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miR-216a may inhibit pancreatic tumor growth by targeting JAK2

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ABSTRACT

This study was aimed to investigate miR-216a expression in pancreatic cancer and determine its effects on proliferation. miR-216a was found downregulated in pancreatic cancer tissues as compared to benign pancreatic lesions. JAK2 was identified as a miR-216a gene target. Further, in vivo treatment of PANC-1 tumors with miR-216a reduced JAK2 protein levels in the tumor and reduced tumor volume. In conclusion, miR-216a may function as a tumor suppressor regulating pancreatic cancer cells by targeting the JAK/STAT pathway. Further studies with a larger number of patient samples are necessary to fully explore the diagnostic and therapeutic potential of miR-216a for pancreatic cancer.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death worldwide [1]. In China, it is the sixth commonest cause of cancer-related mortality [2]. Pancreatic ductal adenocarcinoma (PDAC) is the most common tumor type of the exocrine pancreas, accounting for 85–90% for all of pancreatic tumors. Its late clinical presentation, aggressive nature, and the lack of sensitivity to most treatment options renders the mortality rate virtually equal to its incidence with an overall 5-year survival rate of 6% and a median survival rate approximately 5–6 months [3]. Therefore, identification of novel diagnostic markers and therapeutic targets is crucial.

Previous studies have demonstrated that the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway may play an important role in pancreatic cancer progression as well as invasion and metastasis [4]. For instance, Scholz et al. [5] demonstrated aberrant activation of STAT3 in pancreatic cancer tissues and cell lines. In addition, constitutive activation of STAT3 induced cellular transformation and promoted pancreatic tumorigenesis, possibly through regulating the expressions of target genes, such *as c-Myc*, *Bcl-xL*, *p21WAF1*, *cyclinD1* and *VEGF* [6], and its inhibition led to growth arrest [7]. Conversely, functional inactivation of STAT3 by dominant-negative STAT3 or AG490 inhibited the proliferation and promoted the apoptosis of pancreatic cancer cells [6].

Along with the JAK/STAT pathway, abnormal expression of microRNAs (miRNAs), non-coding single-stranded RNAs of approximately 20–25 nucleotides in length that regulate gene expression at the transcriptional or post-transcriptional level [8], has been implicated in the oncogenic process as both oncogenes and tumor suppressors. Moreover, approximately 50% of miRNAs are located at or close to fragile sites of regions known to be amplified or deleted in human cancer [9], and aberrant miRNA expression or miRNA gene mutation have been well described in leukemia as well as solid tumors, including pancreatic cancer [10–13]. In addition to regulating development, differentiation, apoptosis, proliferation, and viral infection [12], miRNAs can also function as potential oncogenes or tumor suppressor genes, suggesting a direct role in cancer initiation [13]; their role in metastasis, chemosensitivity, and radiosensitivity has also been reported [14]. Furthermore, determining miRNA expression may have diagnostic and prognostic value in pancreatic cancer [12] as dysregulated miR-148a, miR-217, and miR-196a expression was observed in the PDAC precursor, pancreatic entraepithelial neoplasm [15]. In

Abbreviations: 3′-UTR, 3′-untranslated region; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; IL-10, interleukin-10; miRNA, microRNA; NC, negative control; PDAC, pancreatic ductal adenocarcinoma; PDT, photodynamic therapy; STAT, signal transducer and activator of transcription; TBS, Tris buffered saline

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addition, normal pancreatic tissues, chronic pancreatitis, and PDAC tissues could be differentiated through the analysis of miR-217 and miR-196a [10]. In PDAC patients, miR-155, miR-203, miR-210 and miR-222 expression was significantly associated with poorer overall survival [11].

We previously identified miRNAs that were dysregulated in pancreatic cancer, including miR-216a, miR-216b, miR-217, miR-130b and miR-604 downregulation and miR-1246, miR-517*, miR-146a, miR-645 and miR-424* upregulation [16]. Although downregulated miR-216a expression was correlated with tumor T stage [16], its function in pancreatic cancer is unknown. Therefore, the primary objective of the present study was to explore the expression of miR-216a in pancreatic cancer and its effect on pancreatic cell proliferation. These studies may form the basis for identifying novel treatment strategies as well as diagnostic or prognostic markers for pancreatic cancer.

2. Materials and methods

2.1. Tissue samples

Fourteen pancreatic ductal adenocarcinoma tissues (6 females and 8 males of 44–78 y; mean age, 61 y) and six benign pancreatic tissue lesions (3 females and 3 males of age 45–74 y; mean age 60 y) were obtained from patients undergoing pancreaticoduodenectomy at the Guangdong General Hospital from 2007 to 2009. Benign pancreatic tissue was selected in place of normal adjacent tissue from cancer patients to prevent cancer tissue contamination due to unclear borders. Among the six benign pancreatic lesions, two were classified as chronic pancreatitis, two were mucinous cystic neoplasms, one was a serous cystic neoplasm, and one was a solid pseudopapillary neoplasm. All tissue samples were derived from untreated patients undergoing surgery, and written informed consent was obtained before collection. This study was approved by the Internal Review Board of the Guangdong General Hospital, China.

All the samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until analyzed Histopathologic analysis, including hematoxylin and eosin staining, was performed by two professional pathologists. Other clinical data were obtained from the medical records at the Department of General Surgery of Guangdong General Hospital.

2.2. Cell culture

The pancreatic adenocarcinoma cell line, PANC-1, was kindly provided by the Biochip Department of the Guangdong General Hospital obtained from the American Type Culture Collection (ATCC) and was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were tested regularly for mycoplasma using the MycoProbe® Mycoplasma Detection Kit (Minneapolis, MN, USA).

The immortalized but not transformed pancreatic cell line, HPDE6c7, was obtained from Kyushu University, and the pancreatic tumor cell lines, BxPC3, panc-1, CFPAC-1, and Aspc-1, were all purchased from ATCC (Manassas, VA, USA). These cell lines were cultured following the manufacturer's instructions.

2.3. RNA extraction

Total RNA was extracted from the tissue samples using the mirVana™ RNA Isolation Kit (Applied Biosystem, Ambion, TX, USA) following the manufacturer's instructions. RNA concentrations and quality were determined with 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

2.4. miRNA microarray

Microarray analysis was performed using 100 ng of total RNA from histologically confirmed pancreatic cancer tissues and benign pancreatic lesions, and carried out using the Agilent Human miRNA Microarray (V12.0) following the manufacturer's instructions. Briefly, low-molecular weight RNA was dephosphorylated, denatured, and labeled with Cyanine 3-pCp using T4 RNA ligase. After hybridization for 20 h using the miRNA Complete Labeling and Hyb Kit (Agilent) the array was washed twice with Gene Expression Wash Buffers for 5 min each at 37 °C. The microarray was next scanned (Agilent), and the data was extracted using the Agilent feature extraction software version 9.5.3 followed by Quantile normalization using GeneSpring GX10.0 software (Agilent).

2.5. qRT-PCR analysis

Total RNA extraction using Trizol and was purified by chloroform extraction and dissolved in H_2O. The lack of protein contamination was confirmed using a BioPhotometer plus UV/Vis photometer (Eppendorf, Hauppauge, NY) as follows: OD260/OD280 > 1.8. RNA integrity was confirmed using agarose gel electrophoresis. qRT-PCR analysis was undertaken in triplicate using a SYBR Green qPCR SuperMix (Invitrogen) and an ABI PRISM® 7500 Sequence Detection System with the following primers: miR-216a (MIMAT0000273) forward, 5′-TAATCTCAGCTGGCAACT GTGA-3′; miR-216a reverse, 5′-TCACAGTTGCCAGCTGAGATTA-3′ or 5′-CTCAACTGGTGTCGTGGA-3′ (for validation experiments in ASPC-1, PANC, Capanc, and Bxp3, and HPDE6-c7 cell lines.); U6 forward, 5′-CTCGCTTCGGCAGCACA-3′ and U6 reverse, 5′-AACGCTTC ACGAATTTGCGT-3′. The following cycling conditions were used: 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 65 °C for 15 s and 72 °C for 32 s; and melting curve analysis from 60 °C to 95 °C. The relative expression was calculated as follows: $2^{-\Delta\Delta Ct}$, in which −ΔΔCt (tumor cell ΔCt-non-tumor cell ΔCt).

2.6. Plasmid construction

The putative miR-216a target sequence of the *JAK2* 3′ untranslated region (3′-UTR) was 5′-UGAGAUU-3′, corresponding to nucleotides 833–839, as predicted by miRanda (miRBase or microrna.org [17]), TargetScanS [18], and PicTar [19]. The human *JAK2* 3′-UTR, which corresponded to the miR-216a binding site (NCBI access number MI0000292), was amplified by RT-PCR using the following primers (target site is underlined; Invitrogen, Guangzhou, China):

JAK2XhoIF, 5′-CCGCTCGAGAAGAAATGACCTTCATTCTGAGATT-A-3′ and JAK2NotIR, 5′-AAGGAAAAAAGCGGCCGCTAAAGTAAGAAA CTATTTTCTTTTTAATCAAAAC-3′. The human JAK2 3′-UTR containing mutated miR-216a-binding sites was generated using the following primers (mutated target site is underlined; Invitrogen): mutJAK2F, mutJAK2F: 5′ GAACAGTTTTCTTTTAAAATTTATCGATCAA GAATGCCAGGAATATTGTC3′ and mutJAK2R: 5′ GACAATATTCCTGG CATTCTTGATCGATAAATTTTAAAAGAAAACTGTTC 3′.

The amplified products were cloned into the PsiCHECK™-2 vector (Promega, Madison, WI, USA).

miR-216a mimics (MIMAT0000273; UAAUCUCAGCUGGCAACU GUGA) and miR-216a inhibitor, in which the complementary strand was methylated, were synthesized by RiboBio (Guangzhou, China). siRNA duplexes containing non-specific