Melatonin induces cell apoptosis in renal carcinoma cells via regulating mitochondria function, promoting ROS production, and suppression of Akt/mTOR/S6K signaling pathway

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Abstract

Purpose

In recent years, metabolic alteration has been identified as a driver in the development of renal cell carcinoma (RCC), which plays a critical role in cancer cells to adapt to hypoxic environment and cell proliferation. Melatonin (MLT), a neurohormone secreted during the dark hours by the vertebrate pineal gland, induces metabolic reprogramming in cancer cells by suppression of aerobic glycolysis. Whether it can be used as a potential therapeutic tool in RCC is worth exploring.

Methods

In this study, we detected concentration of metabolites in RCC cells through metabolomic analyses using UPLC-MS/MS and the oxygen consumption rate was determined using the Seahorse Extracellular Flux analyzer.

Results

We found that MLT significantly inhibited proliferation and induced apoptosis in RCC cells, moreover, MLT increased ROS level and inhibited the activity of antioxidant enzymes. Furthermore, MLT up-regulated key TCA cycle metabolites while reduced aerobic glycolysis products, and induced higher oxygen consumption rate, more ATP production, and higher membrane potential in RCC cells, indicating that MLT enhances mitochondrial function and re-vert aerobic glycolysis to mitochondrial OXPHOS in RCC cells. Moreover, MLT treatment inhibited the phosphorylation levels of Akt, mTOR, and p70 S6 Kinase in RCC cells, while the application of NAC (inhibitors of ROS) not only restored the phosphorylation of these proteins but also decreased cell apoptosis. Consistently, MLT treatment significantly inhibited growth of RCC cell xenografts in nude mice.

Conclusion

Our results indicate that MLT treatment promotes intracellular ROS production via metabolic reprogramming and reducing antioxidants, which suppressed the activity of Akt/mTOR/S6K signaling pathway and induced apoptosis in RCC cells.

Background

In the 2022 analysis of US cancer statistics, 79,000 cases of kidney cancer were diagnosed(1). Renal clear cell carcinoma (ccRCC) is still the most common phenotype of renal cell carcinoma (RCC) and the main cause of morbidity and mortality(1, 2). Although many effective treatments have been used, the survival rate of patients with RCC has not been effectively improved(2). Dominant therapies are based on
preventing or reversing the course of chemoresistance, which plays a key role in the treatment of RCC. Interestingly, in recent years RCC has been revealed to share a recurrent pattern of mutations to metabolic genes, including VHL, M TOR, ELOC, TSC1/2, FH, SDH, and mitochondrial DNA through the leveraging high-throughput technologies to measure small-molecule metabolites(3, 4), indicating that RCC could be a mitochondrial metabolism-related disease, in which metabolic reprogramming plays a key role in tumorigenesis and metastasis(5). Therefore, exploring new targets in mitochondrial metabolic pathways could be essential for applying new therapy in RCC treatment.

Mitochondria plays a much more significant role in cancer metabolism than previously thought(6), which is the major source of ATP, reactive oxygen species (ROS), and biosynthetic metabolites(7) in cells. In addition, mitochondria also serves as a signaling center in multiple cellular processes including proliferation, differentiation, autophagy, and apoptosis via activating corresponding signaling pathways(8, 9). Mitochondria in cancer cells are characterized by ROS overproduction, which promotes cancer development by inducing genomic instability, modifying gene expression, and participating in signaling pathways(10, 11). On the other hand, the antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), and nonenzymes such as glutathione (GSH) have the ability to remove ROS in cells, and there is a negative correlation with ROS(12). Hence, antioxidant enzymes and mitochondria metabolic homeostasis in cancer cells can be modulated to restrain the growth of tumor cells.

Melatonin (MLT) is the primary neurohormone secreted during the dark hours at night by the vertebrate pineal gland. MLT performs a variety of physiological functions by binding to membrane MT1 and MT2 receptors, while triggering downstream signaling cascades activation(13). Studies have shown that MLT in mitochondria is 100 times more than that in the cytoplasm, which provides an crucial role in balancing ROS balance(14). In particular, the energy source of tumor cells is in the process of aerobic glycolysis (Warburg effect), during which substances in aerobic respiration are inhibited, and the accumulation of lactic acid and increased PH provide a more comfortable environment for the proliferation and metastasis of tumor cells(15). Studies have reported that MLT can metabolize energy in tumor cells, reversing the Warburg effect and thereby inhibiting cancer cell growth(16, 17). Moreover, MLT is a proven anticancer drug that inhibits tumor growth by inhibiting tumor cell proliferation, maintaining gene stability, promoting tumor cell apoptosis and autophagy(16, 18–21). However, the effect on RCC cell growth remains unknown.

In this study, we investigated whether MLT treatment can inhibit the growth of 786-O, 769-P and SW839 RCC cells. Moreover, we detected the metabolomic change and examined mitochondrial function and antioxidant enzyme activity in RCC cells after MLT treatment, to test whether MLT can reprogram cell metabolism, switching from glycolysis to oxidative phosphorylation (OXPHOS), by regulating mitochondrial function and producing more ROS. Whether MLT inhibits Akt/mTOR/S6K pathway and induces cell apoptosis via ROS was determined as well. This study may provide a new strategy for RCC therapy focusing on metabolism reprogramming.

Materials And Methods
Cell Culture

The RCC cells lines 786-O, 769-P and SW839 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). 786-O, 769-P and SW839 were cultured in RPMI 1640 medium (Sigma-Aldrich, Wisconsin, USA), containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Co., Carlsbad, CA, USA). All cells were cultured at 37°C in a saturated aqueous atmosphere of 5% CO\textsubscript{2} and collected at the peak of the logarithmic growth phase for experiments.

Reagents And Antibodies

Melatonin (MLT) (T1659), 3-TYP (T4108) and N-Acetylcarnosine (NAC) (TP1088) were purchased from Topscience (Shanghai, China), and dissolved in DMSO. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was from Servicebio (Wuhan, China). Antibodies against SIRT3 (2627), mTOR (2983), Phospho-mTOR (Ser2448) (5536), Phospho-p70 S6 Kinase (Thr389/Ser371) (9208,9234), Akt (4691), Phospho-Akt (Ser473) (4046), Cleaved-PARP (5625), Caspase3 (9662), Cleaved-caspase3 (9664), β-actin (3700) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MnSOD (ab68155), MnSOD (acetyl K122) (ab214675), Bax (ab32503), Bcl2 (ab32124) were purchased from Abcam (Cambridge, UK).

Mtt Assays

Cell viability was evaluated using the MTT assay. At the end of incubation, both cell types (2500 cells per well) were incubated with MTT (5 mg/ml) (Abcam) for an additional 2 hours. Then the crystal formazan was dissolved in dimethyl sulfoxide (DMSO; 150 µl/well; ECHO Chemical Co. Ltd., Shanghai), and 96-well microplate reader (Bio-Rad, Hercules, USA) was used to measure the absorbance at the wavelength of 490 nm. The morphological changes in the cells were observed under a microscope.

Colony Formation Assay

786-O, 769-P and SW839 RCC cells in logarithmic growth phase were seeded in each well of a 6-well plate (1000 cells/well) and cultured in 1640 medium supplemented with 10% fetal bovine serum in an incubator with 5% CO\textsubscript{2} at 37 °C. 11 days after inoculation, when colonies formed, the medium was discarded, the colonies were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min, stained with crystal violet for 15 min, and the staining solution was slowly washed with running water. After the plates were air-dried, the number of colonies was determined.

Western Blotting Analysis
Western blotting analysis
The expression levels of related proteins were detected by Western blotting. Briefly, cell samples were collected 24 h after MLT treatment and dissolved in 1 × radioimmunoprecipitation buffer with 1% phenylmethylsulfonyl on ice for 20 min. Quantification was performed with a bisphenol acid assay kit (Beyotime, Shanghai, China). After boiling for 10 min at 100 °C, aliquots of 20 µg protein were separated by electrophoresis on 10% or 12% sodium dodecyl sulphate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and then blocked with 5% skim milk for 1 h at room temperature. The cells were incubated with primary antibody on a shaker (4 °C overnight). After washing three times with TBST and incubating with the corresponding secondary antibody for 2 h at room temperature, immunoblots on the membranes were visualized using an enhanced chemiluminescence detection kit (Millipore, Burlington, MA, USA). β-actin was used as a loading control for total homogenates; MnSOD was used as a loading control for isolated mitochondria. Densitometry was performed using ImageJ 1.52k software.

Apoptosis Assay
The apoptotic ratio of cells treated with MLT were analyzed by flowcytometry (BD FACScan flow cytometer, BD Biosciences). Cells were implanted into 6-well plates at a density of 4 × 10^5 cells/well. After Mel for 24 h, the cells were washed twice with precooled PBS, and then collected in 1.5 ml tubes and centrifuged at 500 × g (4°C) for 5 min. Subsequently, the cells were resuspended in 100 µl of binding buffer, and mixed with 4 µl of Annexin V-FITC and 4 µl of propidium iodide (PI) for 15 min. After that, 400 µl of binding buffer was added for dilution and flow cytometry was performed to measure the proportions of apoptotic cells. For inhibition experiments, cells were preincubated with 5 mM NAC (ROS scavenger) for 1 h, washed three times with PBS after MLT and incubated for 24 h. In addition, fluorescence intensity was assessed by the same method as above.

Mitochondrial Sirt3, Mnsod And Ack122 Mn-sod Assay
Mitochondrial extracts were prepared using the Cell Mitochondria Isolation kit (Beyotime Biotechnology) according to manufacturer's instructions after the 786-O cells treated with MLT and 3-TYP for 24 h. Purity of the mitochondrial fraction was assessed by MnSOD (a mitochondrial marker). The levels of SIRT3 and Ack122 Mn-SOD in mitochondrial protein were subsequently detected via Western blot analysis.

Measurement Of Ros Concentration
2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were from KeyGen BioTECH (Nanjing, China). DCFH-DA is a non-fluorescent cell permeable compound, which can be hydrolyzed by intracellular endogenous esterase, and its products are oxidized by intracellular ROS and finally converted into DCF with fluorescent signal. Because DCF cannot freely pass through the cell membrane, its fluorescence intensity
can be used to reflect the amount of ROS produced in the cell. The specific method was to wait for 24 h after the cells were treated with MLT, and incubate with serum-free medium containing 10 µM DCHF-Da for 30 min in the dark. After treatment, the fluorescence intensity of different groups of cells was photographed by fluorescence microscope with the same exposure intensity, and then detected by flow cytometry immediately to prevent the fluorescence intensity from weakening and disappearing.

**Measurement Of Antioxidant Enzyme Activities**

Briefly, cell samples at 24 h after MLT treatment were collected and lysed in 1 × radioimmunoprecipitation assay buffer for 20 min on ice. The supernatant was collected. After been quantified by a bicinchoninic acid assay kit (Beyotime), the levels of GSH-Px, SOD, GSH, and CAT in cells were measured with antioxidant kits (Jiancheng Bioengineering Institute, Nanjing, China). Absorbance at the corresponding wavelength was determined by a microplate reader (Bio-Rad, Hercules, USA).

**Metabolomic Analysis**

After six hours of treatment with MLT, the 786-O cells were flash-frozen in liquid nitrogen. The TCA cycle metabolites, acetyl-CoA, citrate, isocitrate, succinate, pyruvate, and lactate in the samples, were quantitatively determined by ultraperformance liquid chromatography, in combination with triple quadrupole mass spectrometry (UPLC-MS/MS) (Shanghai Applied Protein Technology Co., Ltd). The data acquisition, principal component analysis, heatmap, and pathway impact analysis were performed by Shanghai Applied Protein Technology Co., Ltd.

**Mitochondrial Respiration**

The oxygen consumption rate (OCR) was determined using the Seahorse Extracellular Flux XF-96 analyzer (Seahorse Bioscience, N. Billerica, MA, USA). Cells were seeded in XF 96-well cell culture microplates (Seahorse Bioscience) at a density of 1 × 10^4 /well and incubated overnight at 37°C with 5% CO₂. After 24 hours, the medium in the 96-well plate was carefully aspirated and then MLT was added at various concentrations (2 mM, 4 mM) overnight. Prior to Base Medium (102353-100, Seahorse Bioscience) containing 10 mM glucose, 5 mM pyruvate, and 2 mM glutamine, and the cells were equilibrated for 1 h at 37°C without CO₂. Oxygen concentration in media was measured under basal conditions and after sequential addition of compounds as indicated in corresponding figure legends. Concentration of inhibitors used was as follows: Oligomycin A (1.5 µM), Fccp (2.0 µM), and a mixture of rotenone (500 nM) and antimycin A (500 nM). A minimum of four wells were utilized per condition to calculate OCR and it was normalized by the number of cells.

**Measurement Of Mitochondrial Membrane Potential**
The increase of mitochondrial membrane potential (ΔΨm) was monitored with fluorescent probe JC-1 (Beyotime). 786-O,769-P and SW839 cells were treated with different concentrations of MLT (2,4 mM) for 24 h, and then incubated with JC-1 (10 µM, final concentration) in the dark at 37°C for 20 min. Finally, after washing with JC-1 buffer, cells were collected and analyzed by flow cytometry (BD Biosciences). Then, the fluorescence intensity of different groups of cells was observed immediately with a fluorescence microscope.

**Cell And Tumor Tissue Atp Production Measurement**

The ATP content in 786-O,769-P and SW839 cells was measured using a luciferase-based luminescence enhanced ATP analysis kit (Beyotime). The cells were washed twice with ice-cold PBS, collected in 100 µL of ice-cold ATP release buffer, and centrifuged at 12 000 × g for 5 min at 4°C. Subsequently, the isolated mitochondria (with a protein concentration of 1 mg/mL) were incubated in 0.5 mL of respiratory buffer containing 2.5 mM succinate, 2.5 mM malate, and 2.5 mM ADP for 10 min. The ATP content in cell lysates and mitochondrial suspensions were then measured using a SpectraMax Paradigm multi-mode microplate reader (Molecular Devices). A portion of the tumor tissue was added to 150 µL of lysate per 20 mg and lysed thoroughly by ultrasound. After centrifugation, the supernatant was taken in the same procedure as above.

**Animal Experiments**

Female BALB/c mice, four weeks old, were obtained from Shanghai Experimental Animal Center of Chinese Academic of Sciences (Shanghai, China) and housed in Animal Room (SPF) of Xi'an Jiao Tong University according to standard conditions. All animal experiments were approved by the ethics committee of Xi'an Jiao Tong University (Xi'an, China) and complied with the legal requirements and national guidelines for the care and maintenance of experimental animals. After one week of adaptation, 5×10^6 786-O cells in 100 µL of PBS were injected subcutaneously on the right side of each mouse. Tumor size was measured every other day by means of a Vernier caliper, and tumor volume was estimated according to the following formula: tumor volume = (length × width^2)/2. The body weight was measured with an electronic balance every other day. When tumors reached an average volume of 100 mm^3 (approximately 10–12 days post injection), the mice were randomly divided into two groups: Mice were administered with 0.9% DMSO as a control group or administered with MLT (25 mg/kg) through intraperitoneal (i.p.) injection every other day in the evening (18:00–20:00). Twelve days after treatment, all the mice were sacrificed, and the entire tumor was collected and weighed. During this period, the body weight and tumor size of each animal were monitored as described above.

**Tunel Analysis**
Analysis of the apoptotic marker TUNEL was performed using the TUNEL assay kit from Servicebio (Wuhan, China). The sections were incubated twice with xylene for 15 to 20 min each. The cells were dehydrated with two times of pure ethanol for 10 min each, followed by dehydration with 95%, 90%, 80%, and 70% gradient ethanol for 5 min each. Clear fluid was removed, and markers were made on the target tissue with a hydroresistive pen. Proteinase K working solution was added to cover the target and incubated at 37 °C for 25 min. They were then washed 3 times for 5 min each with PBS (pH 7.4) in a Rocker device. Excess fluid was removed and permeable working solution was added to cover the target tissue, followed by incubation for 20 min at room temperature. The plates were washed three times with PBS (pH 7.4) in a Rocker device. According to the number of sections and tissue size, appropriate amounts of TDT enzyme, dUTP, and buffer were mixed in the TUNEL kit at a ratio of 1:5:50. This reaction solution was prepared as needed before use. This mixture was added to the target tissue placed in a flat wet box and incubated for 2 h at 37 °C. DAPI counterstained nuclei were washed three times with PBS (pH 7.4) for 5 min each in a Rocker apparatus. They were then washed with PBS (pH 7.4) three times for 5 min each in a Rocker device. Discard the liquid slightly and cover with anti-fade mounting medium. Microscopy and image acquisition were performed using a fluorescence microscope (Olympus Optical Co., Tokyo, Japan): DAPI emitted blue light, UV excitation wavelength 330–380 nm, emission wavelength 420 nm; TUNEL emitted green fluorescence with an excitation wavelength of 465–495 nm and an emission wavelength of 515–555 nm.

**Immunohistochemistry (Ihc) Analysis**

Ki-67 and p-Akt antibodies were purchased from Servicebio (Wuhan, China) to analyze the expression level of these two proteins. Control and MLT-treated tumor tissues were fixed with 4% PFA overnight at 4°C. Then, the tissues were washed four times with precooled PBS, gently mounted in optimum cutting temperature (OCT) embedding compound, frozen at −20°C, and then transferred to a −80°C freezer. Tissue sections were prepared at a thickness of 5 µm and placed on histological slides. The sections were fixed in acetone for 12 min and then washed four times in PBS. Then, the sections were blocked and permeabilized with blocking buffer (2% BSA and 0.05% Triton-X in PBS) in a humidified chamber at RT for 1 h. The blocking buffer was drained from the slides, appropriate diluted primary antibodies (Ki-67, p-Akt) were added, and the slides were incubated overnight at 4°C. The slides were then washed three times with PBS and incubated with secondary antibodies for 1 h at RT. The slides were washed again 3 times with PBS and the sections were covered with a coverslip. Immunohistochemical staining was performed using horseradish peroxidase (HRP).

**Statistical analysis**

All data were presented as mean ± standard deviation (SD) of 3 independent experiments. All statistical analyses were performed using GraphPad Prism 5.2 software (GraphPad Software, Inc.). The difference between two groups was analyzed by Student’s t test. The difference among multiple groups was analyzed by one-way analysis of variance. P < 0.05 was used to suggest statistical significance.
Results

MLT Significantly inhibited the proliferation and promoted apoptosis of RCC.

To detect the effect of MLT on cell growth in RCC cells, 786-O, 769-P, SW839 cells were treated with different concentrations (2.0 and 4.0 mmol/L) of MLT for 24 h and MTT assay was performed to detect cell proliferation. As shown in Fig. 1A, 1B, and 1C, the proliferation of all these RCC cells was inhibited by MLT treatment in a dose-dependent manner. The IC50 of MLT for 786-O, 769-P, and SW839 cells was 2.4 mM, 2.7 mM, and 2.0 mM, respectively (Fig. 1A, 1B, 1C). It was also obvious that the number of RCC cells was progressively reduced with the increase of MLT concentration as shown in the microscopic images (Fig. 1D) and quantification (Fig. S1A). Colony formation assay was performed to assess the anti-proliferative effect of MLT, and we found that proliferative ability of RCC cells in the MTL groups showed a greater decrease than that in the control groups (Fig. 1E). To investigate whether MLT induces apoptosis of RCC cells, 786-O,769-P, and SW839 cells were exposed to different concentrations of MLT (2 mM and 4 mM), and changes in the expression of associated proteins (PARP, cleaved-PARP, Caspase-3, cleaved-Caspase-3, Bax, Bcl-2) were detected via western blotting analysis. As shown in Fig. 1F, MLT treatment resulted in the reduction of expression of anti-apoptotic protein Bcl-2, whereas increased pro-apoptotic protein Bax expression. Moreover, the expression levels of cleaved PARP and cleaved caspase-3 were increased by MLT treatment, suggesting that MLT treatment activated the downstream signaling molecules. Consistent with these results, MLT increased the percentage of apoptotic cells in 786-O,769-P, and SW839 cells, as evaluated by Annexin V/PI staining and flow cytometry assay (Fig. 1G; Fig. S1B, S1C, S1D). Taken together, these results indicated that MLT inhibits cell proliferation and promotes cell apoptosis in RCC cells.

Figure 1. MLT inhibited cell proliferation and promoted apoptosis of RCC cells. (A, B, C) Relative cell viability of 786-O, 769-P and SW839 cells treated with MLT at different concentrations for 24 h was measured by MTT assays; (D) 786-O, 769-P and SW839 cells treated with MLT at different concentrations for 24 h. Cells were stained with trypan blue, and the number of the cells is shown for different group; (E) Colony formation test of RCC cells after treated with MLT. (F) The RCC cells were treated with different doses of MLT for 24, and then protein expression was detected by western blotting analysis; (G) 786-O, 769-P and SW839 cells were pretreated with MLT (0 mM, 2 mM, and 4 mM) for 24 h, and cell apoptosis was detected by flow cytometry analysis.

Mlt Treatment Increased Ros Production And Inhibits Antioxidases Activities In Rcc

The mechanistic basis of cell damage induced by MLT is closely associated with the generation of intracellular ROS. It has been reported that MLT affects the deacetylase SIRT3 in the mitochondria of myocardial cells, thereby affecting its substrate Ack122-MnSOD(22). Therefore, we verified the biological function of SIRT3 in RCC cells by extracting mitochondria for western blotting analysis and used the 3-TYP (inhibitor of SIRT3) to advance MTT analysis. Interestingly, the expression level of Ack122-MnSOD...
was increased in RCC cells (Fig. S2A, 2B, 2C), which was mutually refuted with the increase of ROS, and 3-TYP did not reduce cell death (Fig. S2D, 2E, 2F). These results indicate that the inhibition of RCC cell growth by MLT is not through the enhancement of SIRT3 function. Therefore, we investigated the relationship between the increase of ROS and other antioxidant enzymes in MLT-treated RCC cells. Consistent with previous studies, we found that MLT significantly increased the fluorescence intensity of the intracellular ROS probe compared to the other treatments under a fluorescence microscope (Fig. 2B). Moreover, the results of flow cytometry showed that the amount of ROS in the MLT group increased significantly (Fig. 2A, 2C, 2D, 2E). In addition, activities of SOD, GSH, GSH-Px, and CAT were measured in order to detect the antioxidant capacity of MLT in RCC. As shown in Figs. 2F, 2G, 2H, and 2I, the activities of SOD, GSH, and CAT significantly decreased in the group treated with MLT, whereas the activities of GSH-Px increased in the MLT-treated cells. Collectively, MLT treatment increased ROS production and inhibits antioxidases activities in RCC cells.

Figure 2. MLT treatment increased ROS production and inhibited antioxidase activities in RCC cells. (A) ROS production in RCC cells were detected by fluorescence staining. Scale bar = 50 µm. (B, C, D, E) Flow cytometry analysis of the ROS amount and quantitative analysis of intracellular ROS detection. (n = 3, mean ± SD). (F) SOD activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). (G) GSH activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). (H) GSH-PX activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). (I) CAT activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). * p < 0.05; ** p < 0.01, and *** p < 0.001 vs control group.

MLT treatment up-regulated key TCA cycle metabolites while reduced aerobic glycolysis products in RCC cells
The role of MLT in the regulation of cancer cell metabolism remains largely unexplored. Therefore, we wondered whether the increase of ROS in renal cancer cells by MLT treatment was related to the tricarboxylic acid cycle in mitochondria. To further dissect the effects of MLT in cancer metabolism, we carried out metabolomic analyses using UPLC-MS/MS and found obvious differences between the control group and the MLT-treated group (Fig. 3A). We found that after the treatment by MLT for 6 h, key TCA cycle metabolites, including Acetyl-CoA, citrate, isocitrate and succinate were upregulated in RCC cells (Fig. 3B, 3C, 3D), while the levels of pyruvate (Fig. 3E) and lactate were reduced (Fig. 3F). A larger influx of pyruvate into the mitochondria would lead to enhanced oxidative phosphorylation and reduced lactate production, which may lead to mitochondrial ROS production. These results indicate that MLT treatment inhibited aerobic glycolysis but enhanced the TCA cycle in RCC cells.

Figure 3. MLT treatment up-regulated key TCA cycle metabolites while reduced aerobic glycolysis products in RCC cells. (A) Heatmap of the metabolomic analysis in 786-O cells between MLT-treated groups vs. control group. (B, C, D, E, F, G) Metabolomic analysis of intracellular levels of Acetyl-CoA, citrate, isocitrate, succinate, pyruvate and lactate respectively, as detected using UPLC-MS/MS. (n = 3, mean ± SD). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.
Mlt Treatment Enhanced Mitochondrial Function In Rcc Cells

It has been reported that MLT may influence mitochondrial energy metabolism including OXPHOS when it functions as an antioxidant(23). To further determine whether the metabolic reprogramming in RCC cells after MLT treatment was related to their mitochondria function change, functional mitochondrial analyses were carried out using the Seahorse XF96 extracellular flux analyzer in 786-O, 769-P, and SW839 cells (Fig. 4A, 4B, 4C). Consistent with previous findings, elevated OCR corresponding to increased basal respiration with the increasing of MLT concentration was observed in RCC cells (Fig. 4D, 4E, 4F). In addition, the ATP turnover (ATP production), maximal respiration and the proton leak of mitochondrial in RCC were increased after treated with MLT (Fig. S3A, S3B, S3C), indicating that MLT reverses the impaired basic respiratory function and partially restores the maximal respiratory function of the mitochondria in RCC cells. Moreover, flow cytometry analysis by JC-1 staining revealed the enhanced mitochondrial membrane potential in MLT-treated cells (Fig. 4G, 4H), suggesting that the membrane potential and the mitochondrial capacity of RCC cells were significantly increased after MLT application. Consistently, we found that MLT-treated groups significantly increased the RED fluorescence intensity and decreased the GREEN fluorescence intensity of the intracellular JC-1 probe compared to the control groups under a fluorescence microscope (Fig. 4I). This was also verified by the higher level of ATP in RCC cells after treatment with MLT (Fig. 4J, 4K, 4L). Taken together, these findings indicate that MLT treatment enhanced mitochondrial function and inhibited aerobic glycolysis (the Warburg effect) in cancer cells causing them to revert to mitochondrial OXPHOS and increase the ROS production, which may result in the inhibition of cancer cell proliferation and promotion of cell apoptosis.

Figure 4. MLT treatment enhanced mitochondrial function in RCC cells. (A, B, C) OCR level of 786-O, 769-P and SW839 cells pretreated with different dose of MLT for 24h as measured by seahorse analyzer Oligomycin A, Fccp, and Rotenone/Antimycin A (n = 5, mean ± SD). (D, E, F) The basal respiration in 786-O, 769-P, and SW839 cells respectively. (n = 5, mean ± SD). (G, H) The mitochondrial membrane potential changes in 786-O, 769-P, and SW839 cells after treatment with MLT for 24 h, as detected by flow cytometry analysis, and the quantification of JC-1 green is shown in bar graph (H), in which the lower JC-1 green indicates the higher membrane potential. (n = 3, mean ± SD). (I) Fluorescence diagrams of mitochondrial membrane potential with RED and GREEN changes in 786-O, 769-P and SW839 cells after treatment with MLT for 24h, in which the lower JC-1 Green/Red ration indicates the higher membrane potential. Scale bar = 50 µm. (J, K, L) Level of ATP in 786-O, 769-P and SW839 cells after treatment with MLT at different concentrations, respectively. (n = 3, mean ± SD). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.

Mlt Induced Cell Apoptosis Via Regulating The Ros-mediated Suppression Of Akt/mtor/s6k Signaling Pathway

It has long been demonstrated that ROS-mediated cell apoptosis is related to Akt/mTOR/S6K signaling pathways(20). To verify whether cell apoptosis in RCC cells was related to the ROS/Akt/mTOR/S6K signaling pathways, we performed western blotting analysis and found that the expression levels of p-Akt
and p-mTOR, p-p70 S6 Kinase (Thr389/Ser371) in the MLT treatment groups (4 mM) were significantly decreased compared with the control groups (Fig. 5A), which indicated that the Akt/mTOR/S6K signaling pathway was inhibited by MLT treatment. In particular, the protein expression of p-p70 S6 Kinase (Thr389) of 786-O and 769-P was not inhibited, while SW839 was significantly inhibited. Moreover, given the importance of ROS in MLT treatment, the scavenger NAC was used to evaluate the role of ROS in the apoptotic and related pathways. We found that the expression levels of apoptosis associated proteins were reversed (Fig. 5B), and the number of apoptotic cells obtained by flow cytometry was significantly reduced compared with the MLT-treatment groups (Fig. 5C, 5E). Furthermore, the Akt/mTOR/S6K signaling pathway was reactivated by the addition of NAC compared with the MLT-treatment groups (Fig. 5D). In summary, these results indicate that MLT treatment induced cell apoptosis via producing ROS and inhibiting the Akt/mTOR/S6K signaling pathway.

Figure 5. MLT induced cell apoptosis via regulating the ROS-mediated suppression of Akt/mTOR/S6K signaling pathway. (A) Western blot analysis of Akt, p-Akt and mTOR, p-mTOR, p-p70 S6 Kinase (Thr389/Ser371) and β-actin expression, after 786-O, 769-P and SW839 cells were treated with different concentrations MLT for 24 h; (B) Western blot analysis of the expression levels of apoptosis related proteins after 786-O, 769-P and SW839 cells treated with MLT (4 mM) and ROS inhibitor NAC (5 mM). (C, D) The apoptotic cell number changes in 786-O, 769-P and SW839 cells after treatment with MLT (4 mM) and ROS inhibitor NAC (5 mM) as detected by flow cytometry assay, and the quantification of apoptotic cells is shown in bar graph (D) (n = 3, mean ± SD). (E) Western blot analysis of the expression Akt, p-Akt and mTOR, p-mTOR, p-p70 S6 Ki-nase (Thr389/Ser371), and β-actin after RCC cells treated with MLT (4 mM) and ROS in-hibitor NAC (5 mM). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.

**MLT treatment inhibited RCC cell growth in vivo**

To investigate the effect of MLT on RCC growth *in vivo*, 786-O cells were injected into BALB/c nude mice to form subcutaneous xenograft tumor. After treatment with MLT for 12 days, it was no significant difference in body weight between the MLT treatment group and the control group (Fig. 6A). However, the tumor volume was clearly decreased in the MLT-treated nude mice (Fig. 6B). After sacrificing, isolated tumors were photographed and weighed, and we found that net tumor weight was significantly decreased in the MLT-treated group (Fig. 6C, 6D). These results demonstrated that MLT inhibited the growth of RCC cell xenografts in nude mice without affecting their body weight. Similar to the cell experiments, we also investigated the ATP production in the tumor tissues of nude mice after MLT treatment and found that more ATP was generated compared with the control group (Fig. 6E). In addition, cell proliferation (Ki-67), cell apoptosis (TUNEL) and p-Akt expression in tumor tissues were analyzed by H&E, IHC staining assay and immunofluorescent staining assay. It was shown that MLT treatment increased the TUNEL expression *in vivo*, while inhibited expression level of Ki-67 and p-Akt (Fig. 6F, 6G), further confirming that MLT treatment inhibited the activity of Akt/mTOR/S6K signaling pathway, induced cell apoptosis and suppressed cell proliferation of RCC cells *in vivo*. Collectively, these results demonstrate that MLT treatment inhibited the growth of RCC *in vivo*, which is consistent with the *in vitro* experiment results.
Figure 6. MLT treatment inhibited growth of RCC cell xenografts in vivo. (A) The nude mouse body weight was measured every 2 days with/without MLT treatment. (B) The nude mouse tumor volume was measured every 2 days with/without MLT treatment. (C) After sacrificing, tumors were isolated and photographed after treatment with/without MLT for 12 days. (D) After sacrificing, tumors were isolated and weighted after treatment with/without MLT for 12 days. (E) The level of ATP in mouse tumors was measured \( n = 3, \text{ mean} \pm \text{ SD} \). (F) Representative images showing immuno-fluorescence of staining for TUNEL and in RCC cell xenografts (scale bar = 20 µm). (G) Representative images showing immunocytochemical of staining for HE, Ki-67 and p-Akt in RCC cell xenografts (scale bar = 20 µm). ** p < 0.01 and *** p < 0.001.

Figure 7. Schematic diagram of how melatonin causes apoptosis in RCC cells.

**Discussion**

In the last decade, a large number of basic and clinical studies have demonstrated that MLT treatment is effective for a multitude of diseases, including cancer, metabolic diseases, neurodegeneration, metabolic diseases, and diabetes(14, 18, 19, 24, 25). However, the effect of MLT on RCC remains unknown. Herein, we found that MLT significantly inhibited proliferation and induced cell apoptosis in RCC cells in vitro and in vivo. Mechanistically, we found that MLT increased the level of TCA cycle metabolites and oxidative phosphorylation in mitochondria, as well as inhibited the activity of antioxidant enzymes, leading to the reversal of the Warburg effect and the increase of ROS. Furthermore, our results demonstrated that the anti-proliferative effects of MLT in RCC are due to the suppression of Akt/mTOR/S6K signaling pathway mediated by ROS, resulting in enhanced cell apoptosis and inhibited cell proliferation (Fig. 7). These findings indicate that MLT might be a promising drug to inhibit tumor growth by reprogramming mitochondrial function and altering the activity of antioxidant enzymes.

In recent years, it has been elucidated that mitochondria plays a major role in the metabolic reprogramming of tumor cells to rapidly adapt to stress conditions such as hypoxia and nutrient limitation, which is considered a hallmark of cancer(26, 27). The Warburg effect suggests that cancer cells, even in the presence of sufficient oxygen, produce ATP and large amounts of lactic acid by glycolysis(28). The generation of lactic acid creates an acidic environment that provides a more favorable condition for cancer cell proliferation, invasion and metastasis(29). Therefore, inhibition of glycolysis by molecules is considered as an effective strategy to reduce metastasis while reversing the Warburg effect and re-instituting mitochondrial OXPHOS(30–32). As we know, MLT is a well-known endogenously-produced molecule involved in antioxidation(33). Our results indicate that MLT treatment increases TCA intermediates such as Acetyl-CoA, citrate, isocitrate and succinate, but decreased pyruvate and lactate levels (Fig. 3). Pyruvate enters mitochondria from cytosol and it is the key substance linking glycolysis and mitochondrial oxidative phosphorylation. The decrease in lactate and pyruvate after MLT treatment suggested that melatonin can inhibit or even reverse the Warburg effect and metabolic reprogramming in cancer cells. Our findings also indicated that in cancer cells after melatonin treatment, TCA and OXPHOS are significantly increased, while glycolysis was decreased, resulting in more ATP and
ROS production. Moreover, we determined the impact of MLT on the behavior of mitochondria in cellular metabolism, and the enhancement of ATP, the increase of ATP production-coupled OCR, basal respiration, and membrane potential, indicated that mitochondria function was improved by MLT (Fig. 4). Taken together, our findings indicate that MLT treatment triggered the metabolic reprogramming via inhibition of glycolysis, increase of TCA and OXPHOS, and improvement of mitochondria function, which may result in more ATP and ROS production in RCC cells.

The redox state of cellular is determined by the ratio of antioxidants to oxidants(34). Antioxidants include the enzymes SOD, glutathione peroxidase (GPx), and CAT, and nonenzymes such as GSH and vitamins A, C, and E. Oxidants include ROS such as hydrogen peroxide (H₂O₂), superoxide anion radical (O₂•−), and hydroxyl radical (•OH)(35–37). In this study, we found that the activity of antioxidant enzyme SOD, CAT, and GSH decreased by MLT treatment (Fig. 2), which may lead to the reduction of oxidant consumption. On the other hand, MLT treatment enhanced TCA cycle and OXPHOS and improved mitochondria function in RCC cells, which may lead to more ROS production (Fig. 3&4). Thus, in RCC cells less ROS consumption and more ROS production induced by MLT may work together, resulting in the increase of intracellular ROS concentration (Fig. 2). Since MLT-inducing apoptosis in cancer cells is associated with ROS production(38–40), this increase could lead to the inhibition of cell growth of RCC.

In the research of cancer, the exploration of molecular mechanisms is of great help for the treatment of cancer and may form new therapeutic targets. In this regard, among the abnormal molecular mechanism of MLT regulation, several studies have shown that mTOR signaling pathway is crucial with ROS(20, 41). Unexpected Akt activation leads to activation of mTOR, which contributes to cell growth, proliferation and metabolism, and promotes tumourigenesis(42, 43). Activation of mTOR results in increased protein synthesis and cell survival by direct phosphorylation of its effectors, such as the ribosomal S6K(44). Until now, however, the effects of MLT on Akt/mTOR/S6K signaling pathway and redox homeostasis in RCC remain to be explored. ROS can regulate signaling pathways of Akt/mTOR/S6K to different degrees. For instance, low ROS levels promote mTOR activity, whereas high ROS levels inhibit mTOR activity(45). A previous study has shown that combination of MLT and rapamycin promoted cell apoptosis and autophagy in head and neck cancer by inhibiting Akt/mTOR signaling pathway(20). However, it is unclear whether increased ROS mediated apoptosis through Akt/mTOR/S6K signaling pathway in MLT-treated RCC. Therefore, we analyzed RCC treated with MLT by western blotting analysis and found that p-Akt, p-mTOR and p-p70 S6 Kinase (Ser371) protein expression was inhibited. To verify that this process is mediated by ROS, NAC (inhibitors of ROS) was used and we found that p-Akt, p-mTOR and p-p70 S6 Kinase (Ser371) levels were restored. As a consequence, ROS could play a key role in RCC through Akt/mTOR/S6K inactivation. In a nutshell, the increase in ROS was attributed to enhanced mitochondrial function and decreased antioxidant enzyme activity after MLT treatment, while ROS can also be used as inducers of tumor cell apoptotic through the Akt/mTOR/S6K signaling pathway. Overall, our study provides new insights into the mechanisms underlying melatonin's anti-tumor activity in RCC.

A meta-analysis study showed that MLT treatment significantly reduced solid tumor size and improved 1-year mortality (46). According to our findings, MLT also inhibited the growth of RCC in vivo. The growth of
786-O cell-derived tumor xenografts in mice was significantly inhibited by MLT. Consistent with the in vitro results, the tumor tissues of mice treated with MLT produced more ATP than those of controls; moreover, the expression in Ki-67 and p-Akt were decreased, while the TUNEL was increased, confirming that MLT inhibited RCC cell proliferation and promoted cell apoptosis. Considering that mTOR is a clinically accepted treatment target in RCC and low toxicity of MLT because it is an endogenously-produced molecule in human body, MLT might be a promising drug for RCC treatment. However, large scale clinical trials are needed to confirm the efficacy of MLT treatment in RCC patients in the future.

**Conclusions**

In conclusion, our results indicate that MLT treatment promotes intracellular ROS production via metabolic reprogramming and reducing antioxidants, which suppressed the activity of Akt/mTOR/S6K signaling pathway and induced cell apoptosis in RCC cells. Overall, this study provides new insights into the mechanisms underlying anti-tumor effect of MLT in RCC cells, and suggests that MLT might be a promising therapeutic for RCC.

**Abbreviations**


**Declarations**

**Ethics approval and consent to participate**

The animal experimental protocols were approved by Xi’an Jiao Tong University (No: 2021-674), and all efforts were made to minimize the pain of animals and reduce the number of animals used in experiments.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Author Contributions

Conceptualization, K.X., Y.J., and P.G.; Investigation, K.X., Y.J., J.B., D.Z.; Methodology, K.X., Y.J., Y.C., J.M.; Validation, J.M. and Z.Z.; Formal analysis, J.M. and Z.Z. Resources, X.W.; Writing – Original Draft Preparation, K.X.; Writing – Review & Editing, P.G.; Visualization, K.X., Y.J.; Supervision, X.W., P.G.; Project Administration, P.G.; Funding Acquisition, P.G. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data are available in a public repository. All data relevant to the study are included in the article or uploaded as online supplemental information. Additional data related to this paper may be requested from the corresponding authors.

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Figures

Figure 1 (Xue et al.)

A

B

C

D

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Figure 1
MLT inhibited cell proliferation and promoted apoptosis of RCC cells.

(A, B, C) Relative cell viability of 786-O, 769-P and SW839 cells treated with MLT at different concentrations for 24 h was measured by MTT assays; (D) 786-O, 769-P and SW839 cells treated with MLT at different concentrations for 24 h. Cells were stained with trypan blue, and the number of the cells is shown for different group; (E) Colony formation test of RCC cells after treated with MLT. (F) The RCC cells were treated with different doses of MLT for 24, and then protein expression was detected by western blotting analysis; (G) 786-O, 769-P and SW839 cells were pretreated with MLT (0 mM, 2 mM, and 4 mM) for 24 h, and cell apoptosis was detected by flow cytometry analysis.
Figure 2

MLT treatment increased ROS production and inhibited antioxidase activities in RCC cells.

(A) ROS production in RCC cells were detected by fluorescence staining. Scale bar = 50 μm. (B, C, D, E) Flow cytometry analysis of the ROS amount and quantitative analysis of intracellular ROS detection. (n = 3, mean ± SD). (F) SOD activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3,
mean ± SD). (G) GSH activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). (H) GSH-PX activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). (I) CAT activity in 786-O, 769-P and SW839 cells after treatment with MLT 24 h; (n = 3, mean ± SD). * p < 0.05; ** p < 0.01, and *** p < 0.001 vs control group.

Figure 3 (Xue et al.)

MLT treatment up-regulated key TCA cycle metabolites while reduced aerobic glycolysis products in RCC cells.

(A) Heatmap of the metabolomic analysis in 786-O cells between MLT-treated groups vs. control group. (B, C, D, E, F, G) Metabolomic analysis of intracellular levels of Acetyl-CoA, citrate, isocitrate, succinate,
pyruvate and lactate respectively, as detected using UPLC-MS/MS. (n = 3, mean ± SD). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.

Figure 4 (Xue et al.)

MLT treatment enhanced mitochondrial function in RCC cells.
(A, B, C) OCR level of 786-O, 769-P and SW839 cells pretreated with different dose of MLT for 24h as measured by seahorse analyzer Oligomycin A, Fccp, and Rotenone/Antimycin A (n = 5, mean ± SD). (D, E, F) The basal respiration in 786-O, 769-P, and SW839 cells respectively. (n = 5, mean ± SD). (G, H) The mitochondrial membrane potential changes in 786-O, 769-P, and SW839 cells after treatment with MLT for 24 h, as detected by flow cytometry analysis, and the quantification of JC-1 green is shown in bar graph (H), in which the lower JC-1 green indicates the higher membrane potential. (n = 3, mean ± SD). (I) Fluorescence diagrams of mitochondrial membrane potential with RED and GREEN changes in 786-O, 769-P and SW839 cells after treatment with MLT for 24h, in which the lower JC-1 Green/Red ration indicates the higher membrane potential. Scale bar = 50 μm. (J, K, L) Level of ATP in 786-O, 769-P and SW839 cells after treatment with MLT at different concentrations, respectively. (n = 3, mean ± SD). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.
**Figure 5 (Xue et al.)**

**MLT induced cell apoptosis via regulating the ROS-mediated suppression of Akt/mTOR/S6K signaling pathway.**

**(A)** Western blot analysis of Akt, p-Akt and mTOR, p-mTOR, p-p70 S6 Kinase (Thr389/Ser371) and β-actin expression, after 786-O, 769-P and SW839 cells were treated with different concentrations MLT for 24 h;

**(B)** Western blot analysis of the expression levels of apoptosis related proteins after 786-O, 769-P and SW839 cells treated with MLT (4 mM) and ROS inhibitor NAC (5 mM).  

**(C, D)** The apoptotic cell number changes in 786-O, 769-P and SW839 cells after treatment with MLT (4 mM) and ROS inhibitor NAC (5 mM).
mM) as detected by flow cytometry assay, and the quantification of apoptotic cells is shown in bar graph (D) (n = 3, mean ± SD). (E) Western blot analysis of the expression Akt, p-Akt and mTOR, p-mTOR, p-

p70 S6 Kinase (Thr389/Ser371), and β-actin after RCC cells treated with MLT (4 mM) and ROS inhibitor NAC (5 mM). * p < 0.05; ** p < 0.01 and *** p < 0.001 vs control group.

Figure 6 (Xue et al.)

**Figure 6**

**MLT treatment inhibited growth of RCC cell xenografts in vivo.**

(A) The nude mouse body weight was measured every 2 days with/without MLT treatment. (B) The nude mouse tumor volume was measured every 2 days with/without MLT treatment. (C) After sacrificing, tumors were isolated and photographed after treatment with/without MLT for 12 days. (D) After
sacrificing, tumors were isolated and weighted after treatment with/without MLT for 12 days. (E) The level of ATP in mouse tumors was measured (n = 3, mean ± SD). (F) Representative images showing immunofluorescence of staining for TUNEL and in RCC cell xenografts (scale bar =20 μm). (G) Representative images showing immunocytochemical of staining for HE, Ki-67 and p-Akt in RCC cell xenografts (scale bar =20 μm). ** p < 0.01 and *** p < 0.001.

Figure 7 (Xue et al.)

Figure 7
Schematic diagram of how melatonin causes apoptosis in RCC cells.

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