Endothelial Nitric Oxide Synthase Traffic Inducer (NOSTRIN) is a Negative Regulator of Disease Aggressiveness in Pancreatic Cancer

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Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is refractory to available treatments. Delineating critical pathways responsible for disease aggressiveness and therapeutic resistance may identify effective therapeutic targets. We aimed to identify key pathways contributing to disease aggressiveness by comparing gene expression profiles of tumors from early-stage PDAC cases with extremely poor survival (≤7 months) and those surviving 2 years or more following surgical resection.

Experimental Design: Gene expression profiling was performed in tumors in a test cohort of PDAC (N = 50), which included short (≤7 months, N = 11) and long surviving (>2 years, N = 14) patients, using Affymetrix GeneChip Human 1.0 ST array. Key genes associated with disease aggressiveness were identified, using Cox regression, Kaplan–Meier, and pathway analyses with validations in independent cohorts for mechanistic and functional analyses.

Results: Gene expression profiling identified 1,820 differentially expressed genes between short and long survival groups with inflammatory gene network ranking first. Lower expression of endothelial nitric oxide synthase traffic inducer (NOSTRIN) was associated with worst survival indicating its potential inhibitory role in disease progression. NOSTRIN overexpression suppressed migration and invasion of pancreatic cancer cells and enhanced sensitivity to chemotherapeutic drug gemcitabine. NOSTRIN inhibited production of nitric oxide (NO) by suppressing the activation of endothelial nitric oxide synthase (eNOS). Furthermore, miR-221, bound to the 3′UTR of NOSTRIN and suppressed its expression, and an increased miR-221 expression associated with poor survival in PDAC.

Conclusions: Our findings showed that NOSTRIN is a potential negative regulator of disease aggressiveness, which may be targeted for designing improved treatment strategy in PDAC.

Introduction

Pancreatic cancer is the fourth leading cause of death due to cancer with a median patient survival of 6 months and a 5-year survival of a mere 8% (1). Alarmingly, a consistent rise in cancer with a median patient survival of 6 months and a 5-year survival of a mere 8% (1). Among many types, pancreatic ductal adenocarcinoma (PDAC) is the most common (85%–90%) and deadly form of pancreatic cancer. The dismal prognosis in PDAC patients is due to the fact that it is highly refractory to available treatments and is usually diagnosed at an advanced stage of the disease (3, 4). A small number (15%–20%) of PDAC patients are diagnosed at localized, early stages, and qualify for surgical resection with curative intent (5). However, a majority of these surgically resected patients show recurrence within 2 years after surgery and adjuvant therapy, and only 27% survive up to 5 years (seer.cancer.gov). Several clinical prognostic factors, including stage, grade, and resection margin status, are attributed to the outcome in resected patients, and yet variable outcomes exist among resected cases with similar disease status, who survive stage I/II patients with technically perfect resection and negative resection margin (R0) survive less than 6 months, as compared with other resected patients with similar disease status, who survive 5 years or longer. These facts suggest that investigating the potential molecular differences between tumors from early stage, resected patients with long and short survival may provide insights into the molecular mechanism of disease aggressiveness. To understand the underlying mechanism of disease aggressiveness and identify potential molecular targets, we tested the hypothesis that a distinct gene expression profile contributes to the enhanced disease progression in short survival as compared to long survivors.
with the long survival groups of resected patients with PDAC. To test this hypothesis, we compared gene expression profile of stage I/II, resected patients in short (<7 months) and long survival (>2 years) groups. Our findings showed that NOSTRIN is a negative regulator of disease aggressiveness and is regulated by miR-221 in PDAC, and may be targeted for improved disease outcome.

NOSTRIN was identified as a protein, which binds to endothelial nitric oxide synthase (eNOS) and translocates it to the interior compartment of the cell in a vesicular structure, which results in the inhibition of NO production (9, 10). eNOS is a member of the nitric oxide synthase (NOS) family of enzymes, which catalyzes the conversion of arginine to citrulline with the production of nitric oxide (NO), like inducible NOS (iNOS) and neuronal NOS (nNOS), the other members of the family (11, 12). Although, NO performs several critical physiologic functions, it is associated with the development and progression of cancer (13–15).

Materials and Methods

PDAC samples

Pancreatic tumor tissue from resected PDAC cases were collected at University of Maryland Medical System (UMMS), Baltimore, MD, through NCI-UMD resource contract and the Department of General, Visceral and Pediatric Surgery, University Medicine Gottingen (Gottingen, Germany) with the approval of Institutional Review Board. Clinical and demographic information, including age, sex, clinical staging, differentiation grade, resection margin status and survival from the time of diagnosis were also available with patients consent. Tumor histopathology was classified according to the World Health Organization Classification of Tumors by a board-certified pathologist. The characteristics of the patients in test cohort are shown in Supplementary Table S1 and S2. Use of the clinical specimens was reviewed and permitted by the NCI-Office of Human Subject Research (OHSR, Exempt# 4678) at the NIH (Bethesda, MD).

Validation cohort from published microarray data

The correlation of gene expression and survival was validated in Collisson cohort (16). Gene expression data and clinical features of patients were downloaded from Oncomine (17). Clinical features of our test and validation cohorts are shown in Supplementary Table S2.

RNA isolation, cDNA microarray, and quantitative RT-PCR

A standard Trizol protocol (Invitrogen) was used to extract total RNA from frozen tumor samples. RNA extraction from cultured cells was performed using the Total RNA Purification kit (Norgenbiotek) following the manufacturer’s protocol. RNA quality was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies). mRNA expression profiling was performed using the Affymetrix GeneChip Human 1.0 ST arrays according to the manufacturer’s protocol at the microarray core facility of the National Cancer Institute (Frederick, MD). All arrays were normalized and gene expression summaries were created for each gene by using the average of all probe sets for each gene using Partek Genomics Suite 6.5. Further data analysis was performed using the gene-summarized data. The microarray gene expression data has been deposited in the National Center for Biotechnology Information (NCBI) as GSE78229. For quantitative RT-PCR, RNA was first reverse-transcribed using Multi Scribe reverse transcriptase (Life Technology). Gene expression levels were determined by TaqMan assay with probes from Applied Biosystems: NOSTRIN (Hs00976555_m1), IGF2BP3 (Hs00559907_g1), AQP9 (Hs00175573_m1), SLC16A3 (Hs00358829_m1), and 18S rRNA (Hs99999901_s1).

Cell lines and culture condition

Human pancreatic cancer cell lines were purchased from the ATCC and were authenticated by short tandem repeat (STR) analysis. CFPAC-1 cells were grown in Iscove modified Dulbecco’s medium. Panc-1 and Mia-paca 2 cells were grown in DMEM, supplemented with 10% FBS, penicillin-streptomycin (50 IU/mL and 50 mg/mL, respectively) and 2 mmol/L l-glutamine in a humidified incubator containing 5% CO2 at 37°C. All products for cell culture were purchased from Gibco.

Generation of stable NOSTRIN-overexpressing cells using lentiviral vectors

Lentiviral NOSTRIN constructs (EX-E1789-Lv105) were purchased from Genecopoeia. The details are provided in Supplementary Methods.

Western blotting

Cells were washed in PBS, lysed with RIPA buffer (Invitrogen), and standard immunoblotting protocol was used as described in detail in Supplementary Methods.

Measurement of nitric oxide production

Nitric oxide production was measured using Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical) according to the manufacturer’s instruction. Additional detail is provided in the Supplementary Methods.

Drug sensitivity assay

Gemcitabine was purchased from Tocris Bioscience. Mia-paca 2 cells with or without NOSTRIN overexpression were seeded in 96-well plates at a concentration of 2,000 cells/well. Gemcitabine at the indicated concentration was added into each well 24 hours after cell seeding and incubated in 37°C incubator with 5% CO2 for 72 hours. The cytotoxicity was evaluated by WST-8
colorimetric assay (Dojindo). Mean values were calculated from three different wells in triplicates. For colony formation assay, cells were seeded into 6-well plate at 1,000 cells/well, and gemcitabine treatment started 24 hours later until colonies were formed. Colonies were then fixed with methanol and stained with 0.5% crystal violet solution to be visualized.

Luciferase reporter assay
Cells were cultured in 96-well plates and cotransfected with 50 nmol/L of Pri-miR-221 or 50 nmol/L negative control), 100 ng of luciferase reporter NOSTRIN-3’UTR-WT (1532-1541) or NOSTRIN-3’UTR-MUT (1532-1541 Mutant), and 1 ng of pRL-CMV Renilla luciferase reporter. Cells were incubated for 48 hours then washed twice in PBS and harvested for firefly/Renilla luciferase assay using the Dual-Luciferase Reporter Assay System (Promega). Additional details are provided in the Supplementary Methods.

Caspase activity
Caspase-3/7 activity was measured using Apo-one kit (Promega) according to manufacturer’s instructions. Briefly, 2,000 cells were seeded into 96-well plate in triplicates. Chemotherapeutic drug was added 24 hours after seeding and incubated for 18 or 60 hours. Apo-one reagent was added into each well at a 1:1 ratio, and absorbance at 520 nm was measured an hour later using Fluostar microplate reader (Omega). Caspase-3/7 activity induced by chemotherapeutic drugs was calculated relative to untreated group. Results were obtained from three independent experiments.

Xcelligence real-time migration
Real-time cell migration was measured in CIM E-plates (ACEA), and cell index was recorded by Xcelligence system (ACEA) as indicator of cell migration ability. The details are provided in the Supplementary Methods.

Statistical analysis
Kaplan–Meier analysis was performed, using GraphPad Prism 6.0, to evaluate the differences in survival probability in resected pancreatic cancer cases. Univariable and multivariable Cox proportional hazards regression analysis was performed using STATA 11 (StataCorp LP) to investigate the association of NOSTRIN expression level in tumors and other clinical factors with survival in PDAC cases. Final multivariable models were based on stepwise addition and removal of clinical prognostic factors found to be associated with survival in univariate analysis (P < 0.05). These models did not violate proportional hazards assumptions based on Schoenfeld residuals. For these analysis, resection margin status was dichotomized as positive (R1) versus negative (R0); staging as stage I/IIA versus stage IIB, and differentiation grade as G1–G2 versus G3–G4.

Results
Gene expression profiling of tumors from short and long survival groups of resected patients identified NOSTRIN as a prognostic biomarker in PDAC
We compared gene expression profile of tumors from patients with short (≤7 months, N = 11) and long survival (≥2 years, N = 14) in our test cohort of a total of 50 resected PDAC patients, using affymetrix GeneChip Human 1.0 ST arrays (GSE78229; Fig. 1). The clinical characteristics of these patients are described in Supplementary Tables S1 and S2. Comparing gene expression, using ANOVA, identified 1,820 differentially expressed genes between short and long survival groups (Supplementary Table S3). Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) using 1,820 genes ranked inflammatory genes as the top gene network. Next, we assessed the prognostic significance of these 1,820 genes first by Cox regression analysis in the test cohort (N = 50), which resulted in the identification of 201 genes that
were associated with survival (\(P < 0.05\); Supplementary Table S4). Gene ontology analysis classified 66 of 201, as inflammatory genes (Supplementary Table S5). The fact that inflammatory mediators play a significant role in the development and progression of pancreatic cancer (18), and that the inflammatory genes are the top ranking gene network in our dataset, we pursued further investigation on the 66 inflammatory genes. Kaplan–Meier analyses showed that 35 of 66 inflammatory genes were associated with survival (Supplementary Table S6). Four of 35 potentially prognostic genes AQP9, NOSTRIN, IGF2BP3, and SLC16A3, could be validated in publicly available gene expression dataset (GSE 17891; ref. 16; Fig. 2A and B). We then used qRT-PCR to further validate 4 genes in an additional validation cohort-2 (\(N = 37\)), which showed that a decreased expression of NOSTRIN was associated with poorer survival (Fig. 2C). In addition, multivariable analysis showed that NOSTRIN is an
Figure 3.

NOSTRIN suppresses invasiveness of pancreatic cancer cells by inhibiting eNOS-derived NO production. **A**, Western blot analysis showing NOSTRIN overexpression in three PDAC cell lines. **B**, Overexpression of NOSTRIN reduced cell migration in Mia-paca 2 and CFPAC-1 pancreatic cancer cells. Cell migration index was recorded by Xcelligence system for up to 24 hours after cells were seeded in the top wells. Histogram represents the migration index at 24 hours. Cell migration index was calculated by averaging the values in quadruplicate wells. Curves were compared using Student t test. P values less than 0.05 were considered significant. **C**, Panc1 and CFPAC1 cells with NOSTRIN overexpression showed reduced invasion as determined by BD invasion assay. Representative picture showing cell invasion at 24 hours. Average cell number in five randomly selected fields for each cell line is shown in the histogram. Results are compared using Student t test. **D**, Western blot analysis showing that overexpression of NOSTRIN reduced p-eNOS, while the knockdown of NOSTRIN by shRNA increased p-eNOS level. **E**, NOSTRIN overexpression inhibited the production of NO in PANC1 cells. **F**, Exposure to NO by treatment with NO-donor drug Deta/NO, at indicated concentration, increased cell migration in CFPAC and Panc10.05 cell lines. **G**, shRNA-mediated knockdown of NOSTRIN and exposure to NO-donor increased cell migration to comparable levels. Experiments were repeated three times. Error bars, SD.
NOSTRIN regulates PDAC progression

**Independent predictor of prognosis in PDAC** (Supplementary Table S7). Furthermore, a lower expression of NOSTRIN was found in tumors as compared with adjacent nontumor pancreas (Fig. 2D), which could be validated in two additional publicly available datasets (GSE 15471 and GSE 16515; refs. 19, 20; Fig. 2E and F). In addition, immunohistochemical staining revealed that NOSTRIN largely expressed in tumor cells (Supplementary Fig. S1). These findings showed that a lower expression of NOSTRIN is associated with poor survival in resected PDAC patients and may negatively regulate disease progression.

**NOSTRIN suppresses invasiveness of pancreatic cancer cells by inhibiting the activation of eNOS and NO production**

To determine the potential role of NOSTRIN in the progression of pancreatic cancer, we generated pancreatic cancer cell lines with lentiviral-mediated stable expression of NOSTRIN (Fig. 3A). NOSTRIN-overexpressing Mia-paca2 and CFPAC1 pancreatic cancer cell lines showed significantly reduced mobility and invasion (Fig. 3B and C). As mentioned earlier, NOSTRIN has been described to bind eNOS and modulates its intracellular distribution and reduces the release of NO. In addition, eNOS-generated NO may contribute to the development and progression of pancreatic cancer (21). Therefore, we tested the hypothesis that NOSTRIN-induced suppression of pancreatic cancer cell migration and invasion is mediated through the inhibition of eNOS and subsequent reduction in NO level. NOSTRIN-overexpressing cells showed a reduced expression of phosphorylated (activated) eNOS (p-eNOS; Fig. 3D). Consistently, shRNA-mediated knockdown of NOSTRIN enhanced the expression of p-eNOS (Fig. 3D). Phosphorylation of eNOS is required for its activation and subsequent generation of NO (22). We then examined whether NOSTRIN-mediated inhibition of p-eNOS affects the NO production in the pancreatic cancer cells. NOSTRIN-overexpressing cells generated a significantly lower amount of NO, as determined by the level of nitrate/nitrite, stable end products of NO in culture medium (Fig. 3E). Furthermore, treatment of pancreatic cancer cells with NO-donor drug, Deta/NO, enhanced cell migration (Fig. 3F). Consistently, the increase in cell migration, following shRNA-mediated knockdown of NOSTRIN was comparable with the increased cell migration observed following the treatment with NO-donor drug (Fig. 3G). These findings showed that NOSTRIN suppresses pancreatic cancer cell mobility and invasiveness by inhibiting eNOS activation and NO production.

**NOSTRIN promotes gemcitabine-induced apoptotic cell death of PDAC cell lines**

PDAC is highly resistant to available chemotherapeutic drugs. Gemcitabine is the standard-of-care drug both in early-stage resected and advanced cases. We examined whether NOSTRIN modulates the sensitivity of pancreatic cancer cells to gemcitabine. NOSTRIN overexpression significantly reduced the viability and colony-forming ability of pancreatic cancer cells following gemcitabine treatment (Fig. 4A and B and Supplementary Fig. S2). We then examined whether increased sensitivity is due to enhanced apoptosis by determining caspase-3/7 activity. NOSTRIN-overexpressing cells showed significantly enhanced caspase-3/7 activity (Fig. 4C). Consistently, shRNA-mediated knockdown of NOSTRIN sensitized Mia-paca2 cells to gemcitabine (Fig. 4A and B). These findings indicate that NOSTRIN promotes gemcitabine-induced apoptotic cell death in PDAC cell lines.

**Figure 4.**

Overexpression of NOSTRIN sensitizes PDAC cell lines to chemotherapeutic drug, gemcitabine, by enhancing apoptosis. A, Representative pictures of colony formation assay in Mia-paca2 cells with or without NOSTRIN overexpression, following treatment with gemcitabine at 0.005 and 0.01 μmol/L concentration. B, NOSTRIN overexpression sensitized Mia-paca2 cells to gemcitabine. Histogram showing the percentage of viable Mia-paca2 cells with or without NOSTRIN overexpression after 72-hour exposure to gemcitabine. C, NOSTRIN-overexpressing cells showed an enhanced apoptosis, as determined by caspase-3/7 activity, following treatment with gemcitabine. All experiments were repeated three times. Error bars, SD.
activity following treatment with gemcitabine (Fig. 4C). These findings showed that enhanced expression of NOSTRIN sensitizes tumor cells to chemotherapeutic drug gemcitabine.

**NOSTRIN is regulated by miR-221**

To further delineate the role of NOSTRIN in PDAC, we next examined the regulation of NOSTRIN. miRNAs are important regulators of gene expression and are implicated in the development and progression of pancreatic cancer (23, 24). Examination of 3’UTR of NOSTRIN, using Target Scan and MiR-Walk target prediction module identified potential miR-221/222 binding sites. miR-221 is highly expressed in pancreatic cancer (23) and a higher plasma level of miR-221 is positively correlated with distant metastasis (25). We found a negative correlation between NOSTRIN and miR-221/222 expression, as determined by qRT-PCR, in tumors from PDAC cases ($N = 37$; Fig. 5A and Supplementary Fig. S3A). Furthermore, an increased expression of miR-221 associated with poorer prognosis in PDAC patients (Fig. 5B). However, miR-222 expression did not show any association with survival in our sample set (Supplementary Fig. S3B). Therefore, we pursued our further investigation with miR-221.

Overexpression of miR-221 resulted in a decrease in the expression of NOSTRIN both at mRNA and protein level along with an increase in the level of phosphorylated eNOS (p-eNOS; Fig. 5C).
and D). Furthermore, a potential miR-221–binding site was identified in 3’UTR of NOSTRIN, and luciferase reporter–based activity assay showed that miR-221 binds to the 3’UTR and downregulates NOSTRIN-luciferase reporter activity. However, this effect was abolished with mutations in the miR-221-binding site (Fig. 5E and F). These data showed that NOSTRIN is downregulated by miR-221 resulting in an increased level of p-eNOS.

Discussion

Prognosis in patients with PDAC continues to be dismal with the lack of effective treatment in advanced disease. Understanding the underlying mechanism of disease aggressiveness is crucial for identification of desperately needed novel and effective therapeutic targets. Early detection and surgical resection do improve survival as compared with advanced disease but the median survival remains less than 2 years even in resected cases. However, a small percentage of resected patients do show prolonged survival of 5 years and in some cases even 10 years. We hypothesized that comparing gene expression profile of tumors from early-stage, resected patients with long and short survival groups of PDAC patient cohort may provide insight into the mechanistic regulation of disease aggressiveness and identification of candidate therapeutic targets. In this study, we compared mRNA expression profile of short (≤7 months) and long (≥2 years) survival groups of early-stage (I/II), resected patients and validated our findings in multiple independent cohorts of PDAC. Our findings showed that NOSTRIN is a potential negative regulator of disease aggressiveness and is modulated by miR-221 in PDAC.

NOSTRIN was discovered as a protein, which binds to eNOS, regulates its intracellular distribution, and inhibits the generation of NO (9). In addition to NOSTRIN, another protein, called eNOS interacting protein (NOSIP), binds to eNOS resulting in its dislocation from plasma membrane and inhibition of NO production (10, 26). However, in our gene expression analysis of tumors from short and long survival groups of PDAC patient cohort, NOSIP was not differentially expressed. In addition, platelet-endothelial cell adhesion molecule-1 (PECAM-1) regulates eNOS activity and localization through transcriptionally enhancing the expression of NOSTRIN (27). The functional role of NOSTRIN in pancreatic cancer has not been described. Previously, a meta-analysis of gene expression described NOSTRIN as a member of multi-gene signature associated with pancreatic cancer prognosis (28). The findings in our study additionally show that NOSTRIN is an independent predictor of prognosis in resected PDAC. Our examination of the functional role of NOSTRIN in pancreatic cancer revealed that it suppresses the migratory and invasive properties of pancreatic cancer cell lines and increases their sensitivity to chemotherapeutic drug, gemcitabine, by enhancing apoptosis. These findings are consistent with the hypothesis that NOSTRIN is a potential negative regulator of disease aggressiveness.

NO is a free radical with multiple functions including vasodilation, neurotransmission, and inflammatory response. Moreover, NO has been implicated in the development and progression of cancer (12). NO is generated during the conversion of arginine to citruline, a reaction catalyzed by a family of nitric oxide synthases (NOS), which includes neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Both iNOS and eNOS are implicated in the development and progression of pancreatic cancer (21, 29, 30, 31). In a recent study, genetic and pharmacologic inhibition of eNOS resulted in reduced tumorigenesis in a genetically engineered mouse model of PDAC (21). eNOS activity is regulated through phosphorylation in addition to the calcium concentration, and phosphorylated eNOS (p-eNOS) can produce a high and sustained level of NO (22, 32). shRNA-mediated inhibition of eNOS reduced tumor growth of pancreatic cancer cell lines containing highly phosphorylated eNOS (33). Suppression of p-eNOS level and NO production by NOSTRIN in our study indicated that tumor-inhibitory role of
NOSTRIN may be mediated through inhibition of eNOS activation and subsequent decrease in NO production. Treatment with NO-donor compound Deta/NO enhanced migration of pancreatic cancer cell lines. In addition, knocking down NOSTRIN resulted in increased migration of pancreatic cancer cells and was comparable with the increase in cell migration following treatment with NO-donor. These findings suggest that NOSTRIN-induced reduction in the p-eNOS and NO production may negatively regulate the metastatic potential of tumor cells.

Determining the potential mechanisms for the regulation of NOSTRIN may provide insights into developing strategies for its therapeutic targeting. miRNA-mediated regulation of cancer-related genes has been described as one of the important event in the development and progression of cancer (24). A number of miRNAs are implicated in PDAC and are described as both prognostic biomarkers as well as functional components associated with therapeutic resistance and disease aggressiveness (23, 34, 35). Analysis of the 3’UTR of NOSTRIN revealed potential binding site for miR-221, which is earlier described to be highly expressed in PDAC. Our findings showed that miR-221 targets NOSTRIN and reduces its expression. Furthermore, an increased expression of miR-221 in our cohort was associated with poorer survival in resected cases of PDAC. Whereas PECAM/STAT3 signaling pathway transcriptionally enhances the expression of NOSTRIN (27), we did not see any difference in the expression of PECAM in tumors between the long and short surviving PDAC patients following resection. Therefore, it is conceivable that inhibition of miR-221 may enhance NOSTRIN leading to the inhibition of eNOS activity, NO production, and tumor progression in PDAC (Fig. 6).

Taken together, our findings identify NOSTRIN as a potential negative regulator of disease progression, which can be targeted in designing rational treatment strategy in patients with PDAC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.P. Hussain
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