AGR2 silencing contributes to metformin-dependent sensitization of colorectal cancer cells to chemotherapy

ANDREA MARTISOVA[1*], LUCIA SOMMEROVA[1*], KATARINA KURICOVA[2], JAN PODHOREC[1], BORIVOJ VOJTESEK[1], KATERINA KANKOVA[1,2] and ROMAN HRSTKA[1]

[1]Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, 656 53 Brno; [2]Department of Pathophysiology, Faculty of Medicine, Masaryk University, 625 00 Brno, Czech Republic

Received December 21, 2018; Accepted June 19, 2019

DOI: 10.3892/ol.2019.10800

Abstract. There is growing epidemiological evidence indicating an association between diabetes mellitus and the increased incidence of colorectal cancer (CRC). The preferred initial and most widely used pharmacological agent for the treatment of type 2 diabetes is metformin, which in parallel reduces the risk of CRC and improves patient prognosis. AMP-activated protein kinase (AMPK) appears to be tightly associated with the beneficial metabolic effects of metformin, serving as a cellular energy sensor activated in response to a variety of conditions that deplete cellular energy levels. Such conditions include nutrient starvation (particularly glucose), hypoxia and exposure to toxins that inhibit the mitochondrial respiratory chain complex. The aim of the present study was to determine the effect of metformin on CRC cell lines, with different levels of anterior gradient 2 (AGR2) expression, exposed to 5-fluorouracil (5-FU) and oxaliplatin, alone or in combination with metformin. AGR2 has recently emerged as a factor involved in colon carcinogenesis. In *AGR2*-knockout cells, markedly higher levels of phosphorylated-AMPK were observed in comparison with control cells transfected with *GFP*-scrambled guide RNA, which indicated that the presence of AGR2 may interfere with the metformin-dependent activation of AMPK. In addition, metformin in combination with 5-FU and oxaliplatin induced ROS production and attenuated autophagy. This effect was enhanced in *AGR2*-knockout cells.

Correspondence to: Dr Roman Hrstka, Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty Kopec 7, 656 53 Brno, Czech Republic
E-mail: hrstka@mou.cz

*Contributed equally

Abbreviations: AGR2, anterior gradient 2; T2DM, type 2 diabetes mellitus; CRC, colorectal cancer; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; 5-FU, 5-fluorouracil; ROS, reactive oxygen species

Key words: AGR2, diabetes mellitus, colorectal cancer, AMPK, autophagy, ROS

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide; according to GLOBOCAN estimates, there were 1,801,000 new cases of CRC in 2018 worldwide (1). The risk of CRC is increased up to three times by diabetes mellitus, predominately type 2 diabetes mellitus (T2DM) (2-4). T2DM is a chronic metabolic disorder associated with high mortality and morbidity. Patients with diabetes have an increased risk of various malignancies due to numerous putative mechanisms, including hyperinsulinemia, hyperglycemia and inflammation (5). T2DM has been demonstrated to increase CRC aggressiveness and mortality (6,7). The key molecular mechanisms by which T2DM increases the incidence or worsens the prognosis of patients with CRC are associated with nutrient-sensing pathways coupling energy metabolism to signals of cell growth and survival, which are often dysregulated in diabetes, and may be important contributors to cancer development in patients with diabetes (8). The pathophysiology of T2DM involves enhanced oxidative stress induced by high plasma glucose, lipid and cytokine levels, which can trigger endoplasmic reticulum (ER) stress (9).

Anterior gradient 2 (AGR2) protein, coded by the *AGR2* gene, has recently been described as an important regulator of ER stress. AGR2 is inducible by ER stress, which is associated with the acquisition of a pro-inflammatory phenotype (10). The presence of AGR2 has been detected in a wide range of human malignancies, including CRC (11,12). Functionally, AGR2 belongs to the protein disulfide isomerase family with all the key features of an ER-resident protein responsible for maintaining ER homeostasis (13,14). Metformin, which is a biguanide derivative, is a first-line drug used in T2DM treatment worldwide (15). Considering the epidemiological evidence between T2DM and an increased risk of CRC, the impact of metformin therapy on the incidence and outcome of CRC has been intensively studied (16). The beneficial effects of metformin for patients with CRC and diabetes are already supported by recent clinical trials, which reported prolonged overall survival for metformin users compared with nonusers (17). Studies on CRC cell lines have revealed that metformin inhibits cell proliferation and migration by arresting the cell cycle in the G_0/G_1 phase, and by transient downregulation of c-Myc and insulin-like growth factor receptor 1 (18).

A recent study on the effects of metformin on ER stress demonstrated that metformin acts as a modulator of ER stress in patients with T2DM by promoting an adaptive unfolded protein response (19). Studies on metformin in combination with 5-fluorouracil (5-FU) and/or oxaliplatin, which are drugs routinely used during standard chemotherapy of patients with CRC, confirmed its synergistic anti-cancer effects (20). The potential association between metformin and AGR2 expression has been demonstrated by transcriptomic analysis identifying *AGR2* as one of the most downregulated genes in pancreatic cancer cells exposed to combined treatment with metformin and aspirin (21). Since AGR2 has been identified as a putative marker of chemoresistance (22-24), and AGR2 silencing may sensitize tumor cells to ER stress-induced autophagy (13), the aim of the present study was to elucidate the role of AGR2 in CRC cells exposed to metformin in combination with 5-FU and oxaliplatin.

Materials and methods

Cell lines and culture. Human epithelial colorectal adenocarcinoma cell lines DLD-1 and SW480 (American Type Culture Collection) were maintained in high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% pyruvate and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. Unless otherwise stated, cells were grown to 70-80% confluence prior to treatment. In addition, unless otherwise stated, the cells were treated with the following concentrations of drugs: 1 μM bafilomycin A1, (Cell Signaling Technology, Inc.), 5 μM 5-FU (Sigma Aldrich; Merck KGaA), 2 μM oxaliplatin (PLIVA Lachema) and 5 mM metformin (Sigma Aldrich; Merck KGaA).

Transfection. *AGR2*-knockout (KOAGR2) DLD-1 cells were prepared using CRISPR/Cas9 technology. Briefly, the guide RNA oligonucleotide (5'-AGAGATACCACA GTCAAACC-3') that targets exon 2 of the human *AGR2* gene (ENSE00003623642) was designed using Tools for Guide Design (zlab.bio/guide-design-resources). The guide RNA specifically targeted mRNA coding 21-27 aa of the AGR2 N-terminal region, which is important for AGR2 protein-mediated cell adhesion. The *GFP*-scrambled sequence (5'-AACAGTCGCGTTTGCGACTGG-3') served as a control (25). Both sequences were cloned into a LentiCRISPR-v2 vector (cat. no. 52961; Addgene) using *Esp*3I restriction cloning. DLD-1 cells ($1x10^6$ cells) were transfected with LentiCRISPR-v2_AGR2 or LentiCRISPR-v2_scrambled (scr). After 2 days, the cells were exposed to puromycin (2 μg/ml) and the pool of resistant cells was sorted and seeded as single colonies in 96-well plates. KOAGR2 cells were tested for AGR2 expression using western blotting. Two clones with an undetectable expression of AGR2, DLD1 KOAGR2 A9 and F5, were selected for further experiments. SW480 cells ($1x10^6$) were transiently transfected using an Amaxa Nucleofector II (Lonza Group Ltd.) with 2 μg pcDNA3-*AGR2* or empty pcDNA3 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.), which served as a control, and were subsequently selected with G-418 (400 μg/ml; Sigma-Aldrich; Merck KGaA). A pool of resistant cells was tested for positive AGR2 expression with western blot analysis.

To determine the doubling time of the transfected cancer cell lines with manipulated *AGR2* gene expression compared with untransfected cells, equal numbers of cells ($5x10^5$) were seeded into the complete media and maintained under the standard conditions for 48 h. The culture medium was removed, and adherent cells were detached by 0.5% trypsin (Gold Biotechnology, Inc.) and counted using CASY Model TT cell counter (Roche Diagnostics).

Western blot analysis. Cells were washed twice with cold phosphate-buffered saline (PBS). The cells were then scraped into NET lysis buffer [150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0), 50 mM NaF, 5 mM EDTA (pH 8.0)] supplemented with Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Protein concentration was determined using the Bradford method and 15 μg proteins were loaded onto 10% acrylamide gels. Following SDS-PAGE, the samples were transferred to nitrocellulose membranes, blocked with 5% milk diluted in PBS supplemented with 1% Tween for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies against phosphorylated-AMPKα at Thr172 (p-AMPK; 1:1,000; cat. no. 2535; Cell Signaling Technology, Inc.), β-actin and p62 (both 1:1,000; cat. nos. sc-47778 and sc-28359; Santa Cruz Biotechnology, Inc.), microtubule-associated proteins 1A/1B light chain 3 (LC3)-II (1:1,000; cat. no. NB100-2220; Novus Biologicals, LLC) and AGR2 (in house) (26). The membranes were washed and probed with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (1:1,000; cat. nos. P0217 and P0161; Dako) for 1 h at room temperature. Chemiluminescent signals were developed using a mixture of 1:1 ECL-A and ECL-B solutions [ECL-A: 0.5 M EDTA (pH 8), 90 mM coumaric acid, 1 M luminol, 200 mM Tris (pH 9.4) and ECL-B: 0.5 M EDTA (pH 8), 0.123 g $NaBO_3$ x $4H_2O$, 50 mM CH_3COONa (pH 5)] and visualized with the SYNGENE G:BOX Chem XX6 gel doc system (Syngene; Synoptics Ltd.).

Clonogenic assay. Cells were plated at a density of 250 cells/well in a 6-well plate. Following 24-h incubation at 37˚C with 5% CO_2, 500 μM metformin, 5 μM 5-FU and 2 μM oxaliplatin were applied and further incubation for ~10 days was performed. The medium was removed, and the colonies were stained with 1% crystal violet at room temperature for 20 min and counted. Recombinant extracellular AGR2 (eAGR2) was prepared as described previously (27) and was used in the clonogenic assay as an extracellular protein supplied in the medium (1 ng/ml) throughout the experiment.

Determination of mitochondrial depolarization. To analyze mitochondrial depolarization, the JC-1 probe was used. At 24 h post-treatment, ~$2x10^5$ cells were harvested and washed with PBS, stained with 5 μg/ml JC-1 dye (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C and analyzed using a BD FACSAria III flow cytometer (BD Biosciences). Viable population of cells was gated according to forward scatter (FSC) vs. side scatter (SSC). Individual cells were gated by FSC-A vs. FSC-H parameters. The ratio of green (FITC)

to red (phycoerythrin) fluorescence was analyzed by FCS Express version 4 software (De Novo Software) as a loss of mitochondrial potential ($\Delta\psi m$). Treatment with valinomycin (25 μM; Thermo Fisher Scientific, Inc.) for 2 h at 37˚C was used as a positive control for mitochondrial depolarization.

Detection of reactive oxygen species (ROS) production. CM-H2DCFDA was used as an indicator for ROS in CRC cells. Briefly, the cells were seeded in 96-well plates ($4x10^3$ cells/well) and incubated at 37˚C overnight. The next day, the cells were treated with respective drugs diluted in Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich; Merck KGaA) for 6 h at 37˚C. Hydrogen peroxide (50 μM; Penta s.r.o.) served as a positive control, whereas N-acetyl cysteine (10 mM; Sigma-Aldrich; Merck KGaA) was used to block ROS production. Subsequently, the drugs were removed, and 5 μM CM-H_2DCFDA (Thermo Fisher Scientific, Inc.) in HBSS was added for 30 min at 37˚C. The cells were washed twice with HBSS, and the fluorescence was examined using an Infinite 100 plate reader (Tecan Group, Ltd.).

Statistical analysis. Statistical analyses were performed using the Online Web Statistical Calculators for Categorical Data Analysis (https://astatsa.com). One-way ANOVA with post-hoc Tukey HSD calculator was used to determine statistically significant differences between the groups. For western blot analyses, the protein level changes were first normalized to β-actin and were then statistically analyzed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of metformin in combination with 5-FU and oxaliplatin on CRC cells with manipulated AGR2 expression. Two clones with *AGR2* gene knockout, KOAGR2 A9 and KOAGR2 F5, were prepared by CRISPR/Cas9 using DLD-1 cells (Fig. 1A). SW480 cells with no detectable AGR2 expression were used as the second model; an *AGR2*-knock-in SW480 cell line was prepared by stable transfection (Fig. 1A). The effects of AGR2 expression on cell proliferation were tested in DLD-1 and SW480 cells. Consistently with previous studies (28,29), the results demonstrated a positive effect of AGR2 expression on cell proliferation rate in the two models (Fig. 1B). The cells were then exposed to selected drugs to determine their effects on the phosphorylation of AMPK. It has previously been reported that the actions of metformin are attributable to AMPK (30). Metformin treatment exhibited a negligible effect on AMPK phosphorylation in DLD-1 scr cells. Treatment with metformin in combination with 5-FU or oxaliplatin induced a moderate increase in AMPK phosphorylation (Fig. 1C). Notably, a significant increase in the expression of p-AMPK was observed in both KOAGR2 clones treated with 5-FU and oxaliplatin in combination with metformin (Fig. 1C). To confirm the involvement of AGR2 in the regulation of AMPK activation, the same experiment was performed using SW480 cells. A non-significant increase in p-AMPK expression was observed in SW480 cells without AGR2 (SW480-pcDNA3) treated with the combination of metformin and 5-FU (Fig. 1D). Since both A9 and F5 KOAGR2 clones showed very similar p-AMPK/AMPK expression patterns, subsequent experiments were performed with only KOAGR2 clone A9.

Effects of metformin on colony formation. The effects of metformin on the ability of cells to sufficiently form colonies, based on AGR2 expression and in response to standard chemotherapy drugs, were tested using a clonogenic assay. A significant loss in the ability of cells to develop colonies was associated with the absence of AGR2 (Fig. 2). Metformin alone exhibited no effect on the number of colonies developed by DLD-1 and SW480 cells irrespective of AGR2 expression. However, metformin sensitized DLD-1 cells producing AGR2 to 5-FU and oxaliplatin, which was reflected by significantly reduced colony formation compared with untreated ARG2-negative cells (Fig. 2A).

To investigate the impact of eAGR2 on cell chemosensitivity, recombinant eAGR2 protein was added into the culture medium (Fig. 2B). Similar trends were observed as in Fig. 2A; however, with eAGR2, twice the number of colonies was formed compared with cells cultured in medium without eAGR2. In addition, the effect of eAGR2 was stronger in AGR2-negative cells treated with chemotherapy drugs, which exhibited approximately twofold increase in the number of colonies compared with cells maintained in media without eAGR2 (Fig. 2A and B).

SW480-AGR2 cells exhibited a significant decrease in the number of colonies in response to the combination of metformin and 5-FU (Fig. 2C). The presence of eAGR2 protein approximately doubled the number of colonies (Fig. 2D). Taken together, these data support the antiproliferative activity of metformin administered in combination with standard chemotherapy and suggest a significant contribution of eAGR2 to the resistance of tumor cells to chemotherapy.

Effect of metformin combined with standard chemotherapy on autophagy and oxidative stress. Since AMPK has been demonstrated to regulate autophagy, the effects of metformin on LC3-II cleavage were determined (Fig. 3). In agreement with the elevated p-AMPK levels in AGR2-negative cells (Fig. 1), markedly higher signals of LC3-II were detected in DLD-1 KOAGR2 cells in comparison with DLD-1 scr cells (Fig. 3), which indicated that AGR2 may attenuate autophagy. No significant induction of LC3-II was observed in cells exposed to all drugs compared to untreated cells (Fig. 3A). 5-FU treatment was even associated with a sharp decrease in LC3-II, indicating that autophagy may serve an important role in survival of these tumor cells. The role of autophagy may correspond with the high sensitivity of DLD-1 cells to 5-FU, as demonstrated in Fig. 2. Although the amount of LC3-II is clearly correlated with the number of autophagosomes, LC3-II itself is also degraded by autophagy (31). Therefore, it is important to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors e.g. bafilomycin A1. However, the addition of bafilomycin A1 was not associated with increased LC3-II cleavage in cells treated with metformin in combination with 5-FU or oxaliplatin (Fig. 3B), which indicates that autophagy was inhibited. An alternative method for detecting the autophagic flux is the determination of p62/sequestosome-1 degradation, since p62 can bind LC3,

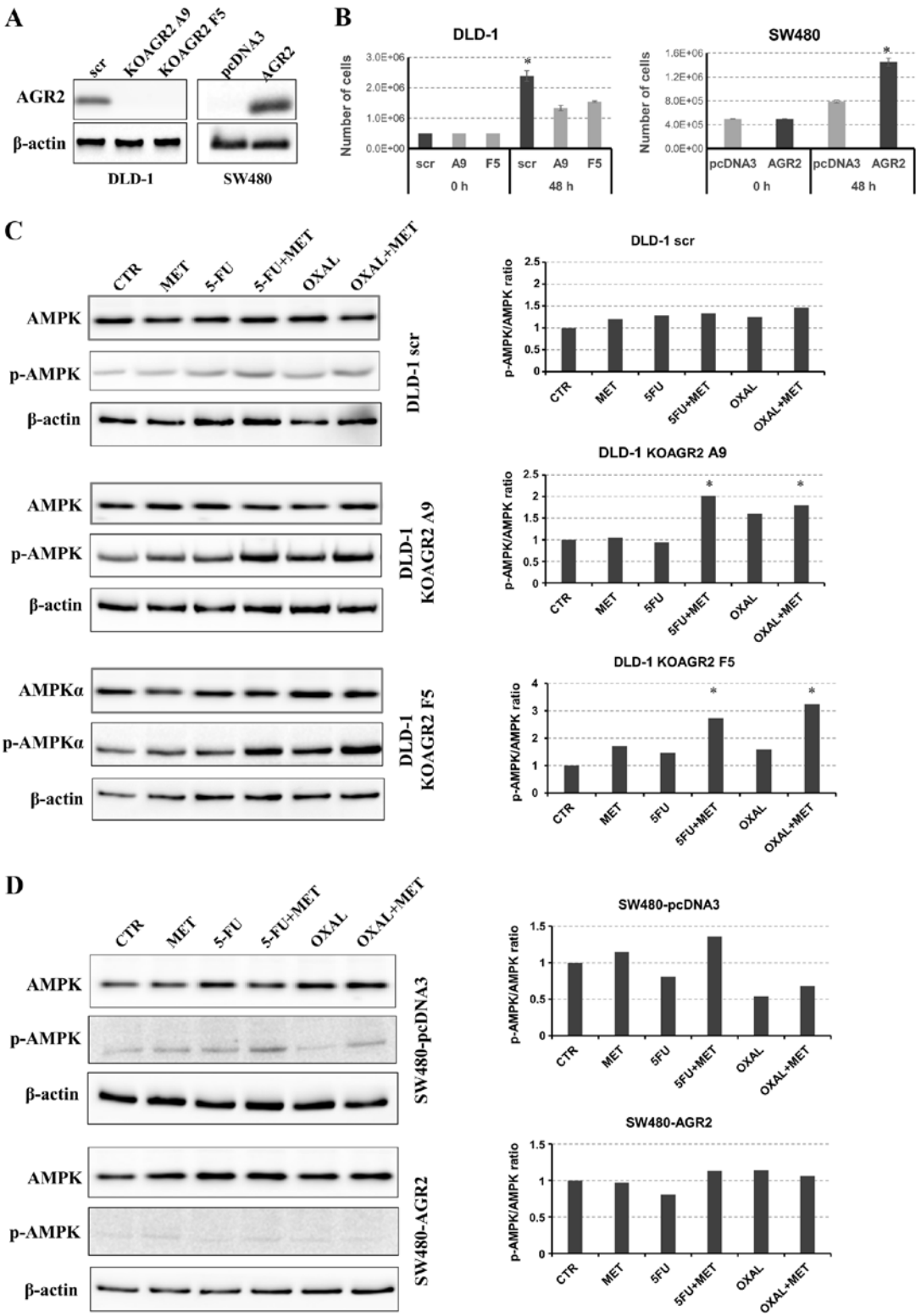

Figure 1. Determination of AMPK expression. (A) Evaluation of AGR2 protein levels in DLD-1 cells with *AGR2* gene knockout and in SW480 cells with established production of AGR2. (B) Proliferation rate of DLD-1 and SW480 cells with different AGR2 expression levels. Cells producing AGR2 showed enhanced proliferation. *P<0.05 vs. A9 and F5 or pcDNA3. (C and D) Western blot analysis of total and p-AMPK levels in (C) DLD-1 cells and (D) SW480 cells treated with MET, 5-FU, OXAL and their combination in relation to CTR cells. β-actin was used as a loading control and for normalization. *P<0.05 vs. CTR. 5-FU, 5-fluorouracil; AMPK, AMP-activated protein kinase; AGR2, anterior gradient 2; CTR, control; KOAGR2, *AGR2*-knockout; MET, metformin; OXAL, oxaliplatin; scr, scrambled guide RNA.

serving as a selective substrate of autophagy (32). However, no significant decrease in p62 levels was observed in response to metformin combined with 5-FU or oxaliplatin treatments; by contrast, an increase in p62 levels was observed in response to 5-FU and oxaliplatin administered alone. Western blot analysis of SW480 cells revealed a reduction in LC3-II isoform

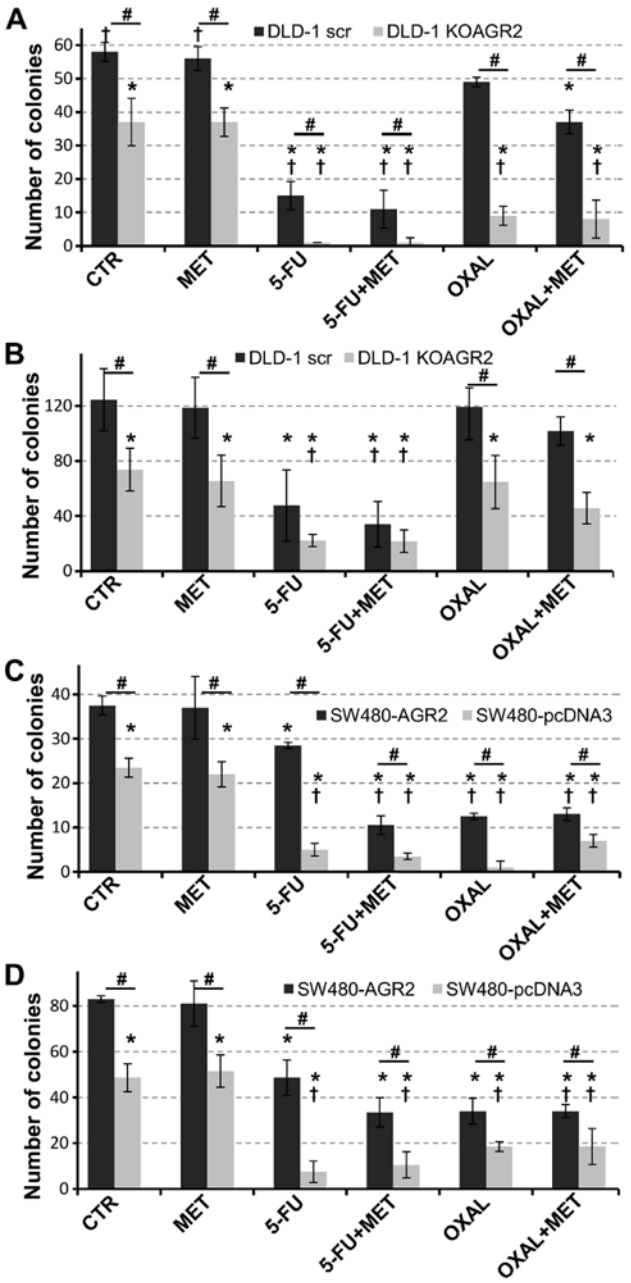

Figure 2. Clonogenic assay. AGR2-positive (black bar) or AGR2-negative (gray bar) colorectal cancer cells were incubated alone (CTR) or with drugs, as indicated. (A and B) DLD-1 cells were maintained either in (A) complete media or (B) complete media supplemented with recombinant AGR2 protein. (C and D) SW480 cells were tested (C) without and (D) with extracellular recombinant AGR2 added into the media. *P<0.05 vs. untreated AGR2-positive cells (DLD-1 scr or SW480-AGR2); †P<0.05 vs. untreated AGR2-negative cells (DLD-1 KOAGR2 or SW480-pcDNA3); #P<0.05 as indicated. 5-FU, 5-fluorouracil; AMPK, AMP-activated protein kinase; AGR2, anterior gradient 2; CTR, control; KOAGR2, AGR2-knockout; MET, metformin; OXAL, oxaliplatin; scr, scrambled guide RNA.

expression in response to metformin and its combinations with 5-FU and oxaliplatin (Fig. 3C). These findings support the hypothesis that autophagy-dependent clearance of misfolded proteins in non-metformin-treated patients with T2DM may be suppressed by metformin treatment (19).

Metformin inhibits mitochondrial respiratory complex I at a cellular level, possibly leading to mitochondrial membrane depolarization and the release of ROS (33,34). Therefore, alterations in mitochondrial membrane potential were investigated in cells exposed to metformin in combination with 5-FU or oxaliplatin. JC-1 staining followed by flow cytometric analysis revealed a significant mitochondrial depolarization in response to metformin alone and in combination with oxaliplatin in AGR2-negative DLD-1 cells compared with in untreated cells (Fig. 4A-C).

The impact of metformin on ROS production was monitored using an indicator for reactive oxygen species in cells, CM-H2DCFDA. All tested drugs administered alone resulted in a low or moderate induction of ROS. However, the combined administration of metformin with 5-FU or oxaliplatin significantly increased ROS production in both DLD-1 scr and DLD-1 KOAGR2 cells. The effect was significantly enhanced in AGR2-negative cells exposed to a combination of 5-FU and metformin, whereas combined treatment with oxaliplatin and metformin reached only a marginal effect (Fig. 4D).

Discussion

CRC is a type of cancer, the relative risk of which is increased by diabetes (35); in addition, the outcomes of CRC are significantly worse in patients with diabetes compared with in non-diabetic subjects (36). Metformin is used for treating patients with T2DM, including those with CRC, as demonstrated in the clinical setting (17). Beneficial effects of metformin are not limited to primary CRC. Metformin also significantly increases the therapeutic effectiveness of standard chemotherapeutics, such as 5-FU or oxaliplatin, on recurring CRC by targeting chemoresistant CRC cells enriched in stem or stem-like cells (37,38). The effects of metformin on CRC cells depend predominately on regulation of the AMPK/mammalian target of rapamycin (mTOR) pathway. Under low energy conditions, AMPK phosphorylates specific enzymes and growth control nodes to increase ATP generation and decrease ATP consumption (39). A decreased level of AMPK in T2DM attenuates the inhibition of protein, fatty acid and cholesterol synthesis to favor cancer cell growth. AMPK regulates these processes by interfering with mTOR, which is a regulator of growth. Thus, under nutrient-rich conditions, AMPK is inactive and mTOR is active, whereas under energy deficient conditions, increased AMPK activity leads to a decrease in mTOR activity, resulting in reduced protein synthesis and cell growth (40). The PI3K/AKT/mTOR axis has also been shown to be involved in the positive regulation of AGR2 expression (41-43). Activation of AKT signaling and impaired expression of its negative regulator phosphatase and tensin homolog has been reported in 60-70% of human colon cancer cases (44). Recent meta-analysis has reported that AGR2 overexpression has an unfavorable impact on overall survival and time to tumor progression in patients with solid tumors (45). Although elevated expression of AGR2 is generally perceived as undesirable due to the prediction of poor outcome, in several tumor types, such as lung, ovarian and colorectal cancer, contradictory findings have been reported; therefore, further analyses and clinical trials on certain types of cancer are required (46,47).

Autophagy may exhibit dual functions in tumors, including CRC; it may contribute to cancer development; however, it may also act as a tumor suppressor by inducing

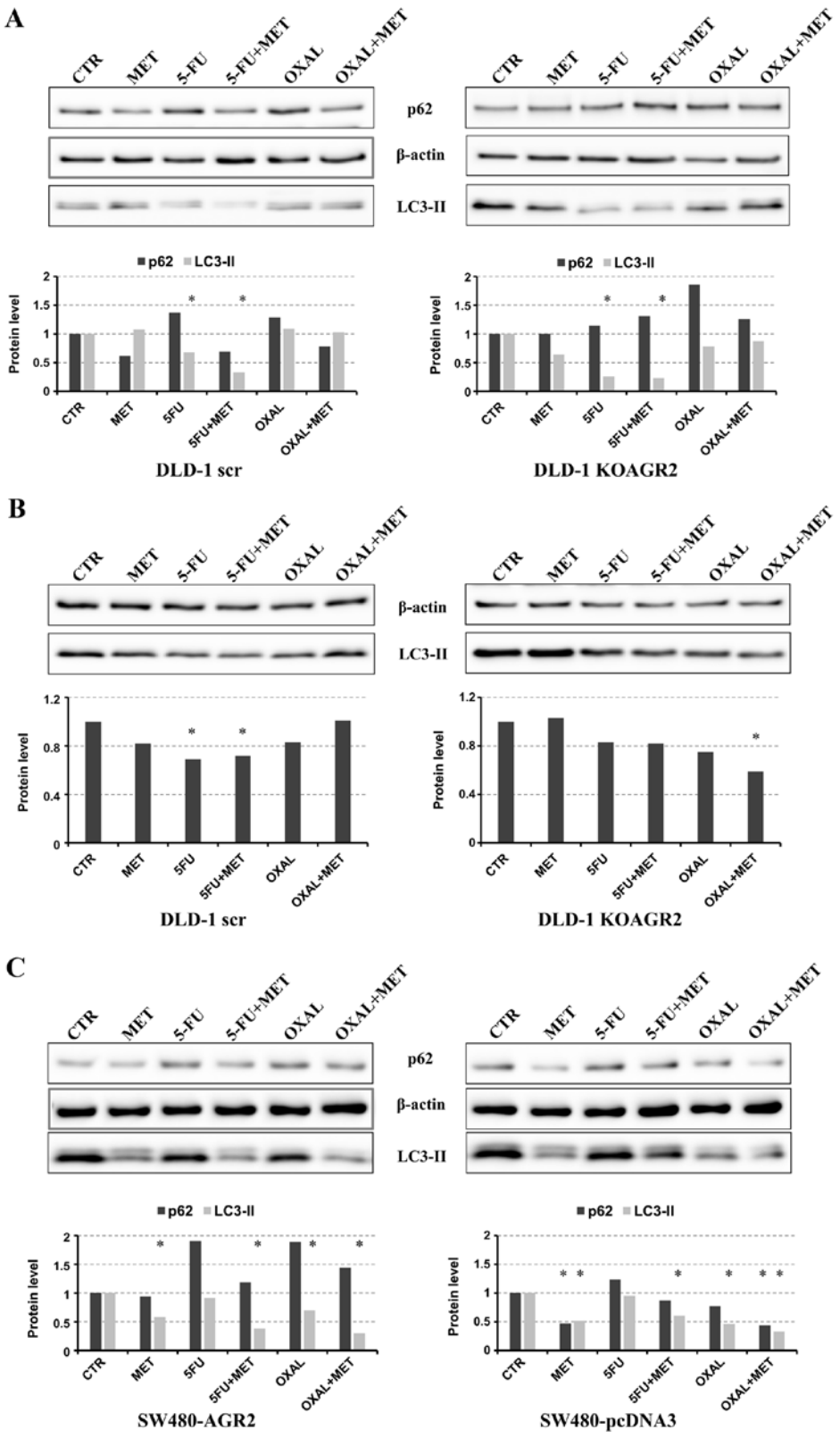

Figure 3. Detection of autophagy-related markers. (A) Western blot analysis of p62 and LC3-II in DLD-1 scr and DLD-1 KOAGR2 cells in response to treatments as indicated. (B) Changes in the LC3-II level following co-treatment with bafilomycin A1. (C) Western blot analysis of p62 and LC3-II in SW480 cells stably transfected with AGR2 expression plasmid or empty construct. *P<0.05 vs. CTRL. 5-FU, 5-fluorouracil; AMPK, AMP-activated protein kinase; AGR2, anterior gradient 2; CTRL, control; KOAGR2, AGR2-knockout; LC3, microtubule-associated proteins 1A/1B light chain 3; MET, metformin; OXAL, oxaliplatin; scr, scrambled guide RNA.

cell death. The decision to trigger either induction of processes leading to cell death or activation of pro-survival functions depends on the stage of the neoplastic process (48). Early stages of CRC carcinogenesis are usually associated with the tumor suppressive role of autophagy, whereas late stages are associated with pro-survival functions (49).

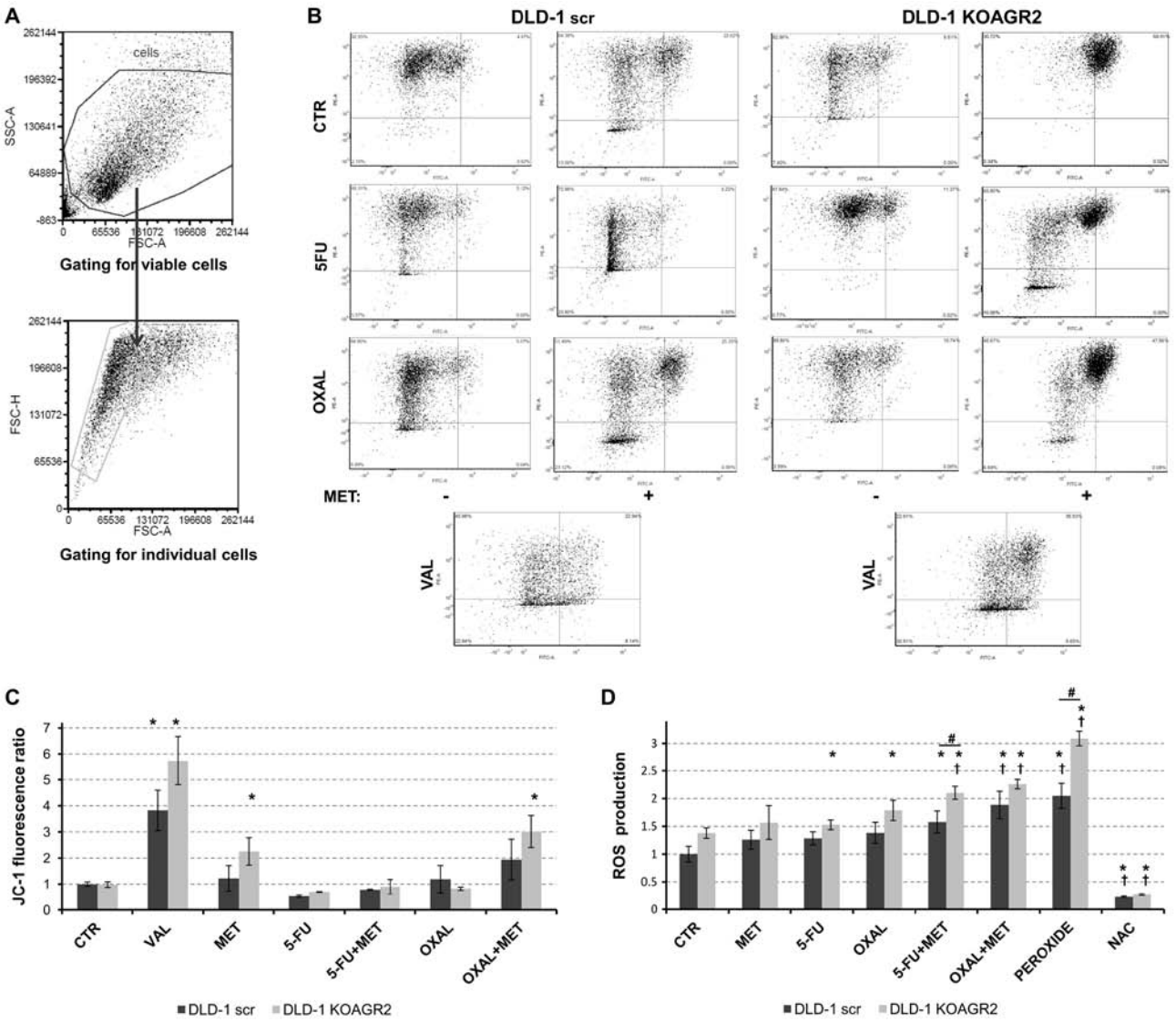

Figure 4. Analysis of mitochondrial depolarization and oxidative stress. (A) Flow cytometry plots showing the gating strategy to determine viable and individual cellular population. (B) Representative plots demonstrating mitochondrial depolarization. (C) At 24 h post-treatment with 5-FU (5 μM), OXAL (2 μM), MET (0.5 mM) and their combination, the JC-1 probe was used to measure the FITC/PE ratio to identify alterations in mitochondrial membrane potential compared with in control cells. 25 μM VAL was used as a positive control. (D) ROS production was determined relative to untreated cells. Hydrogen peroxide was used as a positive control, whereas NAC was used to inhibit ROS development. *P<0.05 vs. untreated DLD-1 scr; †P<0.05 vs. untreated DLD-1 KOAGR2; #P<0.05 as indicated. 5-FU, 5-fluorouracil; CTR, control; MET, metformin; NAC, N-acetyl cysteine; OXAL, oxaliplatin; VAL, valinomycin. At least three independent biological experiments were performed to construct the graphs.

Development of chemoresistance represents an event that is frequently observed in later stages of CRC due to activation of autophagy. For instance, Yang *et al* (50) demonstrated that autophagy was induced in response to oxaliplatin treatment, along with the enrichment of the CRC stem cell population, which increased cancer cell resistance to chemotherapy and prevented apoptosis. Li *et al* (51) reported that the combination of fluorouracil treatment with autophagic inhibitors, such as bafilomycin A1 or 3-methyladenine, enhanced the chemotherapeutic effect of fluorouracil by stimulating cell death.

The results of the present study demonstrated that, compared with in AGR2-positive cells, CRC cells with silenced AGR2 expression exhibited increased levels of autophagy. However, the activation of AMPK in response to chemotherapy combined with metformin did not induce autophagy. A clear decrease in LC3-II was observed in SW480 cells irrespective of AGR2 status. Although the mechanism of metformin as an antineoplastic agent is not yet fully understood (52), its activity is at least partially attributable to AMPK activation through the inhibition of complex I of the mitochondrial respiratory chain, leading to an increased AMP:ATP ratio (30,34), membrane depolarization (33) and the release of mitochondrial ROS (53). Notably, the present study revealed that metformin in combination with 5-FU or oxaliplatin attenuated autophagy in CRC cells, but increased ROS production responsible for decreased cell viability, as demonstrated by the clonogenic assay. This effect was also significantly enhanced by knocking out *AGR2*, supporting its potential catalytic redox activity in the regulation of redox balance in cells, which is a typical feature of protein disulfide isomerases (54). The results of

the present study are also in agreement with a recent study that demonstrated increased ROS production in CRC cells induced by metformin (18). By contrast, a study on breast cancer MDA-MB-231 cells revealed that low doses of metformin inhibited ROS production and inflammatory signaling (55). This discrepancy suggests that metformin may function through distinct mechanisms at lower versus higher concentrations (56).

Another important aspect is the cellular localization of AGR2. Although AGR2 is overexpressed in various types of human cancer and has been reported to promote aggressive tumor features, including the resistance to anticancer treatment (23,24,26,57), little is known regarding the extracellular functions of AGR2 in tumorigenesis. Secreted eAGR2 has been demonstrated to promote cell migration and metastasis of CRC *in vitro* and *in vivo* (12). A comprehensive protein-protein interaction screen identified AGR2 as an interacting partner of the mTOR complex 2 pathway; eAGR2 promoted increased phosphorylation of rapamycin-insensitive companion of mTOR (T1135), whereas intracellular AGR2 antagonized its levels and phosphorylation (58). A subsequent study aiming to distinguish between the roles of intracellular AGR2 and eAGR2 in response to chemotherapy using an *in vitro* prostate cancer model revealed that eAGR2 promoted significant resistance to docetaxel (58). Recently, the interaction between AGR2 and transmembrane p24 trafficking protein 2 has been described to serve a key role in AGR2 dimerization and following autophagy-dependent release of AGR2 in the extracellular milieu (59). These findings support the present results, which indicated that autophagy may contribute to survival of CRC cells exposed to 5-FU and oxaliplatin, and the presence of eAGR2 may significantly enhance cell survival and colony formation.

Notably, 5-FU and oxaliplatin represent the standard therapy option for patients with CRC, although with a limited therapeutic success rate (60). Therefore, compounds sensitizing CRC cells to these routinely used drugs are urgently required to improve therapeutic outcome. Metformin is a promising candidate, as documented by several clinical trials (61,62). The results of the present study demonstrated that metformin augmented the anticancer activity of 5-FU and oxaliplatin, and that the effect was enhanced in CRC cells with disrupted AGR2 expression. However, the non-canonical mechanisms by which metformin activates AMPK and induces oxidative stress are areas that require further investigation.

Acknowledgements

The authors would like to thank Ms. Tamara Kolarova (Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute) for technical assistance.

Funding

The present study was supported by the Ministry of Health, Czech Republic-Conceptual Development of Research Organization (Masaryk Memorial Cancer Institute; grant no. 00209805), by the project MEYS-NPS I-LO1413 and GACR 16-14829S and 19-02014S.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AM conducted the majority of western blotting experiments and prepared the manuscript. LS prepared stable *AGR2* gene knockout cell lines and prepared the manuscript. KKu was responsible for functional analyses. JP participated in autophagy experiments and reactive oxygen species determination. BV provided materials and tools, designed the preparation of stable *AGR2* knockout cell lines and drafted the manuscript. KKa participated in the design of the study and revised the manuscript. RH conceived and approved all experiments, performed functional biological assays and finalized the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A and Bray F: Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer 144: 1941-1953, 2019.
2. de Kort S, Masclee AAM, Sanduleanu S, Weijenberg MP, van Herk-Sukel MPP, Oldenhof NJJ, van den Bergh JPW, Haak HR and Janssen-Heijnen ML: Higher risk of colorectal cancer in patients with newly diagnosed diabetes mellitus before the age of colorectal cancer screening initiation. Sci Rep 7: 46527, 2017.
3. Sun L and Yu S: Diabetes mellitus is an independent risk factor for colorectal cancer. Dig Dis Sci 57: 1586-1597, 2012.
4. van de Poll-Franse LV, Haak HR, Coebergh JW, Janssen-Heijnen ML and Lemmens VE: Disease-specific mortality among stage I-III colorectal cancer patients with diabetes: A large population-based analysis. Diabetologia 55: 2163-2172, 2012.
5. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG and Yee D: Diabetes and cancer: A consensus report. Diabetes Care 33: 1674-1685, 2010.
6. Mills KT, Bellows CF, Hoffman AE, Kelly TN and Gagliardi G: Diabetes mellitus and colorectal cancer prognosis: A meta-analysis. Dis Colon Rectum 56: 1304-1319, 2013.
7. Sharma A, Ng H, Kumar A, Teli K, Randhawa J, Record J and Maroules M: Colorectal cancer: Histopathologic differences in tumor characteristics between patients with and without diabetes. Clin Colorectal Cancer 13: 54-61, 2014.
8. Yang J, Nishihara R, Zhang X, Ogino S and Qian ZR: Energy sensing pathways: Bridging type 2 diabetes and colorectal cancer? J Diabetes Complications 31: 1228-1236, 2017.
9. Ariyasu D, Yoshida H and Hasegawa Y: Endoplasmic Reticulum (ER) stress and endocrine disorders. Int J Mol Sci 18, 2017.

10. Dumartin L, Alrawashdeh W, Trabulo SM, Radon TP, Steiger K, Feakins RM, di Magliano MP, Heeschen C, Esposito I, Lemoine NR and Crnogorac-Jurcevic T: ER stress protein AGR2 precedes and is involved in the regulation of pancreatic cancer initiation. Oncogene 36: 3094-3103, 2017.
11. Brychtova V, Vojtesek B and Hrstka R: Anterior gradient 2: A novel player in tumor cell biology. Cancer Lett 304: 1-7, 2011.
12. Tian S, Hu J, Tao K, Wang J, Chu Y, Li J, Liu Z, Ding X, Xu L, Li Q, *et al*: Secreted AGR2 promotes invasion of colorectal cancer cells via Wnt11-mediated non-canonical Wnt signaling. Exp Cell Res 364: 198-207, 2018.
13. Higa A, Mulot A, Delom F, Bouchecareilh M, Nguyên DT, Boismenu D, Wise MJ and Chevet E: Role of pro-oncogenic protein disulfide isomerase (PDI) family member anterior gradient 2 (AGR2) in the control of endoplasmic reticulum homeostasis. J Biol Chem 286: 44855-44868, 2011.
14. Chevet E, Fessart D, Delom F, Mulot A, Vojtesek B, Hrstka R, Murray E, Gray T and Hupp T: Emerging roles for the pro-oncogenic anterior gradient-2 in cancer development. Oncogene 32: 2499-2509, 2013.
15. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, Peters AL, Tsapas A, Wender R and Matthews DR; American Diabetes Association (ADA); European Association for the Study of Diabetes(EASD): Management of hyperglycemia in type 2 diabetes: A patient-centered approach: Position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetes Care 35: 1364-1379, 2012.
16. Higurashi T and Nakajima A: Metformin and colorectal cancer. Front Endocrinol 9: 622, 2018.
17. Meng F, Song L and Wang W: Metformin improves overall survival of colorectal cancer patients with diabetes: A meta-analysis. J Diabetes Res 2017: 5063239, 2017.
18. Mogavero A, Maiorana MV, Zanutto S, Varinelli L, Bozzi F, Belfiore A, Volpi CC, Gloghini A, Pierotti MA and Gariboldi M: Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. Sci Rep 7: 15992, 2017.
19. Diaz-Morales N, Iannantuoni F, Escribano-Lopez I, Bañuls C, Rovira-Llopis S, Sola E, Rocha M, Hernandez-Mijares A and Victor VM: Does metformin modulate endoplasmic reticulum stress and autophagy in type 2 diabetic peripheral blood mononuclear cells? Antioxid Redox Signal 28: 1562-1569, 2018.
20. Kobiela J, Dobrzycka M, Jędrusik P, Kobiela P, Spychalski P, Śledziński Z and Zdrojewski T: Metformin and colorectal cancer-a systematic review. Exp Clin Endocrinol Diabetes 127: 445-454, 2019.
21. Yue W, Wang T, Zachariah E, Lin Y, Yang CS, Xu Q, DiPaola RS and Tan XL: Transcriptomic analysis of pancreatic cancer cells in response to metformin and aspirin: An implication of synergy. Sci Rep 5: 13390, 2015.
22. Arumugam T, Deng D, Bover L, Wang H, Logsdon CD and Ramachandran V: New blocking antibodies against novel AGR2-C4.4A pathway reduce growth and metastasis of pancreatic tumors and increase survival in mice. Mol Cancer Ther 14: 941-951, 2015.
23. Hrstka R, Bouchalova P, Michalova E, Matoulkova E, Muller P, Coates PJ and Vojtesek B: AGR2 oncoprotein inhibits p38 MAPK and p53 activation through a DUSP10-mediated regulatory pathway. Mol Oncol 10: 652-662, 2016.
24. Hrstka R, Brychtova V, Fabian P, Vojtesek B and Svoboda M: AGR2 predicts tamoxifen resistance in postmenopausal breast cancer patients. Dis Markers 35: 207-212, 2013.
25. Zhang Q, Gu J, Li L, Liu J, Luo B, Cheung HW, Boehm JS, Ni M, Geisen C, Root DE, *et al*: Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. Cancer Cell 16: 413-424, 2009.
26. Hrstka R, Nenutil R, Fourtouna A, Maslon MM, Naughton C, Langdon S, Murray E, Larionov A, Petrakova K, Muller P, *et al*: The pro-metastatic protein anterior gradient-2 predicts poor prognosis in tamoxifen-treated breast cancers. Oncogene 29: 4838-4847, 2010.
27. Ostatná V, Vargová V, Hrstka R, Ďurech M, Vojtěšek B and Paleček E: Effect of His_6-tagging of anterior gradient 2 protein on its electro-oxidation. Electrochim Acta 150: 218-222, 2014.
28. Ma SR, Mao L, Deng WW, Li YC, Bu LL, Yu GT, Zhang WF and Sun ZJ: AGR2 promotes the proliferation, migration and regulates epithelial-mesenchymal transition in salivary adenoid cystic carcinoma. Am J Transl Res 9: 507-519, 2017.
29. Park K, Chung YJ, So H, Kim K, Park J, Oh M, Jo M, Choi K, Lee EJ, Choi YL, *et al*: AGR2, a mucinous ovarian cancer marker, promotes cell proliferation and migration. Exp Mol Med 43: 91-100, 2011.
30. Kim J, Yang G, Kim Y, Kim J and Ha J: AMPK activators: Mechanisms of action and physiological activities. Exp Mol Med 48: e224, 2016.
31. Mizushima N and Yoshimori T: How to interpret LC3 immunoblotting. Autophagy 3: 542-545, 2007.
32. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, *et al*: Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12: 1-222, 2016.
33. Andrzejewski S, Gravel SP, Pollak M and St-Pierre J: Metformin directly acts on mitochondria to alter cellular bioenergetics. Cancer Metab 2: 12, 2014.
34. Owen MR, Doran E and Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 348 Pt 3: 607-614, 2000.
35. Larsson SC, Orsini N and Wolk A: Diabetes mellitus and risk of colorectal cancer: A meta-analysis. J Natl Cancer Inst 97: 1679-1687, 2005.
36. Zanders MM, Vissers PA, Haak HR and van de Poll-Franse LV: Colorectal cancer, diabetes and survival: Epidemiological insights. Diabetes Metab 40: 120-127, 2014.
37. Kim JH, Lee KJ, Seo Y, Kwon JH, Yoon JP, Kang JY, Lee HJ, Park SJ, Hong SP, Cheon JH, *et al*: Effects of metformin on colorectal cancer stem cells depend on alterations in glutamine metabolism. Sci Rep 8: 409, 2018.
38. Nangia-Makker P, Yu Y, Vasudevan A, Farhana L, Rajendra SG, Levi E and Majumdar AP: Metformin: A potential therapeutic agent for recurrent colon cancer. PLoS One 9: e84369, 2014.
39. Herzig S and Shaw RJ: AMPK: Guardian of metabolism and mitochondrial homeostasis. Nat Rev Mol Cell Biol 19: 121-135, 2018.
40. Emami Riedmaier A, Fisel P, Nies AT, Schaeffeler E and Schwab M: Metformin and cancer: From the old medicine cabinet to pharmacological pitfalls and prospects. Trends Pharmacol Sci 34: 126-135, 2013.
41. Hrstka R, Murray E, Brychtova V, Fabian P, Hupp TR and Vojtesek B: Identification of an AKT-dependent signalling pathway that mediates tamoxifen-dependent induction of the pro-metastatic protein anterior gradient-2. Cancer Lett 333: 187-193, 2013.
42. Li Z, Wu Z, Chen H, Zhu Q, Gao G, Hu L, Negi H, Kamle S and Li D: Induction of anterior gradient 2 (AGR2) plays a key role in insulin-like growth factor-1 (IGF-1)-induced breast cancer cell proliferation and migration. Med Oncol 32: 577, 2015.
43. Matoulkova E, Sommerova L, Pastorek M, Vojtesek B and Hrstka R: Regulation of AGR2 expression via 3'UTR shortening. Exp Cell Res 356: 40-47, 2017.
44. Pandurangan AK: Potential targets for prevention of colorectal cancer: A focus on PI3K/Akt/mTOR and Wnt pathways. Asian Pac J Cancer Prev 14: 2201-2205, 2013.
45. Tian SB, Tao KX, Hu J, Liu ZB, Ding XL, Chu YN, Cui JY, Shuai XM, Gao JB, Cai KL, *et al*: The prognostic value of AGR2 expression in solid tumours: A systematic review and meta-analysis. Sci Rep 7: 15500, 2017.
46. Alves MR, E Melo NC, Barros-Filho MC, do Amaral NS, Silva FIB, Baiocchi Neto G, Soares FA, de Brot Andrade L and Rocha RM: Downregulation of AGR2, p21, and cyclin D and alterations in p53 function were associated with tumor progression and chemotherapy resistance in epithelial ovarian carcinoma. Cancer Med, 2018.
47. Riener MO, Thiesler T, Hellerbrand C, Amann T, Cathomas G, Fritzsche FR, Dahl E, Bahra M, Weichert W, Terracciano L and Kristiansen G: Loss of anterior gradient-2 expression is an independent prognostic factor in colorectal carcinomas. Eur J Cancer 50: 1722-1730, 2014.
48. Chen N and Karantza-Wadsworth V: Role and regulation of autophagy in cancer. Biochim Biophys Acta 1793: 1516-1523, 2009.
49. Madia F, Grossi V, Peserico A and Simone C: Updates from the intestinal front line: Autophagic weapons against inflammation and cancer. Cells 1: 535-557, 2012.
50. Yang HZ, Ma Y, Zhou Y, Xu LM, Chen XJ, Ding WB and Zou HB: Autophagy contributes to the enrichment and survival of colorectal cancer stem cells under oxaliplatin treatment. Cancer Lett 361: 128-136, 2015.

51. Li J, Hou N, Faried A, Tsutsumi S and Kuwano H: Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer 46: 1900-1909, 2010.
52. Abdelsatir AA, Husain NE, Hassan AT, Elmadhoun WM, Almobarak AO and Ahmed MH: Potential benefit of metformin as treatment for colon cancer: The evidence so far. Asian Pac J Cancer Prev 16: 8053-8058, 2015.
53. Hur KY and Lee MS: New mechanisms of metformin action: Focusing on mitochondria and the gut. J Diabetes Investig 6: 600-609, 2015.
54. Wang L, Wang X and Wang CC: Protein disulfide-isomerase, a folding catalyst and a redox-regulated chaperone. Free Radic Biol Med 83: 305-313, 2015.
55. Schexnayder C, Broussard K, Onuaguluchi D, Poché A, Ismail M, McAtee L, Llopis S, Keizerweerd A, McFerrin H and Williams C: Metformin inhibits migration and invasion by suppressing ROS production and COX2 expression in MDA-MB-231 breast cancer cells. Int J Mol Sci 19, 2018.
56. Queiroz EA, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Lees SJ, Barbosa AM, Dekker RF, Fortes ZB and Khaper N: Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS One 9: e98207, 2014.
57. Ramachandran V, Arumugam T, Wang H and Logsdon CD: Anterior gradient 2 is expressed and secreted during the development of pancreatic cancer and promotes cancer cell survival. Cancer Res 68: 7811-7818, 2008.
58. Tiemann K, Garri C, Lee SB, Malihi PD, Park M, Alvarez RM, Yap LP, Mallick P, Katz JE, Gross ME and Kani K: Loss of ER retention motif of AGR2 can impact mTORC signaling and promote cancer metastasis. Oncogene 38: 3003-3018, 2019.
59. Maurel M, Obacz J, Avril T, Ding YP, Papadodima O, Treton X, Daniel F, Pilalis E, Hörberg J, Hou W, *et al*: Control of anterior GRadient 2 (AGR2) dimerization links endoplasmic reticulum proteostasis to inflammation. EMBO Mol Med 11, 2019.
60. Meyerhardt JA and Mayer RJ: Systemic therapy for colorectal cancer. N Engl J Med 352: 476-487, 2005.
61. Miranda VC, Braghiroli MI, Faria LD, Bariani G, Alex A, Bezerra Neto JE, Capareli FC, Sabbaga J, Lobo Dos Santos JF, Hoff PM and Riechelmann RP: Phase 2 trial of metformin combined with 5-Fluorouracil in patients with refractory metastatic colorectal cancer. Clin Colorectal Cancer 15: 321.e1-328.e1, 2016.
62. Richard SM and Martinez Marignac VL: Sensitization to oxaliplatin in HCT116 and HT29 cell lines by metformin and ribavirin and differences in response to mitochondrial glutaminase inhibition. J Cancer Res Ther 11: 336-340, 2015.