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Original Articles

Regulation of the Nampt-mediated NAD salvage pathway and its therapeutic implications in pancreatic cancer

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD) is a crucial cofactor for the redox reactions in the metabolic pathways of cancer cells that have elevated aerobic glycolysis (Warburg effect). Cancer cells are reported to rely on NAD recycling and inhibition of the NAD salvage pathway causes metabolic collapse and cell death. However, the underlying regulatory mechanisms and clinical implications for the NAD salvage pathway in pancreatic ductal adenocarcinoma (PDAC) remain unclear. This study showed that the expression of Nampt, the rate-limiting enzyme of the NAD salvage pathway, was significantly increased in PDAC cells and PDAC tissues. Additionally, inhibition of Nampt impaired tumor growth *in vitro* and tumorigenesis *in vivo*, which was accompanied by a decreased cellular NAD level and glycolytic activity. Mechanistically, the Nampt expression was independent of Kras and p16 status, but it was directly regulated by miR-206, which was inversely correlated with the expression of Nampt in PDAC tissues. Importantly, pharmacological inhibition of Nampt by its inhibitor, FK866, significantly enhanced the antitumor activity of gemcitabine in PDAC cells and in orthotopic xenograft mouse models. In conclusion, the present study revealed a novel regulatory mechanism for Nampt in PDAC and suggested that Nampt inhibition may override gemcitabine resistance by decreasing the NAD level and suppressing glycolytic activity, warranting further clinical investigation for pancreatic cancer treatment.

Introduction

The metabolic properties of cancer cells diverge significantly from those of normal cells. Cancer cells abnormally take up more glucose, which they mainly process through aerobic glycolysis, producing high levels of secreted lactate and exhibiting a lowered use of mitochondrial oxidative phosphorylation; this phenomenon is called the "Warburg effect" [1,2]. This metabolic switch generates adenosine 5′-triphosphate (ATP) from enhanced glycolysis, provides substrates for cell growth and division, and produces intermediates for anabolic reactions [2,3]. A better understanding of these altered metabolic pathways and the underlying regulatory mechanisms may ultimately lead to better cancer treatments.

Beside the related enzymes (e.g., Glut1, HK2, LDHA, PKM2) that play key roles in aerobic glycolysis, nicotinamide adenine dinucleotide (NAD) is a crucial cofactor in the redox reactions within cancer cell metabolic pathways [4]. NAD-dependent signaling pathways have been reported to regulate gene transcription, DNA repair, cell cycle progression, apoptosis, and metabolism [4,5]. Mechanistically, the conversion of glyceraldehyde 3-phosphate to 1,3-biphosphoglycerate, which is catalyzed by GAPDH in the glycolytic pathway, is dependent on NAD(H) as a coenzyme [5]. Thus, both the permanent synthesis and circulating utilization of NAD are essential to fueling bioenergetic processes and maintaining balanced redox regulation in cancer cells. NAD can be synthesized by both the salvage and *de novo* pathways in cells [6]. Nampt produces nicotinamide mononucleotide (NMN), a key NAD intermediate, from nicotinamide in the salvage pathway, and Naprt1 produces nicotinic acid mononucleotide (NaMN) from nicotinic acid (NA) in the *de novo* pathway [5,6].

A recent study showed that pancreatic ductal adenocarcinoma (PDAC) cells rely on the NAD salvage pathway and that inhibition of this pathway causes metabolic collapse and cell death [7]. However, the expression profile of the molecules involved in NAD synthesis and the underlying regulatory mechanisms for the key molecules remain unclear. Moreover, gemcitabine, the first-line

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chemotherapy regent for PDAC treatment, has been reported to modulate the expression of hypoxia-inducible factor 1α (HIF1α) in PDAC cells [8,9]. Given that HIF1α plays a key role in reprogramming cancer metabolism by activating the transcription of genes encoding glycolytic enzymes [10], we hypothesized that the combination of gemcitabine and Nampt inhibitor FK866 may have a synergistic cytotoxic effect against PDAC cells by modulating energy metabolism. In this study, we investigated the underlying regulatory mechanisms of the Nampt-mediated NAD salvage pathway and the therapeutic implications for overcoming chemoresistance though Nampt inhibition in pancreatic cancer.

Materials and methods

Cell lines and cell culture

The human PDAC cell lines (AsPc-1, BxPc-3, Colo357, Capan-1, Capan-2, Panc-1, Panc-28) and HEK293T cells were purchased from ATCC (Manassas, VA, USA) and cultured under conditions specified by the supplier. PATC-43, PATC-50, PATC-53, and PATC-66, which were established from patient-derived xenografts, were provided by Dr. Jason B. Fleming (MD Anderson Cancer Center, USA). The gemcitabine-resistant cell line, MiaPaCa-2/GR, immortalized/nontumorigenic HPDE cells, and hTERT-HPNE cells were described elsewhere [11,12]. All cell lines were authenticated by short tandem repeat (STR) fingerprinting before use at the Characterized Cell Line Core of MD Anderson Cancer Center.

Reagents and antibodies

Gemcitabine and FK866 was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in water and DMSO, respectively. The following antibodies were used for immunoblotting analysis: Nampt, Kras, p16, HIF1α and Ki67 (Santa Cruz Biotechnology, Santa Cruz, USA), HK2 and β-Actin (Cell Signaling Technology, Beverly, MA, USA).

NAD level and glycolytic activity analysis

The intracellular level of NAD/NADH was measured with the Fluorescent NAD/NADH Detection Kit (Promega, Madison, USA) according to the manufacturer's instructions. The cellular glucose uptake and lactate production levels were analyzed using the SBA-40C Biosensor (Biology institute of Shandong Academy of Science, Jinan, China), and the cellular ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, USA) according to the manufacturer's instructions.

Vectors and lentiviral transduction

The human miR-206 gene was PCR-amplified from gDNA and cloned into a pLV-puro lentiviral vector (Biosettia, San Diego, USA). Stable cell lines expressing miR-206 or Nampt shRNAs were generated via lentiviral infection of HEK293T cells and selected with 0.5 mg/mL puromycin for 7 days according to a previous report [12]. The oligo sequences for Nampt shRNA were (#2) ggtctccactcatggacat and (#3) cttcgtcacttccttttca.

Luciferase assay

DNA fragments from the 3′-UTR of Nampt that contained the predicted complementary sites of miR-206 were cloned into a pGL3-basic vector (Addgene, Cambridge, USA). MiR-206 mimic and miR-206-mut mimic were purchased from RiboBio (Guangzhou, China). Ten thousand cells were seeded in triplicate in 48-well plates and allowed to settle for 24 h. Then, the pGL3-Nampt-3′UTR reporter plasmids (100 ng) plus 5 ng of pRL-TK renilla plasmid (Promega, Madison, USA), which had increasing levels (10 nM and 50 nM) of negative control (NC), miR-206 mimic, or miR-206-mut mimic, were co-transfected into PDAC cells using the Lipofectamine LTX reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Luciferase and Renilla signals were measured 24 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, USA) according to the protocol provided by the manufacturer.

Animal study

The orthotopic pancreatic tumor-bearing NOD/SCID mouse model was established as described previously [13]. Female BALB/c nude mice (4–5 weeks of age, 16–18 g) were purchased from the Guangdong Province Laboratory Animal Center (Guangzhou, China). Two weeks after they were injected with cells, MiaPaCa-2 or Panc-1 orthotopic tumor-bearing NOD/SCID mice were randomly assigned (n = 5 per group) to receive the following on a weekly schedule: group 1 served as the control; group 2 received 50 mg/kg intraperitoneal gemcitabine once a week; group 3 was treated with 25 mg/kg intraperitoneal FK866 on 4 consecutive days per week (twice per day); and group 4 was treated with both of the treatments used in groups 2 and 3. All mice were weighed every 5 days and observed for tumor growth. The tissue was then fixed in formalin, embedded in paraffin, cut into 6-μm sections, and subjected to IHC and H&E staining. After deparaffinization, sections were analyzed by either IHC using anti-Ki67 or H&E staining with Mayer's hematoxylin solution. Apoptosis was detected using a fluorescence-labeled *in situ* cell death detection kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Our animal protocol was approved by our Institutional Animal Care and Use Committee.

The details for cell viability, colony formation, qRT-PCR, apoptosis and immunohistochemistry (IHC) assays are described in the Appendix: Supporting Materials and Methods.

Statistical analysis

For comparison of the significant differences between more than two groups, one-way analysis of variance (ANOVA) and Newman Keul's multiple comparison tests were used. All other statistical analyses were evaluated using Student's unpaired *t*-test with Prism GraphPad software (San Diego, CA, USA). All data are presented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$. A P value < 0.05 was considered statistically significant.

Results

Nampt is overexpressed in PDAC cells and human PDAC tissues

NAD synthesis is mediated by two distinct mechanisms, the salvage and *de novo* pathways, which are catalyzed by Nampt and Naprt1, respectively [6] (Fig. 1A). To determine whether the two pathways are involved in pancreatic tumorigenesis, we first investigated the expression profile of Nampt and Naprt1 in human PDAC tissues. qPCR analysis showed that the mRNA level of Nampt, not Naprt1, was significantly increased in human PDAC tissues compared with adjacent normal tissues (N = 21) (Fig. 1A). The increased expression of Nampt in PDAC was also supported by multiple cancer microarray data sets available from Oncomine (Fig. 1B). Furthermore, immunoblotting analysis showed that Nampt expression was notably increased in 8 primary PDAC tissues compared with the paired adjacent normal tissues (Fig. 1C). To further determine the clinical relevance of this finding, we analyzed Nampt expression in a pancreatic tissue microarray, including 90 PDAC and 87 normal pancreatic acinous tissues. We observed that 78.9% (71/90) of the PDAC tissues exhibited a high level of Nampt protein, whereas only approximately 18.4% (16/87) of the normal pancreatic tissues were positive for Nampt expression. Statistical analysis showed that the increase in Nampt expression in PDAC patients is significant (Fig. 1D). The expression profiles of Nampt and Naprt1 were also analyzed in PDAC cell lines. As expected, both the mRNA and protein levels of Nampt were increased in PDAC cells compared with nontumorigenic HPNE and HPDE cells (Fig. 1E); however, there were no significant differences in Naprt1 expression in these cells (Fig. S1A). Together, these results suggest that Nampt expression is significantly increased in PDAC cells and human PDAC tissues, and the Nampt-mediated NAD salvage pathway may play a key role in PDAC tumorigenesis.

Intracellular NAD depletion through inhibition of Nampt expression decreases PDAC cell growth in vitro *and* in vivo

As expected, we found that PDAC cells had enhanced glycolytic activity, which was demonstrated by the increased glucose uptake and lactate production level compared with those in nontumorigenic cells (Fig. S1B). NAD serves as a coenzyme of GAPDH in the glycolytic pathway [5]. Because Nampt has an important role in generating NAD to support elevated glycolysis in cancer cells, we posited that intracellular NAD depletion, achieved through inhibition of Nampt, may suppress glycolytic activity and inhibit PDAC cell growth. To test this possibility, we stably knocked down Nampt expression in MiaPaCa-2 and Panc-1 cells with high Nampt expression using lentiviral Nampt shRNAs and then evaluated the resulting cellular NAD level and glycolytic activity in these cells. As shown in Fig. 2A–B, Nampt depletion resulted in a significant decrease in the cellular