in breast, ovarian and pancreatic cancer cell lines, we observed significant inhibition of MTC proliferation with micromolar concentrations applied for 72 hours. Inhibition of growth was associated with down-regulation of Cyclin D1, but not induction of apoptosis. Our findings are similar to previously reported observations in breast, prostate and ovarian cancer cell lines showing that metformin induces blockade of cell cycle progression in G0/G1 phase [2, 34]. In MTC cells, treatment with metformin resulted in inhibition of mTOR signaling pathway as demonstrated by down-regulation of p-p70S6K and p-pS6. Activation of p70S6K appears to be necessary for translation of specific set of mRNA, encoding ribosomal protein and translation elongation factors [30, 32]. It has also been demonstrated that expression of other genes important for cell cycle progression, such as Myc and Cyclin D may be controlled by the p70S6K pathway. These findings could explain the reason why inhibition of this pathway induces blockade of proliferation in MTC derived cancer cells.

We demonstrated that metformin inducible inhibition of mTOR/S6K signaling was associated with increased AMPK phosphorylation only in TT cells but not in MZ-CRC-1 cell line. Previous studies, in breast and ovarian cancer cell lines demonstrated that the inhibitory effects of metformin on cancer cell growth were mediated via activation of AMPK [9, 15]. In contrast, studies using prostate cancer cells showed that metformin inhibits...
cell growth independently of its effect on AMPK [2]. In the current study, pharmacological inhibition of AMPK activity or AMPK silencing did not prevent metformin inducible inhibition of growth and down-regulation of mTOR/S6K signaling in MTC cells. These data suggest that in addition to AMPK, other mechanisms could be involved in metformin inducible down-regulation of mTOR/S6K signaling and inhibition of cell growth in MTC cells. We also demonstrated that treatment with metformin was associated with inhibition of MEK/ERK signaling in TT and MZ-CRC-1 cells. Taking into consideration the role of both MEK/ERK and mTOR/S6K cascades in development of MTC, the dual inhibition of these signaling pathways by metformin could represent an attractive strategy for the treatment of MTC.

Since local and distant metastases are common in MTC patients, we also examined the effects of metformin on cancer cell properties that are required for development of metastases (migration and resistance to anoikis). We found that MTC derived cells were intrinsically resistant to anoikis and maintained activation of AKT/mTOR/p-70S6K and ERK signaling after detachment from extracellular matrix. Treatment with metformin sensitized MTC cell to anoikis and dramatically impaired their ability to survive in non adherent conditions. Resistance to anoikis is a critical element of the metastatic cascade and our findings suggest the possible utility of metformin as an agent that may prevent metastases.

These findings are specifically promising since we found increased expression of metformin molecular targets in a series of human MTC samples. We found that activation of mTOR signaling is common in human MTCs. Positive staining with anti-p-p70S6K antibody in human MTC cells that invaded vessels corroborated in vitro results and suggested the role of mTOR signaling in MTC cell resistance to anoikis. Our results are similar to recently published findings [26] demonstrating activation of mTOR/p70S6K and ERK signaling after detachment from extracellular matrix. Similar to mTOR signaling, metformin induced down-regulation of p70S6K, it is tempting to speculate that detection of p-p70S6K in samples from MTC patients could represent a marker of tumor cell response to the treatment with metformin.

In the current study, growth inhibitory effects of metformin were observed at concentrations that were significantly higher than those achievable in human. The therapeutic concentrations of metformin in human serum range from 5 to 20 μM [8]. It is noted that in animal models, anti-tumor effects of physiological concentration of metformin (3–13 μM) were observed only after chronic exposure (for up to 13 weeks). The inhibition of tumor growth was attributed not only to direct effects of metformin on cancer cells, but also to its indirect effects through diminished levels of circulating growth factors. It is possible that a combination of direct effects of metformin on MTC cells with its systemic insulin lowering effects may result in inhibition of MTC growth with concentration that are achievable in human.

Retrospective data from population-based studies demonstrated that treatment with metformin reduced cancer risk in humans and improved outcome of different cancers. On the basis of these studies, metformin is now being used in various clinical trials including in a neoadjuvant setting and in combination with other drugs. Our in vitro data showed that metformin inhibits growth and induces anoikis in MTC derived cells. Analysis of human MTC samples demonstrated expression of metformin molecular targets in MTC cells. In summary, these data suggest that the anti-diabetic drug metformin can inhibit growth and prevent development of metastases in MTC.

Conclusions

1. Metformin reduced cancer risk in humans and improved outcome of different cancers.
2. Metformin is now being used in various clinical trials including in a neoadjuvant setting and in combination with other drugs.
4. Analysis of human MTC samples demonstrated expression of metformin molecular targets in MTC cells.
5. These data suggest that the anti-diabetic drug metformin can inhibit growth and prevent development of metastases in MTC.

References


Получено 08.08.13
ВЛИЯНИЕ МЕТФОРМИНА НА КЛЕТКИ МЕДУЛЯРНОГО РАКА ЩИТОВИДНОЙ ЖЕЛЕЗЫ

Резюме. Медуллярный рак щитовидной железы (МРЩЖ) связан с активацией mTOR сигнальных путей. Недавние исследования показали, что антидиабетический препарат метформин снижает пролиферацию раковых клеток путем AMPK-зависимого ингибирования mTOR.

Цель исследования — оценка влияния метформина на клетки МРЩЖ.

Материал и методы. Проведена оценка роста, жизнеспособности, миграции и устойчивости к аноикису клеток МРЩЖ с использованием двух клеточных линий (ТТ и MZ-CRC-1). Экспрессия молекулярных мишеней метформина исследована в клеточных линиях МРЩЖ и в 14 образцах человеческой ткани, пораженной МРЩЖ.

Результаты. Определено, что метформин ингибирует рост и снижение экспрессии циклина D1 в клетках МРЩЖ. Лечение метформином ассоциировалось с пригнётовым ростом клеток МРЩЖ в клеточных линиях. Метформин-индуцибельная AMPK-активация отмечена только в клетках МРЩЖ. Применение ингибитора AMPK или глушителей AMPK не влияло на ингибирующий эффект метформина в клеточных линиях МРЩЖ. Не выявлено влияния метформина на миграцию клеток МРЩЖ.

При иммунологическом исследовании во всех случаях опухолей МРЩЖ по сравнению с соответствующей нормальной тканью железы выявлена избыточная экспрессия циклина D1. Активация mTOR/p70S6K наблюдается в 8 из 14 (57,1 %) обследованных опухолей.

Выводы. Полученные данные свидетельствуют о том, что угнетение роста клеток МРЩЖ под действием метформина связано со снижением экспрессии pERK сигнальных путей. Выявление молекулярных мишеней метформина в клетках МРЩЖ определяет потенциальную возможность применения препарата в лечении пациентов с данной патологией.

Ключевые слова: метформин, аноикис, медуллярный рак щитовидной железы.