MiR-126 Acts as a Tumor Suppressor in Pancreatic Cancer Cells via the Regulation of ADAM9

Shin Hamada1, Kennichi Satoh1,2, Wataru Fujibuchi3, Morihisa Hirota1, Atsushi Kanno1, Jun Unno1, Atsushi Masamune3, Kazuhiro Kikuta1, Kiyoshi Kume1, and Tooru Shimosegawa1

Abstract
The epithelial-mesenchymal transition (EMT) is a critical step for pancreatic cancer cells as an entry of metastatic disease. Wide variety of cytokines and signaling pathways are involved in this complex process while the entire picture is still cryptic. Recently, miRNA was found to regulate cellular function including EMT by targeting multiple mRNAs. We conducted comprehensive analysis of miRNA expression profiles in invasive ductal adenocarcinoma (IDA), intraductal papillary mucinous adenoma, intraductal papillary mucinous carcinoma, and human pancreatic cancer cell line to elucidate essential miRNAs which regulate invasive growth of pancreatic cancer cells. Along with higher expression of miR-21 which has been shown to be highly expressed in IDA, reduced expression of miR-126 in IDA and pancreatic cancer cell line was detected. The miR-126 was found to target ADAM9 (disintegrin and metalloproteinase domain-containing protein 9) which is highly expressed in pancreatic cancer. The direct interaction between miR-126 and ADAM9 mRNA was confirmed by 30 untranslated region assay. Reexpression of miR-126 and siRNA-based knockdown of ADAM9 in pancreatic cancer cells resulted in reduced cellular migration, invasion, and induction of epithelial marker E-cadherin. We showed for the first time that the miR-126/ADAM9 axis plays essential role in the inhibition of invasive growth of pancreatic cancer cells.

Introduction
Early invasion to surrounding tissue or rapid dissemination to distant organ is characteristic feature of pancreatic cancer. Only 20% of patients are eligible for surgical resection and the prognosis is unfavorable even in those patients (1). Epithelial-mesenchymal transition (EMT) plays important role during invasion and metastasis which requires involvement of multiple cytokines and signaling pathways (2). Targeting the EMT-inducing machinery in pancreatic cancer cells could be a novel therapeutic strategy.

In addition to cytokines and signaling molecules, a novel paradigm has been brought into the cancer research field. Now it is recognized that noncoding RNA including miRNA regulates various biologic processes including EMT (3). miRNA is a small RNA consisting of 20 to 23 nucleotide which targets 30 untranslated region (UTR) sequence of mRNA for destabilization (4). One miRNA is able to target multiple mRNAs which indicates that miRNA could orchestrate gene regulation of biologic processes. The role of miRNA in cancer progression has been extensively studied on the basis of these evidences. Several miRNAs are differentially expressed in cancer cells compared with normal cells. Their expression status determines the biologic behavior of cancer cells. The miR-21 is highly expressed in pancreatic cancer cells than in the normal pancreatic ductal cells (5). Furthermore, transient overexpression of miR-21 attenuates the antiproliferative effect of gemcitabine. The miR-145 is downregulated in breast cancer tissue compared with normal tissue (6). Reexpression of miR-145 in breast cancer cell lines results in decreased cellular migration and invasion.

In this study, we carried out comprehensive analysis of miRNA expression profiles in invasive ductal adenocarcinoma (IDA), intraductal papillary mucinous adenoma (IPMA), intraductal papillary mucinous carcinoma (IPMC), and human pancreatic cancer cell line to elucidate indispensable miRNAs which are differentially expressed in IDA and pancreatic cancer cell line. The miR-126 was significantly downregulated commonly in IDA and pancreatic cancer cell line and its direct interaction with novel...
target gene ADAM9 was identified by 3′-UTR assay. The miR-126/ADAM9 axis was confirmed to regulate cellular migration and invasion of pancreatic cancer cells for the first time which would delineate novel therapeutic target of advanced pancreatic cancer.

Materials and Methods

Cell line and cell culture

The human pancreatic cancer cell lines ASPC-1, MiaPaca-2, BxPC3, Panc-1, and KLM-1 were obtained from American Type Culture Collection or Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Sendai, Japan) and maintained as described previously (7, 8).

Tissue samples and microdissection

Normal pancreatic tissue samples were obtained from patients who underwent surgical resection of organ with pancreatic tissue due to the non-pancreatic disease in our institute. Tissue samples of IDA, IPMA, and IPMC were obtained from patients who underwent surgical resection in our institute. Tissue samples of IDA were also obtained from patients who received fine-needle aspiration (FNA) biopsy for diagnosis. Histologic diagnosis of each sample was confirmed by pathologists who were not informed about current research. For microdissection, tissue samples were immediately embedded in Tissue-Tek O.C.T. compound medium (Sakura), frozen in liquid nitrogen, and stored at −80°C. The frozen section in 8 μm thick was made using cryostat (Jung CM3000; Leica). Five IDA samples, 6 IPMA samples, and 6 IPMC were subjected to laser-captured microdissection using a Leica CIR MIC system (Leica Microsystems). Sample collection and usage were conducted under written informed consent obtained from each patient before surgery.

RNA extraction

Total cellular RNA was extracted from microdissected or bulk sample or pancreatic cancer cell line using the mirVana miRNA Isolation Kit (Applied Biosystems).

Microarray and statistical analysis

We used the Agilent Human miRNA microarray v2.0 (Agilent Technologies) to compare the expression profiles of miRNA. Each array contains 723 human miRNA and 76 viral miRNA. One hundred nanogram of total RNA from each sample was labeled by cyanine-3-cytidine bisphosphate using miRNA Complete Labeling Reagent and Hyb kit (Agilent Technologies), then array slide was hybridized with labeled miRNA for 20 hours. Hybridized array slides were scanned by Agilent Microarray Scanner (Agilent Technologies), and fluorescent signal of each spot was corrected using Agilent Feature Extraction v.9.1.3.1 (Agilent Technologies). The array data were deposited in the Gene Expression Omnibus database (GSE29542).

The significantly upregulated or downregulated miRNAs in IDA sample or pancreatic cancer cell line compared with IPMA or IPMC sample were elucidated by statistical analysis of array data. The raw data of each sample were normalized by robust multichip average method. These normalized data were adjusted to the linear model, and each miRNA was ranked using Bayesian t test. Differentially expressed miRNAs were determined at δ (false discovery rate) = 0.01. Normalization, linear model fitting, and elucidation of differentially expressed miRNAs were conducted using AgiMiRNA package (9) in Bioconductor-2.7 on R version 2.12.0.

Real-time reverse transcriptase PCR

The expression level of hsa-miR-126 in each normal pancreas, FNA sample, or cancer cell line sample was quantified by real-time reverse transcriptase PCR (RT-PCR) using TaqMan MicroRNA Assays (Applied Biosystems) and StepOnePlus real-time PCR system (Applied Biosystems). The expression level of hsa-miR-126 in each sample was normalized by expression level of U6.

Immunohistochemistry and in situ hybridization

Normal pancreatic tissue or IDA tissue collected at the time of surgery were fixed in 10% paraformaldehyde overnight and embedded in paraffin wax. Immunohistochemistry of ADAM9 was conducted as described previously using ADAM9 antibody (2099 Cell Signaling Technology, Inc.) and the Histofine Kit (Nichirei; ref. 10). In situ hybridization of hsa-miR-126 was conducted using miRCURY LNA microRNA ISH Optimization Kit [formalin-fixed, paraffin-embedded (FFPE); EXIQON] and DIG-labeled hsa-miR-126 detection probe (EXIQON) at final concentration of 50 nmol/L. The proteinase-K treatment, hybridization of probe, and stringent washing were conducted according to the manufacturer’s protocol. The hybridized DIG-labeled probe was detected using Sheep anti-DIG-AP (11 093 274 910, Roche Applied Science) and NBT/BCIP ready-to-use tablets (Roche Applied Science).

Expression of miRNA in pancreatic cancer cell line

Transient expression of hsa-miR-126 in Panc-1 and ASPC-1 cells was conducted using the Pre-miR miRNA Precursor (Applied Biosystems) at final concentration of 200 nmol/L. Control treatment was conducted using the Pre-miR miRNA Precursor Molecules Negative Control (Applied Biosystems). Transfection of the miRNA precursor was conducted using Lipofectamine 2000 (Invitrogen).

RNA interference

The transient knockdown of ADAM9 was conducted by using the ON-TARGET plus SMARTpool siRNA against human ADAM9 (Dharmacon) at the final concentration of 100 nmol/L. As a negative control, the ON-TARGET plus Non-targeting pool siRNA (Dharmacon) was used at the same concentration. The siRNA transfection was conducted by using Lipofectamine 2000 Transfection Reagent (Invitrogen).
Cell growth assay

Panc-1 and ASPC-1 cells were transfected with hsa-miR-126 precursor or negative control precursor, ADAM9 siRNA or negative control siRNA and incubated for 24 hours. Then, 6,000 cells per well were plated in 96-well plate in triplicate. The bromodeoxyuridine (BrdUrd) incorporation assay was conducted 72 hours after.

Migration assay and invasion assay

Cellular migration and invasion were assessed by two-chamber assay using the 8-μm pore, 24-well BD
FALCON Cell Culture Insert and BD BioCoat Matrigel Invasion Chamber (BD Biosciences), respectively. Panc-1 and ASPC-1 cells were transfected with hsa-miR-126 precursor or negative control precursor, ADAM9 siRNA or negative control siRNA and cultured for 72 hours. Then, 10,000 cells per chamber for Panc-1, 50,000 cells per chamber for ASPC-1 were plated, and cellular migration and invasion were assessed 24 hours after plating. Migrated or invaded cells were counted in random 5 high-power fields.

**Scratch assay**

Scratch assay was conducted as described previously (2). Briefly, Panc-1 or ASPC-1 was transfected with indicated miRNA precursor or siRNA at final concentrations of 200 or 100 nmol/L, respectively. Cells were grown until confluent state and then the monolayer of the cells was scratched using sterile tips. Cellular migration toward the scratched area was assessed 24 hours after.

**Western blotting**

Western blot analysis was done as described previously (2). The primary antibodies used in this study were as follows: α-tubulin (T5168; Sigma-Aldrich), E-cadherin (610181; BD), N-cadherin (610920; BD), and ADAM9 (2099; Cell Signaling Technology, Inc.). As a secondary antibody, horseradish peroxidase (HRP)-conjugated anti-mouse antibody (GE Healthcare) or HRP-conjugated anti-rabbit antibody (7074; Cell Signaling Technology) was used. Reactive bands were detected using ECL plus Western blotting detection reagents (GE Healthcare). The specific bands were subjected to densitometry analysis.

**3′-UTR assay**

Potential target site of hsa-miR-126 in the 3′-UTR sequence of ADAM9 mRNA was identified using TargetScan (http://www.targetscan.org/). The DNA sequence which corresponds to this 3′-UTR sequence of ADAM9 mRNA was subcloned into the pmirGLO vector (Promega; pMG-A9). The DNA sequence of ADAM9 3′-UTR in which the seed sequence for the hsa-miR-126 binding was mutated was also subcloned into the pmirGLO vector (Promega; pMG-A9M). Panc-1 and ASPC-1 cells were transfected with hsa-miR-126 precursor or negative control precursor and incubated for 24 hours. Then, 100,000 cells per well were plated into 12-well plates and transfected with 100 ng per well of pMG-A9 or pMG-A9M reporter vector. Twenty-four hours after transfection, luciferase assay was conducted using Dual-Luciferase Reporter Assay Kit (Promega).

**Statistical analysis**

Statistical analysis was conducted using Excel (Microsoft). The difference between 2 groups was analyzed by Mann–Whitney U test. The differences between multiple groups were analyzed by ANOVA with Fisher least significant difference method. The value of \( P < 0.05 \) was considered as statistically significant.

**Results**

**Comprehensive analysis of the differentially expressed miRNAs in IDA samples**

Microdissection was done as shown in Fig. 1A. Each sample’s miRNA expression profile including pancreatic cancer cell line was assessed by microarray. By comparing the miRNA expression profiles of IDA samples or cancer cell lines versus IPMA or IPMC samples by cluster analysis after the normalization (Fig. 1B), differentially expressed miRNAs were elucidated (Fig. 1C). Those miRNAs which were differentially expressed commonly in IDA samples or cancer cell lines compared with the IPMA or IPMC samples are summarized in Supplementary Tables S1 and S2. The miR-21 was upregulated in IDA and cancer cell lines and previous reports have suggested its correlation with gemcitabine resistance or patients’ outcome (5, 11). In addition, the tumor suppressor miR-145 was downregulated in IDA and cancer.
cell lines (12). These results indicate the validity of current experimental design. Among those differentially expressed miRNAs, we chose miR-126, as recent reports suggested the tumor suppressive role in other type of cancer (13, 14).

**Effect of miR-126 reexpression in pancreatic cancer cell lines**

Cellular proliferation after the transfection of miR-126 precursor was not altered in Panc-1 and ASPC-1 (Fig. 2A). However, cellular migration or invasion after the transfection of miR-126 precursor was attenuated in both cell lines by up to 10% to 50% (Fig. 2B). Decreased cellular migration in miR-126–transfected Panc-1 or ASPC-1 was also confirmed by scratch assay (Fig. 2C). In addition, expression of E-cadherin in Panc-1 or ASPC-1 after the transfection of miR-126 precursor was increased whereas expression of N-cadherin was decreased (Fig. 2D). These effects of miR-126 were considered to oppose EMT of cancer cells. The possible miR-126 target genes by database analysis are summarized in Supplementary Table S3. ADAM9 was included in those genes and previous report has shown its frequent expression in pancreatic cancer and correlation with poor prognosis (15). Potential seed sequence in ADAM9 3′-UTR which could interact with miR-126 was

![Figure 4](image-url)
identified with evolutionary conservation (Fig. 2E). Among a large number of ADAM family members, the database analysis identified only ADAM9 as a predicted miR-126 target, based on the evolutionarily conserved 3'-UTR seed sequence which defines the miRNA-targeting specificity (16–18).

Expression of miR-126 attenuates ADAM9 expression
To confirm that ADAM9 is a direct target of miR-126, we assessed the expression levels of ADAM9 in miR-126–transfected Panc-1 or ASPC-1. The expression levels of ADAM9 in miR-126–transfected cells were decreased (Fig. 3A). Expression of miR-126 and ADAM9 in normal pancreas or pancreatic cancer tissue was assessed by in situ hybridization and immunohistochemistry, respectively (Fig. 3B). Normal pancreas expresses miR-126 and lacks ADAM9 expression. Expression of miR-126 in pancreatic cancer was diminished, whereas cancerous glands were positive for ADAM9 staining. Quantitative real-time RT-PCR also confirmed higher expression of miR-126 in normal pancreas tissues than in pancreatic cancer tissues (Fig. 3C).

ADAM9 knockdown attenuates cellular migration and invasion
As a next step, we assessed the biologic role of ADAM9 expression in Panc-1 and ASPC-1 by siRNA-based knockdown experiment. Transient transfection of siRNA against ADAM9 efficiently repressed ADAM9 expression in Panc-1 and ASPC-1 (Fig. 4A). Knockdown of ADAM9 did not alter cellular proliferation of Panc-1 or ASPC-1 (Fig. 4B). Cellular migration and invasion of Panc-1 or ASPC-1 was attenuated until 50% to 60% of control treatment (Fig. 4C) and decreased cellular migration in ADAM9 siRNA–transfected Panc-1 or ASPC-1 was also confirmed by scratch assay (Fig. 4D). Along with these biologic changes, E-cadherin expression was increased and N-cadherin was decreased by ADAM9 knockdown (Fig. 4E). On the basis of these findings, we concluded that ADAM9 regulates cellular migration and invasion in pancreatic cancer cells as a target gene of miR-126.

miR-126 regulates ADAM9 expression by direct interaction with ADAM9 3'-UTR
The interaction of miR-126 and ADAM9 mRNA 3'-UTR was assessed by 3'-UTR assay. The 3'-UTR sequence of wild-type ADAM9 mRNA (pMG-A9) or mutated sequence (pMG-A9M) was subcloned into the pmirGLO vector (Fig. 5A). Reexpression of miR-126 decreased reporter activity of pMG-A9 in Panc-1 or ASPC-1 compared with the control treatment (Fig. 5B). Mutation in the seed sequence (pMG-A9M) abrogated these effects which indicates the direct interaction of miR-126 and ADAM9 3'-UTR.

Discussion
IPMC or IPMA manifests better prognosis than IDA as its noninvasive growth. Once the invasive growth of IPMC occurs, the prognosis is as poor as IDA (19). Various cytokines and signaling pathways contribute to this complex biologic process which requires mobilization of cancer cells from the site of origin. However, the contribution of miRNA in invasion of pancreatic cancer and metastasis is not yet fully understood. We tried to elucidate miRNAs whose differential expressions are indispensable in invasive growth of cancer cells by comparing the miRNA expression profiles of IPMA, IPMC, IDA, and pancreatic cancer cell lines. This approach confirmed the differential expression of several miRNAs such as miR-21 or miR-145 which are involved in the cancer cell invasion in previous studies (5, 6).

In this study, we focused on miR-126 as its role in pancreatic cancer was not yet clarified, whereas its expressions in endothelial cells and immune cells are retained in tumor (20). Previous reports suggested that miR-126 acts as a tumor suppressor in lung, gastric, and breast cancer by targeting EGFL7, Crk, or SLC7A5 which yields growth advantages (13, 14, 21, 22). These evidences suggested possible tumor suppressive role of miR-126 in pancreatic cancer. Transient expression of miR-126 attenuated cellular migration or invasion, and the database analysis identified ADAM9 as a potential target of miR-126 based on the evolutionarily conserved 3'-UTR sequence. ADAM9 is uniformly expressed in pancreatic cancer cells, but its upstream regulator was not identified (15). Recent report identified that ADAM9 promotes cancer cell invasion by modulating tumor–stromal cell interaction (23), and our observation indicated that
ADAM9 could also function in cell autonomous manner. Transient expression of miR-126 and siRNA-based knockdown of ADAM9 revealed similar effects in pancreatic cancer cells which confirmed the role of ADAM9 as a downstream effector of miR-126.

Present study provided a novel therapeutic target, miR-126/ADAM9 axis in pancreatic cancer. Other differentially expressed miRNAs identified in this study also could be useful targets. Because the detection of miRNA in blood sample is technically possible (24), an miRNA which predicts that invasive pancreatic cancer could lead to the early detection of curable disease. As another approach, these differentially expressed miRNAs could be applied to individualize pancreatic cancer patients before and during the therapy. These approaches will be beneficial to develop effective therapy against pancreatic cancer. Further study is necessary to dissect the complex regulation of pancreatic cancer progression by miRNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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