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Original Article

Differential Expression of Multiple Genes in Association with *MADH4/DPC4/SMAD4* Inactivation in Pancreatic Cancer

Dengfeng Cao[1], Raheela Ashfaq[3], Michael G. Goggins[1], Ralph H. Hruban [1, 2], Scott E. Kern[2] and Christine A. Iacobuzio-Donahue[1, 2]

Departments of Pathology[1] and Oncology[2], The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins Hospital, Baltimore, MD and Department of Pathology[3], University of Texas Southwestern Medical Center, Dallas, TX

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Abstract: The Gene Logic Inc. Gene Express® tools and Affymetrix GeneChip® arrays were utilized to discover genes differentially expressed in pancreatic cancers with *MADH4/DPC4/SMAD4* gene inactivation. cDNA was prepared from thirteen pancreas cancer cell lines with known *MADH4* status (5 with wild-type *MADH4* and 8 with inactivated *MADH4*) and hybridized to the complete Affymetrix Human Genome U133 GeneChip® set (arrays U133 A,B) for simultaneous analysis of 45,000 gene fragments corresponding to 33,000 known genes. 25 known genes were identified as down-regulated at least three fold in the *MADH4* mutant cancer cell lines. 9 were decreased in expression at least 5 fold, and 1 in particular (*ID3*) was decreased 23 fold. Only 2 of the 25 down-regulated genes (*ID1* and *ID3*) have been previously reported as *MADH4*-dependent targets, and the remaining 23 genes represent potential novel direct or indirect *MADH4* downstream targets. Immunolabeling for Id1 and Id3 did not show a relationship with known *MADH4* status in pancreatic cancer tissues, suggesting additional regulation of these two genes than activation by MadH4. Further investigations to validate and to determine the significance of these candidate target genes in pancreatic carcinogenesis and progression are warranted.
Key Words: *DPC4*, *SMAD4*, pancreas cancer, *ID1*, *ID3*, gene expression

Introduction

The importance of the transforming growth factor beta (TGF-β)/Activin/Smad signaling pathway is underscored by its suppression of tumorigenesis through activation of downstream genes. Cell functions reportedly regulated by the TGF-β/Activin pathway include cell proliferation, differentiation and death following binding by specific ligands to TGF-β/Activin receptors. Upon binding of ligand (TGF-β, nodal, activin, or bone morphogenic protein BMP), type I and type II TGF-β receptors form heteromeric complexes in which the type II receptor phosphorylates the type I receptor. The phosphorylated type I receptor then activates other Smad proteins (Smad1 and Smad5 in BMP signaling, Smad2 and Smad3 in TGF-β/activin/nodal signaling) with which Madh4 protein, as the common co-Smad, forms complexes through its activation domain. These Smad complexes are then translocated from the cytoplasm to the nucleus, where Madh4 initiates transcription of specific genes by binding to the Smad-binding element (SBE) through its N-terminal DNA-binding domain. Madh4 may also act as a general transcriptional regulator by binding to transcriptional co-activators and co-repressors. Through these mechanisms, the tumor suppressive role of the TGF-β/Activin signaling pathway is fulfilled [1, 2].

MADH4/DPC4/SMAD4 is a tumor suppressor gene located on chromosome 18q, isolated by mapping of a consensus region of homozygous deletions in pancreatic and biliary cancer [3, 4]. The *MADH4* gene is inactivated in 55% of pancreatic duct adenocarcinomas and to a lesser extent in other tumor types [4, 5]. Considering the high rate of *MADH4* inactivation in pancreatic cancers, it is

attractive to consider the genes differentially expressed in accord with *MADH4* status in an unbiased survey [6-9]. We utilized the Gene Logic Inc. BioExpress™ platform and Affymetrix U133 GeneChip® set to determine the changes in gene expression associated with *MADH4* gene inactivation in a series of well-characterized pancreatic cancer cell lines to define the downstream effects associated with *MADH4* gene inactivation. Candidate genes were validated by immunohistochemical labeling of an independent series of resected pancreas cancer tissues. This approach not only allowed an independent confirmation of previously reported target genes, but also identified additional candidates for further study. In addition, we provided *in vivo* data of the functional consequences of two *MADH4* target genes in pancreas cancer tissues.

Materials and Methods

Cell Lines

Human pancreatic cancer cell lines AsPc1, CAPAN1, CAPAN2, CFPAC1, COLO357, Hs766T, MiaPaCa2, and Panc-1 were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Panc2.13, Panc4.14, Panc6.03, Panc8.13 and Panc3.27 cells lines were a generous gift from Dr. Elizabeth Jaffee, and are also generally available from the ATCC. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) with the exception of CAPAN1 and CAPAN2 cell lines that were cultured in RPMI 1640 medium (Life Technologies, Inc, Gaithersburg, MD) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air.

Determinations of 18q Loss of Heterozygosity and Sequencing of MADH4

The determination of the *MADH4* gene status in pancreas cancer cell lines AsPC1, CAPAN1, CAPAN2, CFPAC1, COLO357, Hs766T, MiaPaCa2, and Panc-1 has been previously described [4, 5]. For pancreas cancer cell lines Panc2.13, Panc4.14, Panc6.03, Panc8.13 and Panc3.27, the determination of 18q LOH and sequencing of exons 1-11 were performed as described in previous studies [4, 5].

mRNA Extractions and Oligonucleotide Arrays

Sample preparation and processing was performed as described in the Affymetrix GeneChip® Expression Analysis Manual (Santa Clara, CA). Briefly, each frozen tissue was crushed to powder by using the Spex Certiprep 6800 Freezer Mill (Metuchen, NJ). Total RNA was then extracted from the cell pellets using TRIzol (Life Technologies, Rockville, MD) and cleaned using RNeasy™ columns according to manufacturer's protocol (Qiagen). Double-stranded cDNA was synthesized following SuperScript Choice system (Life Technologies). The resultant cDNA was purified using Phase Lock Gel, phenol/chloroform extraction and precipitated with ethanol. The cDNA pellet was collected and dissolved in appropriate volume. cRNA was synthesized using a T7 MegaScript In-Vitro Transcription (IVT) Kit (Ambion, Austin, TX) using biotinylate-11-CTP and 16-UTP ribonucleotides (Enzo Diagnostics Inc., Farmingdale, NY) were as labeling reagents. The labeled cRNAs were purified using RNeasy columns (Qiagen) followed by incubation in fragmentation buffer (40 mM Tris-Acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 35 minutes at 94ºC. Fragmented cRNA (10-11 microgram/probe array) was used to hybridize to human GeneChip® set (HG_U133 A and B) at 45ºC for 24 hours in a hybridization oven with constant rotation (60 rpm). The chips were washed and stained using Affymetrix fluidics stations. Staining was performed using streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to streptavidin (Vector Laboratories, Burlingame, CA), and finally with streptavidin phycoerythrin conjugate. Probe arrays were scanned using fluorometric scanners (Hewlett Packard Gene Array Scanner; Hewlett Packard Corporation, Palo Alto, CA). The scanned images were inspected and analyzed using established quality control measures.

Statistical Analysis

The GeneExpress® Software System Fold Change Analysis tool was utilized to identify gene fragments expressed at least 3 fold less in the pancreatic cancer cell lines with *MADH4* gene inactivation as compared to those with wild-type *MADH4* gene status. For each gene fragment, the ratio of the geometric means of the expression intensities in the cell lines with and without *MADH4* gene inactivation was

calculated, and the fold change then calculated on a per fragment basis. Confidence limits were calculated using a two-sided Welch modified t-test on the difference of the means of the logs of the intensities.

Tissue Microarrays and Immunohistochemistry

Tissue microarrays of resected primary pancreas cancer tissues were created as previously described [10]. For detection of Madh4 protein, slides were treated with 1X sodium citrate buffer (diluted from 10x heat-induced epitope retrieval buffer, Ventana-Bio Tek Solutions, Tucson, AZ) before steaming for 20 minutes at 80ºC. Slides were then cooled 5 minutes before incubating with a 1:100 dilution of monoclonal antibody to Madh4 protein (clone B8, Santa Cruz Biotechnology, CA,) using a Bio Tek-Mate 1000 automated stainer (Ventana-Bio Tek Solutions). For detection of Id1 and Id3 proteins, serial sections were deparaffinized by routine techniques before placing in 200ml Target Retrieval Solution, pH 6.0 (Dako, Envision Plus Detection Kit, Carpinteria, CA) for 20 minutes at 100ºC. After cooling for 20 minutes, slides were quenched with 3% H_2O_2 for 5 minutes, before incubating with a 1:100 dilution of monoclonal antibody to Id1 or Id3 proteins (Id1, clone C-20, cat# sc-488 and Id3, clone C-20, cat # sc-490, Santa Cruz Biotechnology, CA) for 30 minutes using the Dako Autostainer. Anti-Madh4, anti-Id1 or Id3 antibodies were detected by adding secondary antibody followed by avidin-biotin complex and 3,3'-diaminobenzidine chromagen. All sections were counterstained with hematoxylin, and staining was evaluated by two of the authors (D.C. and C.I.D.). Immunohistochemical labeling of Madh4, Id1 and Id3 were scored as intact (positive normal labeling of nuclei) or lost (abnormal loss of labeling of nuclei). Only sections in which internal controls (lymphocytes, stromal cells, acinar epithelium, etc) present on the same slide showed intact nuclear labeling were used.

Results

The MADH4 Status in the Pancreatic Cancer Cell Lines

The *MADH4* gene status of the 13 pancreas cancer cell lines has been previously determined [3-5]. 5 were wild type for *MADH4*, and 6 harbored a homozygous deletion of *MADH4*, and 2 had an intragenic mutation in association with loss of the remaining allele (**Table 1**).

Table 1 *MADH4* status in pancreatic cancer cell lines.

Cell Lines	*MADH4*
Capan2	WT
MiaPaca2	WT
Panc1	WT
Panc4.14	WT
Panc8.13	WT
AsPC1	LOH/Mut
Capan1	LOH/Mut
CFPAC1	HD
Colo357	HD
Hs766T	HD
Panc2.13	HD
Panc6.03	HD
Panc3.27	HD

WT, wild-type; LOH/Mut, loss of heterozygosity in association with an inactivating mutation; HD, homozygous deletion

Identification of Differentially Expressed Genes in Association with MADH4 Inactivation

We identified 45 fragments expressed at least three fold less in the *MADH4*-inactivated pancreatic cancer cell lines compared to those cell lines with functional *MADH4*, of which 28 fragments correspond to known genes and 17 to expressed sequenced tags (**Table 2**). Two genes (matrix metalloproteinase 28 or *MMP 28* and tripartite motif-containing 31) are represented by two fragments each, resulting in 26 known genes identified. *MADH4* was included within this gene fragment set, providing an internal validation of the sensitivity of the U133 GeneChip® in detecting significant changes in gene expression (**Table 2**). Therefore, 25 known genes were identified as down regulated by at least three fold in the *MADH4*-inactivated pancreatic cancer cell lines. Two fragments in particular, inhibitor of DNA-binding 3 (*Id3*) and adipose specific 2, were decreased by more than 15 fold. We also found 80 gene fragments corresponding to 35 unique genes overexpressed in *MADH4* inactivated cells as compared to those with wild type status. However, as our hypothesis is that *MADH4* inactivation results in decrease in the expression of *MADH4* target genes, we chose to focus only on those genes decreased in expression in cell lines with inactivated *MADH4*

Table 2 Genes downregulated in *MADH4*-inactivated pancreas cancer

Fragment Name	Sequence Cluster Accession	Gene Name	Fold decrease	P value
207826_s_at	Hs.76884	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	22.98	0.02712
203571_s_at	Hs.74120	Adipose specific 2	16.28	0.02954
215444_s_at	Hs.91096	Tripartite motif-containing 31	9.09	0.0036
206463_s_at	Hs.272499	Short-chain alcohol dehydrogenase family member	6.93	0.02763
207254_at	Hs.2217	Solute carrier family 15 (oligopeptide transporter), member 1	6.87	0.02189
240356_s_at	Hs.230463	Unknown	6.85	0.00965
235220_at	Hs.13297	Unknown	6.80	0.02356
239272_at	Hs.231958	Matrix metalloproteinase 28	6.57	0.02049
231721_at	Hs.219421	Junctional adhesion molecule 3	5.80	0.01565
206785_s_at	Hs.177605	Killer cell lectin-like receptor subfamily C, member 2	5.71	0.05077
217562_at	Hs.106642	Unknown	5.70	0.02968
215514_at	Hs.21195	Unknown	5.62	0.01168
209071_s_at	Hs.24950	Regulator of G-protein signaling 5	5.36	0.04159
241745_at	Hs.131164	Unknown	5.35	0.00555
238824_at	Hs.145569	Unknown	5.23	0.04093
225155_at	Hs.292457	Unknown	4.80	0.04318
202527_s_at	Hs.75862	MAD, mothers against decapentaplegic homolog 4	4.69	0.00768
226973_at	Hs.7991	Chromosome 20 open reading frame 102	4.42	0.02751
227190_at	Hs.26216	Unknown	4.33	0.01363
205613_at	Hs.26971	B/K protein	4.28	0.00762
221583_s_at	Hs.348348	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	4.16	0.00995
219587_at	Hs.183362	Hypothetical protein FLJ20535	4.06	0.03145
204898_at	Hs.20985	Sin3-associated polypeptide, 30kD	3.91	0.00018
235428_at	Hs.104480	Unknown	3.84	0.01548
230643_at	Hs.149504	Unknown	3.82	0.0319
208170_s_at	Hs.91096	Tripartite motif-containing 31	3.81	0.0163
239273_s_at	Hs.231958	Matrix metalloproteinase 28	3.77	0.02607
35626_at	Hs.31074	N-sulfoglucosamine sulfohydrolase (sulfamidase)	3.74	0.00036
210431_at	Hs.333509	Alkaline phosphatase, placental-like 2	3.67	0.04665
244489_at	Hs.169875	Thrombospondin 3	3.62	0.00174
212705_x_at	Hs.118463	Transport-secretion protein 2.2	3.53	0.00679
229480_at	Hs.29079	Unknown	3.51	0.02714
228904_at	Hs.156044	Unknown	3.39	0.01859
213843_x_at	Hs.187958	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	3.38	0.0289
208937_s_at	Hs.75424	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	3.36	0.00783
214993_at	Hs.323815	Unknown	3.36	0.01325
206314_at	Hs.1148	Zinc finger protein	3.28	0.02745
240717_at	Hs.44410	Unknown	3.23	0.02952
233766_at	Hs.297650	Unknown	3.17	0.04165
239725_at	Hs.16727	Unknown	3.16	0.04325
201185_at	Hs.75111	Protease, serine, 11 (IGF binding)	3.13	0.03968
205433_at	Hs.1327	Butyrylcholinesterase	3.12	0.00058
205047_s_at	Hs.75692	Asparagine synthetase	3.07	0.025
212813_at	Hs.334703	Hypothetical protein FLJ14529	3.05	0.03675

Gene fragments identified as 3.0 fold or more decreased in *MADH4* inactivated versus *MADH4* wild type cell lines

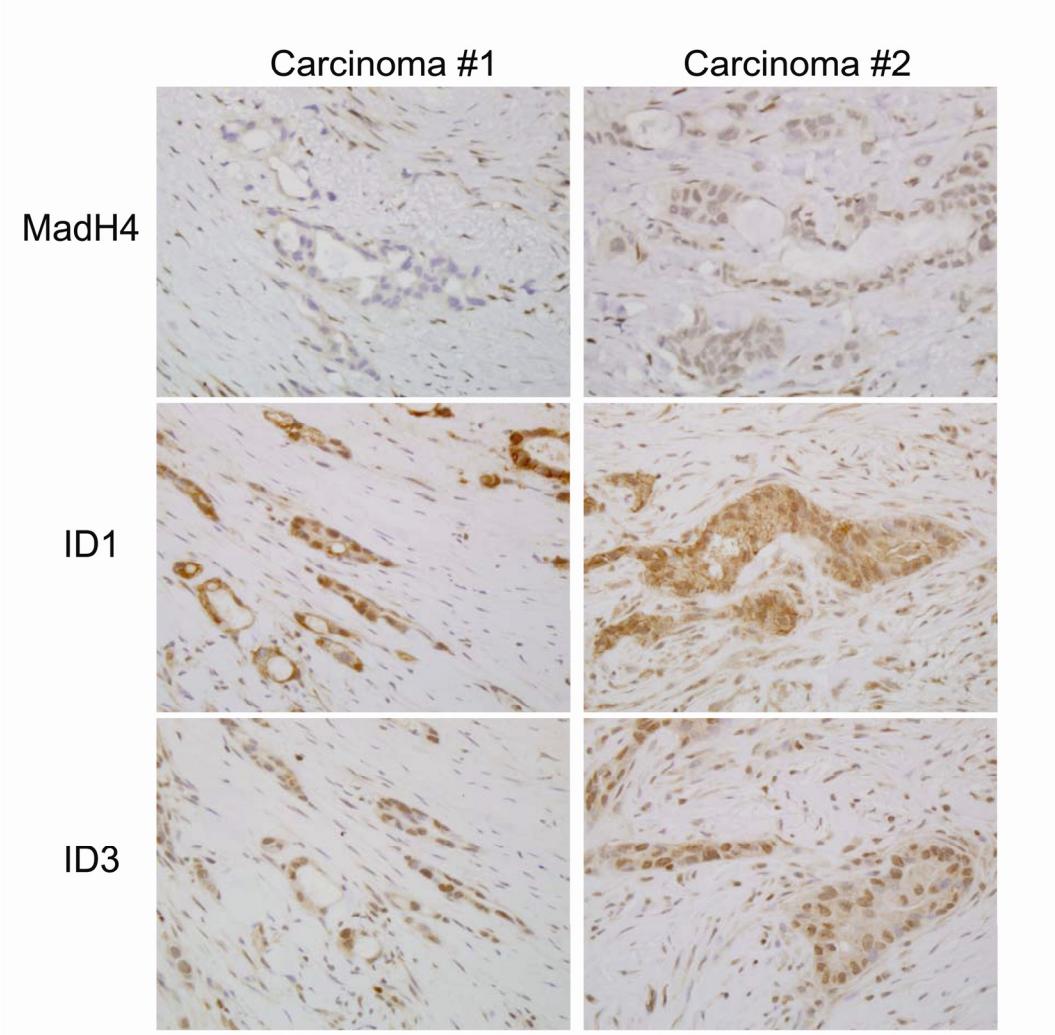

Figure 1 Immunohistochemical labeling of Madh4, Id1 and Id3 proteins in a pancreatic cancer with loss of *MadH4* (Cancer #1) and intact *MadH4* (Cancer #2). Equally intense nuclear labeling for both Id1 and Id3 are seen in both cancers (magnification 200X).

For each of the 25 genes identified, a literature search was performed using the online NCBI database PUBMED with the known gene name together with the terms "*MADH4/DPC4/SMAD4*". Of the 25 genes, *ID1* and *ID3* were previously reported to be *MADH4* target genes [6, 11, 12]. The remaining 23 genes represent new potential *MADH4* target genes.

Immunohistochemical Labeling of the Madh4 Target Genes Id1 and Id3 in Pancreatic Cancer

The expression of Madh4 and its known targets Id1 and Id3 were examined in 67 cases of infiltrating pancreatic duct adenocarcinoma from resection specimens. Among the 67 cases, 37 cases showed intact labeling for Madh4 protein (55%) and 30 cases showed loss of Madh4 protein labeling (45%). Although Id1 and Id3 have both been reported to be Madh4 target genes in functional studies [6, 11, 12], and our findings support this notion, their expression *in vivo* in relation to MadH4 has not been explored. Therefore, we determined the immunolabeling pattern of these two proteins in relation to Madh4 status.

Immunolabeling for Id1 and Id3 proteins indicated that normal duct epithelial cells, acinar and islet cells were all strongly positive for Id1 and Id3 proteins in all cases. Surprisingly, among the 67 pancreatic cancers

analyzed, all 67 (100%) showed strong positive and diffuse immunolabeling for Id1 and Id3 proteins (**Figure 1**). Moreover, there was no difference in immunolabeling intensity or cytologic location among carcinomas with intact Dpc4 versus lost Dpc4.

Discussion

Genetic alterations of multiple genes have been implicated in the pathogenesis of pancreatic duct adenocarcinoma [13]. Inactivation of the *MADH4* gene is perhaps the most significant genetic alteration identified to date in pancreatic cancers, identified in ~55% of cases analyzed. Patients whose pancreatic cancers have inactivation of *MADH4* gene have significantly shorter survival after Whipple procedure than those with intact *MADH4* gene [14], indicating that *MADH4* gene inactivation may play an important role in the biological aggressiveness of pancreatic duct adenocarcinoma. However, the downstream genes and functional consequences of its inactivation related to this aggressiveness are unclear.

In this study using the Gene Logic Inc. BioExpress™ platform and Affymetrix GeneChip® arrays, we identified 25 genes whose expression are down-regulated at least three fold in pancreatic cancer cell lines with *MADH4* gene inactivation. Two of these genes identified, *ID1* and *ID3*, are known MadH4 target genes based on evaluation of downstream signaling in well-defined systems [6, 11, 12]. However, unlike those studies, we did not find a relationship of Id1 or Id3 protein expression with MadH4 status in cancer tissues. Id1 and Id3 showed robust nuclear expression in all pancreatic cancers analyzed, irrespective of MadH4 status in those same tissues, suggesting additional levels of regulation of these two genes in addition to their activation by Smad proteins. Id proteins are negative regulators of basic helix-loop-helix transcription factors by forming heterodimers with them, thus preventing transcription of genes related to cellular processes such as cell cycle regulation, angiogenesis, or development and differentiation [15-20]. Deregulation of Id proteins has been reported in several other human tumors such as squamous cell carcinoma, breast cancer, colorectal cancer, esophageal carcinoma and prostate cancer [21-25]. Maruyama *et al* reported that Id proteins (Id1, Id2 and Id3) are over expressed in some pancreas cancers as compared to normal pancreatic duct epithelium [26]. However, as MadH4-dependent transcription is reliant on activation by specific ligands [12], immunohistochemical labeling of these proteins alone may not be entirely representative of this relationship.

Among the 25 genes identified, *ID1* and *ID3* have been previously reported as targets of Madh4 signaling [6, 11, 12], while the remaining 23 are potential new candidate Madh4 target genes. These 25 genes represent a variety of cellular functions ranging from extracellular matrix modeling (*TSP-3*, *MMP28*) [27-29], cell adhesion (junctional adhesion molecule 3), membrane transport (transport-secreting protein 2.2), signal transduction (regulator of G-protein signaling 5, serine proteinase 11), intracellular transport (oligopeptide transporter, neuro-transmitter transporter and tripartite motif-containing 31), metabolism (sulfamidase, butylocholinesterase, asparagines synthetase) and transcription regulation (*ID1* and *ID3*, SIN3-associated protein, zinc finger protein). The variety of the functions displayed by these downregulated genes lends support to the potentially diverse biological processes regulated by TGFβ/Activin signaling pathway including cell cycle regulation [7, 30], angiogenesis [31], or extracellular matrix modeling [32]. Future studies are required to confirm these findings using specific measures of MadH4 binding to target gene promoter sequences, such as gel electrophoretic mobility shift assay.

The identification of downstream targets of Madh4 that deviate in association with *MADH4* status in tissue may have therapeutic implications in pancreatic cancers. For example, Su *et al* developed a high-throughput screening system to identify compounds that augment Madh4 signal transduction pathways in pancreatic cancer cell lines [33]. Thus, the level of expression of downstream targets may be used as a surrogate marker for restoration of Madh4-dependent signaling in pancreatic cancer. Alternatively, Madh4-dependent genes may themselves be targets for therapy of pancreatic cancers. For example, histone deacetylases (HDAC1, HDAC2) form a repressor complex (also called SIN3 complex) with proteins such as SIN3, SAP30 and N-CoR to target promoters of other genes [34]. Proteins in this complex are attractive drug

targets. Our identification of a SIN3-associated polypeptide, a member of the SIN3 complex, suggests that use of histone deacetylase inhibitors may have utility as an anticancer agent for pancreatic cancers with inactivation of the *MADH4* gene.

In summary, we have identified 25 downregulated genes in pancreatic cancer cell lines with MADH4 inactivation. Identification of these genes offers insight into the role played by Madh4 in pancreatic cancer, as well as providing potential novel targets for the development of molecular therapeutics in pancreatic cancer.

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Please address all correspondences to Christine A. Iacobuzio-Donahue, M.D., Ph.D., The Johns Hopkins Hospital, Division of Gastrointestinal/Liver Pathology, 1550 Orleans Street, CRB2 Rm 343, Baltimore, MD 21231. Tel: 410-955-3511; Fax: 410-614-0671; Email: ciacobu@jhmi.edu

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