

Twist1-Overexpression Induces Glycolytic Reprogramming and Atp Production In 293t Cells

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ABSTRACT

Reprogramming of glucose metabolism in cancer cells triggers tumor development. Most cancer cells provide the energy needed from aerobic glycolysis called the "Warburg effect" to promote uncontrolled proliferation and invasion. Therefore, direct regulator molecules of aerobic glycolysis remain active research targets. Epithelial mesenchymal transition (EMT) is the main mechanism that promotes cancer invasion and poor prognosis. One of the key effector molecules of the EMT is TWIST1. In this study, we sought to investigate the role that TWIST1 has in driving glycolytic programming and cellular energy charges in 293T cells by overexpressing TWIST1 in 293T cells. Plasmid vectors were successfully transfected by lipofectamine 2000 and mRNA expressions of interested genes were assessed. Glucose, lactate and pH levels of culture supernatants were determined by radiometer analyzer. The cellular energy charge of the cells were calculated from ATP, ADP and AMP data analyzed by HPLC.

Here, we found that TWIST1 transcription factor, which has highly conserved sequences, is an important regulator for aerobic glycolysis. We found that TWIST1 increases the expression of glycolytic genes such as HK2, PKM2, LDH α , PFKM and G6PD, the production of lactate and extracellular acidification in 293T cells. We have also determined that TWIST1 promotes aerobic glycolysis metabolism by providing cellular energy exchange. TWIST1 overexpression reduced AMP/ATP and ADP/ATP ratios in 293T cells, with further increase seen in ATP production. By this work, we confirmed that TWIST1 is closely related to the glycolysis pathway and is an important regulator of the Warburg effect.

Key Words: TWIST1, Glycolysis, ATP

Introduction

Cancer cells alter their metabolism programme to promote survival, proliferation, and longtime maintenance. Cancer cells promote aerobic glycolysis process to generate energy for themselves. The main feature of this alteration is increased fermentation of intracellular glucose to produce lactate. Reprogramming the energy metabolism is considered as a hallmark of cancer (1, 2).

Glycolysis is the formation of pyruvate or lactic acid from glucose following glucose uptake by glucose transporters. Each reaction is catalyzed by a specific enzyme for high energy (ATP) formation. Following glucose uptake, glycolysis is arranged at several steps such as initially glucose phosphorylation is catalyzed by hexokinase II (HK2), then fructose-6-phosphate converted into fructose-1,6-bisphosphate by phosphofructokinase (PFK) and phosphoenolpyruvate (PEP) to pyruvate is catalyzed by pyruvate kinases (PK) (3, 4).

HK2, an isoform of hexokinases, plays an essential role in rearranging cellular glycolytic metabolism. HK2 interacts with the voltage-dependent anion channels (VDAC) of the mitochondrial external membrane and creates the HK2-VDAC complex. By this way, glucose is activated for the first step of glycolytic pathway (5, 6). Then, PFK converts fructose-6-phosphate+ATP into fructose-1,6-bisphosphate+ADP. Another rate limiting enzyme PK catalyzes the final speed limiting step of glycolysis which produce pyruvate. With increasing rates of glucose in cytosol PKM2 (M2 isoforms) monomers forms functional tetramers. Tetrameric structure can easily disband by decreasing glucose levels due to its allosteric activation (4). Under normal physiological conditions, pyruvate enters the Krebs cycle and decarboxylated to generate acetyl-CoA. Under hypoxic conditions lactate dehydrogenase (LDH), reversible catalyzes pyruvates to lactate and the oxidation of NADH to NAD⁺ (7). Also, the pentose phosphate pathway is an alternative to glycolysis in cell metabolism. G6PD is the main enzyme that catalyzes the first step of this

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pathway and its expression is increased in cancer cases. The most important role of the G6PD enzyme is to regulate redox potential and extracellular pH levels (8).

TWIST1 gene, encoding a basic helix-loop-helix (bHLH) transcription factor, is localized in 7p21 and consists of two exons and an intron. Only the first exon coding the protein (9). *TWIST1* transcription factor is known to serve as a primary regulator in epithelial mesenchymal transition (EMT) process and so triggers invasion and metastasis of cancer cells (10). Nonetheless, it still remains unclear the possible role of *TWIST1* on energy metabolism of cancer cells.

In this study, we showed that *TWIST1* transcriptionally upregulated glycolytic rate limiting step enzymes and cellular energy levels, consequently causes acidosis through promoting the production of lactic acid.

Materials and Methods

Cell culture: 293T cells which contains the SV40 T-antigen, (ATCC, VA, USA) were cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco; USA), %1 penicillin/streptomycin/amphotericin B (Gibco, USA). The cells were cultured in a humidified incubator with 5% CO₂ incubator at 37°C.

Cell transfection: The empty plasmid vector (pcDNA3.1) (Invitrogen, USA) encoding human *TWIST1* cDNA (pcDNA3.1+*TWIST1*) and empty vector as a control were transfected to the cells by using lipofectamine2000 (Thermo Fisher Sci., USA). Briefly, 293T cells were seeded and cultured in 6-well plates (2x10⁵ cells/well), when reached at 70% confluency cells were transfected with 2,5 µg/well plasmid vectors via lipofectamine2000 in accordance with the manufacturer's instructions. Then the cells were harvested after 48h to evaluate further gene expression analysis.

Semi-quantitative RT-PCR: Total RNA was obtained from control and *TWIST1* positive 293T cells by using RNeasy mini kit (Qiagen) and total RNA measured by Multiskan GO spectrophotometer (Thermo Fisher Sci.). Reverse transcription were performed first-strand with an oligo(dT) primer using the QuantiTect reverse transcription kit (Qiagen). *TWIST1*, Hexokinase 2 (HK2), Pyruvate Kinase M2 (PKM2), Lactate Dehydrogenase-α (LDH-α), Phosphofructokinase M (PFKM), Glucose 6-phosphate dehydrogenase (G6PD) and the internal control gene β-actin (ACTB) were amplified using the following primers; *TWIST1* forward: 5'-

GGCTCAGCTACGCCTTCTC-3', *TWIST1* reverse: 5'-TCCTTCTCTGGAAACAATGACA-3'; HK2 forward: 5'-AAGGCTTCAAGGCATCTG-3', HK2 reverse: 5'-CCACAGGTCATCATAGTTCC-3'; PKM2 forward: 5'-CCACTTGCAATTATTTGAGGAA-3', PKM2 reverse: 5'-GTGAGCAGACCTGCCAGACT-3'; LDH-α forward: 5'-TTGGTCCAGCGTAACGTGAAC-3' LDH-α reverse: 5'-CCAGGATGTGTAGCCTTTGAG-3'; PFKM forward: 5'-GCATCCCATTTGTGGTCATTC-3', PFKM reverse: 5'-GTCACAGGTTGTGCAGATAGT-3'; G6PD forward: 5'-ACCTCATGGTGCTGAGATTTG-3', G6PD reverse: 5'-GCTCCTTGAAGGTGAGGATAAC-3'; ACTB forward: 5'-CATGTACGTTGCTATCCAGGC-3', ACTB reverse: 5'-CTCCTTAATGTCACGCACGAT-3'.

Detection of glucose consumption and lactate release: Glucose and lactate levels in the culture media of control (pcDNA3.1) and *TWIST1* positive (pcDNA3.1+*TWIST1*) 293T cells were detected by Radiometer ABL800 FLEX analyzer (Radiometer Inc., Denmark). After transfection of the cells with empty and also *TWIST1* positive vectors for 48 h, the culture supernatants were collected separately to safePICO syringes. Then, the each sample is automatically scanned and analyzed by the FLEXQ module of the ABL800 FLEX analyzer.

Measurement of ATP, ADP, AMP levels: Firstly, the standard curve was generated by Bradford reagent (BioRad) and the protein lysates were obtained from each experimental groups; control (pcDNA3.1) and *TWIST1* positive (pcDNA3.1+*TWIST1*) 293T cells. The protein concentration of the samples were calculated from the standard curve obtained by ELISA reader (BMG Labtech ClarioStar). Protein lysates neutralized with 1 mol⁻¹ K₂HPO₄, centrifuged for 15min at 10.000g at 4°C, then filtrated through a syringe filter (0.2µm). The cell lysates were stored at -80°C until the analyses were performed. The ATP, ADP and AMP levels of samples were detected by the HPLC at 254 nm. The solution of mobil phase (160 mM KH₂PO₄ and 100 mM KCl, pH:6.5) was degassed by filtration through a syringe filter (0.45µm). The HPLC settings were adjusted to the appropriate operating conditions for analysis. Then the ATP, ADP and AMP standards were prepared at different concentrations (10, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 mM) and loaded

Table 1. Effects of TWIST1 overexpression on pH, glucose and lactate levels in TWIST1-positive (pcDNA3.1+TWIST1) and control (pcDNA3.1) 293T cells culture supernatants

	pH	Glucose(mg/dl)	Lactate(mmol/L)
PcDNA3.1	7.7±0.2	278±3	3.1±0.08
PcDNA3.1+TWIST1	7.1±0.5	265±2	3.5±0.1

to the HPLC system to determine its retention times. The time of peaks identification, the appropriate mobile phase component and the appropriate flow rate were determined. The ATP, ADP and AMP concentrations were deduced from the standard curve. The cellular energy charges were calculated as follow; $(ATP + 0.5ADP) / (ATP + ADP + AMP)$ (11).

Statistical analysis: Data analysis and statistical significance were determined by Mann-Whitney U test via using GraphPad Prism8.0 software (GraphPad Software Inc., USA). Each experiments was performed in triplicate. *p* values<0.05 were evaluated statistically significant.

Results

TWIST1 upregulates glycolytic genes in 293T cells: Glycolytic pathway enzymes regulate energy metabolism by aerobic glycolysis in cancer. This metabolic change provides a great advantage for tumor progression and metastasis (12). Therefore, in our study, we investigated whether TWIST1 directly regulates glycolytic gene expressions that cause metabolic change. For this, we overexpressed TWIST1 ectopically in 293T cells showing low TWIST1 expression endogenously. Then we determined the change of expressional regulation of *HK2*, *PKM2*, *LDH- α* , *PFKM*, *G6PD* genes compare to control. According to the results, ectopic expression of TWIST1 transcriptionally increased the expression of *HK2* by 4-fold, *PFKM* by 1,3-fold, *PKM2* by 1,4-fold, *LDH- α* by 2-fold and *G6PD* by 2,6-fold in 293T cells (Figure 1). After normalization according to internal control (*ACTB*), fold change of interested gene were calculated as the mean expression value of TWIST1-overexpressed transfected cells compare to non-transfected control cells.

Overexpression of TWIST1 promotes lactate production and glucose consumption: Through oncogene activation, such as TWIST1 and HIF1, cancer cells increase lactate production as the end product despite the normoxic state (13,14). In glycolytic tumors, lactate production was found to be increased (15). Atypical lactate production is considered as an interpretation of the Warburg Effect and is mostly based on glycolysis (16).

Lactate is the major factor for acidification of the tumor microenvironment, where the common pH levels are between 5.5–7.0 in cancers (17). In this context, we determined whether TWIST1 had an effect on extracellular glucose, lactate and as a consequence pH levels of supernatants of TWIST1 positive and control 293T cells. As a result, average lactate levels increased from 3.1 mmol/L to 3.5 mmol/L, pH values changed from 7.7 to 7.1 and glucose levels of culture supernatants decreased from 278 mg/dL to 265 mg/dL in the supernatant of TWIST1 positive cells compare to control cells. By these data it could be said that overexpression of TWIST1 promotes aerobic glycolysis in 293T cells (Table 1).

Enhanced cellular energy charges and changed ATP, ADP and AMP levels by TWIST1 overexpression: Glucose is the only source that can be used for both biosynthesis and energy production. Biomolecules cannot be produced without an energy source within the cells. ATP is needed for growth signals and their activations. In addition, ATP is essential to the function of DNA or RNA synthesis enzymes. Therefore, cancer cells must have a large source of ATP (18). Glycolysis produces ATP at a faster rate than oxidative phosphorylation although it has lower efficiency. This higher ATP production is consider to help and promote cancer cells proliferation. In addition to this, other metabolic products such as higher glycolytic ratio, lactate and H⁺ support acidification of the extracellular environment in tumors (19). The balance of intracellular levels of ATP, ADP and AMP is very important in energy homeostasis. AKT signalling pathway decreasing the AMP/ATP ratio in cancer cells (20, 21). To restore this intracellular energy balance AMP-activated protein kinase (AMPK) increases the AMP/ATP and ADP/ATP ratios (22, 23). Herein, we determined whether TWIST1 had a regulatory role on intracellular AMP/ATP, ADP/ATP and cellular energy charge ratios. According to our results, TWIST1 increased ATP production by about 3.5-fold, thereby reducing the AMP/ATP ratio by approximately 4-fold and the ADP/ATP ratio by approximately 2-fold. In addition, ectopic

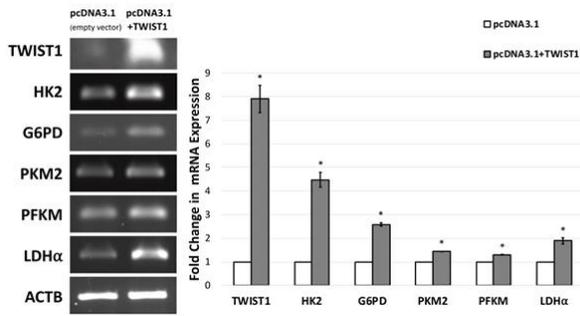


Fig. 1. TWIST1 upregulates main glycolytic genes in TWIST1-positive 293T cells. TWIST1 overexpression ($p=0.021$) transcriptionally upregulates hexokinase II (HK2) ($p=0.0098$), glucose-6-phosphate dehydrogenase (G6PD) ($p=0.0012$), pyruvate kinase M2 (PKM2) ($p=0.0065$), phosphofructo-kinase M (PFKM) ($p=0.041$) and lactate dehydrogenase alpha (LDH α) ($p=0.042$) with β -actin (ACTB) as a control were analyzed by RT-PCR. Results are shown as mean \pm SEM. * $p < 0.05$.

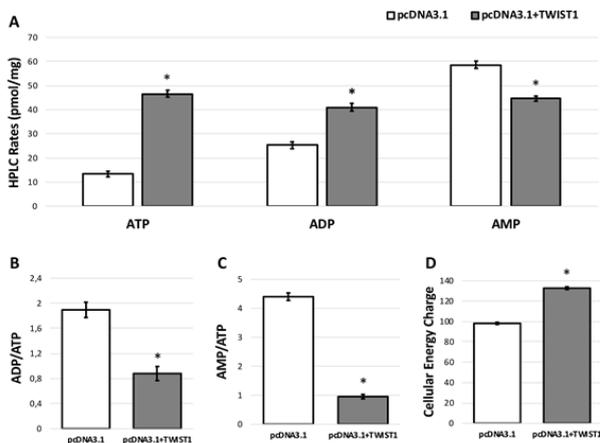


Fig. 2. Overexpression of TWIST1 enhances ATP production and cellular energy charge ($p=0.0017$). Rate of AMP ($p=0.0088$), ADP ($p=0.0098$) and ATP ($p=0.002$) measured using by HPLC system. The AMP/ATP ($p=0.012$) and ADP/ATP ($p=0.018$) rates were decreased and cellular energy charge was increased in TWIST1-positive (pcDNA3.1+TWIST1) 293T cells compare to control (pcDNA3.1). Results are shown as mean \pm SEM. * $p < 0.05$

TWIST1 overexpression increased the cellular energy charge by 33% in 293T cells (Figure 2). By this way, it could be said that TWIST1 could influencing the growth of the cells with the increase in energy yield.

Discussion

In the 1920s, Otto Warburg determined that cancer cells were more active in terms of the glycolysis pathway than normal cells. This metabolic reprogramming means that cancer cells

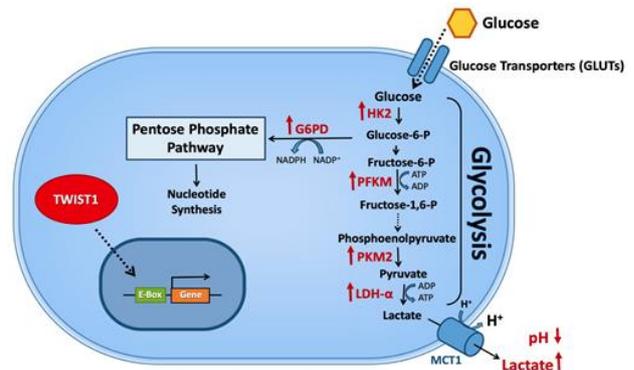


Fig. 3. Schematic diagram of the effects of TWIST1 transcription factor on aerobic glycolysis pathway. TWIST1 regulates glycolytic genes are directly responsible for the aerobic glycolysis

prefer aerobic glycolysis pathway rather than oxidative phosphorylation. (24, 25). In this study we evaluated the role of TWIST1 on transcriptional regulation of main step-limiting glycolytic genes, lactate production and cellular energy levels of 293T cells.

Previously, many essential transcription factors for aerobic glycolysis, such as HIF1 α and c-Myc were documented (26, 27). In many cancers, HIF1 α mediates increased glycolysis and expressions of it's enzymes including LDH α (26). Also, c-Myc could directly regulate the transcriptional expression of several glycolytic genes, including GLUT1, HK2, PKM2 and LDH α . It's well known that, c-Myc is an important oncogene that cooperate cancer metastasis and development by activating TWIST1 (28, 29). TWIST1 was highly expressed in human cancers as an oncogene, and its expression is closely related to poor prognosis (30).

In the present study, we determined that TWIST1 enhances aerobic glycolysis through upregulating the expression levels of the glycolytic genes; HK2, PFKM, PKM2, LDH- α , and also G6PD (Figure 3). In detail, HK2 irreversible catalyzes the first step of glycolysis which is phosphorylation and activation reaction of glucose to glucose-6-P. It's expression level was low in normal tissues, but was increased in tumor (5, 6). In addition, overexpression of HK2 enhances tumor growth, metastasis and lactate production (31). Another glycolytic gene PFKM is the isoform of the phosphofructokinase gene identified in skeletal muscles. In glycolysis, this enzyme irreversibly converts its substrate fructose-6-P into fructose-1,6-bisP. Whole genome analysis studies showed that PFKM has been shown to play an important role in the development of breast cancer patients diagnosed with early stage (32). Also, PKM2 activity increases in the presence of fructose-1,6-

bisP as a consequence of PFKM activity. PKM2 monomers are transformed into tetramer form in the event of glucose uptake. PKM2 catalyzes ATP production by ADP formation via transferring the phosphoryl group from phosphoenolpyruvate, which is the final glycolytic step. Because of this, PKM2 plays significant roles in embryogenesis, tissue repair, and cancer development (33). Another of the important genes is LDH α , catalyzes the interconversion among lactate and pyruvate, also the oxidation of NADH to NAD $^{+}$. This is the last step of anaerobic glycolysis (7). LDH α is mostly expressed in skeletal muscle and its expression abnormally increased in tissue damage, hypoxia, and cancer (34-37). In addition, there is the pentose phosphate pathway, an alternative to glycolysis in energy metabolism. G6PD is the basic enzyme that catalyzes the first step of this pathway. G6PD regulates redox potential and extracellular pH levels (38). Its expression was increased in various cancers including leukemia, gastrointestinal, kidney, breast, and liver cancers (39- 42). In this respect, G6PD is an important metabolic factor in cancer development (43).

Acidosis and lactate accumulation enhances in tumor microenvironment. Lactic acid is the major factor responsible for the acidosis (44, 45). Lactate is necessary for important carcinogenesis steps such as angiogenesis and cell migration of cancer cells (16). Oxystressed tumor microenvironment triggers epithelial mesenchymal transition process via overexpression of TWIST1 and LDH activity (46). In this study, we examined the lactate level in the supernatants of TWIST1-overexpressed 293T and control cells. As a result, we found that TWIST1 could lead to enhance lactate releasing to the cell microenvironment.

Cancer cells use much more glucose to get more ATP needed for metabolic activities. To explain the aerobic glycolysis process, many genes, enzymes and factors continue to be explored (47). Based on this, we examined the changes of intracellular levels of ATP, ADP and AMP depending on TWIST1. In TWIST1-positive 293T cells, ATP production was increased and AMP/ATP and ADP/ATP ratios were decreased. With these results, it could be deduced that TWIST1 regulated energy metabolism in 293T cells.

Eventually, these data showed that TWIST1 supports aerobic glycolysis (Warburg effect) in cancer cells. Therefore, it could be assumed that TWIST1 is one of the main factors could change the bioenergetic characteristics of cancer cells.

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