Na^+/H^+ exchanger regulatory factor 1 overexpression suppresses the malignant phenotype of MIAPaCa-2 pancreatic adenocarcinoma cells by downregulating Akt phosphorylation

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Abstract. Na^+/H^+ exchanger regulatory factor 1 (NHERF1) is reported to be associated with the development of numerous types of tumor; however, its effects on the metastasis of pancreatic adenocarcinoma are not fully understood. In the present study, it was revealed that the expression level of NHERF1 in pancreatic adenocarcinoma is decreased compared with normal pancreatic tissue based on the analysis of a protein expression database. The present study was undertaken in order to investigate the potential effects of NHERF1 overexpression on the malignant phenotype of MIAPaCa-2 pancreatic adenocarcinoma cells. NHERF1 was stably overexpressed in this cell line, and Cell Counting Kit-8, wound healing and Transwell assays were used to detect the proliferative and migratory abilities of the cells. NHERF1 overexpression suppressed proliferation in the MIAPaCa-2 cell line compared with empty vector-transfected (negative control) cells. Additionally, NHERF1 overexpression significantly inhibited the migration of MIAPaCa-2 cells. The results of a western blot analysis identified that NHERF1 overexpression markedly decreased the expression of phosphorylated-protein kinase B (p-Akt), while no significant difference was observed between untransfected and negative control cells. Taken together, these results suggested that NHERF1 may be able to inhibit the proliferation and migration and alter the malignant phenotype of pancreatic adenocarcinoma cells via reduction of p-Akt levels. These findings indicate a potential novel approach to the treatment of pancreatic adenocarcinoma.

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Abbreviations: NHERF1, Na^+/H^+ exchanger regulatory factor 1; PDAC, pancreatic ductal adenocarcinoma; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B

Key words: Na^+/H^+ exchanger regulatory factor 1, proliferation, migration, Akt phosphorylation

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer-associated mortality in the USA, and pancreatic ductal adenocarcinoma (PDAC) is a highly metastatic disease with a high mortality rate (1). The majority of patients are diagnosed at a late stage and, despite recent advances in chemotherapeutic approaches, the prognosis of pancreatic adenocarcinoma is extremely poor compared with other types of cancer (2,3). Tumor growth and migration are largely responsible for the high mortality rate of patients with pancreatic adenocarcinoma; therefore, it is important for researchers to investigate methods for preventing pancreatic adenocarcinoma cell proliferation and migration in order to improve the treatment of this disease (3,4).

Na^+/H^+ exchanger regulatory factor 1 (NHERF1; also known as sodium-hydrogen antiporter 3 regulator 1 or ERM-binding protein 50) is a multi-functional scaffolding protein that has different functions in a variety of types of cancer through its interactions with oncogenic or tumor-suppressive proteins (4,5). In breast cancer, NHERF1 has been demonstrated to inhibit proliferation by influencing the transduction of growth signals induced by epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor, and by modulating the expression of phosphatase and tensin homolog (6,7). By contrast, in prostate cancer, the expression of NHERF1 has been demonstrated to be increased, suggesting that NHERF1 may be associated with the carcinogenic potential of this cancer type (8). However, the function of NHERF1 in the proliferation and migration of pancreatic adenocarcinoma cells remains unresolved.

The objective of the present study was to determine the effects of NHERF1 expression on proliferative and migratory abilities of pancreatic adenocarcinoma cells by overexpressing NHERF1 in MIAPaCa-2 cells. The results revealed that NHERF1 may be able to inhibit the proliferative and migratory abilities of pancreatic adenocarcinoma cells by downregulating the phosphorylation of protein kinase B (Akt).

Materials and methods

Immunohistochemical (IHC) data, plasmids and cell lines. The IHC-based protein expression data, including high-resolution images, were downloaded from the Human Protein Atlas web portal, using *NHERF1* as the search term (pancreatic cancer database; www.proteinatlas.org). All IHC images from pancreatic cancer and normal tissues were collected, and the sum of the integrated optical density (IOD) values of images were analyzed using ImagePro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The mean IOD values of these images were counted, which reflected the relative NHERF1 expression level in pancreatic cancer and normal pancrease tissues, respectively. The pBK-CMV-HA-NHERF1 wild-type (wt) plasmid and the empty vector plasmid (pBK-CMV-HA) were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and G418 resistance was encoded by the plasmid. MIAPaCa-2 human PDAC cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Identification of stably transfected cells. MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in an incubator with 5% CO_2. DMEM was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). For stable overexpression of NHERF1, MIAPaCa-2 cells were transfected with 2 μg pBK-CMV-HA-NHERF1 wt plasmid or the pBK-CMV-HA plasmid (negative control) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Stably transfected cells were selected using 300 μg/ml G418 (Amresco, LLC, Solon, OH, USA) for 4 weeks and then maintained in maintenance culture medium containing G418 (150 μg/ml).

Western blotting. Cells were collected and total protein was extracted from cells stably expressing NHERF1 and from empty vector-transfected cells using radioimmunoprecipitation lysis buffer (Beijing CoWin Biotech Co., Ltd., Beijing, China) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (100X; Thermo Fisher Scientific, Inc.). Protein levels were quantified using bicinchoninic acid assays (Beijing CoWin Biotech Co., Ltd.). Subsequently, 30 μg protein from each sample was subjected to SDS-PAGE (10% gel). Proteins were then transferred to nitrocellulose membranes (Sigma-Aldrich; Merck KGaA). The membranes were blocked with 5% skimmed milk (dissolved in TBST) for 1 h at 25˚C, prior to incubation with rabbit anti-human primary antibodies against NHERF1 (1:1,000 dilution, cat. no. ab88238), and GAPDH (1:5,000 dilution, cat. no. ab70699) (both from Abcam, Cambridge, UK), Akt (1:1,000 dilution, cat. no. 9272; Cell Signaling Technology, Inc., Danvers, MA, USA) or phospho-Akt (p-AKT) (phospho-Ser473, 1:2,000 dilution, cat. no. 4060; Cell Signaling Technology, Inc.) overnight at 4˚C, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution, cat. no. ab6721; Abcam) for 1 h at room temperature. Detection was facilitated using an enhanced chemiluminescence western blot kit (Beijing CoWin Biotech Co., Ltd.) and images were analyzed using ImageJ software (version 1.62; National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay. Cells stably expressing NHERF1 were plated at a density of $5x10^3$ cells/well in 96-well plates at 37˚C in an incubator with 5% CO_2. Cell proliferation was then assessed every 24 h for 96 h using a Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. For each sample at each time-point, 6 wells were analyzed, and the experiment was repeated independently three times.

Wound healing assay. Cells were plated at $3x10^5$ cells/well in 6-well plates and grown to 100% confluence. Scratches were created with 1-ml pipette tips in the cell monolayer, and an image was immediately captured (0 h). Subsequent images were captured every 12 h, and the migration (scratch width) relative to 0 h was calculated using Image-Pro Plus analysis software (version 6.0; Media Cybernetics).

Transwell assay. For the Transwell assay, DMEM containing 10% fetal bovine serum was added to the lower chamber of Transwell culture plates. Subsequently, three groups of cells (untransfected, empty vector and NHERF1-overexpressing) were seeded into the upper chambers of Transwell 24-well culture plates in serum-free DMEM. Following incubation for 24 h at 37˚C, the cells on the upper membrane were removed with a cotton swab, and the cells that had migrated through the membrane were fixed in 4% paraformaldehyde for 15 min at 25˚C, and stained with 0.5% crystal violet for 15 min at 25˚C. The mean number of cells that had traversed the membrane was calculated in five random fields under a light microscope (magnification, x400).

Statistical analysis. All experiments were repeated at least three times. SPSS software (version 21.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used to analyze data. IHC data were analyzed using an independent samples t-test. Growth curves, Transwell assay and wound healing assay results were analyzed using a repeated-measures analysis of variance with Fisher's least significant difference post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of NHERF1 is downregulated in pancreatic adenocarcinoma tissues. To identify the expression level of NHERF1 in pancreatic adenocarcinoma tissues, sum of the IOD values of NHERF1 expression levels in pancreatic adenocarcinoma tissues (n=3) and adjacent normal pancreatic tissues (n=11) were compared using a Human Protein Atlas Database dataset. The results confirmed that the expression of NHERF1 was significantly downregulated in pancreatic adenocarcinoma tissues compared with adjacent tissues ($P<0.01$; Fig. 1), indicating that decreased NHERF1 expression may be associated with pancreatic cancer progression.

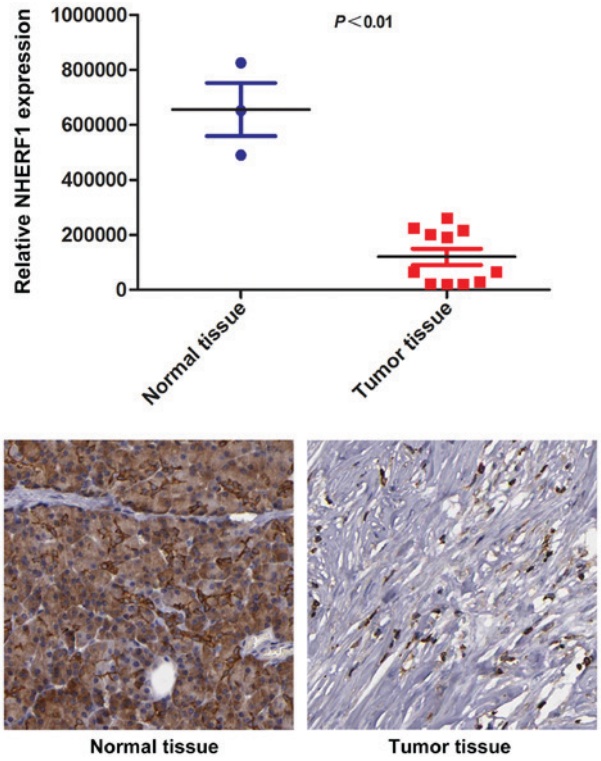

Figure 1. Expression of NHERF1 in pancreatic adenocarcinoma and adjacent tissues. The Human Protein Atlas web portal was used to analyze the expression of NHERF1 in pancreatic adenocarcinoma tissues and adjacent tissues. Data are presented as the sum of the integrated optical density values, relative to those of blank images. Representative images showing immunohistochemical staining of NHERF1 (brown) are presented (magnification, x200). NHERF1, Na^+/H^+ exchanger-regulatory factor 1.

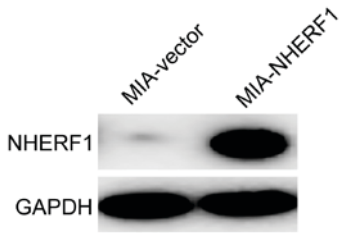

Figure 2. Expression of NHERF1 in MIAPaCa-2 cells transfected with a NHERF1 plasmid. Western blotting was used to detect NHERF1 expression in MIA-vector and MIA-NHERF1 cells. GAPDH was used as a loading control. NHERF1, Na^+/H^+ exchanger regulatory factor 1; MIA-vector, MIAPaCa-2 cells transfected with empty vector; MIA-NHERF1, MIAPaCa-2 cells stably expressing NHERF1.

Generation of PDAC cells overexpressing NHERF1. As presented in Fig. 2, NHERF1 was overexpressed in MIAPaCa-2 cells transfected with the NHERF1 plasmid compared with the cells transfected with the empty vector. These cells were used to verify whether NHERF1 is able to attenuate the malignant phenotype in subsequent experiments. Cells transfected with NHERF1 were designated MIA-NHERF1 and cells transfected with the empty vector were designated MIA-vector.

NHERF1 overexpression inhibits the proliferative ability of MIAPaCa-2 cells. The ability of NHERF1 to modulate the proliferation of PDAC cells was analyzed using a CCK-8 assay. The results suggested that NHERF1 overexpression significantly inhibited the proliferative capacity of MIAPaCa-2 cells following 48 h of incubation, compared with that of control cells (P<0.05; Fig. 3). There were no significant differences observed between MIA-vector and untransfected MIAPaCa-2 cells.

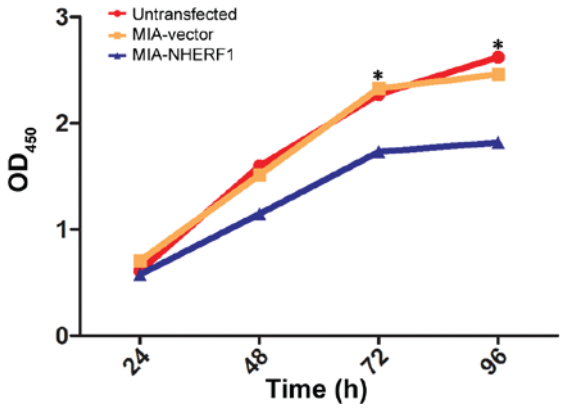

Figure 3. Anti-proliferative effects of NHERF1 overexpression in MIAPaCa-2 cells. A CCK-8 assay was performed to analyze the proliferative ability of untransfected control cells as well as MIA-NHERF1 and MIA-vector cells at different time-points. NHERF1, Na^+/H^+ exchanger regulatory factor 1; CCK-8, Cell Counting Kit-8; OD450, optical density at 450 nm; *P<0.05 compared with the untransfected and MIA-vector groups. MIA-vector, MIAPaCa-2 cells transfected with empty vector; MIA-NHERF1, MIAPaCa-2 cells stably expressing NHERF1.

NHERF1 overexpression inhibits the migratory ability of MIAPaCa-2 cells. A proliferation assay indicated that NHERF1 exhibited the ability to inhibit tumor cell proliferation. Since proliferation and migration are closely associated with tumor progression (9), the ability of NHERF1 to suppress cell migratory ability was investigated. The wound-healing assay revealed that, after 24 h, the wound width was significantly decreased in the MIA-vector and untransfected MIAPaCa-2 group, while the width reduction was significantly inhibited in the MIA-NHERF1 group (P<0.05), as presented in Fig. 4A. Consistently, in the Transwell assay, NHERF1 overexpression significantly decreased the number of migrated cells compared with the vector control group (P<0.05) (Fig. 4B). These results suggested that NHERF1 overexpression was able to inhibit the migratory abilities of MIAPaCa-2 cells.

NHERF1 overexpression reduces the Akt phosphorylation in MIAPaCa-2 cells. The activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway contributes to cell growth and survival of numerous types of cancer (10,11), including pancreatic adenocarcinoma (12), and NHERF1 has been demonstrated to regulate the PI3K/Akt signaling pathway in breast cancer (13). Thus, the activation of Akt was examined in the present study. The results identified markedly decreased levels of p-Akt in the MIA-NHERF1 group compared with the Mia-vector group, whereas there was no notable difference between the MIA-vector and untransfected MIAPaCa-2 cells. These results suggest that NHERF1 may inhibit Akt phosphorylation in pancreatic cancer cells (Fig. 5).

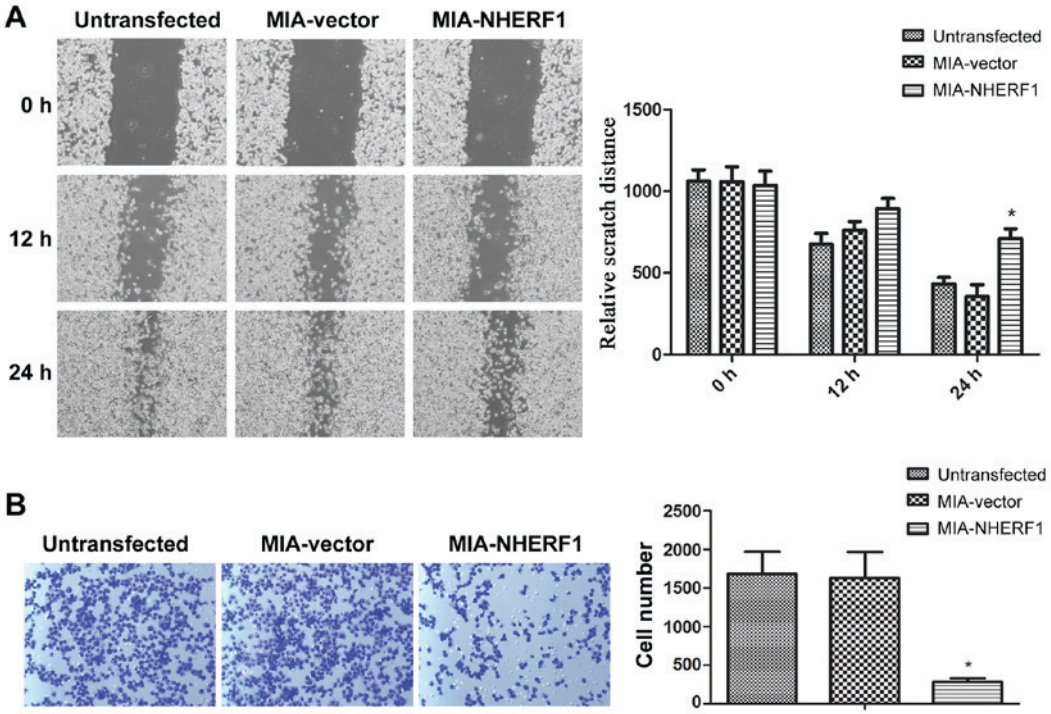

Figure 4. Anti-migratory effects of NHERF1 overexpression in MIAPaCa-2 cells. (A) Representative images of the wound-healing assay, which demonstrated that NHERF1 inhibited the migratory potential of MIAPaCa-2 cells. Quantitative analysis (right) identified that NHERF1 significantly suppressed MIAPaCa-2 cell migration. (B) Representative images of the Transwell migration assay, which revealed that NHERF1 inhibited the migration of MIAPaCa-2 cells. Quantitative analysis (right) confirmed that this effect was statistically significant. *P<0.05 compared with the untransfected and MIA-vector groups. Images were captured using light microscopy (magnification, x400). NHERF1, Na^+/H^+ exchanger regulatory factor 1; MIA-vector, MIAPaCa-2 cells transfected with empty vector; MIA-NHERF1, MIAPaCa-2 cells stably expressing NHERF1.

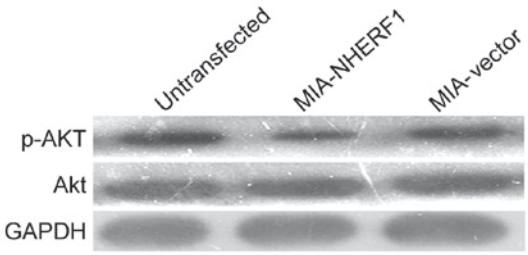

Figure 5. Effect of NHERF1 on the phosphorylation of Akt in MIAPaCa-2. Western blotting was used to detect the expression of Akt and p-Akt in untransfected, MIA-vector and MIA-NHERF1 cells. Mia-NHERF1 cells exhibited markedly decreased expression of p-Akt (Ser473) compared with MIA-vector and untransfected MIAPaCa-2 cells, but no difference was observed among these three groups. The expression level of Akt was not changed; however, NHERF1 was significantly increased (P<0.05). Akt, protein kinase B; p, phosphorylated; NHERF1, Na^+/H^+ exchanger regulatory factor 1; MIA-vector, MIAPaCa-2 cells transfected with empty vector; MIA-NHERF1, MIAPaCa-2 cells stably expressing NHERF1.

Discussion

In the present study, it was demonstrated that the expression level of NHERF1 was downregulated in pancreatic adenocarcinoma tissues, and that NHERF1 overexpression may inhibit the proliferative and migratory abilities of MIAPaCa-2 PDAC cells *in vitro*, while downregulating Akt phosphorylation. Therefore, NHERF1 may represent a metastasis-suppressing protein in pancreatic adenocarcinoma. The data suggest that NHERF1 expression may be able to inhibit the malignant phenotype of pancreatic adenocarcinoma cells via downregulating the expression of p-Akt.

Abnormalities in NHERF1 expression have been demonstrated to be associated with the occurrence, development and metastasis of cancer (6,14). NHERF1 is hypothesized to directly or indirectly affect adenocarcinoma behaviors via interaction with other proteins and signal transduction (15). NHERF1 is known to form a protein complex with EGFR (16), thereby mediating the internalization and signal transduction of EGFR to regulate oncogenic processes. NHERF1 also serves as a binding partner for G protein-coupled estrogen receptor (GPER), and its overexpression promotes the stability and activation of GPER in estrogen receptor-positive invasive breast cancer (17). Furthermore, a previous report outlined a complex function of NHERF1 in intestinal morphology and presented evidence for its *in vivo* tumor-suppressive function upstream of the Wnt-β-catenin and Hippo-YAP signaling pathways (18). However, the physiological function of NHERF1 in pancreatic cancer has largely remained unresolved.

Components of the PI3K/Akt/mTOR signaling pathway are commonly upregulated in malignant tumors, and increased Akt phosphorylation is commonly observed (19). The activation of Akt signaling is associated with cell proliferation, migration and invasion (20). Notably, it has been reported that NHERF1 inhibits the migration and invasion of human breast cancer cells via the PI3K/Akt signaling pathway (13). However, it remains unknown known whether or not NHERF1 may be able to attenuate the malignant phenotype of pancreatic cancer cells via inhibition of Akt phosphorylation.

In pancreatic adenocarcinoma tissues, NHERF1 exhibited relatively low endogenous expression. NHERF1 expression was increased using stable transfection with a pBK-CMV-HA-NHERF1 wt plasmid. Analysis indicated that NHERF1 overexpression inhibited the proliferation of MIAPaCa-2 cells, and also suppressed cell migration. Cell proliferation and migration are associated with tumor development and substantially contribute to the mortality of patients with tumors (2-4). Thus, the results of the present study are relevant to the understanding of tumor development and therapeutics. With regard to the molecular mechanism underlying the inhibition of cell proliferation and migration by NHERF1, two possible mechanisms are hypothesized. The first is that NHERF1 interacts with molecular partners, which serve important functions in proliferation and migration; for example, NHERF1 is reported to interact with EGFR and regulate EGFR signaling (21). The other hypothesis is that NHERF1 may be associated with the downregulation of Akt phosphorylation. Further experimental studies are required to confirm these hypotheses, and this may be a focus of future study.

NHERF1 participates in cell signaling and has multiple physiological functions. The results of the present study demonstrate that NHERF1 can regulate the malignant behaviors (proliferative and migratory abilities) of MIAPaCa-2 PDAC cells by downregulating Akt phosphorylation, and support that NHERF1 may function as a metastasis-suppressing protein in pancreatic adenocarcinoma. Thus, NHERF1 may be a potential therapeutic target for the treatment of pancreatic adenocarcinoma.

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