SIRT6-Autophagy-Warburg effect axis in papillary thyroid cancer

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Research

Keywords: Autophagy, ER-stress, Reactive oxygen species, SIRT6, Warburg effect

Posted Date: February 17th, 2020

DOI: <https://doi.org/10.21203/rs.2.23666/v1>

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Abstract

Background: Our previous study has demonstrated SIRT6 promotes aggression and epithelial-mesenchymal transition (EMT) in papillary thyroid cancer (PTC). In this study, we focused on the regulatory axis between SIRT6-Autophagy-Warburg effect.

Methods: Autophagy activation was confirmed by Western Blotting, quantitative PCR, immunofluorescence and transmission electron microscopy. Warburg effect was examined by Seahorse XF96 analysis and PET imaging. Regulation of SIRT6 in negatively regulates reactive oxygen species (NRROS) was confirmed by chromatin immunoprecipitation.

Results: We innovatively confirmed overexpression of SIRT6 depleted histone H3 lysine 56 acetylation (H3K56ac) of NRROS in vitro, thus increased reactive oxygen species (ROS). Then, accumulated ROS activated endoplasmic reticulum stress (ER stress), and subsequently induced autophagy. Furthermore, the overexpression of SIRT6 inhibited Glucose Transporter 1 (Glut1) via autophagic degradation, thus ulteriorly suppressed Warburg effect. Treatment of ROS scavenger N-acetyl-L-cysteine (NAC, 5mM) or autophagy inhibitor chloroquine (CQ) both rescued the inhibition of Warburg effect. In addition, higher concentration of NAC (15mM) deepened the inhibited Warburg effect. This concentration-dependent bilateral effects of NAC in this process had been confirmed owe to regulation of AMPK signaling pathway. Finally, we further determined above mechanism in vivo via subcutaneous xenografts in nude mice applied with 18F-FDG PET/CT.

Conclusions: We identified a SIRT6-ROS-ERstress-Autophagy-Glut1-Warburg effect axis in PTC, which may provide new target for therapy. In addition, NAC (low concentration) and CQ which previously been considered as tumor inhibitors, have been shown to promote tumorigenesis in PTC with high SIRT6 expression via activation of Warburg effect.

1. Background

Thyroid cancer is one of the common malignant tumors, including papillary, follicular, undifferentiated and medullary pathological type. In which, papillary thyroid cancer (PTC) accounted for 85% of the total(1). The incidence of PTC has increased year by year recently(2). In the past 10 years, the early detection of PTC has improved the patient survival rate, but the overall survival rate of thyroid cancer in nearly 10% patients has not been significantly improved. Therefore, searching for new targets and new diagnostic markers for PTC is imminent(3).

Sirtuins is nicotinamide adenine dinucleotide (NAD⁺) - dependent histone deacetylase. The Sirtuin family consists of 7 members (SIRT1-7), each of which has different location and function in tissue cells. They play an important role in cell proliferation, metabolism of various substances, regulation of aging, and regulation of tumor growth. Among them, SIRT6 gene is located on chromosome 19 p13.3, whose encoded protein contains 355 amino acids. SIRT6 is a nuclear histone deacetylase that specifically targets H3K56ac and H3K9ac(4, 5). Our previous study had infirmed SIRT6 is upregulated in PTC and

enhances tumor aggressiveness via BRAF/ERK/Mcl-1 pathway and EMT(6, 7). Meanwhile, SIRT6 was found associate with autophagy in bronchial epithelial cell and Warburg effect in cancer cell(8, 9). Whereas rare researches focused on SIRT6 induced autophagy in cancer.

Malignant tumor is not only a genetic disease, but also an energy metabolic disease. Even when the oxygen supply is sufficient, the tumor cells obtain energy mainly by glycolysis, which is also called as aerobic glycolysis or Warburg effect. This abnormal glycolysis of tumor cells promotes glucose uptake and lactate production, which is conducive to tumorigenesis and tumor progression. The regulation of Warburg effect is a complex network involves transcription factors, glycolysis enzymes genes, PI3K/AKT pathway, ROS and so on(10).

Autophagy is a process of phagocytosis of cytoplasmic proteins or organelles into vesicles and fusion with lysosomes to form autophagic lysosomes, which degrade the contents of the lysosomes, thereby realizing the metabolic needs of the cells themselves and the renewal of some organelles. Most malignant tumors are positively or negatively correlated with autophagy in many stages of occurrence, development and metastasis. Previous study of autophagy on thyroid cancer mainly focused on cell apoptosis and chemosensitivity(11, 12). Actually, the effect of autophagy on tumors has more directions. Accumulating evidences infirm autophagy interacts with Warburg effect. In cervical cancer, Warburg effect activated HIF1- α was found induce autophagic pathway(13). In colorectal cancer, oridonin was found induce autophagy via inhibition of Warburg effect(14). In ovarian cancer, quercetin induces protective autophagy and apoptosis through ER stress via the p-STAT3/Bcl-2 axis(15). The specific mechanism of interaction of autophagy and Warburg effect in PTC is not clear; Therefore, we focused on the regulatory axis of SIRT6-Autophagy-Warburg effect.

2. Material And Methods

2.1 Cell Culture and reagents

Human PTC cell lines TPC-1 and K1 were purchased from the University of Colorado Cancer Center Cell Bank. All cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO₂ atmosphere.

ROS scavenger N-acetyl-L-cysteine (NAC), histone deacetylase HDAC inhibitor Trichostatin A (TSA), AMPK pathway activator AICAR, autophagy inhibitor chloroquine (CQ), ER-stress inhibitor 4-phenylbutyrate (4-PBA), proteasome inhibitor MG-132 were all purchased from Cell Signaling Technology. The work concentration and time were confirmed by instructions combined with experimental requirements.

2.2 Generation of SIRT6 stably upregulated cell lines

The cDNA of human SIRT6 was purchased from Origene (RC202833, Rockville, MD, USA) and cloned into the pCDH-CMV-MCS-EF1-Puro lentiviral vector to construct the pCDH-SIRT6 overexpression plasmid. In accordance with the instructions of the product manual, Lipofectamine 3000 (Invitrogen, Inc.) was used

to co-transfect the target plasmid or the empty vector, psPAX2, PMG.2G into the HEK293T tool cells to obtain a SIRT6 overexpressed lentivirus or negative control lentivirus. Then, the lentivirus (multiplicity of infection, MOI = 10) was used to infect PTC cell lines. The SIRT6-upregulated cell lines TPC1-SIRT6/K1-SIRT6 and empty vector control cell lines TPC1-NC/K1-NC was screened by puromycin (1 µg/mL, 72 h). The overexpression of SIRT6 was confirmed by Western blotting and RT-qPCR.

2.3 Western Blotting Analysis

The total cellular proteins from each group were extracted using RIPA lysis buffer with 1% phenylmethanesulfonyl fluoride (PMSF). Then, equal amounts (20 µg) of protein determined by BCA protein assay kit (Thermo Fisher Scientific, USA) were separated using 10% SDS-PAGE gels. The proteins were then transferred to PVDF membranes (0.45 mm, Solarbio, China). The membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated with primary antibodies at 4°C for 12 h. The following antibodies were tested: SIRT6, ATF4, CHOP(1:1000, Proteintech, USA), LC3B, ATG5, Beclin1, Glut1, NOX2, NRROS (1:1000, Abcam, USA), p-Akt, Akt, p-mTOR, mTOR, p-Erk1/2, Erk1/2, p-AMPK, p-eIF2α (1:1000, Cell Signaling Technology, USA) rabbit polyclonal antibodies. β-actin rabbit polyclonal antibody (1:4000, Proteintech, USA) was used as loading controls and normalization. The secondary antibodies were anti-mouse or anti-rabbit antibody and conjugated to horseradish peroxidase (HRP) (1:4000, Proteintech, USA). The secondary antibodies were used at a 1:4000 dilution and were incubated for approximately 1 h at room temperature. The bands were visualized with ECL reagents (Thermo Fisher Scientific, USA) and developed by Omega Lum G (Aplegen, USA).

2.4 RNA extraction, reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was extracted by Trizol Regent (Invitrogen) from PTC cells. cDNA was obtained from total RNA with PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan). The SIRT6 expression was assessed by Real-time quantitative PCR, which was carried out in triplicate by a SYBR Premix Ex Taq™ kit (Takara Bio) and ABI 7900HT Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers used are shown in Table 1. The comparative cycle threshold values ($2^{-\Delta\Delta Ct}$) were adopted to analyze the final results.

2.5 Immunofluorescence (IF)

For cell IF, coverslips were laid flat on the bottom of a 6-well plate after cleaning, disinfection, and 24 h ultraviolet irradiation. Then, the cells were seeded on the coverslips at a density of 1×10^6 in each well, cultured in an incubator. The coverslips were rinsed with PBS for 5 min 3 times and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization of the cells in 0.3% TritonX-100/PBS for another 20 min. Next, the coverslips were rinsed with PBS again for 5 min 3 times and blocked by incubating cells in 5% BSA for 60 min. Then, the cells were stained with LC3 (1:100, Abcam, USA) rabbit polyclonal antibody, followed by a 12-hour incubation at 4°C. After washing the uncombined antibody with PBS for 5 min 3 times, the cells were incubated with Alexa 498-coupled Goat Anti-Rabbit IgG secondary antibody (1:200, ProteinTech Group) for 1 h at room temperature in the darkness. Finally, DAPI

was used as a counterstain to label the nuclei. The stained cells were then acquired and photographed with a laser confocal microscopy.

For tissue IF, tissues were made into paraffin sections, deparaffinized and blocked as described in IHC part. The SIRT6 rabbit antibody was incubated overnight at 4°C, followed by HRP-conjugated rabbit secondary antibody for 1 h and Cy5-conjugated goat anti-HRP antibody (1:200; ProteinTech Group) for 10 min at room temperature sequentially. Then, sections were brought to a boil in 1 mM EDTA followed by 15 minutes at a sub-boiling temperature for removing antibodies that have been incorporated into tissues. Similar as SIRT6, Glut1 and p62 followed HRP-conjugated rabbit/mouse secondary antibodies were incubated by Cy3-conjugated goat anti-HRP antibody and FITC-conjugated goat (1:200; ProteinTech Group) anti-HRP antibody (1:200; ProteinTech Group) respectively. Finally, DAPI was used as a counterstain to label the nuclei. The stained sections were then acquired and photographed with a fluorescent microscope.

2.6 Chromatin immunoprecipitation (ChIP) assay

ChIP was performed according to the manufacturers' instructions by using Magna ChIP Kit (Millipore, Darmstadt, Germany). Chromatin samples were immunoprecipitated with antibodies against a negative control normal mouse IgG, H3K56ac (Active motif, USA) or SIRT6 (Abcam, USA) respectively. Subsequently, IP production was performed with RT-qPCR as described above. The primers of NRROS promoter were as follows: Forward: 5'-GGACTTGGCTCCTGTTCTCTT-3' and reverse: 5'-TGACGTGGACCACCATAGTC-3'.

2.7 Transmission electron microscopy (TEM)

4×10^6 cells were harvested and fixed at 4°C by mixing with 2.5% glutaraldehyde/PBS for 2 h. The cell pellet was embedded with 1% agarose and washed in 0.1 M PBS for three times, 15 min each. The samples were further fixed with 1% OsO₄ in PBS (pH 7.4) for 2 h and washed 3 times in PBS, and were then dehydrated with a series of ethanol concentrations (50, 70, 80, 90, 95, 100%) for 15 min intervals. The samples were subsequently incubated in a mixture of alcohol and isoamyl acetate for 30 min, followed by incubation with pure isoamyl acetate for 1 h. Finally, the samples were coated with gold-palladium, cut into ultrathin sections, and observed using a transmission electron microscope (TEM; Hitachi HT7700, Tokyo, Japan).

2.8 Glucose uptake, lactate production and ATP content assays

For glucose uptake and lactate production assays, 5×10^5 cells were seeded onto 6- well plates and cultured at 37 °C. After 24 h, 5 µl cell culture supernatant was collected in 96- well plates and mixed with 200 µl Glucose (HK) Assay Reagent (Sigma-Aldrich, CA, USA) or 100 µl Lactate Assay Regent (Sigma-Aldrich, CA, USA). After 20 minutes incubation at 37°C, the absorbance of 340 nm (OD₃₄₀) was determined with microplate reader. At the same time, standard curve is established through glucose and lactate standard. OD₃₄₀ was substituted into standard curve to get glucose or lactate level. For ATP

content assay, 2×10^3 cells were seeded onto 96-well plates and incubated for 48 h. Culture medium was removed and ATPLite luminescent assay (PerkinElmer, Inc., Waltham, MA, USA) was added. After 30 minutes incubation at 37°C, luminescence was monitored. At the same time, standard curve is established through ATP standard. Luminescence intensity was substituted into standard curve to get ATP level. Glucose uptake, lactate production and ATP content were all normalized by cell number.

2.9 Extracellular Acidification Rate and Oxygen Consumption Rate Assays

The extracellular acidification rate (ECAR) and cellular oxygen consumption rate (OCR) of cells were measured using the Seahorse XFe 96 Extracellular Flux Analyzer (Seahorse Bioscience) via Glycolysis Stress Test Kit and Cell Mito Stress Test Kit respectively (Agilent Technologies). Both assays were performed according to the manufacturer's instructions. Briefly, 10^4 cells were seeded per well into a Seahorse XF 96 cell culture microplate for 24 h, accompanied with treatment of CQ or NAC performing as described above. For ECAR, glucose (10 mM), the oxidative phosphorylation inhibitor oligomycin (1 μ M), and the glycolytic inhibitor 2-DG (50 mM) were sequentially injected into each well at the indicated time points; and for OCR, oligomycin (1 μ M), the reversible inhibitor of oxidative phosphorylation FCCP (p-trifluoromethoxy carbonyl cyanide phenylhydrazine, 1 μ M), and the mitochondrial complex I inhibitor rotenone plus the mitochondrial complex III inhibitor antimycin A (Rote/AA, 1 μ M) were sequentially injected. All data was normalized by protein concentration via BCA assay as described above.

2.10 ROS detection and inhibition

For ROS detection, 5×10^5 cells were seeded onto 6-well plates and cultured at 37 °C for 24 h. Then, 1 ml FBS free medium diluted 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, Shanghai, China) was added in the dish after removing of old culture medium. After incubation for 20 min at 37°C, cells were washed with FBS free medium for 3 times. Then, fluorescence intensity of cell was detected with microplate reader. For ROS inhibition, 5 mM NAC was added in culture medium for 3 h. The inhibition of ROS was confirmed with DCFH-DA.

2.11 Subcutaneous xenografts of nude mice

5-week-old male Balb/c-nu mice were provided by the Beijing Vital River Laboratory Animal Technology Co. Ltd. All detailed experimental procedures were approved by the Institutional Animal Care and Utilization Committee of Fudan University Pudong Animal Experimental Center. All the mice (n = 30) were equally and randomly divided into the TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 + CQ, TPC1-SIRT6 + NAC (low), TPC1-SIRT6 + NAC (high) group. 2×10^7 TPC1-NC or TPC1-SIRT6 cells suspended in 100 μ l PBS were injected subcutaneously from the axilla of each nude mice. After 2 weeks, TPC1-SIRT6 + NAC group was treated with NAC (low: 50 mg/kg; high: 150 mg/kg) dissolved in 100 μ l normal saline via intraperitoneal injection (once every two days, for 21 days), TPC1-SIRT6 + CQ group was treated with CQ (50 mg/kg) under the same condition, TPC1-NC and TPC1-SIRT6 group were treated with 100 μ l normal saline as

placebo. The long (L) and short (S) diameter of the tumors were measured with vernier caliper every 3 days (tumor volume = $L \times S^2 / 2$). The growth curve of subcutaneous tumors was drawn on the basis of the measured tumor volume. All mice were killed 2-week treatment and subcutaneous tumors were removed completely.

2.12 PET imaging of glucose uptake in mice

PET imaging of mice was performed using an animal PET scanner (Siemens Corp.). Mice were injected intravenously with 3.7 MBq (100 mCi) of ^{18}F radio-labeled fluorodeoxyglucose (^{18}F -FDG) after anesthetization with pentobarbital. Five-minute emission scans were performed to obtain attenuation correction data in the prone position at 60 minutes after injection, and delay scans of 10 minutes were acquired at 2 hours.

2.13 Patients and specimens

PTC specimens were collected from July 2018 to July 2019. Patients with the following criteria were excluded from participation: had received adjuvant chemotherapy or radiotherapy prior to surgery; had additional cancers diagnoses. All patients were classified according to the 7th edition of the TNM staging system [23]. Postoperative adjuvant therapies were performed, according to standard schedules and doses. All participating patients gave their written informed consent. This study was approved by the Ethical Committee of Shanghai Pudong Hospital.

2.14 Statistical analysis

All the experiments were performed 3 times at least. SPSS software (version 19.0, IBM Corp., Armonk, NY, USA) was used for statistical analysis of all the experimental data. GraphPad Prism (version 7, GraphPad Software, La Jolla, CA, USA) was used to determine the statistical results. All data are expressed as the mean + standard deviation (mean + sd). The statistical analysis of the data from 2 groups was performed using a t-test. The comparisons of multiple groups were performed by one-way ANOVA and then an LSD-t test. $P < 0.05$ was considered to be significant.

3. Results

3.1 SIRT6 induces autophagy in PTC

SIRT6 stably upregulated PTC cell line TPC1-SIRT6 was successfully generated and confirmed with Western Blotting and RT-qPCR. Both mRNA (Fig. 1A) and protein (Fig. 1B) expressions of SIRT6 in the TPC1-SIRT6 group were significantly upregulated compared with the TPC1-NC group. Meanwhile, the mRNA expressions of autophagy associated gene: Beclin1, Atg7, Vps34, Bnip3, Atg4b all showed

significant upregulation in TPC1-SIRT6 compared with TPC1-NC. Similarly, In western blotting analysis, the ratio of LC3 β / α , expression of Beclin1 and ATG5 showed significantly upregulation, whereas P62 showed significantly downregulation in TPC1-SIRT6 compared with TPC1-NC. In addition, TPC1-SIRT6 showed stronger fluorescence intensity of LC3 compared with TPC1-NC (Fig. 1C, D). Finally, TEM was performed to observe autophagosome directly (Fig. 1E). We successfully found significantly more autophagosomes in TPC1-SIRT6 rather than in TPC1-NC. All these indicated SIRT6 induces autophagy in TPC-1 and similar results was indicated in K1 cell line (Figure S1B, H).

3.2 SIRT6 promotes autophagy via ROS induced ER-stress

To explore mechanism of SIRT6 induced autophagy, Western Blotting was performed to detect two most common pathways of autophagy: Akt/mTOR and Erk/mTOR. Although p-Akt was inhibited in TPC1-SIRT6, there was no significant alteration in its downstream p-mTOR. ERK/mTOR signaling pathway was not activated at all. Meanwhile two most common transcriptional factor associated with autophagy: p53 and c-Myc were detected, and no expression alteration was found similarly (Fig. 2A). Previous research demonstrated ROS induced ER- stress promotes autophagy, thus we further detected ROS of cells with 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA)(16). A significant upregulation of ROS was found in TPC1-SIRT6 compared with TPC1-NC. Then, the upregulated ROS of TPC1-SIRT6 could be inhibited by 5/15 mM ROS inhibitor NAC, whereas ER stress inhibitor 4-PBA (2 mM for 24 h) had no effect (Fig. 2B). We also found ER-stress associated p-eIF2 α /ATF4/CHOP pathway was activated in TPC1-SIRT6 compared with TPC1-NC. NAC (5 mM for 3 h) but not CQ (50 μ M for 3 h) successfully inhibited p-eIF2 α /ATF4/CHOP pathway at different level (Fig. 2C). These results indicated SIRT6 induced ER-stress via upregulating ROS, and autophagy maybe its downstream reaction. To further study association of SIRT6 induced ER-stress and autophagy, TPC1-SIRT6 was treated with NAC and 4-PBA to detect autophagy associated gene again. Both NAC (5 mM) and 4-PBA successfully rescued mRNA expression of autophagy associated gene in TPC1-SIRT6(Fig. 2D). Similar in Western blotting analysis, ratio of LC3 β / α , expression of Beclin1, Atg5, p62 rescued in both TPC1-SIRT6 + NAC and TPC1-SIRT6 + 4-PBA group compared with changes of TPC1-SIRT6 (Fig. 2E). Similar results were indicated in K1 cell lines (Figure S1A, B, D). Thus far, we have confirmed SIRT6 induced ER-stress via upregulating ROS, and subsequently promoted autophagy. However, we were still wondering how SIRT6 increase ROS. As SIRT6 is a member of the class III histone deacetylases (HDAC) that have been demonstrated inhibited H3K56ac and H3K9ac. We consulted the CHIP-sequence datasets GSE102813 which studied SIRT6 depletion in BRAF^{V600E} melanoma cells(17). Main ROS negative regulators were assessed and we found SIRT6 binds to the promoter of NRROS accompanied with a significant H3K56ac peak. Negative results were found in other ROS scavengers such as superoxide dismutase 2 (SOD2) and glutathione peroxidase (GSH-px, GPX1) (Fig. 2F). NRROS is a recent reported ROS regulator, which inhibits ROS via interacting with NOX2(18). We found overexpression of SIRT6 significantly oppressed the expression of NRROS whereas promoted expression of NOX2. Thus, treatment with HDAC inhibitor trichostatin A (TSA, 400 nM for 12 h) could rescue its effect in both NRROS and NOX2 (Fig. 2G). Furthermore, RT-qPCR showed SIRT6 inhibited NRROS at transcriptional level, and TSA rescued its expression (Fig. 2H). Similar as trend of NRROS, TSA

successfully rescued inhibited ROS in TPC1-SIRT6 and K1-SIRT6 (Fig. 2I). To research specific mechanism at transcriptional level, CHIP analysis was performed. The promoter region of NRROS showed significant enrichment in each group with anti-SIRT6 CHIP, and overexpression of SIRT6 promoted the enrichment. Furthermore, overexpression of SIRT6 depleted H3K56ac at the promoter of NRROS (Fig. 2J). All these indicated SIRT6 binds to the promoter of NRROS and inhibited H3K56ac, thus oppressed its expression at transcriptional level, and inhibition of NRROS negatively increased ROS.

3.3 SIRT6 inhibits Warburg effect via autophagic degradation of Glut1

RT-qPCR and Western Blotting were performed to research association in vitro. The mRNA expressions of key Warburg effect genes: Glut1, PKM, LDHA was significantly decreased in TPC1-SIRT6 compared with TPC1-NC; whereas GAPDH, HK2 and PGK1 showed no change (Fig. 3A). The protein expression of Glut1 significantly decreased in the TPC1-SIRT6, whereas HK2, GAPDH showed no change (Fig. 3B). We further detected glucose uptake, lactate production from cell culture medium, and found an obvious decrease in the TPC1-SIRT6 group compared with the TPC1-NC group. Then, ATP content of two cell lines were measured by ATPLite luminescent assay. A same trend of lower ATP content was detected in the TPC1-SIRT6 group than the TPC1-NC group (Fig. 3C). All these indicated SIRT6 inhibited Warburg effect in vitro. To further explore role of ROS in SIRT6 induced autophagy and Warburg effect inhibition, TPC1-SIRT6 was treated with autophagy inhibitor CQ (50 μ M for 3 h) or ROS inhibitor NAC (5 mM for 3 h). Both CQ and NAC rescued Warburg effect gene mRNA expression at different level performed by RT-qPCR (Fig. 3A). In Western blotting analysis, we found expression of Glut1 was upregulated in NAC and CQ treated TPC1-SIRT6 compared with control TPC1-SIRT6. Though we have not found obvious difference in expression of HK2 or GAPDH between TPC1-SIRT6 and TPC1-NC groups previously. Interestingly, NAC and CQ treatment both significantly upregulated GAPDH and HK2 expression compared with control TPC1-SIRT6 (Fig. 3B). Furthermore, glucose uptake, lactate production and ATP content were measured and we found both NAC and CQ rescued these measurements at different level (Fig. 3C). Similar results were also got in K1 cell lines and these indicated SIRT6 inhibited Warburg effect via ROS induced autophagy (Figure S1C, D, E, F, G). As Glut1 showed been strongly depleted by SIRT6, we suggested the depletion came from autophagic degradation. To verify our conjecture, lysosome inhibitors CQ(50 μ M for 6 h) and protease inhibitor MG-132(10 μ M for 6 h) was used to treat PTC cells. As expect, CQ but not MG-132 significantly promoted the expression of Glut1, which indicated autophagy-lysosome pathway may be the main way for degradation of Glut1(Fig. 3D). Subsequently, each group of cells were treated with Cycloheximide (CHX), a protein synthesis inhibitor at 50 μ g/ml for 24 h. Then CHX was removed simultaneously and protein was extracted at different time (0, 15, 30, 60 min) after the withdraw to research the degradation of Glut1. We found the expression of Glut1 in TPC1-SIRT6 was far below TPC1-NC at each time point (except 60 min), whereas CQ significantly reverse the expression in TPC1-NC (Fig. 3E). Interestingly, the degradation rate of Glut1 in TPC1-SIRT6 showed slower compared with TPC1-NC and TP1-SIRT6 + CQ (Fig. 3F). We hypothesized that the low expression of GLUT1 in TPC1-SIRT6 triggers other feedback regulation to antagonize the SIRT6 induced degradation. IF was performed to

further determine the relationship between Glut1 degradation and SIRT6-induced autophagy. In TPC1-NC, Glut1 was membrane expression and LC3 showed diffuse and weak distribution in cytoplasm. Whereas in TPC1-SIRT6, Glut1 showed cytoplasmic expression, as well as co-location with stronger expressed LC3(Fig. 3G). These indicated SIRT6 inhibited membrane expression of Glut1 via autophagy-lysosome pathway. In addition, Similar results were also got in K1 cell lines (Figure S2A, B). All these results indicated SIRT6 inhibited Warburg effect via ROS induced autophagy. This inhibition may due to the autophagic degradation of Glut1.

3.4 Higher inhibition of ROS oppositely restrained Warburg effect via suppressing AMPK pathway

To further explore mechanism of ROS in Warburg effect, TPC1-SIRT6 was treated with higher concentration of NAC. In RT-qPCR, 15 mM NAC further restrained Warburg effect associated gene in TPC1-SIRT6 oppositely, as 5 mM NAC rescued inhibited Warburg effect associated gene (Fig. 4A). In Western blotting, with the increase of NAC concentration, autophagy gradually weakened. Similar to RT-qPCR result, 15 mM NAC restrained Warburg effect associated protein, which was oppositely to 5 mM NAC (Fig. 4B). Furthermore, 15 mM was more than enough to inhibited glucose uptake, lactate production and ATP content of TPC1-SIRT6 (Fig. 5C). In Western blotting, we found p-AMPK did not decrease at 5 mM concentration, but when the concentration reached 10–15 mM, p-AMPK decreased significantly, thus inhibiting AMPK signaling pathway. Therefore, AMPK activator AICAR (2 mM, 3 h) was used to treat TPC1-SIRT6 with NAC (15 mM, 3 h) simultaneously. AICAR successfully activated AMPK pathway and increased ratio of LC3 β / α . Furthermore, Warburg effect associated gene, glucose uptake, lactate production, ATP content rescued at different level. Increase of Extracellular Acidification Rate (ECAR) and decrease of Oxygen Consumption Rate (OCR) are two main indexes reflecting Warburg effect(19). As expect, overexpression of SIRT6 significantly decrease ECAR whereas increase OCR, which indicated SIRT6 repressed Warburg effect. Both CQ and NAC(5 mM) rescue effect of SIRT6. In addition, Higher concentration of NAC (15 mM) oppositely aggravated inhibition of Warburg effect in TPC1-SIRT6(Fig. 4D, E). Similar results were also confirmed in K1 cells (Figure S2C, D).

3.5 CQ and NAC promoted SIRT6 upregulated PTC growth in mice model

To confirm results acquired above in vivo, subcutaneous xenografts model was generated for different treatment. SIRT6 showed significant inhibition of tumor growth compared with NC. Both 50 mg/kg CQ and 50 mg/kg NAC rescued the inhibition of tumor growth in TPC1-SIRT6. Whereas, 150 mg/kg NAC oppositely oppressed tumor growth compared with both TPC1-SIRT6 and TPC1-NC (Fig. 5A, C). The trend of tumor weight was similar as the growth curve (Fig. 5D). Furthermore, we performed PET/CT before sacrifice of mice, a prospective clinical imaging diagnostic method in various cancer. As expected, TPC1-SIRT6 group showed depleted signal (namely depleted Warburg effect) compared with TPC1-NC. As trend of tumor growth, 50 mg/kg CQ and 50 mg/kg NAC rescued the depletion, 150 mg/kg NAC oppositely

aggravated the depletion (Fig. 5B). After mice sacrificed, the xenografts were performed with IF. As expected, TPC1-SIRT6 showed weaker expression of p62 and Glut1 compared with TPC1-NC. Both CQ and NAC (50 mg/kg) rescued expression of p62 and Glut1 in TPC1-SIRT6. Oppositely, 150 mg/kg NAC further aggravated inhibition of Glut1 (Fig. 5E). These results in vitro were in good agreement with those obtained in vitro.

3.6 Inspection of SIRT-Autophagy-Warburg effect axis in clinical specimens

To research SIRT6-Autophagy-Warburg effect axis in clinical PTC patients, 501 cases in TCGA database was analyzed by TIMER web tools (<https://cistrome.shinyapps.io/timer/>)(20). We found SIRT6 showed significant co-expression with Atg4b, Atg7 and Atg10 (Fig. 6A). Whereas showed negative co-expression with Warburg associated gene: HK2, PGK1 and HIF1A in clinical PTC patients (Fig. 6B). In addition, SIRT6 also showed significant co-expression with NRROS(LRRC33), which completely tally with results above (Fig. 6C). Subsequently, protein expression of SIRT6, p62, Glut1 in several cases were performed with Western Blotting and IHC. SIRT6 showed negative co-expression with p62 and Glut1 (Fig. 6D, E).

4. Discussion

Our previous study had demonstrated that SIRT6 was upregulated in PTC and promoted tumor invasion and migration via inducing epithelial mesenchymal transformation(21). We took a note that SIRT6 can induce both Warburg effect and autophagy in other disease from other researches. However, in tumors, especially in thyroid cancer, the role of SIRT6 on these two effects have been less studied. Therefore, we determined whether SIRT6 induce Warburg effect and autophagy in PTC firstly.

We successfully generated SIRT6-upregulated PTC cells TPC1-SIRT6 and negative controls TPC1-NC. In order to study effect of SIRT6 on autophagy, Western blotting, RT-qPCR was performed to detect key autophagy gene expression. ratio of LC3 β / α , expression of Beclin1 and ATG5 showed significantly upregulation, whereas P62 showed significantly downregulation in TPC1-SIRT6 compared with TPC1-NC. IF and TEM was further performed to observe autophagy flux and autophagosomes, and stronger autophagy flux, more autophagosomes were observed in TPC1-SIRT6. All these results indicated SIRT6 induced autophagy in TPC-1. In the further study on the mechanism of SIRT6 induced autophagy, we speculated that signaling pathways play an important role in this interaction at first. Two main common pathways: Pi3k/Akt/mTOR, MAPK/Erk/mTOR associated with Warburg effect and autophagy were detected. Although p-Akt was inhibited in TPC1-SIRT6, there was no significant change in its downstream p-mTOR. ERK/mTOR signaling pathway was not activated at all. We then turned our attention to oxidative stress, and ROS production of TPC1-SIRT6 showed significant increase compared with TPC1-NC. ER stress is closely related with ROS, as well as autophagy(22). ER stress is characterized by incorrect folding and aggregation of unfolded proteins in endoplasmic reticulum lumen and disturbance of calcium balance. It can activate signal pathways such as unfolded protein response, endoplasmic reticulum overload response. PERK, ATF6, Ire1 are three key regulation pathways of ER- stress(23). ROS-

related ER stress is most correlated with PERK signaling pathway, loss of PERK may cause defects in cell death sensitivity in pathological conditions linked to ROS-mediated ER stress(24). In addition, previous studies had shown that PERK, eIF2 α , ATF4, CHOP all work as regulation factors of autophagy(25–27). Therefore, we further detected downstream of PERK pathway: eIF2 α /ATF4/CHOP, and obvious activation was found in TPC1-SIRT6 compared with TPC1-NC. To determine causality between ROS and ER stress, TPC1-SIRT6 was treated with ROS inhibitor NAC and ER-stress inhibitor 4-PBA respectively. ROS inhibitor successfully repressed activation of eIF2 α /ATF4/CHOP, whereas, ER-stress inhibitor had no effect in ROS. This indicated upregulation of ROS is the upstream of ER stress. Furthermore, both ROS and ER stress inhibitor restrained upregulation of autophagy in TPC1-SIRT6. All these results confirmed SIRT6 induced ER stress via PERK pathway, then upregulates ROS production and finally induces autophagy. Thus far, we have confirmed SIRT6 induced ER-stress via upregulating ROS, and subsequently promoted autophagy. However, we were still wondering how SIRT6 increase ROS. As SIRT6 is a member of the class III histone deacetylases that have been demonstrated inhibited H3K56ac and H3K9ac(28). We suggested SIRT6 targeted ROS regulator at transcriptional level via H3K56ac or H3K9ac. We consulted the CHIP-sequence datasets GSE102813 which studied SIRT6 depletion in BRAF^{V600E} melanoma cells. SIRT6 binds to the promoter of NRROS accompanied with H3K56ac peak. Interestingly, BRAFV600E is also frequent mutation in PTC similar as melanoma(17). In addition, NRROS is a recent reported ROS negative regulator, which inhibits ROS via interacting with NOX2. Coincidentally, NRROS and NOX2 located in endoplasmic reticulum, which may meet ER-stress described above. Through Western blotting, RT-qPCR and CHIP analysis, we succeeded in verifying our suspect. SIRT6 binds to the promoter of NRROS and inhibited H3K56ac, thereby inhibited its expression at transcriptional level. Oppression of endoplasmic reticulum located NRROS protein promoted ROS and ER-stress sequentially.

In order to study effect of SIRT6 on Warburg effect, Western blotting, RT-qPCR was performed to detect key Warburg effect gene expression. Glut1, PKM, LDHA was significantly decreased in TPC1-SIRT6 compared with TPC1-NC; whereas GAPDH, HK2, PGK showed no change. Then we further detect glucose uptake, lactate production and ATP content of PTC cells. All these measurements decreased significantly in TPC1-SIRT6 compared with TPC1-NC. Therefore, we inquired SIRT6 inhibited Warburg effect in TPC-1. There are many similarities between Warburg effect and autophagy effect on the biological function of tumors. Previous studies have also reported that there is a close relationship between Warburg effect and autophagy effect. In hepatocellular carcinoma, phosphorylation of autophagy associated gene ATG4B at Ser34 promotes Warburg effect in by inhibiting mitochondrial function and participating in metabolic reorganization(29). In prostate cancer, co-targeting hexokinase 2-mediated Warburg effect and ULK1-dependent autophagy cause tumor regression in xenograft, leads to a near-complete tumor suppression and remarkably extends survival in Pten –/p53-deficiency-driven mouse model(30). In A549 alveolar adenocarcinoma cells, the downregulation of Warburg effect gene PKM2 induced apoptosis and autophagy and this autophagy protected the cells from apoptotic cell death(31). In summary, Warburg effect and autophagy are closely related to the occurrence and development of tumors, and their regulatory networks overlap to some extent. However, there are few studies on the relationship between Warburg effect and autophagy in thyroid cancer. As we had inquired to SIRT6 inhibited Warburg effect

and induced autophagy in PTC, we further focused our attention in interaction mechanism between SIRT6 induced autophagy and Warburg effect.

To further study interaction of Warburg effect and autophagy, TPC1-SIRT6 were treated with NAC(5 mM) and autophagy inhibitor CQ respectively to detect Warburg effect indicators. Both NAC and CQ could rescued inhibited Warburg effect in TPC1-SIRT6. Taken together, SIRT6 induced autophagy via upregulating ROS, and upregulated autophagy further inhibited Warburg effect. As autophagy-lysosome pathway a main mechanism for protein degradation, we suggest SIRT6 inhibited Warburg effect protein via autophagic degradation. As expect, lysosome inhibitor CQ but not proteasome inhibitor MG-132 rescue the expression of Glut1. Furthermore, SIRT6 depleted membrane expression of Glut1 and promoted its co-location with autophagosome marker LC3. Therefore, we infer SIRT6 inhibited Warburg effect via autophagic degradation of Glut1.

Here we find some contradictions with previous studies. ROS is a class of oxygen atoms or clusters formed by oxygen and is always reported as activator of Warburg effect via AMPK pathway(32, 33). Whereas in our research, upregulated ROS was accompanied with inhibited Warburg effect. We further increased concentration of NAC for treatment of TPC1-SIRT6. Interestingly, 15 mM NAC further inhibited autophagy, as well as restrained Warburg effect. We found that NAC at 5 mM had no effect on AMPK pathway, but at 10–15 mM, NAC significantly inhibited AMPK pathway. After treated TPC1-SIRT6 + NAC (15 mM) with AMPK activator, Warburg effect rescued again. Furthermore, we also found AMPK activation could increase autophagy directly.

Warburg effect is an important basis for rapid growth of tumors and PET/CT examination. The uptake rate and concentration of contrast media (such as 18F-FDG) in tumors with active Warburg effect are higher(34). Therefore, we learnt effect of SIRT6-ERstress-Autophagy-Warburg effect axis in growth of xenografts via animal model combined 18F-FDG PET/CT. Overexpression of SIRT6 significantly inhibited Warburg effect as well as tumor growth; however, CQ rescued the inhibition. Previous studies have suggested that chloroquine is a drug that treats cancer by inhibiting autophagy or other mechanisms(35, 36). Whereas our research found CQ promotes tumor growth via enhancing Warburg effect in SIRT6 upregulated PTC cell. Therefore, CQ should be more cautious applied in the treatment of PTC patients and SIRT6 expression needs to be assessed. Similarly, NAC plays a dual role in the Warburg effect and growth of tumors. 50 mg/kg NAC significantly enhanced Warburg effect and tumor growth, whereas 150 mg/kg NAC restrained Warburg effect and tumor growth instead. In previous studies, NAC has been considered as an anti-cancer drug, which inhibits the growth of tumors through anti-oxidative stress and other mechanisms(37, 38). However, above mishap happened in TPC1-SIRT6. Therefore, the application of NAC treatment of PTC patients with overexpression of SIRT6, we may need to increase the concentration to prevent counteraction.

5. Conclusions

From all we have discussed above, we can safely draw the conclusion that SIRT6 promotes autophagy in PTC cell via increasing ROS production, and upregulated autophagy further inhibited Warburg effect via degradation of Glut1. Furthermore, AMPK pathway acts as a negative feedback inhibitor in this process (Fig. 7).

Abbreviations

ATGs: autophagy associated genes; CQ: chloroquine; EMT: Epithelial-Mesenchymal Transition; ER-stress: Endoplasmic Reticulum stress; Glut1: Glucose Transporter 1; H3K56ac: histone H3 lysine 56 acetylation; NAC: N-acetyl-L-cysteine; NRROS: negatively regulates reactive oxygen species; PTC: papillary thyroid cancer

Declarations

Ethics approval and consent to participate

All procedures involving human participants were performed in accordance with Shanghai Pudong Hospital ethical committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All patients provided their written informed consent. The study protocol was approved by the Pudong Hospital Committee on human research.

Consent for publication

Written informed consent for publication was obtained from all the participants.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

Funding

This study was funded by Specialty Feature Construction Project of Pudong Health and Family Planning Commission of Shanghai (Min Ye, Grant No.PWZzb2017-21)

Authors' contributions

ZY and RH contributed to the experiments performing and data analysis. WY contributed to the species collection and animal model. ZM and MY contributed to the design of the study. All authors read and approved the final version of the manuscript.

Acknowledgements

Not applicable

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Table

Gene	Forward Primer	Reverse Primer
SIRT6	GCACCGTGGCTAAGGCAAGG	GTGATGGACAGGTCGGCGTTC
Beclin1	CAAGATCCTGGACCGTGTCA	TGGCACTTTCTGTGGACATCA
Bnip3	TCCACTTCAGACACCCTA	CTCAGTCGCTTTCCAATA
Vsp34	GGACCTTCTGACCACGAT	GCAACAGCATAACGCCTC
ATG7	TGTATAACACCAACACACTCGA	GGCAGGATAGCAAAACCAATAG
ATG4B	AGAGCCCGTTTGGATACT	GTCGATGAATGCGTTGAG
Actin	GGGACCTGACTGACTACCTC	TCATACTCCTGCTTGCTGAT
PKM	ACTGGCATCATCTGTACCATTG	AGCCACATTTCATTCCAGACTTA
LDHA	GGTTGGTGCTGTTGGCATGG	TGCCCCAGCCGTGATAATGA
HK2	CGACAGCATCATTGTAAAGGAG	GCAGGAAAGACACATCACATTT
Glut1	CTGGCATCAACGCTGTCTTC	GCCTATGAGGTGCAGGGTC
Eno1	GCCTCCTGCTCAAAGTCAAC	AACGATGAGACACCATGACG
PGK1	TTCTGTTCTTGAAGGACTGTGT	CTTTAACCTTGTTCCCAGAAGC
GAPDH	GTCAAGGCTGAGAACGGGAA	AAATGAGCCCCAGCCTTCTC
NRROS	AGTCTGCAAGTTGGTGGGTG	GGTCTTGAGAGGGTTGGCAT

Table 1 The Primers of RT-qPCR

Supplementary Figure Legends

Supplementary Figure 1. A. The protein expression of PERK/ATF4/CHOP pathway of each group in K1 cells. B. The expression of autophagy associated protein of each group in K1 cells. C. The expression of Warburg effect associated protein of each group in K1 cells. D. ROS production of each group in K1 cells detected by DCFH-DA. E. Glucose uptake of each group in K1 cells. F. Lactate production of each group in K1 cells. G. ATP content of each group in K1 cells. H. Autophagosome monitored by IF of LC3 in K1 cells. (All compared with K1-SIRT6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Supplementary Figure 2. A. Expression of Glut1 at different time after withdraw of 24h CHX treatment, and normalized by Actin. (*k1-NC vs k1-SIRT6, $k^*p < 0.05$; # k1-SIRT6 vs k1-SIRT6+CQ, $\#p < 0.05$). B. Expression and location of Glut1 and LC3 performed by IF in K1 cells. C. ECAR of each group in K1 cells detected by Seahorse Xf96. D. OCR of each group in K1 cells detected by Seahorse Xf96. (* $p < 0.05$, *** $p < 0.001$)

Figures

Figure 1

SIRT6 induces autophagy in PTC cells. A. mRNA expression of autophagy associated genes (Beclin1, Atg7, Vps34, Bnip3, Atg4b) in TPC1-SIRT6 and TPC1-NC detected by RT-qPCR. B. Protein expression of autophagy associated genes (p62, Beclin1, LC3B) detected TPC1-SIRT6 and TPC1-NC by Western Blotting. C. D. The immunofluorescence of LC3 in TPC1-SIRT6 and TPC1-NC. E. Autophagosome observation via TAE in TPC1-SIRT6 and TPC1-NC (Arrow: Autophagosome). (** $p < 0.01$, *** $p < 0.001$).

Figure 4

SIRT6 promotes autophagy via ROS induced ER-stress. A. The protein expression of common autophagy associated pathways (Akt/mTOR, Erk/mTOR) and transcriptional factors (p53, c-Myc) in TPC1-SIRT6 and TPC1-NC detected by Western blotting. B. ROS production of TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ (50μM for 3h) and ER-stress inhibitor 4-PBA (2mM for 24h). C. Protein expression of ER stress associated PERK/ATF4/CHOP pathway in TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with CQ and

ROS inhibitor NAC (5mM for 3h). D. mRNA expression of autophagy associated genes (Beclin1, Atg7, Vps34, Bnip3, Atg4b) in TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ and 4-PBA. E. Protein expression of autophagy associated genes (LC3B, p62, Atg5, Beclin1) in TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with CQ and NAC. F. CHIP-sequence of SIRT6 depletion in BRAFV600E melanoma cells extracted from GSE102813. G. Protein expression of NRROS and NOX2 in TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with histone deacetylation inhibitor Trichostatin A (TSA, 2mM for 24h). H. mRNA expression of NRROS in TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with TSA. I. ROS production of TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with TSA. J. Interaction of SIRT6 and H3K56ac with promoter region of NRROS in PTC cells (TPC1-NC, TPC1-SIRT6, K1-NC, K1-SIRT6) detected by CHIP analysis. (*p<0.05, **p<0.01, ***p<0.001)

Figure 7

SIRT6 inhibits Warburg effect via autophagic degradation of Glut1. A. mRNA expression of Warburg effect associated genes (Glut1, GAPDH, PKM, LDHA, PGK1, HK2) in TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ and NAC. B. Protein expression of Warburg effect associated genes (Glut1, HK2, GAPDH) in TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ and NAC. C. Glucose uptake, lactate production and ATP content of TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ and NAC. (*p<0.05, **p<0.01,

***p<0.001). D. Protein expression of Glut1 in TPC-1 and K1 treated with CQ and proteinase inhibitor MG-132(10μm for 6h). E. F. Protein expression of Glut1 at different time (0, 15, 30, 60min) after withdraw of 24h CHX treatment in TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with CQ. (*TPC1-NC vs TPC1-SIRT6, **p<0.01, ***p<0.001; # TPC1-SIRT6 vs TPC1-SIRT6+CQ, ###p<0.001). G. Expression and location of Glut1 and LC3 in TPC1-NC and TPC1-SIRT6.

Figure 10

Higher inhibition of ROS oppositely restrained Warburg effect via suppressing AMPK pathway. A. mRNA expression of Warburg effect associated gene (Glut1, GAPDH, PKM, LDHA, PGK1, HK2) in TPC1-SIRT6 treated with different concentration of NAC (0, 5, 15mM for 3h) as well as AMPK pathway activator AICAR (2mM for 3h). B. Protein expression of Warburg effect associated protein (Glut1, HK2, GAPDH), LC3B, p-AMPK in TPC1-SIRT6 treated with different concentration of NAC as well as AMPK pathway activator AICAR. C. Glucose uptake, lactate production and ATP content in TPC1-SIRT6 with different concentration of NAC treatment as well as AMPK pathway activator AICAR. (All compared with TPC1-SIRT6+NAC 0mM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). D. ECAR of TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with CQ and NAC (low: 5mM for 3h, high: 15mM for 3h) detected by Seahorse Xf96. E. OCR of TPC1-NC, TPC1-SIRT6 and TPC1-SIRT6 treated with CQ and NAC (low: 5mM for 3h, high: 15mM for 3h) detected by Seahorse Xf96. (* $p<0.05$, *** $p<0.001$)

Figure 13

CQ and NAC promoted SIRT6 upregulated PTC growth in mice model. A. Subcutaneous xenografts derived from each group: TPC1-NC, TPC1-SIRT6, TPC1-SIRT6 treated with CQ, TPC1-SIRT6 treated with NAC (low: 50mg/kg, high: 150mg/kg). B. PET/CT image performed before sacrifice of mice in each group described above. C. Tumor growth curve of each group described above. D. Tumor weight of each group

described above. E. Expression and location of SIRT6, p62, Glut1 performed by IF in each group described above (*p<0.05, ***p<0.001).

Figure 16

Inspection of SIRT-Autophagy-Warburg effect axis in clinical specimens. A. Co-expression of SIRT6 and autophagy associated genes (ATGs) analyzed by TIMER web tools based on TCGA database. B. Co-expression of SIRT6 and Warburg effect associated genes analyzed by TIMER web tools based on TCGA

database. C. Co-expression of SIRT6 and NRROS(LRRC33) analyzed by TIMER web tools based on TCGA database. D. Protein expression of SIRT6, p62 and Glut1 in PTC specimens performed by Western Blotting. E. Protein expression and location of SIRT6, p62 and Glut1 in PTC specimens performed by IHC.

Figure 7

Figure 19

SIRT6-Autophagy-Warburg effect in PTC.

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