Protein Phosphatase 2a Inhibits Gastric Cancer Cell Glycolysis by Reducing MYC Signaling

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Abstract
Aerobic glycolysis, also known as the Warburg effect, has emerged as a hallmark of cancer and is associated with tumor progression and unfavorable clinical outcomes in cancer patients. PP2A is a highly conserved eukaryotic serine/threonine protein phosphatase that functions as a tumor suppressor in a variety of human cancers. However, the relationship between PP2A and the Warburg effect in gastric cancer has yet to be fully understood. In this study, the expression profile of two endogenous inhibitors of PP2A, SET and CIP2A, in gastric cancer, were analyzed by real-time quantitative polymerase chain reaction. Loss-of-function and gain-of-function studies were performed to investigate the roles of PP2A in gastric cancer cell proliferation and glycolysis. Cell biological, molecular, and biochemical approaches were employed to uncover the underlying mechanisms. The results showed that SET and CIP2A were overexpressed in gastric cancer and associated with a decreased PP2A activity. Pharmacological activation of PP2A with FTY-720 and DT-061 in two gastric cancer cell lines significantly reduced gastric cancer cell proliferation and glycolytic ability. Importantly, inhibition of PP2A activity by genetic silencing of PPP2R5A resulted in a growth advantage, which can be largely compromised by the addition of the glycolysis inhibitor 2-Deoxy-D-glucose, suggesting a glycolysis-dependent effect of PP2A in gastric cancer. Mechanistically, the well-known transcription factor and glycolysis regulator c-Myc was discovered as the functional mediator of PP2A in regulating cell glycolysis. Ectopic expression of a phosphorylation-mutant c-Myc resistant to PP2A (MycT58A) restored the inhibitory effect of FTY-720 and DT-061 on lactate production and glucose uptake. Furthermore, there was a close association between SET and CIP2A expression and c-Myc gene signatures in gastric cancer samples. Collectively, this study provides strong evidence of the involvement of PP2A in the Warburg effect and indicates that it could be a novel antitumor strategy to target tumor metabolism in gastric cancer.

Keywords PP2A · Warburg effect · Gastric cancer · SET · CIP2A

Introduction
The glucose is converted into lactate regardless of whether oxygen is present in cancer cells, a phenomenon called aerobic glycolysis, also known as the Warburg effect. The Warburg effect, an emerging hallmark of cancers, can enable tumor progression through multiple mechanisms [1, 2]. Firstly, an increased glycolytic rate for adenosine triphosphate (ATP) generation and biosynthesis (the Warburg effect) sustains a high proliferation rate [3, 4]. Secondly, increased lactate generation from the Warburg effect can acidify the tumor microenvironment to enhance disruption of tissue structure and immune cell evasion. Therefore, the enhanced Warburg effect is closely associated with tumor progression and blocking the glycolytic phenotypes of cancer cells significantly attenuated tumor growth, metastasis, and drug resistance [5–7].

Protein Phosphatase 2A (PP2A) is a highly conserved eukaryotic serine/threonine protein phosphatase with functions that counter-balance kinase-mediated phosphorylation [8]. PP2A is a heterotrimeric enzyme comprised of a...
scaffolding subunit (A), a regulatory subunit (B) and a catalytic subunit (C) [9]. PP2A is frequently inactivated in human cancers and is considered a tumor suppressor [10, 11]. Therefore, strategies for improving PP2A activity have been regarded as a promising therapeutic intervention. PP2A activity is tightly regulated by several endogenous inhibitors especially SET (I2PP2A, inhibitor 2 of PP2A) [12] and cancerous inhibitors of PP2A (CIP2A), which are overexpressed in a variety of human cancers [10]. SET is first identified as an inhibitor of PP2A through isolation from bovine kidney and is upregulated in many cancers such as leukemia and breast cancer [13]. CIP2A is an oncoprotein that prevents PP2A-mediated dephosphorylation of c-Myc at Ser 62 and contributes to the stabilization of c-Myc [10]. PP2A has been shown to regulate many cellular processes in gastric cancer including, but not limited to, growth, stemness, and apoptosis [14–17]. However, the link between PP2A and the Warburg effect remains largely unknown.

In this study, we show that highly expressed SET and CIP2A lead to reduced PP2A activity in gastric cancer. Pharmacological activation of PP2A with FTY-720 or DT-061 decreases the colony formation ability and glycolytic capacity of gastric cancer cells. PP2A activation decreases phosphorylated c-Myc levels and c-Myc transcriptional activity. Taken together, our study suggests that PP2A plays an important role in the Warburg effect by modulating c-Myc signaling.

Materials and Methods

Gene Expression Analysis

For analysis of SET and CIP2A expression in gastric cancer samples, data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) were used and analyzed by the GEPIA2 database [18], which is available at the website http://gepia2.cancer-pku.cn/#index. TCGA RNA-seq data of gastric cancer samples (n = 408) was used for correlation analysis and the correlation coefficient was determined by the Spearman method.

Cell Culture Condition and Reagents

The gastric cancer cell lines used in this study were obtained from American Type Culture Collection (ATCC, VA, USA) or the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Gastric cancer cells were cultured in RPMI-1640 medium or DMEM (Gibco, USA) with 10% (v/v) of fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) streptomycin-penicillin (Sigma-Aldrich, Shanghai, China) as supplementations. All gastric cancer cells were placed at 37 °C in an atmosphere containing 5% CO₂. FTY-720 (SET inhibitor, S5002), DT-061 (PP2A activator, S8774), and 2-Deoxy-D-glucose (2-DG, S4701) were all brought from Selleck (Shanghai, China).

Cell Transfection

Two specific siRNAs against PPP2R5A were employed to inhibit PPP2R5A expression in gastric cancer cells. MycT58A was cloned into pcDNA3.1 for ectopic expression. For siRNA experiment, the siRNAs of PPP2R5A were obtained from Genepharma Biotechnology (Shanghai, China); the antisense sequences were: PPP2R5A-#1: TTAGTTGAAACC-TACTCAACCA; PPP2R5A-#2: TTTAATTATATTA-TACTGATGA. Scramble non-target siRNAs were used as negative controls. Cell transfection was performed with 15 μmol siRNAs by Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol. Cells were collected 48 h after transfection and used for further experiments.

Detection of PP2A Activity

The PP2A activity in response to FTY-720, DT061, and PPP2R5A knockdown was detected by PP2A Immunoprecipitation Phosphatase Assay Kit (Millipore, USA) according to the manufacturer’s protocol. Briefly, gastric cancer cells with indicated treatment were lysed by NP-40 lysis buffer supplemented with protease inhibitor cocktail. Then, cell lysates were mixed with PP2A antibody and protein A slurry. After washing with TBS and assay buffer three times, phosphopeptide was added and allowed to incubate for 10 min at room temperature. Finally, Malachite green solution was added, followed by absorbance detection at 650 nm.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA in gastric cancer cells was isolated using TRIzol reagent (Invitrogen, USA) and then reversely transcribed to cDNA with the PrimeScript RT Master Mix Kit (RR036A; Takara). Next, cDNA was subjected to PCR detection with 2 × SYBR Green PCR Master Mix (Takara). All real-time qPCR reactions were performed in triplicate. In this study, the primer sequences used were shown as follows: HK2 forward, 5′-TTGACCAGGAGATTGACATGGG-3′; HK2 reverse, 5′-CAACCGCATAAGCACCTCA-3′; PFKL forward, 5′-GCTGGGGGCACCTATCCT-3′; PFKL reverse, 5′-TACGTGTCGAGTAGGTCCG-3′; LDHA forward, 5′-ATGGAATACACTGAGACCTCA-3′; LDHA reverse, 5′-CCAACCCCAACTCCTATCT-3′; GPDH forward, 5′-CTGGCGTACACTGAGCACC-3′; GPDH reverse,
5-AAGTGGTCGTTGAGGGCAATG-3'. GAPDH was used as an internal control.

**Western Blotting Analysis**

Total protein samples from gastric cancer cells were obtained using lysis buffer (Sangon, Shanghai, China) supplemented with 1% protease and phosphatase inhibitors. After centrifugation at 4 °C for 30 min, Bicinchoninic Acid Protein Assay kits (Thermo Fisher Scientific, USA) were used to determine the protein concentrations in the light of specifications. Finally, protein samples were incubated with 1 × SDS PAGE solution and denatured in boiling water for 10 min. The denaturized proteins were separated on 6–12% PAGE gels and transferred onto 0.2 μm polyvinylidene fluoride (PVDF) membranes, followed by blocking with 5% skimmed milk for 1 h at room temperature, then incubated with primary antibodies. In this study, the following antibodies were used for western blotting analysis: anti-PPP2R5A (Abcam, ab89621, 1:2000 dilution), anti-c-Myc antibodies (Cell Signaling Technology, #5605, 1:1000 dilution), anti-p-c-Myc (S62) antibodies (Cell Signaling Technology, #13748, 1:1000 dilution), anti-β-actin antibody (Sigma, A2228, 1:5000 dilution). After incubation with corresponding species-specific secondary HRP-conjugated antibodies, the target protein bands were visualized by an ECL imaging system.

**Glucose Uptake and Lactate Production**

The gastric cancer cell supernatants were collected 48 h after cell transfection. After appropriate dilution, the glucose and lactate levels in the cell supernatants were measured using the commercial glucose assay kit (Biovision, Milpitas, CA, USA) and lactate assay kit (Biovision, Milpitas, CA, USA), respectively.

**Cell Proliferation Assay**

The plate colony formation assay was used to measure cell proliferation ability. Briefly, about 500 suspended gastric cancer cells were seeded in the 6-well plates and cultured for 2 weeks. Next, cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The experiment was performed in triplicate. Finally, the number of colonies with more than 50 cells was calculated.

**Measurement of c-Myc Transcriptional Activity**

The c-Myc transcription factor assay Kit (Abcam, ab207200) was used to quantify c-Myc activation in nuclear
extracts from gastric cancer cells. In brief, HGC-27 and MGC-803 cells were subjected to treatment with 5 μM FTY-720 or 10 μM DT-061 for 24 h, followed by detection of c-Myc transcriptional activity according to the manufacturer’s instructions.

**Statistical Analysis**

All data were represented as mean ± standard deviation (SD). GraphPad 6.0 (GraphPad Software Inc., San Diego, CA) and Microsoft office 2016 EXCEL (USA) were used for statistical analysis. The correlation of gene expression was evaluated by Spearman’s correlation. P-values were calculated by two-tailed unpaired Student’s t-test or one-way ANOVA followed by Tukey’s tests. P-value less than 0.05 was considered to be statistically significant.

**Results**

**Increased SET and CIP2A Expression are Associated with Reduced PP2A Activity in Gastric Cancer**

To investigate the role of PP2A in gastric cancer, we first determined the expression of SET and CIP2A, two known oncogenic inhibitors of PP2A. Consistent with previous...
reports, data obtained from TCGA and GTEx cohorts showed that SET and CIP2A expression levels were high in gastric cancer tissues compared with normal samples (Fig. 1A). By real-time qPCR analysis, we found that SET and CIP2A mRNA expression was significantly increased in 87.5% (7/8) and 75% (6/8) of the gastric cancer cell lines,
The effect of FTY-720 (1 μM, 2 μM and 5 μM) on the lactate production of HGC-27 and MGC-803 cells. B The effect of DT-061 (1 μM, 5 μM and 10 μM) on the lactate production of HGC-27 and MGC-803 cells. C The effect of FTY-720 (1 μM, 2 μM and 5 μM) on the glucose uptake of HGC-27 and MGC-803 cells. D The effect of DT-061 (1 μM, 5 μM and 10 μM) on the glucose uptake of HGC-27 and MGC-803 cells. E Real-time qPCR analysis of glycolytic genes (HK2, PFKL and LDHA) in HGC-27 and MGC-803 cells in the presence or absence of treatment with 5 μM FTY-720 or 10 μM DT-061. F Correlation analysis of SET and CIP2A expression and glycolytic genes (HK2, PFKL and LDHA) in gastric cancer; data were acquired from TCGA cohort. *p < 0.05; **p < 0.01 respectively (Fig. 1B). By measuring the PP2A activity, we found that 75% (6/8) of the gastric cancer cell lines had an increased PP2A activity compared with the nonmalignant GES1 cells (Fig. 1C). Of note, SET or CIP2A expression was closely associated with a reduced PP2A activity in gastric cancer cell lines (Fig. 1D). Therefore, decreased PP2A activity in gastric cancer appears to be induced by aberrant SET and CIP2A expression.

**FTY-720 and DT-061 Suppress Gastric Cancer Proliferation in a Dose-Dependent Manner**

To better understand the role of PP2A in gastric cancer, we used a known SET inhibitor FTY-720 that activates PP2A and a small-molecule activator of PP2A DT-061 to treat HGC-27 and MGC-803 cells [11]. Based on previous reports [19–21], different concentrations of FTY-720 (1, 2, and 5 μM) and DT-061 were used (1, 5, and 10 μM). As shown in Fig. 2A, B, FTY-720 and DT-061 treatment for 24 h led to a significant increase in PP2A activity in HGC-27 and MGC-803 cells. In the long-term observation of cell proliferation (14 days), plate colony formation assay showed that FTY-720 and DT-061 suppressed the colony formation ability of HGC-27 and MGC-803 cells in a dose-dependent manner (Fig. 2C, D), suggesting the tumor-suppressive role of PP2A in gastric cancer.

**PP2A Activation Inhibits the Warburg Effect in Gastric Cancer**

Occasionally, we found that cell culture medium was acidified much slower after FTY-720 and DT-061 treatment for 24 h. Therefore, we speculated a potential role of PP2A in regulating lactate production, a classical character of the Warburg effect. To test this hypothesis, we measured the lactate levels in the culture medium of HGC-27 and MGC-803 cells after treatment with different concentrations of FTY-720 and DT-061. After normalization with total cell protein level, we observed that the lactate level was significantly reduced by FTY-720 in HGC-27 and MGC-803 cells in a dose-dependent manner (Fig. 3A). Likewise, similar effects were also induced by DT-061 (Fig. 3B). To further confirm the impact of PP2A on the Warburg effect, we tested the glucose uptake upon FTY-720 and DT-061 treatment in gastric cancer cells. As a result, both FTY-720 and DT-061 resulted in a marked reduction of glucose uptake in HGC-27 and MGC-803 cells (Fig. 3C, D). Moreover, using real-time qPCR, we found that glycolytic genes including HK2, PFKL and LDHA were also significantly downregulated by FTY-720 or DT-061 treatment (Fig. 3E). Consistently, correlation analysis revealed that there was a high correlation between SET and CIP2A expression and glycolytic genes (HK2, PFKL and LDHA) in gastric cancer samples (Fig. 3F).

**Growth Advantage Induced by PP2A Inhibition is Glycolysis-Dependent in Gastric Cancer**

Because that PP2A can inhibit gastric cancer proliferation and reduce the glycolytic ability, we next tested whether PP2A affects tumor growth via modulation of tumor glycolysis. To address this hypothesis, we aimed to genetic manipulation of PP2A activity. Because the regulatory B subunit of PP2A complex dictates its subcellular localization and substrate specificity, we therefore genetically silenced PPP2R5A, a regulatory B subunit of the major PP2A protein complex [22], in two gastric cancer cell lines, MKN28 and SGC-7901. The knockdown efficiency was shown in Fig. 4A. PPP2R5A knockdown led to a significant decrease in PP2A activity (Fig. 4B). Interestingly, lactate production (Fig. 4C) and glucose uptake (Fig. 4D) of MKN28 and SGC-7901 were remarkably upregulated by PPP2R5A knockdown. Moreover, plate colony formation assay showed that PPP2R5A knockdown boosted cell proliferation of MKN28 and SGC-7901 cells, which can be largely abrogated by addition of the known glycolysis inhibitor 2-DG (Fig. 4E). Collectively, these data above suggest that PP2A couples cell glycolysis to tumor cell proliferation in gastric cancer.

**PP2A Modulates c-Myc Expression to Suppress Gastric Cancer Glycolysis**

PP2A complex dephosphorylates serine 62 (S62) of c-Myc, rendering c-Myc as a substrate for FBXW7-mediated ubiquitination and subsequent degradation by the 26S proteasome. Importantly, c-Myc is a known transcriptional factor of the Warburg effect [23]. Therefore, we reasoned that PP2A targets c-Myc to modulate gastric cancer cell glycolysis. By western blotting, we found that PP2A activation by FTY-720 or DT-061 decreased the S62-phosphorylated c-Myc and total c-Myc levels in HGC-27 and MGC-803 cells (Fig. 5A). In contrast, S62-phosphorylated c-Myc and total c-Myc levels were...
reduced upon genetic inhibition of PPP2R5A in MKN28 and SGC-7901 cells (Fig. 5B). As the second line of evidence, c-Myc transcriptional activity was also suppressed by FTY-720 and DT-061 treatment (Fig. 5C). To determine whether aberrant expression of c-Myc can restore the suppressive effect induced by PP2A activation, we ectopic introduced MycT58A, a phosphorylation-mutant resistant to PP2A in HGC-27 and MGC-803 cells. As shown in Fig. 5D, the inhibitory effect of FTY-720 and DT-061 treatment on lactate production and glucose uptake was largely restored by MycT58A. Furthermore, correlation analysis showed that SET or CIP2A expression was positively associated with c-Myc gene signature in gastric cancer samples (Fig. 5E). Collectively, c-Myc is a critical mediator for PP2A-induced suppressive effects on cell proliferation and glycolysis in gastric cancer (Fig. 5F).
Discussion

PP2A plays diverse roles in human cancers [11, 24, 25]. In this study, we investigated (i) the expression pattern of two cellular inhibitors of PP2A, SET and CIP2A, in gastric cancer, (ii) the effect of PP2A activation on tumorigenic potential and the Warburg effect, (iii) whether increased growth advantage induced by PP2A inhibition is glycolysis-dependent, (iv) the underlying molecular mechanism by which PP2A regulates the Warburg effect. Our results suggest that PP2A is profoundly implicated in the Warburg effect and be exploited as potential target for gastric cancer therapy.

It is well documented that increased expression of PP2A-inhibitory proteins such as SET and CIP2A contributes to decreased PP2A activity in cancers [26]. SET protein levels are highly expressed in various human tumors, including chronic myeloid leukemia, pancreatic cancer, and colorectal cancer; in gastric cancer, SET is reported to maintain cancer cell stemness by suppressing PP2A activity and stabilizing E2F1 protein [27]. Moreover, CIP2A is overexpressed in gastric cancer and predicts a poor prognosis; CIP2A knockdown is shown to negatively affect clonogenicity and senescence of tumor cells [28–30]. In this report, we confirmed that SET and CIP2A expression was highly expressed in gastric cancer samples and their dysregulation led to reduced PP2A activity.

SET can be targeted by FTY-720 (Fingolimod), a sphingosine analogue, which is an FDA-approved immunosuppressant used for the treatment of multiple sclerosis [8]. FTY720 is able to disrupt the interaction between SET and PP2A, thus resulting in elevated PP2A activity.

Fig. 5 PP2A modulates c-Myc expression to suppress gastric cancer glycolysis. A Western blotting analysis of c-Myc and p-c-Myc (S62) levels in HGC-27 and MGC-803 cells in the presence or absence of treatment with 5 μM FTY-720 or 10 μM DT-061. B Western blotting analysis of c-Myc and p-c-Myc (S62) levels in si-Control and si-PPP2R5A MKN28 and SGC-7901 cells. C Measurement of the effect of 5 μM FTY-720 or 10 μM DT-061 treatment on c-Myc transcriptional activity in HGC-27 and MGC-803 cells. D The effect of 5 μM FTY-720 or 10 μM DT-061 treatment on lactate production or glucose uptake in HGC-27 and MGC-803 cells transfected with or without MycT58A. E Correlation analysis of SET and CIP2A expression and c-Myc gene signatures in gastric cancer; data were acquired from TCGA cohort. F Model illustrating the mechanism by which PP2A regulates c-Myc to inhibit the Warburg effect and cell proliferation in gastric cancer. *p < 0.05; **p < 0.01
FTY720 has been reported to increase the expression of Cip1/p21, p27, and BH3-only proteins to induce gastric cancer cell apoptosis and have an additive effect in killing cancer cells when in combination with Cisplatin [31]. Consistently, we revealed that FTY720 suppressed gastric cancer cell proliferation in a dose-dependent manner. Moreover, activation of PP2A with DT-061, a phenothiazine derivative, induced similar tumor-suppressive effects in gastric cancer cells. Apart from previous reports regarding the roles of PP2A in gastric cancer, we for the first time identified a novel link between PP2A and the Warburg effect. FTY720 and DT-061 treatment significantly boosted the Warburg effect of gastric cancer cells as evidenced by increased lactate release, glucose uptake, and expression of glycolytic genes. Moreover, PP2A inactivation by knockdown of PPP2R5A facilitated cell growth and blocking glycolysis with 2-DG largely abrogated this effect. Therefore, our findings further broaden the roles of PP2A in gastric cancer and suggest a glycolysis-dependent effect of PP2A.

Prior works have highlighted c-Myc as a critical substrate of PP2A complex in cancers [24, 32–34]. For instance, PP2A activation by a novel SET antagonist OP449 decreases S62 phosphorylation of c-Myc and reduces c-Myc activity and expression of downstream target genes in breast cancer [35]. In MYC-driven cancer, DT-061 treatment inhibited c-Myc expression via proteasome-mediated degradation, resulting in tumor growth inhibition [36].

Given the important role of c-Myc in regulating the Warburg effect, we also tested c-Myc changes in the presence of PP2A activation. Expectedly, S62 phosphorylation of c-Myc was reduced by FTY720 or DT-061 treatment in gastric cancer cells and ectopic expression of c-MycT58A was competent to restore the inhibitory effect of PP2A on the Warburg effect. This finding was also consistent with a previous report that PP2A can induce remarkable downregulation of c-Myc and pyruvate kinase M2 isoform to inhibit the Warburg effect [33]. Moreover, many reports have documented that the key glycolytic enzymes including PKM2 and LDHA can be regulated by phosphorylation. Previously, PP2A has been reported to inhibit PFKFB2-induced glycolysis to promote termination of liver regeneration [34]. Therefore, PP2A might regulate glycolysis through multiple mechanisms. In this study, we cannot fully rule out the possibility of PP2A-dependent phosphorylation of glycolytic enzymes. Future work comprehensively illustrating the full spectrum of substrates of PP2A and whether these substrates are involved in the regulation of the Warburg effect may be very important.

In conclusion, our results reveal that dysregulation of PP2A-inhibitory proteins leads to reduced PP2A activity. We propose that PP2A regulates c-Myc activity to transcriptionally repress the expression of glycolytic genes and the Warburg effect. Given the important role of the Warburg effect in facilitating tumor growth, our data further supports the pursuit of PP2A as a target for cancer therapy in gastric cancer.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

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