**Original Article**

**MCT1 promotes tumor progression through regulating epithelial-mesenchymal transition in pancreatic cancer**

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**Abstract:** Enhanced glycolytic flux in cancer cells, known as Warburg effect, leads to increased intracellular lactate that is exported by monocarboxylate transporters (MCT), which play critical functions in tumor initiation and progression. However, the role of MCT1 in pancreatic ductal adenocarcinoma (PDAC) is poorly explored. In this study, data from two ONCOMINE databases revealed that MCT1 mRNA was frequently up-regulated in PDAC tissues compared to normal pancreas. By immunohistochemical analysis in a PDAC tissue microarray, intense immunoreactivity of MCT1 protein was commonly observed in PDAC tissues but not normal pancreas. Kaplan-Meier survival analysis showed that high level of MCT1 was associated with poor prognosis in PDAC patients. Then genetic silencing of MCT1 in PDAC cells resulted in pronounced decrease in MCT1 protein level and lactate level in culture medium. Specially, knockdown of MCT1 markedly inhibited cell proliferation and invasive capacity as demonstrated by colony formation and transwell assay. Mechanistically, silencing of MCT1 partially compromised epithelial-to-mesenchymal transition phenotype. And suppression EMT process by a pharmacological inhibitor, SB431542, completely blocked MCT1-mediated oncogenic activities. Collectively, these findings provide evidence for use of MCT1 as potential therapeutic target in treatment of PDAC.

**Keywords:** MCT1, epithelial-mesenchymal transition, pancreatic cancer

**Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal of solid malignancies with an overall 5-year survival rate less than 6% [1, 2]. Due to failure to early diagnosis before distant metastasis and drug resistance of current therapies, the poor prognosis of PDAC poses a serious health problem at the beginning of the 21st century [3, 4]. Although genetically engineered mouse models of PDAC have better recapitulated tumor initiation and progression, as well as a multitude of oncogenic pathways, limited therapeutic agents are developed and fail to improve the prognosis of PDAC in recent three decades [5, 6]. Thus, it is imperative to fully interrogate this deadly disease.

Cancer cells exhibit a shift in glucose metabolism from oxidative phosphorylation (OXPHOS) to glycolysis, known as the Warburg effect, to facilitate tumor progression [7, 8]. Accumulating evidences suggest that PDAC cells exhibit enhanced glucose consumption as demonstrated by $^{18}\text{F}$-fluorodeoxyglucose ($^{18}\text{F}$-FDG) accumulation [9]. This reprogrammed metabolic alternation enables PDAC cells to adapt to nutrient-deficiency environments induced by intense desmoplasia and poor vascularity, to facilitate cell proliferation through providing building blocks, to promote cell invasion through lactate-mediated acidification of tumor microenvironment, and to protect cells from oxidative stress by generating NADPH and glutathione [10]. As noted, increased glycolytic flux in PDAC cells leads to increased levels of intracellular lactate, which is exported by monocarboxylate transporters (MCTs) [11, 12]. MCTs are critical players in the maintenance of the glycolytic metabolism through transporting lactate and regulating extracellular pH homeostasis [13, 14]. Specially, MCT1-4, are known to mediate the proton-dependent transport of monocarboxylic acid across the plasma membrane in the presence of immunoglobulin-like molecule CD147 [15-17]. Increased MCT1 has been demonstrated in multiple human cancers, including colorectal cancer [18, 19], cervical cancer [20], glio-
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ma [21], lung cancer [22], and melanoma [23], however, the expression profile and cellular functions of MCT1 in PDAC remain largely unknown.

Conversion of cancer cells with an epithelial phenotype into a mesenchymal phenotype, known as epithelial-mesenchymal transition (EMT), is critical for tumor progression [24, 25]. Cancer cells undergoing EMT are characterized by increased migratory and invasive capacity [25]. Transforming growth factor-β (TGF-β) has been widely used as EMT inducers [26, 27], because this extracellular mediator is overexpressed in many types of carcinomas, especially PDAC [28]. However, little is known about the correlation between glycolysis and EMT.

Given those notions, we reasoned that MCT1 would regulate PDAC progression through EMT. Here, we showed that MCT1 was widely expressed in PDAC and predicted a poor prognosis. Genetic silencing of MCT1 inhibited lactate production, cell proliferation, and invasion. And the suppressive role induced by knockdown of MCT1 might associate with EMT process.

Materials and methods

Cell culture

Human pancreatic cancer cell lines AsPC1, PANC1, BxPC-3, SW1990 and HPAC were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely cultured in DMEM or RPMI-1640 medium containing D-glucose (4.5 g/l), (Invitrogen, USA), supplemented with 10% FBS (Gibco, NY, USA) and 1% penicillin/streptomycin (Invitrogen, USA), in a 37°C humidified atmosphere with 5% CO₂. SB431542 was purchased from Selleck (Shanghai, China).

Immunohistochemistry

The commercial pancreatic cancer tissue microarray (TMA) was purchased from Shanghai Outdo Biotech Inc. After deparaffinizing, rehydrating, antigen retrieval, and blocking endogenous peroxidases, the sections were washed in 1 × PBS for three times, blocked in 5% normal goat serum, followed by incubation of anti-MCT1 antibody (Abcam, USA) at 4°C overnight. After washing in 1 × PBS for three times, sections were incubated in horseradish peroxidase-conjugated secondary antibodies. Visualization was performed by 3, 3-diaminobenzidine tetrahydrochloride (DAB) and all sections were counterstained with hematoxylin. A combination of a proportion score and an intensity score was used to determine MCT1 staining. A total score was obtained by the combining both scores and divided in four levels: negative, low, moderate and high. Scoring was calculated independently by two pathologists. Negative and weak staining was regarded as low expression, while moderate and strong staining was regarded as high expression.

Quantitative real-time PCR

Total RNA was extracted and reversely transcribed using PrimeScript RT-PCR kit (Takara, Japan). Measurement of gene expression was performed by quantitative real-time PCR (ABI PRISM 7700 Sequence Detector, Applied Biosystems). Relative mRNA expression levels were normalized to the β-actin mRNA levels and calculated using the 2^ΔΔCt method. Sequences of primers are provided upon request.

siRNA transfection

BxPC-3 and SW1990 cells were transfected with 100 nmol/L siRNA against MCT1, or control siRNA from GenePharma (Shanghai, China) together with Lipofectamine 2000 (Invitrogen, USA) into a 6-well dish as recommended by the manufacturers. Cells were incubated for 24 hours before plating for cell invasion assays, and RNA isolation.

Measurement of lactate level

Lactate level in the culture medium was measured with Lactate Assay kit (Source Bioscience Life Sciences) according to the manufacturer’s instruction.

Colony formation assay

A total of 1000 si-MCT1 or si-Ctrl cells were seeded into a 6-well dish, and allowed growth for 10-12 days. Culture medium was replaced every two days. Formed colonies were fixed by 4% paraformaldehyde, stained by 0.1% (w/v) crystal violet and counted. Each experiment was performed in triplicate and repeated twice.

Cell invasion assay

Cell invasion assay was performed using Transwell model according to the manufacturer’s
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Instructions (BD Biosciences, USA). Briefly, BxPC-3 and SW1990 MCT1-silenced and control cells were plated into matrigel-coated chambers for 48 h. Invaded cells were fixed and stained by 0.1% (w/v) crystal violet. Membranes were photographed in a stereomicroscope and invaded cells were counted.

Luciferase assays

Cells ($1 \times 10^4$) were seeded in triplicate in 96-well plates and allowed to attach for 24 h. Luciferase reporter plasmids (100 ng) or 100 ng control luciferase plasmid plus 1 ng pRL-TK Renilla plasmid (Promega) were transfected into indicated cells using Lipofectamine 2000 (Invitrogen). Luciferase and Renilla signals were measured 24 h after transfection using a Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instruction.

Statistical analysis

All data are presented as the mean ± SD. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc., USA). Kaplan-Meier method was used to evaluate survival curves and differences between survival curves were tested by the log-rank test. The student’s t-test or one-way ANOVA was used for comparison between groups. All P-values less than 0.05 were considered statistically significant.

Results

Up-regulated MCT1 predicts a poor prognosis of PDAC

Data mining of ONCOMINE database revealed that MCT1 mRNA expression was significantly over-expressed in tumor tissues compared to their normal counterparts (Figure 1A). To further evaluate the expression pattern of MCT1 at protein level, we tested MCT1 expression in a tissue microarray, which contains 44 cases of normal pancreas and 81 cases of PDAC. As shown in Figure 1B, compared to normal pancreas, intense staining of MCT1 was frequently observed in PDAC tissues. By analysis of these PDAC cases with follow-ups ($n = 36$), we found that high MCT1 protein expression was closely associated with decreased overall survival compared those with low MCT1 expression (Figure 1C).
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Genetic silencing of MCT1 inhibits the invasive capacity of PDAC cells

In order to test if the oncogenic roles previously demonstrated were similar in PDAC cells, knockdown of MCT1 expression was performed using specific siRNAs in two MCT1 high expressed cells, BxPC-3 and SW1990 (Figure 2A). As shown in Figure 2B, a pronounced decrease in MCT1 expression was observed upon MCT1 siRNAs in both cell lines. Expectedly, extracellular lactate was decreased by MCT1 down-regulation (Figure 2C). As demonstrated by colony formation assay, si-MCT1 cells showed reduced proliferative ability, especially in SW1990 cells (Figure 2D). Likewise, a marked decrease of the invasive capacity of PDAC cells was observed upon silencing of MCT1 (Figure 2E). Taken together, these data indicate that MCT1 is critically involved in development and progression process of PDAC.

MCT1 is involved in epithelial-to-mesenchymal (EMT) transition

EMT is associated with the increased metastatic invasive potential and we found a faint morphological change in SW1990 upon silencing of MCT1. Therefore, to determine the underlying mechanisms involved in MCT1-mediated invasive potential, we focused on EMT. As shown in Figure 3A, E-cadherin, the epithelial cell marker, was significantly up-regulated in si-MCT1 cells compared to si-Ctrl cells. Reversely, knockdown of MCT1 decreased the expression of N-cadherin, a mesenchymal cell marker, in both BxPC-3 and SW1990 cells (Figure 3B). And furthermore, other mesenchymal cell markers such as vimentin, snail, fibronectin and twist should also be detected. The result showed that vimentin and fibronectin were markedly reduced by silencing of MCT1, while the expression level of snail and twist was faintly affected by MCT1 alteration (Figure 3C). Moreover, luciferase reporter assay showed that silencing of MCT1 decreased the activity of TGF-β signaling, an indicator of EMT, in both BxPC-3 and SW1990 cells (Figure 3D). Importantly, pharmacological inhibition of the EMT process by SB431542 fully blocked the oncogenic roles of MCT1 on cell proliferation (Figure 3E) and invasion (Figure 3F). Collectively, these data above suggest that MCT1 induced malignant phenotypes might be mediated by altered EMT process.
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Discussion

Alternations in glucose metabolism have been emerged as a hallmark of cancer [29]. The Warburg effect, characterized by enhanced glucose consumption and lactate production, draws increasing attention in targeted therapy in recent years [30]. To protect cells from lactate-induced intracellular acidified microenvironment, lactate must be transported from the cell. MCT1, as a critical factor in facilitating the diffusion of lactate across the plasma membrane, is commonly expressed in tumor tissues compared to normal tissues and being promising therapeutic targets [31, 32]. In current study, we determined the expression profile, cellular functions and related mechanisms of MCT1 in PDAC.

By data mining of ONCOMINE database and immunohistochemical analysis of a PDAC tissue microarray, we demonstrated that MCT1 expression was upregulated in PDAC tissues at both mRNA and protein level compared with normal pancreas. Notably, patients with a higher MCT1 expression had a poor prognosis in relative to those had a lower MCT1 expression. Myc oncoproteins drive aerobic glycolysis through regulating expression of glycolytic enzymes, including lactate dehydrogenase A (LDHA) that generates lactate. It is also well revealed that Myc directly activated MCT1 transcription by binding to specific recognition sites [33, 34].

Although Myc determines the metabolic phenotype and plasticity of PDAC, however, whether Myc contributes to the elevated MCT1 in PDAC remains further investigation.

Then by genetic silencing of MCT1 in PDAC cells, we observed significant reduction in colony formation ability and invasive capacity. Consistent with previous in vitro and in vivo reports

Figure 3. MCT1 is involved in epithelial-to-mesenchymal (EMT) transition. E-cadherin (A) and N-cadherin (B) expression in si-Ctrl and si-MCT1 cells was detected in BxPC-3 and SW1990 cells by Western blotting. (C) Expression of several mesenchymal markers in si-Ctrl and si-MCT1 cells were detected in BxPC-3 and SW1990 cells by qRT-PCR. (D) Luciferase assay analyses of the BxPC-3 and SW1990 cells transfected with the TGF-β reporters in the presence of 50 nM MCT1 specific siRNAs. (E, F) Cell proliferation (E) and invasive potential (F) of BxPC-3 and SW1990 cells were measured in the presence 10 μM SB431542. *P < 0.05, **P < 0.01.
using MCT pharmaceutical inhibitors [35, 36], our findings as a proof of principle, support the hypothesis that MCT1 could be promising therapeutic targets in PDAC. Pharmaceutical inhibition of lactate transport showed a marked decrease in glycolytic rate, cell proliferation, migration and invasion in glioma and breast cancer cells [37, 38]. Indeed, knockdown of MCT1 also led to a decrease in tumor cell aggressiveness, as well as decreased lactate transport [39, 40]. Thus, targeting tumor glucose metabolism by MCT1 blockade may become an effective therapeutic option for PDAC cells with enhanced glycolysis.

Finally, to demonstrate the mechanism underlying MCT1-mediated functions. In osteosarcoma, the antitumor effects of targeting MCT1 might be related to the NFκB pathway [41]. In myeloma cells, targeting MCT caused downregulation of homing receptor CXCR4 and abrogated SDF-1-induced migration [42]. Here, we focused on EMT, as the morphological change observed in SW1990 cells. We found MCT1 knockdown partially compromised EMT phenotype, indicating EMT might involve in MCT1-related invasive capacity. Inconsistent with our observations that silencing of MCT1 reduced cell proliferation, however, another hallmark of tumor cells undergoing EMT is decreased proliferation [43, 44]. This discrepancy in proliferation might be explained by reduced Warburg effect, which enables cancer cells with biosynthetic building blocks for proliferation.

In conclusion, our data identify MCT1 as a key regulator of glycolysis and tumor progression, and suggest that patients with PDAC may benefit from targeted therapies focused on MCT1-related signaling pathway.

Disclosure of conflict of interest

None.

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