Pathological And Prognostic Role Of Mdig In Pancreatic Cancer

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PATHOLOGICAL AND PROGNOSTIC ROLE OF MDIG IN PANCREATIC CANCER

By

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THESIS

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Of Wayne State University,
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For the degree of

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Approved by:

__________________________________________
Advisor

__________________________________________
Date
DEDICATION

To my beloved family & friends
ACKNOWLEDGMENT

I would like to dedicate this work to the persons who loved me unconditionally, my parents: P.S Ashok Kumar and Chitra Ashok Kumar for their endless support and encouragement in every single step in my life. I know you have put lot of effort and time to raise me in the environment that values education and standards. My elder sister and brother in law, Srividya Ravi and Ravi Viswanathan, I love you both! Thank you for always supporting and guiding me. I appreciate your help, encouragement and inspiration all throughout my two years of research. You both will always be special to me. Sincere thanks to my friends Kaustubh, Katyani, Jayasurya and Anuska for helping me throughout my research.

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Abbreviation

Mdig: Mineral Dust induced Gene
IHC: Immunohistochemistry
BRACA1: Breast Cancer susceptibility gene 1
PanIN: Pancreatic Intraepithelial Neoplasia
PDAC: Pancreatic Ductal Adenocarcinoma
PA: Pancreatic adenocarcinoma
CDK: Cyclin Dependant Kinase
MAPK: Mitogen Activated Protein Kinase
LOH: Loss of heterozygosity
(NF-κB): Nuclear Factor Kappa B
ATCC: American Type Culture Collection
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.
PVDF: Polyvinylidene difluoride
RT-PCR: Reverse transcription polymerase chain reaction
mRNA: Messenger ribonucleic acid
OD: Optical Density
DAB: Diamino benzidine
1. Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in both men and women in United States.[1] In 2017, it is estimated that 53670 Americans will be diagnosed and 43090 patients will die of pancreatic cancer [2]. Most patients get diagnosed with pancreatic ductal adenocarcinoma and the 5-year survival rate after surgical resection is 20% and for those with metastatic disease, the survival rate is 2%[3]. Late detection and diagnosis has been the critical reason for poor survival rate in pancreatic cancer patients, 85% of patients present with advanced disease that is unresectable.[1]

African Americans have appreciably higher rates than whites. Heritable, germline mutations in p16, BRCA2[4], and other genes appear to be associated with a total of 5%–10% of all pancreatic cancers, and penetrance of these mutations for the disease may be fairly low. Chronic pancreatitis of various types is associated with another 3%–4%. Apart from germline mutations or pancreatitis, the other major risk factor associated with pancreatic cancer is cigarette smoking. Past studies suggest that patients with pancreatic cancer who smoked cigarettes were approximately at 40% increased hazard for death compared with those who never smoked, and risk tends to increase according to the frequency or duration of smoking[5]. Dietary studies have shown a fairly steady pattern of increase hazard with meat or cholesterol intake and decreased risk with fruit or vegetable consumption, although causal inferences regarding these associations are still uncertain [6].
Pancreatic cancer rates in US by sex, race and ethnicity. In all the group blacks have higher incidence rate of pancreatic cancer. According to sex, men have higher incidence rate than women.
Fig 2. Pancreatic cancer mortality rates and incidence among men and women, by regions, GLOBOCAN 2012 estimates.[7]
2.1 Environmental agent affecting pancreatic cancer

In spite of decades of research, the etiology of pancreatic cancer still remains ambiguous [8]. The cause of cancer is complex web of multiple factors[9]. Environment, lifestyle, diet, viral agents, genetics, and occupational exposures all can contribute to pathogenesis of tumor. Cancer causation is extraordinarily intricate. The agents and mechanism which contributes to pancreatic cancer are still not known. One of the major risk factor associated with pancreatic cancer is tobacco smoking, which contributes to one third of cases [10]. A personal history of chronic pancreatitis, diabetes, and high body-mass index, as well as family history of cancer has higher incidence rate of causing pancreatic cancer [11].

Earlier studies indicate that people who are occupationally exposed to various chemicals and metals such as benzinene and β-napthylamine, pesticides, asbestos, benzene, chlorinated hydrocarbons, chromium, and nickel [12, 13] tend to have higher risk of pancreatic cancer. The mechanism by which such exposures may lead to pancreatic cancer is still ambiguous. It is assumed that these substances through bloodstream may reach the pancreas and in this process, tumor suppressor proteins get inactivated, exert genotoxic effects leading to altered methylation and formation of DNA adducts [14, 15].

A meta-analysis of work related to occupational exposures and incidence of pancreatic cancer covering 92 studies [12, 16] reported that workers who were occupationally exposed to nickel and chlorinated hydrocarbon solvents tend to have higher risk of pancreatic cancer, as well as to other substances including chromium and organochlorine pesticides showed marked increase in risk to pancreatic cancer. Several work related to occupational studies have linked
carcinogenic substances like chromium, chlorinated hydrocarbons, nickel to pancreas carcinogenesis [17, 18]. Farmers who are highly exposed to pesticides have also been reported to show increased risk of pancreatic cancer [16, 19-21] although not consistent [16, 22, 23]. Studies relating to asbestos and benzene exposure leading to pancreatic cancer are subtle and ambiguous. [12, 13, 24]. Epidemiologic data suggest that although the incidence of pancreatic cancer is low in Japan compared with that of United States, Japanese who have immigrated to the United States have an increased risk than that of the native white population of this country.[25]

One of the major risk factor associated with pancreatic cancer is cigarette smoking which contributes to nearly 20% of pancreatic cancer cases [26, 27]. In a study of 12 prospective cohorts and one case-control study, people who smoked cigarettes had 80% increased risk of pancreatic cancer compared with non-smokers, and the risk increased with smoking duration, intensity, and cumulative smoking dose [28]. Nevertheless, few studies have examined survival probability who smoked cigarette and among patients with pancreatic cancer. There is a significant association in reduction in survival among patients who smoked cigarette and had pancreatic cancer. Furthermore, reduction in patient survival was observed with high levels of plasma cotinine, the major circulating metabolite of nicotine.[27]

In recent years there have been numerous case control studies to determine whether coffee or alcohol effects pancreatic cancer risk [14, 29]. The results obtained till now are fairly inconsistent. Many attributes may contribute to these inconsistent results. Poor survival rate leads to reduce participation rate in clinical studies. Moreover, in population-based case-control
studies, heavy alcohol and coffee drinkers may have lower participation rates than nondrinkers [30]. Thus, environmental factors appear to play a vital role in the etiology of pancreatic cancer.

### 2.2 Prognosis: Pancreatic cancer

Pancreatic cancer (PC) is a devastating disease and the fourth, fifth and seventh leading cause of cancer-related death in the United States, European Union and China, respectively [31-33]. Large population-based studies in western countries indicated an overall 5-year survival rate less than 5% and a median survival of 3–6 months [34-36]. The median survival rate was 4 months and the 2-year survival rate increased from 4.6% in 1988 to 7.7% in 2000 according to the Surveillance, Epidemiology, and End Results Registry(SEER) from 1988 through 2002 [34].

A post resection prognosis study indicates that median survival times in pancreatic cancer patients after R0 resection are between 17 and 28 months whereas after R1/R2 resection the median survival is between 8 and 22 months [37-41]. The survival probability by Kaplan Meier analysis after R0 resection indicates a 5-, 3-, and 1- year survival of 20%, 35%, and 80% respectively [42-45]. Less than 2% of people survived, in a long-term follow-up as the basis for estimating the prognosis [46, 47]. The identifying symptoms of local relapse are loco regional microscopic tumor cell residues, recurrence of pain due to the cancer infiltrating the nerves, and lymph node metastases [48]. This phenomenon is very common and escapes CT diagnosis if there is no substantial tumor formed. The most common cause of pancreatic cancer death is due to progressive organ metastasis and not the local relapse.
Fig 3. Five-year survival rate at diagnosis

Most pancreatic cancer patients get diagnosed when cancer has metastasized to other parts and five year survival rate is less than 3% [2].

Fig 4. Percent of pancreatic cancer cases by stage at diagnosis.

Cancer stage at diagnosis. Cancer stage has strong correlation to length of pancreatic cancer patient survival and also determines treatment options [2].
Pancreatic cancer incidence rate is slightly high in men than women and most frequently diagnosed among age 65-74 [2].

### 2.4 Mdig in cancer

The mineral dust-induced gene (mdig) was first identified from alveolar macrophages obtained from people with chronic lung disease resulting from occupational to mineral dust [49]. This gene was independently characterized in human glioblastoma cell lines with an over expression of c-myc oncogene and named as myc-induced nuclear antigen 53 (mina53)[50] or nucleolar protein 52 (NO5) [51]. The location human mdig/MINA gene is on chromosome 3 (3q12.1) that encodes a protein that weights 53 kDa. The protein is mainly localized in the nucleus, although
some of them are expressed in the nucleolus. Mdig can be induced by a number of environmental hazards, such as arsenic, silica, coal dust, and particulate matter PM 2.5[52]. The mdig gene encodes a 53 kDa protein that is involved in cell proliferation, neoplastic transformation, epigenetics, and immune regulation, and is over expressed in a variety of human neoplasms [52].

The mdig encodes a protein with a conserved JmjC domain and is indicated in cell growth regulation, possibly through its effect on tri-methylation of lysine 9 on histone H3 (H3K9me3) and hydroxylase activity on ribosomal proteins[53].

Mdig/MINA protein has four isoforms produced due to alternative splicing. Isoform 1 has been chosen as the ‘canonical’ sequence. Isoform 2 with a mass of 31.8 kDa, isoform 3 with a mass of 23.9 kDa and isoform 4 with a mass of 52.6 kDa has been reported in the literature. Mdig belongs to the JmjC family of proteins with one JmjC domain. JmjC domain functions by histone demethylation mechanism. Proteins containing JmjC domain are mainly involved in the regulation of chromatin remodeling and are predicted to be metallo-enzymes adopting the cupin fold. Electro-spray ionization-mass spectrometry guided disulphide cross-linking technology are used to identify the substrate complexes for mdig/MINA. MINA (Tyrosine 209C) residue readily cross-links and crystallizes in complex with RPL27A (G37C).

Fig 6. Position of Jmjc domain (purple background) in mdig protein [53]
Fig 7. Expression of mdig in different human tissues [53]

2.3 Alternative splicing in cancer

In human 92%-94% of genes undergo alternative splicing which results in proteome diversity [54]. In this process introns are removed from the nascent RNA and joins to exon to form mature RNA, which ultimately get translated into proteins [55]. Spliceosome acts as a catalyst for removal of introns, which assembles at the borders between introns and exons, called as ‘splice junctions’. Rearrangement of exons may take place, skipping of some parts; mutual exclusion or inclusion of intron resulting in mature RNA [56]. These processes are collectively termed as Alternative Splicing. This further translates into protein which is highly diverse and complex, Alternative splicing results in extensive variety in cell function due to amplification of protein interactions and signaling.
2.3.1 Alternative splicing of mdig mRNA in lung cancer cell line.

In our earlier studies alternative spliced transcripts of *mdig* mRNA was found in lung cancer cell lines [49]. The presence of alternative spliced mRNA has shown implication in different cancer types due to aberrant splicing and abnormal protein production [57]. During this process introns are removed from the nascent RNA and joins to exon to form mature RNA, which ultimately get translated into proteins. The alternative splice variants of mdig mRNA was produced as consequence of alternative promoters or alternative splice mRNA involvement. To characterize the nature of alternative splice mdig in H441 cells, amplification of RT-PCR primer sets I and III was purified and sequenced [49]. After DNA sequencing and analysis of data it was found that, in *mdig2* ([GenBank Access number: AY456380](https://www.ncbi.nlm.nih.gov/nuccore/AY456380)) the entire region of exon 2 (471 bp) was absent but contains a 207 bp region that illustrate a new alternative exon inserted between exon 5 and exon 6. JmjC homology domain is absent in new protein. Schematic representations of exon of mdig mRNA in A549 and H441 cells is shown in figure [49].
Fig 8. Alternative splicing of mdig mRNA in lung cancer cell line.

(a) Schematic representation of RT–PCR primers in correlation to their exons in the mdig gene. (b) Expression level of mdig mRNA in A549 cells (left) and H441 cells (right). (c) DNA sequencing of primer extension demonstrated a new region
of \textit{mdig} mRNA in H441 cells, due to use of an alternative exon (5') with a size of 207 bp [49].

In cancer, the process of splicing is mainly disrupted leading to both functional and non-functional end-products [58]. In recent years, alternative splicing specific to Cancer contributing to disease progression and its pathological implications have been well described however, the deregulation pattern due to alternative splicing found on a genome-wide scale have not yet fully elucidated [59-62]. It’s important to understand molecular mechanisms which determine the choice of particular splicing events associated with these processes, which will eventually help in interpreting how normal development occurs and how cancers progresses and metastasize.

A research study carried by Hanahan and Weinberg in 2000 which describes about tumor growth and development, tumor obtains additional oncogenic properties as cell progress through various processes [63]. And also mentions about eight processes required for development of tumor and progression to metastases [64]. Important attribute for tumor growth is unlimited proliferation of cells, but other processes such as growth factor, self sufficiency, immune escape, and invasion and antiapoptotic mechanisms are also vital properties acquired by tumors which eventually progress to form threatening malignancies. It’s now possible to understand this mechanism with the help of high-throughput proteomic and genomic techniques which allows identifying the mutations in oncogene for each cancer type [56]. As increasing number of cancer study taking places, a clear co-ordination of hallmarks of cancer is being put together.

Aberrant splicing is widely seen in most types of cancer [59]. Functions of aberrant splicing events in healthy tissues is not known [65] whereas in cancer related genes functional diversity is a result of alternative splicing[66].
2.3.2 Aberrant splicing effecting hallmarks of cancer

Aberrant splicing is known to affect widely accepted hallmarks of cancer [56, 64]. Alternative splicing of genes affects the apoptosis and metastasis. Aberrant splicing induced angiogenesis are one of the best described events in cancer. Mainly, a splicing switch is observed between cancer and healthy tissue samples of pro and anti-angiogenic isoforms of VEGFA [67]. Resistance to apoptosis is shown to confer with the up regulation of anti-apoptotic transcript variants of BCLXL [68, 69]. Furthermore, CD44 is involved in metastasis with more than 20 alternative splicing events [59].

Many alternatively spliced genes are involved in regulation of more than one cancer hallmark. One such example is RAC1B isoforms which is involved in inflammation and genomic instability by increasing the cellular levels reactive oxygen species due to increase cell invasion and proliferation potential of cancer cells [70-73]. Splicing of TP53 isoforms is implicated in both apoptosis and cell proliferation [74]. Further, expression of BRAF proto-oncogene is known to affect its kinase domain [75]. As aberrant splicing is involved in number of stages in cancer development, as a result many research studies have claimed aberrant splicing itself is a hallmark of cancer [58, 61, 65, 66, 76-79].
Fig 9. Cancer hallmark and alternative splicing.

Alternative splicing of genes involved in development of cancer (blue background). Some genes (orange background) are involved in several events in cancer development [76, 79-88].
2.5 Progression model for pancreatic adenocarcinoma

Fig 10.

**Progression model for pancreatic adenocarcinoma.**

Stage wise development of pancreatic ductal cells to infiltrating cancer. In the initial stages, mutation of *K-ras* gene occurs, *p16* gene inactivates at an intermediate stage, inactivation of *p53*, *DPC4* and *BRCA2* occur relatively late [89, 90].
Earlier studies suggest that pancreatic cancer develops in a step-wise progression. Histologic and genetic alterations occur in parallel series which leads to invasive PDAC [89, 91]. The standardized pathologic classification system describes an increasing degree of cytologic and architectural atypia from PanIN-1 (lowest grade) to PanIN-3 (highest grade) lesions[92]. In the K-ras oncogene, duct lesions with minimal cytologic and architectural atypia also have been shown to have point mutations. In pancreatic carcinogenesis K-ras mutation occurs in early stage[93]. P16 inactivation is an intermediate event and inactivations of p53 and DPC4 appear to be late events in this progression model.[1, 94]

2.6 Oncogene and tumor suppressor genes altered in PDAC

K-ras in PDAC, studies of resected tumors suggest that this mutation is present in nearly all cases[95]. In the pathogenesis of PDAC, K-ras mutation is indeed believed to occur in early stage [92]. K-ras gene is located on chromosome 12 which encodes a member of the Ras family of GTP-binding proteins that transduces cellular growth, differentiation, and survival signals[96].

In pancreatic carcinoma, 95% of p16 tumor suppressor gets inactivated [97]. P16 is located on chromosome 9, where it encodes a protein that inhibits entry into the S phase of the cell cycle by inhibiting cyclin-dependent kinase (CDK) 4/6-dependent phosphorylation of retinoblastoma (RB) protein. p16 inactivation leads to unregulated cell growth by inappropriate progression through the cell cycle[98]. Inactivation of p16 mechanism leads to intragenic mutation plus loss of heterozygosity (LOH), promoter hypermethylation and homozygous deletion.
In addition to p16 inactivation, about 50% to 75% of pancreatic cancer show dysfunction of p53, one of the most important tumor suppressors [99]. As a transcription factor whose gene is located on 17 chromosomes, p53 regulates a variety of signaling pathways linked to cell cycle arrest, apoptosis, and DNA repair. Accordingly, functional inhibition of p53 will lead to loss of cell cycle “check-point” [98, 100].

2.7 Signal transduction pathways in PDAC

Mitogen-Activated Protein Kinase (MAPK) Cascade activating K-ras mutations and growth factor receptor (eg, EGFR)-ligand interactions are relevant to activation of this cascade in pancreatic cancer. Ras activation results in activation of serine/threonine kinases which ultimately activates downstream effector extracellular signal-related kinase (ERK). ERK-mediated phosphorylation of its substrates assist cell proliferation, survival, and differentiation [101]. Activation of this pathway results in increased survival, growth and invasion of pancreatic cancer cells [102].

Ras and other growth factor-activated tyrosine kinase has shown implication in activation of Mammalian Target of Rapamycin (mTOR)/Phosphoinositide 3-Kinase (PI3K)/AKT/ Signaling Cascade [101]. Activation of this pathway culminate to overexpression in PDAC and cell mediated proliferation, chemo resistance and survival. [102].

Constitutive activation of Nuclear Factor Kappa B (NF-κB) Signaling Cascade is studied in nearly all pancreatic cancer cell lines and PDAC tissues[103]. NF-κB–regulated genes assist in angiogenesis, chemo resistance, cell survival and invasion [102]. Due to the number of signaling
pathways leading to PDAC, pathogenesis of this disease have become complicated which ultimately leads to poor survival of pancreatic cancer patients.[104]

In the present study, we evaluated the expression of mdig in pancreatic adenocarcinoma cell lines at protein and mRNA levels. Using immunohistochemistry (IHC) approach, we detected expression of mdig in human pancreatic cancer tissues and assessed its association with clinicopathological features and prognosis. We showed that about 12% and 38% of total pancreatic malignant tissue were found to be strongly and moderately positive for mdig. We also demonstrated the presence of the alternatively spliced mdig mRNAs in pancreatic cancer cell lines we tested. Through R2: Genomics Analysis, we found opposite predictive power of different exon regions for the survival of the pancreatic cancer patients, which possibly suggested diversified roles of the alternatively spliced mdig mRNAs in the pathogenesis and prognosis of the pancreatic cancer.
2. Materials and methods

Cell culture

The human pancreatic adenocarcinoma (PA) cell line Bxpc3, Aspc1 and MIAPaca2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Aspc1 and MIAPaca2 cells were cultured in Dulbecco's Modified Eagle's Medium - high glucose (DMEM) with 10% fetal bovine serum (Invitrogen), 1% penicillin–streptomycin. Bxpc3 cells were cultured in RPMI-1640 medium (HyClone) with 10% fetal bovine serum and 1% penicillin–streptomycin and in presence of 5% CO₂. Arsenic chloride (As³⁺, Sigma-Aldrich, MO) was used in 1, 5, 10µM.

Western blotting

Total cellular proteins were prepared by lysing the cells in RIPA buffer (Millipore, Billerica, MA) supplemented with phosphatase/protease inhibitor cocktail and 1 mM PMSF through sonication and centrifugation, followed by quantification using a Micro BCA Protein Assay Reagent Kit (Thermo Scientific, Pittsburgh, PA). Before loading on 10% SDS-PAGE gels, the proteins were boiled in LDS sample buffer (Invitrogen) containing 1 mM dithiothreitol. The proteins were separated and transferred onto PVDF membranes (Invitrogen). Membranes were probed with the primary antibody at a dilution of 1:1000 or 1:2000(according to signal intensity) overnight at 4C. The secondary antibody with HRP was applied at the dilution of 1:2000. The mdig antibody was purchased from Invitrogen and GAPDH was purchased from Cell Signaling Technology, Inc.
**RT-PCR**

Total RNA was prepared by lysing the cells with TRIzol reagent (Invitrogen). Access Quick RT-PCR System (Promega) was used for reverse transcription and PCR. Sample composed of 1 μg of total RNA and 0.3 μM sense and anti-sense primer. The primer sequences for mdig are: left primer: 5′-TCATGTCCGGCCTAAGAGAC-3′; right primer: 5′-GGCATTTGATTCTGCAAAAGG-3′, which amplify a 1509 bp cDNA fragment covering the entire coding region of the mdig mRNA. The primer sequences for GAPDH are: sense: 5′-CTGAACGGGAAGCTCACTGGCATGGCCT-3′; antisense: 5′CATGAGGTCCACCACCCTGTGCTGTAG-3′.

**Immunohistochemistry**

The tissue microarray slide BC14012 was purchased from US Biomax, Inc. (Rockville, MD) and deparaffinization was performed using Xylene and was hydrated in series of alcohol. Slides were incubated with 1.5% H₂O₂ for 20 min at room temperature, to quench the endogenous peroxidase activity. Non-specific binding of immunoglobulin was blocked by incubating slides in a solution consisting of 5% goat serum and 0.2% triton-X 100 in PBS for 2 h at room temperature. Then the slides were incubated with monoclonal antibody against Mdig (mouse anti-Mina 53, Invitrogen) in 1:100 dilutions at 4 °C overnight. Proceeding day goat anti-mouse secondary antibody was applied at 1:200 dilution and incubated at room temperature for 2 h. Followed by incubation with an ABC reagent (Vectastain Elite ABC kit) at room temperature for 45 min and the chromogen was developed with diaminobenzidine (DAB). Hematoxylin was used as counterstain and it was mounted with entellan. All incubation steps were carried out in a humidified chamber and all washing steps were performed with 1 × PBS. Bright field optics of the Nikon Eclipse Ti-S Inverted microscope (Mager Scientific, Dexter MI, USA) was used to capture the images.
Migration and Invasion assay

Cell migration and invasion were determined using BD BioCoat™ Matrigel™ Invasion and Migration Chambers according to the manufacturer's instruction. After incubating for 24 h, the cells in the upper chambers were scrubbed out using cotton tipped swab. Diff-Quik staining was performed on the lower surface of the membrane. The migrated and invasive cells were counted under a microscope.

Cell viability assay

For cell viability assay 1×10^5 cells/well were seeded in 24 well plates (Corning Inc., Corning, NY, USA). After 24 hours the cells were treated with 1, 5 and 10µM arsenic (As3+) in triplicate. After 24 hours of treatment the medium was aspirated and 0.5% para formaldehyde was used for 10 minutes to fix the cells. The cells were washed twice in gentle stream of water. For staining 200µl 0.5% crystal violet was added in each well. The optical density (OD) of treated cells was determined at a wavelength of 450nm with a micro plate reader.

Survival analysis and statistics

The measurement errors for quantitative experiments were determined using standard deviation (SD). Comparison between mdig expression and clinicopathologic variables were determined by chi-square test. P value of ≤ 0.05 were considered statistically significant. Data were analyzed using IBM SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). R2: Genomics Analysis and
Visualization Platform (http://r2.amc.nl) a public data set was used to analyses Kaplan meier survival curve.
3. Results

4.1 Expression of Mdig in Human Pancreatic Adenocarcinoma Cell Lines.

An over expression of mdig has been observed in a number of human cancers, implying its important role in the pathogenesis of human malignancies. To investigate whether mdig is over expressed in pancreatic cancer, the level of mdig expression was determined by western blot technique (Fig 11). In three pancreatic cancer cell lines, Bxpc3, Aspc1 and MIAPaca2, we found that mdig protein was detected in these cell lines cultured in either presence or absence of serum. Several repeating experiments suggested that relative to other two cell lines, the AsPc1 cells expressed lower level of mdig protein.

![Expression of mdig in human pancreatic cancer cell lines.](image)

**Fig 11. Expression of mdig in human pancreatic cancer cell lines.** The indicated cells were cultured in the medium with or without 5% of serum for 24 hours. Total cell lysates were used for Western blotting using antibodies against mdig and GAPDH. Two representative results were shown.
4.2 Pancreatic Adenocarcinoma Cell lines Express Alternatively Spliced Mdig mRNAs.

In an effort to determine the mRNA levels of mdig among these pancreatic cancer cell lines, a traditional RT-PCR was applied for the detection of mdig mRNAs. The PCR primer set was derived from exon 1 and exon 10 regions of mdig gene, respectively, which amplifies a 1,510 bp fragment of mdig mRNA encompassing the entire open reading frame. A marginal increased expression of the full length mdig mRNA was noted among these cell lines cultured in the serum-free medium (Fig. 12). We had previously reported that there are alternatively spliced mdig mRNAs in which the entire exon 2 region was spliced out with or without inclusion of an alternative exon 5’ in lung epithelial cells and lung cancer cells. Such alternatively spliced mdig mRNAs with sizes around 500 to 650 bp were detected in all of these pancreatic cancer cell lines (Fig. 12). In contrast to the full length 1,510 bp mdig mRNA, the expression level of the alternatively spliced mdig mRNAs was decreased in the cells cultured in serum-free medium, suggesting different regulatory mechanisms for the expression of the full length and alternatively spliced mdig mRNAs.
Fig 12. Expression and Alternative Splicing of mdig mRNA in Pancreatic Cancer Cell Line.

Total RNAs were the cells cultured in the conditions as described in Fig. 1. RT-PCR was performed using the primer set that amplifies full length mdig mRNA with a size of 1510bp. The PCR products were separated on 1% agarose gel. Arrows indicates full length and the alternatively spliced mdig mRNAs.

4.3 Correlation of Mdig Expression and the Pathogenesis of Pancreatic Cancer.

We and others had demonstrated an association of mdig expression and the pathogenesis of lung cancer, breast cancer and other cancers. To determine whether there is a correlation between mdig expression and the clinical characteristics of the human pancreatic cancer, we measured the protein level of mdig among the pancreatic tissues through immunohistochemistry.
on a tissue microarray containing 42 cases of pancreatic cancer tissues and 6 normal pancreatic tissues. Based on the staining intensities of mdig, we found that in the normal pancreatic tissues 34% was moderately positive and 66% was weakly positive, no strongly positive staining of mdig was detected (Fig 13a). However, in the pancreatic malignant tissue, we found 12% strong positive, 39% moderately positive, 31% weakly positive and 18% negative (Fig 13b).
Fig 13. Immunohistochemical Analysis of Mdig Protein in the Human Pancreatic Tissue and Pancreatic Adenocarcinoma Tissue Microarray.

Paraffin embedded tissue microarray slide was immunostained for mdig protein as described in Materials and Methods. Red arrows depict the cells positive for mdig. Scale = 50 μM; Magnification = 40X. A. Normal pancreatic tissues; B. Pancreatic adenocarcinoma.
Fig 14. Quantification of mdig expression in Pancreas malignant adenocarcinoma and normal pancreatic tissue

4.4 Correlations between Mdig Expression and Clinicopathological Features in Pancreatic Cancer Patients

To further evaluate mdig expression and the pathogenesis of pancreatic cancer tissues, we determined the protein levels of mdig by IHC analysis among the pancreatic cancer tissues with well-defined clinicopathological features. The association between mdig expression and clinicopathological variables in pancreatic cancer patients was analyzed additionally by chi-
square test (Table 1). Among these variables, we found that cancer grades 2 and 3 exhibited a significantly strong mdig expression than the pancreatic cancer tissues at grade 1 ($p = 0.03$) (Fig 4). No other clinicopathological variables showed a significant correlation with mdig expression.

<table>
<thead>
<tr>
<th>Variables</th>
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<th>P-value</th>
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<td></td>
<td></td>
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<td>23</td>
<td>7</td>
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Table 1. Expression levels of mdig and the clinicopathological variables of the pancreatic cancer patients. Pearson chi-square tests was performed to determine the p-value
Fig 15. Quantification of Mdig expression in pancreatic adenocarcinoma tissues at different stages.

4.5 Increased Invasion and Migration in Pancreatic Cancer Cell Lines

Cell invasion and migration is an important component in the spread of pancreatic cancer. In the early stage of disease, cancer spread is thought to occur after tumor cells infiltrate the peritoneal activity and gain access to blood vessels. By using Boyden chamber assay, the capabilities of the cells invasion and migration in vitro were determined. In migration assay, the inserted filter was uncoated. For invasion assay, the filter membrane of the inserts was pre coated with Matrigel. Bxpc3 and MIAPaca2 showed increased invasion and migration compared to AsPac1. (Fig 16a and 16b)
Increased invasion and migration in Bxpc3 and MIApaca2 cell lines compared to Aspc1.
4.6 Effect of Arsenic on Viability in Pancreatic Cancer Cell Lines

To investigate the potential effect of arsenic on survival, three different pancreatic cancer cell lines (Bxpc3, Aspc1, and MIAPaca2) were used. In earlier studies we showed mdig was able to induce expression in lung cancer cell lines. Similarly, we tried to study the effect of arsenic on cell viability in human pancreatic cancer cell line. We treated each of the cell line with arsenic (1, 5 and 10µM) for 12 and 24 hours led to marked dose-dependent decrease of cell viability as determined using crystal violet exclusion. As shown in figure (Fig 17a and 17b) arsenic induced a decrease in cell number in dose and time dependent manner. The viable cell count was least at higher arsenic concentration.

Fig.17a  After 12 hours of arsenic treatment
4.7 Correlation Between Exon Specific Mdig Expression and Overall Survival in Pancreatic Cancer Patients. Our earlier studies suggested that increased expression of mdig predicts poorer overall survival of breast cancer and lung cancer patients. Studies by other groups also indicated poorer survival of the hepatocellular carcinoma with higher level of mdig \[105\]. To reveal the potential prognostic value of mdig expression for the pancreatic cancer patients, we used R2 genomics visualization tool in a Mixed Tumor Pancreas (Exon) dataset to investigate the changes in the patient survival rate with respect to individual mdig exon that was detected by 19 probes provided by Affymetrix (Fig 18). Kaplan-meier survival graphs were generated from this data set and were compared based on their survival outcomes. Among these 19 survival analysis based on the individual probes for different exons, 15 probe sets indicated a poorer survival of the patients with higher mdig expression. However, 4 probes, probe sets 8089044, 8089049, 8089056, and 8089058, which correspond to exon 2, alternative exon 5, and exon 10, predicted

**Fig 17b.** Cell viability of Bxpc3, Aspc1, MIAPaca2 cells after 12 and 24 hours of arsenic treatment.
better survival of the patients with higher mdig expression (Fig. 19). Since missing exon 2 and inclusion of an alternative exon 5 had been found in an alternatively spliced mdig mRNA we identified previously, the survival profiles of the different mdig exons suggested an unique or opposite role of the alternatively spliced mdig relative to the full length mdig in the pathogenesis of the pancreatic cancer.

**Fig 18.** The levels of mdig expression predict survival of the patients with pancreatic cancer. Diagram of mdig gene structure and the positions of the probe detected.
Fig 19. The levels of mdig expression predict survival of the patients with pancreatic cancer. Kaplan-Meier survival analysis of the selected probe sets as indicated in the R2: Genomics Analysis and Visualization Platform database.

4.8 Heat map analysis

Heat map clustering of 90 pancreatic cancer patients is presented in (Fig 20.) Along the X-axis probe Id’s for MINA/ mdig which overlaps the exonic sequence for mdig is presented. Patient samples which are marked red indicate dead and samples which are marked green are alive. Complete patient information is available in (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi).
Interestingly, majority of patients who died with pancreatic cancer had high level mdig expression. Hence it suggests that mdig contributes to pathogenesis of pancreatic cancer. In all Heat map clustering doesn’t provides significant information with respect to exon specific mdig expression and overall survival of pancreatic cancer patients.

Fig 20. Heat map clustering of pancreatic cancer patient samples.
X axis represents probe sets which lies in mdig regions. Y axis represents pancreatic cancer patient information. Green indicates (<0) low expression of mdig and red indicates (>0) high expression of mdig. The mdig probe Id’s marked RED indicates poor survival rate and BLUE indicates better survival rate.
4. Discussion

There has been a decline in the death rate for the cancers in lung, colorectal, breast, and prostate, since 2003. In contrast, the death rate of pancreatic cancer was increasing in the same time period. The five year survival rate of pancreatic cancer is only about 6% since 1970s [106]. We and others had shown an increased expression of mdig in a number of human cancers, including lung cancer, colon cancer, and breast cancer, which implies important contribution of mdig towards the pathogenesis of human cancers [53]. However, there are no reports studying the relationship of mdig expression and the clinicopathological features and/or prognosis of the pancreatic cancer. In the current study, we provide evidence showing that high level of mdig expression was a salient feature in human pancreatic cancer cell lines and tissue. By western blot analysis, we observed that all the three pancreatic cancer cell lines, BxPc3, Aspc1 and MIAPaca2, expressed high level of mdig protein, although there was a slight difference in the expression levels of mdig among these three cell lines studied. Bxpc3 and MIAPaca2 showed high mdig expression levels compared to AsPc1. The RT-PCR analysis showed mdig mRNA expression in all three cell lines. Interestingly, we were able to detect some strong bands of mdig mRNAs resulted from alternative splicing. The alternatively spliced mdig mRNAs might have distinctive prognostic values against the normally spliced mdig mRNA on the overall survival of the pancreatic cancer patients.

Cell migration is an important component in the spread of pancreatic adenocarcinoma (PA). Therefore we tried to study cell migration property in PA cell lines. In trans well migration assay Bxpc3 cells had 2.5-fold greater motility than Aspc1 cells. MIApaca2 cell lines had better motility than Aspc1 but less than Bxpc3. Another important phenotype of PA cells is its invasive
properties, as pancreatic cancer is highly aggressive and invasive by nature, with almost all patients presenting with metastasis at the time of diagnosis. Indeed, the presence of metastases is thought to be responsible for the poor prognosis of this disease[107]. Therefore we also tried to study the difference in cell invasion property in PA cell lines. Bxpc3 cells had 1 fold greater invasive property than Aspc1 cells. MIApaca2 cell lines had better invasive ability than Aspc1 but less than Bxpc3.

Despite extensive studies, the etiology and mechanisms of the pancreatic cancer are still elusive. Some epidemiologic studies suggested a higher incidence rate of pancreatic cancer among African Americans than Whites. Several heritable, germline mutations in p16 and/or BRCA2 may be associated with the development of pancreatic cancer [4]. It was estimated that 95% of pancreatic carcinoma showed inactivation of p16 tumor suppressor [97].

P16 is located on chromosome 9, where it encodes a protein that inhibits entry into the S phase of the cell cycle by inhibiting cyclin-dependent kinase (CDK) 4/6-dependent phosphorylation of retinoblastoma (RB) protein. Inactivation of p16 leads to an uncontrolled cell growth due to a fast cell cycle transition [98]. Intragenic mutation, loss of heterozygosity (LOH), homozygous deletion, and promoter hypermethylation, may be responsible for p16 inactivation. In addition to p16 inactivation, about 50% to 75% of pancreatic cancer show dysfunction of p53, one of the most important tumor suppressors [99]. As a transcription factor whose gene is located on 17 chromosomes, p53 regulates a variety of signaling pathways linked to cell cycle arrest, apoptosis, and DNA repair. Accordingly, functional inhibition of p53 will lead to loss of cell cycle “check-point” [98, 100].

Mutations in K-ras gene were frequently observed in a number of human cancers. This mutation was also found in almost all resected cases of pancreatic cancer [95]. It was believed
that K-ras mutation occurs in the early stage of cancer development [92]. Because of the proximity of K-ras gene with other ras family of GTP-binding proteins on chromosome 12, genetic abnormality of K-ras gene may be additionally associated with the functional disruption of other GTP-binding proteins critical for cell growth, differentiation and survival.

Chronic pancreatitis of various types was considered as a contributing factor for about 3%–4% of pancreatic cancer. Apart from germline mutations or pancreatitis, other major risk factors associated with pancreatic cancer include cigarette smoking, obesity, diabetes, cirrhosis of the liver, etc. Earlier studies suggested an approximately 40% increased hazard for death of the pancreatic cancer patients who smoked cigarettes compared with those who never smoked, and risk tends to increase according to frequency or duration of smoking [5]. Dietary studies have shown a fairly steady pattern of increase hazard with meat or cholesterol intake and decreased risk with fruit or vegetable consumption, although causal inferences regarding these associations are still uncertain [6].

5. Conclusion

In the current report, we provided evidence showing association of mdig expression and the pathogenesis of the pancreatic cancer. Previous studies suggested that increased expression of mdig predicts poor survival of the patients with breast cancer, lung cancer and hepatocellular carcinoma [105, 108, 109]. By using R2 genomics visualization tool, the probe-exon-based analysis also suggested an inverse relationship between the levels of mdig expression and the survival of the pancreatic cancer patients. However, 4 out of 19 probes showed that increased mdig expression, in fact, predicts a better overall survival of the pancreatic cancer patients, which most likely indicated a different or opposite function of the alternatively spliced mdig mRNAs.
6. Future direction

It remains to be further investigated on how different isoforms of mdig resulted from the normal and alternative splicing affect the initiation, progression and pathogenesis of the pancreatic cancer. It will also be important to determine whether mdig can serve as a biomarker for diagnosis, prognosis and molecular targeting therapy for pancreatic cancer.
Bibliography


Pancreatic cancer is a highly aggressive malignant disease having very limited therapeutic options that ultimately results in its poor prognosis. It is still elusive on the etiology and tumorigenic mechanisms of pancreatic cancer. In the present report, we provide evidence showing involvement of the mineral dust-induced gene (mdig) in the pathogenesis and prognosis of the pancreatic cancer. Using immunohistochemistry approach on human pancreatic cancer tissue microarray, we found differential expression of mdig in pancreatic adenocarcinoma and normal pancreas. Based on the staining intensities of mdig in these tissue samples, we found that 12% of the cancer tissues were strongly positive for mdig, 39% and 31% were moderately and weakly positive respectively. Several alternatively spliced mdig mRNAs were detected in the selected pancreatic cancer cell line. Through R2 platform for the patient survival analysis (http://r2.amc.nl), we found that enrichment of some specific exon of mdig predicates different survival rate of the pancreatic cancer patients. In summary, our findings may help in assessing the role of mdig in the pathogenesis of the pancreatic cancer and the prognosis of the pancreatic cancer patients.
Keywords: Pancreatic cancer, mdig, alternative splicing, immunohistochemistry, survival rate.
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Srinivas Ashok Kumar obtained his Bachelors in pharmacy from Pune University, India in 2014. He worked as Drug Safety Associate in Tata Consultancy limited from 2014 to 2015. He joined Wayne State University, Department of Pharmaceutical Science to pursue his studies as a Master’s student. He joined Dr. Fei Chen’s lab in December 2015 as a rotational student. From January he started working in his lab and learned many technique. His main project focused on Pathological and Prognostic Role of Mdig in Pancreatic Cancer. During his research in Wayne State University, Srinivas earned Wayne State Graduate Scholarship for winter 2017.