Original Paper

Effects of Nicotinamide N-Methyltransferase on PANC-1 Cells Proliferation, Metastatic Potential and Survival Under Metabolic Stress

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Key Words

NNMT • PANC-1 • Proliferation • Migration • Invasion • Metabolic stress

Abstract

Background: Aberrant expression of Nicotinamide N-methyltransferase (NNMT) has been reported in pancreatic cancer. However, the role of NNMT in pancreatic cancer development remains elusive. Therefore, the present study was to investigate the impact of NNMT on pancreatic cancer cell proliferation, metastatic potential and survival under metabolic stress. **Methods:** Pancreatic cancer cell line PANC-1 was transfected with NNMT expression plasmid or small interfering RNA of NNMT to overexpress or knockdown intracellular NNMT expression, respectively. Rate of cell proliferation was monitored. Transwell migration and matrigel invasion assays were conducted to assess cell migration and invasion capacity. Resistance to glucose deprivation, sensitivity to glycolytic inhibition, mitochondrial inhibition and resistance to rapamycin were examined to evaluate cell survival under metabolic stress. **Results:** NNMT silencing markedly reduced cell proliferation, whereas NNMT overexpression promoted cell growth moderately. Knocking down NNMT also significantly suppressed the migration and invasion capacities of PANC-1 cells. Conversely, NNMT upregulation enhanced cell migration and invasion capacities. In addition, NNMT knockdown cells were much less resistant to glucose deprivation and rapamycin as well as glycolytic inhibitor 2-deoxyglucose whereas NNMT-expressing cells showed opposite effects although the effects were not so striking. **Conclusions:** These data suggest that NNMT plays an important role in PANC-1 cell proliferation, metastatic potential and survival under metabolic stress.

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Introduction

Pancreatic cancer remains one of the most lethal malignancies with a 5-year survival rate of less than 7% [1]. The dismal prognosis is largely due to the fact that the disease is difficult to detect at an early and curative stage [2, 3]. While surgical resection is currently the only possible cure, up to 80% of patients diagnosed with locally deteriorated or distant metastatic disease are not appropriate for resection [4]. Improvements in diagnosis and treatment pancreatic cancer are urgently needed [5].

Nicotinamide *N*-methyltransferase (NNMT) catalyzes the *N*-methylation of nicotinamide into 1-methylnicotinamide (NMN) and is involved in the biotransformation of many drugs and xenobiotics [6, 7]. It is predominantly expressed in liver and weakly expressed in other tissues like kidney, lung, skeletal muscle, placenta, heart and brain [6]. Abnormal expression of NNMT has been found in numerous cancers, including glioblastoma [8], hepatocellular carcinoma [9], thyroid carcinoma [10], gastric cancer [11], renal cell carcinoma [12], colorectal cancer [13], oral squamous cell carcinoma [14], bladder cancer [15], non-small cell lung cancer [16], and nasopharyngeal carcinoma [17]. These results suggest that NNMT may serve as a potential biomarker for tumor diagnosis and a new therapeutic target.

Although NNMT mRNA was reported overexpressed in tissues and pancreatic juice of pancreatic cancer patients compared with normal controls [18, 19], the biochemical and cellular roles of deregulation of NNMT in pancreatic cancer remain unknown. Our previous study showed that a dramatic up-regulation of NNMT was associated with miR-1291 triggered human pancreatic carcinoma cell line PANC-1 cell metabolism and tumorigenesis suppression [20]. Furthermore, NNMT mRNA level was inversely correlated with tumor size in the xenograft mouse model with inoculation of miR-1291-expressing and control PANC-1 cells [20]. These results suggest that NNMT may play an important role in PANC-1 cell metabolism. However, whether NNMT has direct impact on PANC-1 cell metabolism has not been addressed. Therefore, the aim of this study was to investigate the effect of NNMT on biological behaviors of PANC-1 cells, including cell proliferation, metastatic potentials and cell survival under metabolic stress.

Materials and Methods

Reagents and materials

Dulbecco's modified Eagle's medium (DMEM), penicillin sodium and streptomycin sulfate solution were purchased from Mediatech (Manassas, VA). Fetal bovine serum (FBS) was bought from Lonza (Walkersville, MD). The pcDNA3.1 (+) vector was purchased from Invitrogen (Shanghai, China) and the ORF region of NNMT gene was synthesized by Invitrogen (Shanghai, China), then cloned into pcDNA3.1(+) via BamHI (MBI) and EcoRI (MBI). The specific primer pairs used were the following: forward primer, 5'-CACCATGGAATCAGGCTTACCTCC-3' and reverse primer, 5'-TCCTCTCCAAGCAGGGTCTGCTCAGCTTC-3'. Control siRNA and siRNA against NNMT were from Ribobio (Guangzhou, China). Transfection reagent LipofectAMINETM 2000 and Lipofectamine RNAiMAX were obtained from Invitrogen (Shanghai, China) and Invitrogen (Grand Island, NY), respectively. Trizol was purchased from Life Technologies (Carlsbad, CA). PrimeScript RT reagent kit and SYBR Premix Ex Taq II (Tli RNaseH Plus) kit were obtained from Takara Biotech (Kyoto). Rabbit NNMT polyclonal antibody was provided by Abgent (San Diego, CA). Rabbit β -Tubulin monoclonal antibody was obtained from cell signaling technology (Beverly, MS). Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and solvents were of the highest grade commercially available.

Cell line and culture conditions

Human pancreatic carcinoma PANC-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% FBS, 100 IU/ml penicillin sodium and 100 μ g/ml streptomycin sulfate in a 5% CO₂ atmosphere at 37°C.

Plasmid transfection

NNMT expression plasmid, pcDNA[™]3.1(+)-NNMT was constructed by Invitrogen (Shanghai, China). Briefly, the cells were grown in 6-well plates at 80%-90% confluence and then transfected with pcDNA[™]3.1(+)-NNMT or pcDNA[™]3.1(+) vector (Invitrogen, Shanghai) using Lipofectamine 2000 reagents (Invitrogen, Shanghai) following the instruction of the manufacturer. pcDNA3.1: PANC-1 cells transfected with empty pcDNA3.1 vector; pcDNA3.1-NNMT: PANC-1 cells transfected with NNMT expression plasmid.

siRNA transfection

The knockdown of NNMT was performed using siRNA inhibition assay. siRNA against NNMT, 5'-CGUCGUCACUGACUACUCAdTdT-3' (forward) and 3'-dTdTGACAGACUGACUGAUGAGU-5' (reverse), and a control siRNA were obtained from Ribobio (Guangzhou, China). Briefly, the cells were grown in 6-well plates at 40%-50% confluence and then transfected with predesigned siRNA oligonucleotides (final concentration 50 nM) using Lipofectamine RNAiMAX transfected reagents (Invitrogen, Grand Island, NY) as instructed by the manufacturer. si-Control: PANC-1 cells transfected with control siRNA; si-NNMT: PANC-1 cells transfected with siRNA against NNMT.

RNA extraction and real-time quantitative PCR

According to the protocol of the transfection kit, the NNMT knockdown or over-expression efficiency can be validated at 24~96 h post-transfection by RT-qPCR and Western Blot. Thus, in this study we harvested the cells at 24, 48 and 72 h post-transfection to validate NNMT silencing or over-expression efficiency. Total RNAs were extracted from cell samples using Trizol reagent (Invitrogen, Grand Island, NY) and quantified with NanoDrop (Thermo Scientific, Rockford, IL) at 260 nm and 280 nm. cDNA was synthesized from 1 µg total RNA using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotech, Kyoto).

To quantitatively examine NNMT gene expression, real-time quantitative PCR analysis was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TaKaRa Biotech, Kyoto) in Applied Biosystems 7500 (Applied Biosystems, Foster City, CA) Real-Time PCR System. Cycling conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by 95 °C for 15 s and 60 °C for 60 s. β-actin was used to normalize mRNA expression in PANC-1 cells. Gene-specific PCR primers for human NNMT and β-actin were designed based on the DNA sequences in the GenBank (National Center for Biotechnology Information, Bethesda, MD. <http://primerdepot.nci.nih.gov>). The primer sequences were as follows: for NNMT, 5'-AGCTGGAGAAGTGGCTGAAG-3' (forward), 5'-TGGACCCTTGACTCTGTTCC-3' (reverse); for β-actin, 5'-GCACAGAGCCTCGCCTT-3' (forward), 5'-CCTTGCACATGCCGGAG-3' (reverse).

Expression of NNMT mRNA was calculated using C_T , the number of cycles to reach a threshold, in triplicate. And it then was normalized relative to the reference gene β-actin by subtracting its average C_T . The relative level of NNMT over β-actin was calculated using the formula $2^{-\Delta CT}$, where $\Delta C_T = C_T$ (NNMT) - C_T (β-actin), and then compared between different groups.

Western blot analysis

In this study cells were harvested at 24, 48 and 72 h post-transfection to validate NNMT silencing or over-expression efficiency. Cell extracts were prepared with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Biocolor Bioscience Technology Company, Shanghai, China). Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL). A total of 30 µg protein was loaded and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel, and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, USA). After regular blocking and washing, membranes were incubated with rabbit polyclonal antibody (1:500 dilution) against NNMT (Abgent, San Diego, CA) or rabbit monoclonal antibody (1:1000 dilution) against β-Tubulin (Cell signaling technology, Beverly, MS) for 1h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz) for 1 h. Protein-antibody complexes were detected using an electrochemiluminescence (ECL) kit (Engreen Biosystem, Beijing, China) and exposed to an X-ray film (GE Healthcare, Piscataway, NJ, USA). The intensity of protein bands was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA). Each western blot analysis was performed three times.

Cell proliferation analysis

PANC-1 cells were transfected and cultured in 6-well plates at a density of 2×10^4 cells/well. Cell numbers were counted daily by hemocytometer from day 1 to day 6 after transfection. All counts were carried out on triplicate samples.

Migration and invasion assay

Transwell migration and matrigel invasion assays were employed to evaluate cell migration and invasion capacities, respectively. Briefly, cells were harvested at 72 h post-transfection and cells suspension of 3×10^4 was added to cell culture inserts (Corning, Tewksbury, MA) containing a polycarbonate filter with 8 μ m diameter pores blocked with 2.5% BSA for transwell migration assay or coated with 1:5 dilution matrigel 100 μ l/well for matrigel invasion assay. Cells were incubated for 17 h under standard culture conditions. Tumor cells remaining on the topside of the membrane or gel were removed, and cells that had migrated or invaded to the underside were fixed and stained with Diff-Quik (PolySciences, Warrington, PA). Five fields per insert were photographed and the number of cells was counted under microscope.

Glucose deprivation, glycolytic inhibition, mitochondrial inhibition, rapamycin sensitivity

Resistance to glucose deprivation, sensitivity to glycolytic inhibition and rapamycin were examined to evaluate cell survival under metabolic stress. 3×10^4 or 1.5×10^4 cells/well were seeded into 96-well plates one day before plasmid or siRNA transfection, respectively. Unless otherwise mentioned, cells were cultured in a 5% CO₂ atmosphere at 37°C. According to the protocol of transfection kit, cells were subjected to different metabolic stress as follows at 24 h post-transfection. For glucose deprivation, cells were cultured in 100 μ L glucose-free DMEM, or DMEM supplemented with indicated glucose solutions for 3 days. For glycolytic inhibition, 2-deoxyglucose (2-DG) (Sigma-Aldrich, St. Louis, MO) was prepared in PBS as stock solutions and then diluted to the required concentrations in standard medium. Transfected cells were cultured in indicated 2-DG solutions for 3 days. For mitochondrial inhibition, rotenone (Sigma-Aldrich, St. Louis, MO) was prepared in DMSO as stock solution and then diluted to 0.4 μ M with standard medium. Transfected cells were incubated with 5 mM 2-DG in the absence or presence of 0.4 μ M rotenone for 3 days. For rapamycin sensitivity, the stock solution of rapamycin (Chemietek, Indianapolis, IN) was prepared in DMSO as stock solution and then was diluted to required concentrations with standard medium. Transfected cells were cultured in indicated rapamycin solutions for 3 days. The appropriate vehicle controls were used for all experiments involving chemical treatments. For all experiments, cell growth was determined by sulforhodamine B colorimetric assay.

Sulforhodamine B (SRB) colorimetric assay

Briefly, treated cells were fixed with cold 10% trichloroacetic acid (TCA) for 1 h at 4°C, followed by cell washing with water. 100 μ L 0.4% SRB was added to each well followed by incubation for 30 min at room temperature, and plates were then washed by with 1% acetic acid. SRB stains were solubilized in 10 mM Tris at room temperature and absorbance was read at 515 nm. Relative cell growth was determined according to relative absorbance of treated and control cells.

Statistical analysis

The two-tailed independent-samples Student's t-test was applied to analyze differences between experimental groups. The data with *P* values < 0.05 were considered to be statistically significant. All experiments were repeated triplicate with similar results.

Results*Efficiency of plasmids and siRNAs transfection*

NNMT expression plasmid or siRNA against NNMT was transiently transfected into human pancreatic cell line PANC-1 to overexpress or knockdown intracellular NNMT expression, respectively. The NNMT mRNA and protein levels at 24, 48, and 72 h post-transfection were analyzed by real-time PCR and western blot analysis to validate the transfection efficiency. Both NNMT mRNA and protein levels were significantly increased

Fig. 1. Gain and loss of NNMT expression in PANC-1 cells 48 h after transfection with NNMT expression plasmids and siRNA, respectively. (A-B) Real-time PCR analysis showed that *NNMT* mRNA was significantly up-regulated or down-regulated after transient transfection with NNMT expression plasmid or siRNA targeting NNMT, respectively. (C-D) Western blot analysis confirmed NNMT protein levels were significantly up-regulated or down-regulated post-transfection. (E-F) Quantification of band intensity. Data are the mean $\pm$ s.d. (n = 3) * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

or decreased at 24, 48, and 72 h post transfection. As shown in Figure 1, at 48 h post transfection, mean NNMT mRNA expression was 25,000-fold higher in NNMT expression plasmid transfected cells compared to the control; while 65% lower post-siRNA transfection (Fig. 1A and B). In addition, NNMT expression plasmid increased NNMT protein levels about 7 folds at 48 h post-transfection, while si-NNMT treated PANC-1 cells manifested a 60% reduction in NNMT protein expression compared to the control cells (Fig. 1C, D, E, F),

Fig. 2. The effect of NNMT on cell proliferation. (A) NNMT up-regulation enhanced cell proliferation, (B) whereas NNMT knockdown led to a significant suppression. Data are the mean $\pm$ s.d. ($n=3$) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

indicating intracellular NNMT level was successfully up-regulated or down-regulated after transfection of NNMT expression plasmid or siRNA against NNMT, respectively.

Effect of NNMT on cell proliferation

To reveal the effect of NNMT on the proliferation of PANC-1 cells, cell growth was monitored over time. The results demonstrated that up-regulation of NNMT modestly promoted the growth of PANC-1 cells compared to the control (Fig. 2A). From day 1 to day 5 after transfection with NNMT plasmid, the cell amounts were increased by 45%, 40%, 60% and 30% of the controls, respectively. In contrast, NNMT knockdown significantly inhibited cell proliferation (Fig. 2B). The growth curve of si-NNMT PANC-1 cells was lagged behind that of the control. Starting from day 2 of the assay, NNMT-deficient PANC-1 cells manifested significant lower growth rate than that of the control cells. On day 5 and day 6, the cell amounts were remarkably decreased to 30%, 35% of the control group, respectively.

Effect of NNMT on cell migration and invasion capacity

To study possible biological functions associated with NNMT expression, transwell migration and matrigel invasion assays were performed 72 h post transfection. NNMT-expressing PANC-1 cells enhanced migration and invasion abilities by 2.5-fold and 2-fold respectively compared with control cells (both $P < 0.01$, Fig. 3A, C, E). In contrast, NNMT-deficient cells displayed a 70% reduction in migration (Fig. 3B and D). In addition, the number of control cells invaded through matrigel-coated membrane was more than 4 folds that of NNMT-deficient cells (Fig. 3B and F). This further underscored NNMT knockdown had inhibitory effect not only on migration, but also on invasion capacity as well. Taken together, these data suggested that NNMT has important effect on PANC-1 cell metastatic potentials such as cell migration and invasion capacity.

Effect of NNMT on cell survival under metabolic stress

Since one of the hallmarks of cancer cell is metabolic reprogramming, whether NNMT had effect on PANC-1 cells survival under metabolic stress was examined. Our data showed that NNMT-expressing cells were less sensitive to the glucose deprivation in comparison to control vector-transfected cells. At 1, 0.5, 0 mM of glucose, 30%, 30%, 40% more cells were survived compared with control cells (All $P < 0.05$, Fig. 4A). In contrast, NNMT-deficient cells had a reduction of cell number by 35%, 40%, 50% compared with control cells at 1, 0.5, 0 mM of glucose (All $P < 0.05$, Fig. 4B).

Sensitivity to 2-DG, a glycolysis inhibitor, was further measured. At intermediate dose of 2-DG (0.1, 1, 5 mM), NNMT-expressing cells demonstrated moderate better survival compared to control cells, with cell number increased by 70%, 20%, and 40%, respectively

Fig. 3. The effect of NNMT on migration and invasion capacities of PANC-1 cells. (A) NNMT overexpression significantly increased cell migration and invasion capacities, (B) whereas NNMT depletion led to opposite effects. (C-F) Average migrated cells or invaded cells. Five fields per insert were photographed and the number of cells was counted under microscope. Data are the mean $\pm$ s.d. (n = 3) * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

(All P < 0.05, Fig. 4C). In contrast, NNMT-deficient cells showed hindered proliferation with reductions of 20% and 25% of cell amount at 1 and 5 mM 2-DG (Both P < 0.05, Fig. 4D). These data further highlighted the important role of NNMT in PANC-1 cell survival under metabolic stress.

Further results from the mitochondrial inhibition study showed that NNMT-overexpressing cells demonstrated significantly better survival compared to control cells in the presence of 2-DG, but there was no significant difference when the cells were co-incubated with 2-DG and rotenone, although cell survival in both groups significantly decreased in the presence of rotenone (Fig. 4E). These results suggested that an increased dependence upon

Fig. 4. The effect of NNMT on PANC-1 survival under metabolic stress. (A-B) NNMT-expressing PANC-1 cells were more resistant to glucose deprivation, whereas NNMT-deficient PANC-1 cells were more sensitive. (C-D) NNMT up-regulation led to an increased resistance to glycolytic inhibition of PANC-1 cells, whereas the resistance was sharply reduced in NNMT-deficient cells. (E) NNMT-expressing PANC-1 cells undergoing glycolysis showed no significant different cell survival compared to the control cells in the presence of rotenone. (F-G) NNMT-expressing cells demonstrated enhanced resistance to rapamycin; in contrast, NNMT-knockdown caused a significant reduced resistance. Data are the mean $\pm$ s.d. (n = 3) ns, not significant, * P < 0.05, ** P < 0.01, *** P < 0.001 versus control.

oxidative phosphorylation may play a role in the increased survival of NNMT-overexpressing cells undergoing glycolysis inhibition.

In order to determine whether NNMT expression might alter rapamycin sensitivity, treated and control PANC-1 cells were cultured in DMEM containing increasing concentrations of rapamycin. Our data showed that NNMT-expressing cells manifested better cell survival than control cells, but the effects were not so striking as that of NNMT-deficient cells. In

contrast, knockdown NNMT by siRNA interference conferred sensitivity to rapamycin (Fig. 4F). NNMT-deficient PANC-1 cells showed a significantly poorer survival in comparison to control cells, with reductions of cell number by 50%, 50%, 40% at 10, 50, 100 nM of rapamycin, respectively (All $P < 0.01$, Fig. 4G)

In summary, these data indicated that abnormal overexpression of NNMT might enhance the survival of PANC-1 cells subjected to various conditions of metabolic stress.

Discussion

Pancreatic cancer has the worst prognosis among all types of malignancies and remains the fourth leading cause of cancer related death in the United States and worldwide [1]. The poor outcome is mainly due to the lack of sensitive diagnostic biomarkers, its strong resistance to therapeutic agents, and high metastatic potential [3]. Therefore, understanding of pancreatic cancer biology and identification of reliable detection biomarkers are urgently needed. As a phase II metabolizing enzyme, NNMT is involved in the biotransformation of many drugs and xenobiotic compounds [6, 7, 21]. Abnormal NNMT expression has been reported in many human neoplasms including brain, nasopharynx, oral cavity, thyroid, lung, liver, stomach, kidney, bladder and colon [8-18]. Furthermore, a recent study showed that NNMT overexpression was associated with increased radioresistance via regulating nicotinamide metabolism [22]. And Parsons et.al have found that NNMT expression reduced cell death by increasing ATP synthesis and complex I activity [23], and was able to induce changes in cell morphology via ephrin-B2 and Akt signaling in human neuroblastoma cells [24]. And very recently, a study found NNMT depletion induced cell apoptosis via mitochondria-mediated pathway in human breast cancer cells [25]. These studies suggest that NNMT promotes tumorigenesis and therefore may serve as a promising anti-tumor target.

In addition, NNMT was overexpressed in pancreatic cancer tissues [18] and up-regulated in the pancreatic juice of patients with pancreatic cancer [19]. Very recently, our study revealed that NNMT is associated with microRNA-1291 altered pancreatic carcinoma cell metabolome and suppressed tumorigenesis [20]. However, the biochemical and cellular roles of deregulation of NNMT in pancreatic cancer remain unknown. The present study shows the effect of NNMT on regulating pancreatic cell proliferation, cell mobility, and tolerance under metabolic stress, suggesting its crucial role in pancreatic cancer cell behavior and pancreatic tumor development.

Firstly, the current data showed that overexpression of NNMT moderately enhances PANC-1 cell growth and promotes pancreatic cell proliferation, whereas depletion of NNMT has a markedly inhibitory effect on cell proliferation, suggesting NNMT expression contributes to PANC-1 cell proliferation. Consistently, Wu et al. [26] reported that knockdown of NNMT by siRNA resulted in a 40% cell number reduction in bladder cancer cells when compared with controls. And Pozzi et al. [27] revealed a cell growth inhibitory effect due to sh-RNA mediated NNMT depletion in KB cells by MTT analysis and soft-agar colony forming assay. Interestingly, the high level of NNMT triggered by miR-1291 over-expression in PANC-1 cells resulted in a significant reduction of cell proliferation on day 6 and day 7 of the growth curve, which was a little bit different from the current study when NNMT was changed directly instead of miR-1291 trigger, suggesting different roles of NNMT under normal and abnormal (miR-1291 overexpression) physiological conditions.

Cell migration and invasion capacities are critical for tumor progression and metastasis. In the present study, NNMT overexpression significantly induced the migration and invasion potentials in PANC-1 cells; conversely, siRNA-treated NNMT depletion efficiently repressed the cell migration and invasion capacity. The results suggested that NNMT function as a mediator in regulating PANC-1 cell mobility. In line with our study, several studies reported NNMT are necessary in mediating cell mobility in other types of cancer [26, 28]. Wu et al. [26] showed NNMT expression correlated positively with cell migration and tumor stage in

bladder cancer and was necessary in regulating cell migration. Tang et al. [28] demonstrated that short hairpin RNA-mediated depletion of NNMT expression efficiently inhibited the metastasis of ccRCC cells in vitro and in vivo. Interestingly, they observed this NNMT-dependent cellular invasion was mediated via MMP-2 activation as well as the PI3K/Akt signaling pathway. However, a contradictory to these findings is that NNMT mRNA expression inversely correlates with lymph node metastasis in oral squamous cell carcinoma tissues, as revealed by Sartini et al. [14]. Similarly, findings in our previous study show that miR-1291 triggered NNMT overexpression reduces the migration and invasion ability of PANC-1 cells [20], which was different from the situation when NNMT was upregulated directly but not triggered by miR-1291.

Metabolic transformation is a process that tumor cells adapt their metabolism under environmental stress to gain survival/growth advantages, which is considered a hallmark of cancer [29]. The effect of NNMT on PANC-1 cell survival under conditions of metabolic stress was further investigated. In this study, NNMT-overexpressing PANC-1 cells demonstrated increased resistance to metabolic stress including glucose deprivation or glycolysis inhibition and were less sensitive to rapamycin, although the effects were not so striking as that of NNMT-deficient cells. In contrast, PANC-1 cells lacking NNMT showed significantly poorer survival under such harsh environment. Further study showed that when NNMT-overexpressing and control PANC-1 cells were incubated with 2-DG in the presence of rotenone, a mitochondrial inhibitor, there was no significant difference in cell survival between the NNMT-overexpressing and control cells. These results suggested that the increased survival of NNMT-overexpressing PANC-1 cells undergoing glycolysis inhibition may be due to the increased dependence upon oxidative phosphorylation, which was in accordance with previous findings in human neuroblastoma cells [23]. The results indicated NNMT is a gene that involves in the process of cancer metabolic transformation, which may play a critical role in pancreatic cancer progression.

As a type of hypovascular malignancy, blood supply is insufficient in pancreatic cancer tissues [30]. Thus, cancer cells may encounter fluctuations in glucose and energy supply due to abnormal angiogenesis and rapid tumor growth [31, 32]. This stringent conditions in tumor microenvironment exert selection pressures on cancer cells, and therefore lead to the progression of their aggressive and metastatic features [33]. The precise mechanism of how NNMT regulates metabolic transformation of pancreatic cancer is largely unknown, although the serine/threonine kinase Akt signaling pathway has been assumed to be involved.

Serine/threonine kinase Akt, also known as Protein Kinase B (PKB), has emerged as a central node in diverse cellular processes including cell survival, proliferation, metabolism, apoptosis, angiogenesis and migration [34-36]. Akt was reported aberrantly overexpressed in PANC-1 and other human pancreatic cancer cell lines, and a high expression of Akt was found associated with their remarkable tolerance for nutrient starvation [37, 38]. Several recent studies have shown that Akt signaling pathway may be linked with NNMT deregulation in cancer. Win et al. [17] observed that NNMT overexpression was positively correlated with Akt phosphorylation and implied worse prognosis in patients with nasopharyngeal carcinoma. Thomas et al. [24] found NNMT expression regulated neuron morphology in vitro via the sequential activation of the EFNB2 and Akt cellular signaling pathways. And very recently Zhang et al. [25] reported phosphorylation of Akt and ERK 1/2 was decreased in NNMT shRNA treated human breast cancer cells. However, whether and how Akt pathway is involved with NNMT deregulation in cancer metabolic transformation warrant further investigation.

In summary, the present study demonstrated that modulation of NNMT expression/function affected pancreatic cell proliferation, migration and invasion as well as cell survival under metabolic stress conditions. Up-regulation of NNMT moderately enhanced cell proliferation, whereas down-regulation of NNMT markedly inhibited cell growth. Furthermore, NNMT-expressing PANC-1 cells showed greater migration and invasion capacities. In addition, NNMT overexpression modestly improved cell survival, whereas NNMT depletion markedly suppressed cell survival under metabolic stress conditions. These

results suggest a crucial role for NNMT in the regulation of pancreatic cancer cell functions and NNMT could be a promising target for treating pancreatic cancer.

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Disclosure Statement

The authors declare no conflict of interest.

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