Universidade Federal do Rio Grande do Sul
Instituto de Biociências
Programa de Pós-Graduação em Genética e Biologia Molecular

AVALIAÇÃO DA EXPRESSÃO DOS GENES *HDAC1*, *HDAC2, HDAC3* E *HDAC7* E SEUS POSSÍVEIS MECANISMOS DE SILENCIAMENTO NO ADENOCARCINOMA DUCTAL PANCREÁTICO.

Cleandra Gregório Silva

Dissertação submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do grau de Mestre em Genética e Biologia Molecular.

Orientadora: Profª Drª Patricia Ashton Prolla

Porto Alegre, janeiro de 2016.

Este trabalho foi desenvolvido no Laboratório de Medicina Genômica do Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre (HCPA). O estudo foi financiado pelo Fundo de Incentivo à Pesquisa e Eventos (FIPE) do HCPA e pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico através do edital Genoprot (nº 559814/2009-7). Todos os experimentos apresentados nesta dissertação estão incluídos em projeto de pesquisa aprovado por seus aspectos éticos e metodológicos pelo Comitê de Ética e Pesquisa do Grupo de Pesquisa e Pós-Graduação (GPPG) do Hospital de Clínicas de Porto Alegre, sob os registros de n° 11-0510, n° 10-0162 e n°14-06526.

DEDICATÓRIA

A todos os pacientes que lutam contra o câncer.

AGRADECIMENTOS

À Professora Patricia Ashton Prolla, pela orientação durante a graduação e mestrado e pelo enorme aprendizado. Sua postura é um exemplo a ser seguido na carreira acadêmica e científica.

À Bárbara Alemar, pela amizade, orientação e inestimável apoio. Agradeço a parceria desenvolvida durante os quatro anos de trabalho em conjunto e o grande papel que teve durante meu amadurecimento acadêmico.

Ao Dr. Alessandro Osvaldt, pela grande colaboração no desenvolvimento do estudo. Também pelo seu incentivo e confiança em mim depositada.

À Professora Úrsula Matte, pelas considerações pertinentes na interpretação dos resultados.

À minha família, por acreditar na minha capacidade e apoiar minhas escolhas. A conclusão desse sonho é consequência do suporte e amor que vocês sempre me deram.

A todos os participantes do grupo de pesquisa em pâncreas.

A todos os integrantes do Laboratório de Medicina Genômica, agradeço o carinho e ao apoio. Em especial para o Igor Araujo, Gustavo Stumpf e Fernanda Vianna pela paciência e grande ajuda na etapa final deste trabalho.

Aos funcionários do Centro de Pesquisa Experimental do HCPA, Patrícia Koehler e Jeferson Beck e Everaldo Almeida pelo suporte e atenção.

Aos órgãos que fomentaram este estudo: CNPq e FIPE-HCPA, pelos recursos destinados ao projeto.

SUMÁRIO

LISTA DE ABREVIATURAS

ADP - Adenocarcinoma Ductal Pancreático (**PDAC** – *Pancreatic Ductal Adenocarcinoma*)
AUC - *Area under (ROC) curve*
BCL2 - *B-cell CLL/lymphoma 2*
BRCA2 - *Breast cancer type 2 susceptibility protein*
CA19-9 - Antígeno Carboidrato 19-9
CDKN2 - *Cyclin-dependent kinase inhibitor 2A*
c-MYC - *Myelocytomatosis oncogene*
DNA - Ácido Desoxirribonocléico (*Deoxyribonucleic Acid*)
DPC4 - *Decapentaplegic homolog 4*
EGF5 - Fator de crescimento epidermal 5 (*Epidermal growth factor 5*)
eIF4 - *Eukaryotic initiation factor 4*
ELISA - *Enzyme Linked Immuno Sorbent Assay*
ERBB2 - *erb-b2 receptor tyrosine kinase 2 gene*
FDA- *Food and Drug Administration*
GEO - *Gene Expression Omnibus*
HAT - Acetilase de Histona
HDAC - Desacetilase de Histona
HDACi - Inibidor de Desacetilase de Histona (Histone Deacetylase Inhibitors)
HIF-1α - *hypoxia inducible factor 1, alpha subunit*
IHQ - Imunohistoquímica
KRAS - *v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog*
miRISC - Complexo de Silenciamento Induzido por RNA
miRNA - Micro RNA
mRNA - RNA mensageiro (*messenger RNA*)
NCM - Neoplasias Císticas Mucinosas
NFKb - *Nuclear factor of kappa light polypeptide gene enhancer in B-cells*
NIPan - Neoplasias Intraepiteliais Pancreáticas (**PanIN** – *Pancreatic Intraepithelial Neoplasia*)
NIPM - Neoplasias Intraductais Papilíferas Mucinosas (**IPMN** -

Intraductal papillary mucinous neoplasm)

PCR - Reação em Cadeira de Polimerase (*Polimerase Chain Reaction*)

Pol-II - RNA Polimerase II

qRT-PCR - PCR quantitativo por transcrição reversa (*quantitative Reverse Transcription PCR*)

RB - Retinoblastoma

RNA - Ácido Ribonucléico (*Ribonucleic acid*)

SIR2 - *NAD-dependent histone deacetylase SIR2*

SIRT1 - *Sirtuin 1*

SMAD4 - *SMAD family member 4*

Smad7- SMAD family member 7

Stat3 - *Signal Transducer and Activator of Transcription 3*

TF - Fator de Transcrição

TGF - Fator de Transformação do Crescimento (*Transforming growth factor*)

TP53 - *Tumor Protein p53*

UICC - União Internacional Contra o Câncer

WB - *Western Blotting*

LISTA DE TABELAS

LISTA DE QUADROS

LISTA DE FIGURAS

RESUMO

O adenocarcinoma ductal pancreático (ADP) é uma doença altamente letal e agressiva. Alteração no perfil de acetilação das histonas envolvendo desacetilases de histonas (HDAC), assim como modificações da expressão de miRNAs podem levar ao desenvolvimento tumoral. Neste estudo, foi avaliada a expressão das *HDAC1, HDAC2, HDAC3* e *HDAC7* em ADP e amostras de tecido pancreático não tumoral (TN) usando análises experimentais e de banco de dados. Os níveis de expressão foram correlacionados com características clínico-patológicas dos pacientes e foi realizada uma investigação *in silico* de miRNAs reguladores de efeito das HDACs. Os níveis de expressão das HDACs foram avaliadas por qRT-PCR a partir de 25 amostras de ADP e 23 amostras de TN e a análise da expressão diferencial (ED) e correlação entre HDACs e miRNAs em ADP foi realizada utilizando perfis de expressão de seis microarranjos do *Gene Expression Omnibus*. Potenciais relações miRNA-HDACs foram coletadas em bases de dados de interação de miRNAs. Um valor de $P<0,05$ foi considerado estatisticamente significativo. Encontramos expressão reduzida em ADP comparado com TN para todas as HDACs analisadas, com $P<0,05$ para *HDAC1*, *2* e *3*. Entretanto, os *fold-changes* foram muito baixos e provavelmente sem relevância biológica, e a expressão da *HDAC2* e *HDAC7* foi correlacionada com a idade ao diagnóstico. Nenhuma outra correlação entre a expressão das HDACs e características clínico-patológicas foi identificada. Análises de ED sugeriram significativa superexpressão das *HDAC1, 2* e *7* e subexpressão da *HDAC3*, contudo todas apresentaram *fold-changes* pequenos. As análises dos bancos de dados identificaram 728 miRNAs como reguladores das HDACs. Interseções entre os conjuntos de miRNAs (GSE41369 e GSE43796) e aqueles recuperados da análise de expressão diferencial indicaram cinco miRNAs que influenciam a *HDAC1* (miR-188-5p, miR-539, miR-708, miR -4269 e miR-3616-3p) e três que influenciam a *HDAC2* (miR-4307, miR-944 e miR-195). A expressão das HDACs provavelmente não é um biomarcador de prognóstico robusto para o ADP, uma vez que a expressão diferencial entre os grupos é sutil. Ainda, este e estudos anteriores indicam nenhuma ou pouca associação entre a expressão HDACs e características clínico-patológicas relacionadas

com o prognóstico. Finalmente, miRNAs provavelmente não estão exercendo um papel central na regulação da HDACs no ADP.

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal and aggressive disease. The disruption of histone acetylation through histones deacetylases (HDACs) and expression regulation by miRNAs can lead to tumor development. In this study we assessed *HDAC1, HDAC2, HDAC3* and *HDAC7* expression in PDAC and non-tumoral tissue (NT) samples using experimental and databases analysis, correlated their expression levels with clinical and pathological features in patients and performed *in silico* investigation of HDACs regulation by miRNAs. Expression levels of HDACs were measured by qRT-PCR from 25 PDAC and 23 NT. An analysis of differential expression (DE) and correlation of HDACs and miRNAs in PDAC was performed using six *Gene Expression Omnibus* microarray datasets. Potential miRNA-HDACs relationships were collected from miRNA interaction databases. A $P<0.05$ was considered statistically significant.

We found reduced expression in PDAC compared with NT for *HDAC1*, *HDAC2* and *HDAC3*, with $P<0.05$. Expression levels of *HDAC7* did not significantly differ between groups. However, fold-changes were very small and probably not biologically relevant. Only *HDAC2* and *HDAC7* were associated with age at diagnosis and no other associations between HDAC expression and clinical features were identified. DE analysis suggested significant up-regulation of *HDAC1*, *HDAC2* and *HDAC7*, and down-regulation of *HDAC3*, albeit all of them associated with small fold changes. Databases analysis identified 728 miRNAs that could be HDACs regulators. Intersections among the set of miRNAs found in differential expression analysis of GSE41369 and GSE43796 and those retrieved from target prediction identified five miRNAs targeting *HDAC1* (miR-188-5p, miR-539, miR-708, miR-4269 and miR-3616-3p) and three targeting *HDAC2* (miR-4307, miR-944 and miR-195). HDACs expression is likely not a robust prognostic biomarker in PDAC since differential expression between groups is subtle. Also, this and previous studies indicate no or only very few associations between HDACs expression and clinicopathological features related to prognosis. Finally, miRNAs are probably not exerting a central role in HDAC regulation in PDAC.

CAPÍTULO I – INTRODUÇÃO

I.1. Epidemiologia do câncer de pâncreas

As neoplasias malignas são uma das principais causas de óbito no mundo, e sua incidência e mortalidade só perdem para as doenças cardiovasculares. No Brasil, as estimativas para o ano de 2016 indicam a ocorrência de 596 mil novos casos de câncer. O panorama do crescimento das doenças crônico-degenerativas como o câncer é o resultado de diversos fatores como, por exemplo, o envelhecimento da população e o estilo de vida. As ações de promoção e recuperação da saúde com foco em patologias crônicas neoplásicas possibilitam o rastreamento e diagnóstico de tumores em uma grande parcela da população (INCa, 2015a).

Entre os tumores gastrointestinais, o câncer de pâncreas é o segundo mais comum, perdendo apenas para tumores do colón (Song e Lee, 2013). Segundo estatísticas mundiais, este tumor é o 12° com maior incidência e a sétima causa de morte por câncer (Ferlay *et al.*, 2015). Nos Estados Unidos, as estimativas para 2015 indicaram que a incidência foi de 48.960 novos casos e esse tipo tumoral foi a quarta causa de morte relacionada ao câncer (Siegel, Miller e Jemal, 2015). No Brasil, esta doença corresponde a apenas 2% de todos os casos novos de câncer diagnosticados e a 4% do total de óbitos decorrente deste tipo de doença (INCa, 2003). As altas taxas de mortalidade associadas ao câncer de pâncreas decorrem da dificuldade de detecção, alta agressividade e diagnóstico tardio, pois não há sintomas específicos e a sua biologia tumoral é muito agressiva (Wray *et al.*, 2005). O único tratamento curativo existente para este tipo tumoral é a ressecção cirúrgica. Contudo, cerca de 80 a 85% dos pacientes apresentam tumores irressecáveis e/ou metástases que contraindicam a ressecção (Neuzillet, Sauvanet e Hammel, 2011; Vincent *et al.*, 2011). O adenocarcinoma ductal pancreático (ADP) é o tipo histológico de tumor pancreático mais frequente e de pior prognóstico, compreendendo 90% dos tumores nesse órgão (Neuzillet, Sauvanet e Hammel, 2011).

I.2. Aspectos clínicos do adenocarcinoma ductal pancreático

A maior parte dos casos de ADP é diagnosticada em estágios bem avançados da doença, sendo que a taxa de sobrevida em cinco anos é de 20%

para pacientes com doença localizada e apenas de 2% para aqueles com metástases à distância (Simianu *et al.*, 2010; Kim e Ahuja, 2015). O estadiamento de todas as neoplasias é utilizado para avaliar a extensão, determinar o planejamento terapêutico e indicar o prognóstico da doença. A ferramenta de avaliação do estadiamento recomendada pela União Internacional contra o Câncer (UICC) é a Classificação TNM. Este sistema baseia-se na extensão anatômica da doença, considerando as características do tumor primário (T), dos linfonodos das cadeias de drenagem linfática do órgão em que o tumor se localiza (N), e a presença ou ausência de metástases à distância (M). Cada tumor recebe graduações em cada categoria e a compilação dessas informações confere um estadiamento geral. Com base nessas informações, o tumor é classificado de inicial/localizado (estágio 0 ao IIb) à irressecável (estagio III e IV). O Quadro 1 apresenta as definições de estádio TNM do câncer de pâncreas (INCa, 2015b).

Estádio T (Tumor primário)				
Tx	Tumor primário não pode ser acessado			
T0	Não há evidência de tumor primário			
Tis	Tumor *in situ*			
T1	Tumor limitado ao pâncreas e ≤ 2cm			
T2	Tumor limitado ao pâncreas e > 2cm			
T3	Tumor se estende além do pâncreas sem envolvimento do plexo celíaco ou artéria mesentérica superior			
T4	Tumor se estende além do pâncreas e envolve o plexo celíaco ou artéria mesentérica superior			
Estágio N (Linfonodos regionais)				
Nx	Linfonodos regionais não podem ser acessados			
N0	Ausência de metástase nos linfonodos regionais			
N1	Presença de metástase nos linfonodos regionais			
Estágio M (Metástase à distância)				
Mx	A presença de metástase à distância não pode ser avaliada			
M0	Ausência de metástase à distância			
M1	Presença de metástase à distância			
Grupamento por Estádios				
Estádio 0	Tis	N0	M0	Localizado
Estádio IA	T1	N0	M0	Localizado
Estádio IB	T2	N0	M0	Localizado
Estádio IIA	T3	N0	M0	Localmente invasivo
Estádio IIB	T1 - 3	N1	M0	Localmente invasivo
Estádio III	T4	Qualquer N	M0	Irressecável
Estádio IV	Qualquer T	Qualquer N	M1	Irressecável

Quadro 1.Classificação TNM de tumores malignos: pâncreas[1]
1 União Internacional Contra o Câncer (UICC) (INCa, 2015b).

Segundo a Sociedade Americana de Oncologia Clínica aproximadamente 45-55% dos pacientes são diagnosticados na fase irressecável (III e IV), na qual o tumor se disseminou para além da área do

pâncreas e de outros órgãos, como o fígado, ou áreas distantes do abdômen (ASCO, 2014). A agressividade e letalidade do ADP se devem a diversos fatores. O primeiro deles é o fato do pâncreas estar localizado na porção posterior do abdômen (retroperitônio), o que dificulta a detecção precoce por métodos convencionais de investigação e exame clínico (Molin e Maitra, 2012). O segundo fator é a apresentação de sinais e sintomas inespecíficos. As manifestações dependem em grande parte da localização do tumor e estágio da doença: em cerca de 65% dos casos o tumor está localização na cabeça do pâncreas, 15% no corpo/cauda e 20% envolvem o órgão de uma forma difusa. A localização tumoral na cabeça do pâncreas frequentemente leva a obstrução da via biliar, desencadeando a colestase, cuja síndrome clinica se expressa por icterícia, colúria e acolia. Outros sintomas incluem dor abdominal, glicemia alterada, astenia, anorexia e perda de peso, sendo fundamental o diagnostico diferencial com pancreatite crônica e diabetes (Hidalgo, 2010; Simianu *et al.*, 2010). O terceiro fator é que os tumores pancreáticos comumente apresentam metástases hepáticas, na cavidade peritoneal e pulmões, mesmo em estágios precoces. De fato, foi observado por Rhim e colaboradores (2012) que o desenvolvimento de metástases pode acontecer concomitantemente ao aparecimento da lesão intraepitelial pancreática (lesões precursoras do ADP) em um modelo animal. Tais dados podem explicar por que pacientes que realizam cirurgia de ressecção total do tumor com boas margens e sem nenhum sinal de metástase no momento da cirurgia apresentam recidivas relacionadas ao sítio primário poucos meses após o procedimento (Rhim *et al.*, 2012)**.** Por fim, o quarto fator crucial para a letalidade do ADP é a sua resistência aos tratamentos quimioterápicos e drogas alvo-moleculares desenvolvidos até o momento, o que pode estar ligado à sua baixa vascularização, à sua heterogeneidade molecular e às subpopulações de células tronco presentes nesses tumores (Hingorani *et al.*, 2005; Li *et al.*, 2007; Olive *et al.*, 2009).

I.3. Biologia molecular do adenocarcinoma ductal pancreático

O conhecimento dos mecanismos moleculares associados ao ADP aumentou significativamente nos últimos anos. Hoje, sabe-se que existem genes fortemente relacionados ao desenvolvimento e à progressão desse tipo

de tumor. As lesões neoplásicas se dão em função de mutações em diferentes famílias de genes: genes supressores de tumor, de manutenção e reparo celular e genes relacionados à proliferação, também chamados de oncogenes. Essas alterações moleculares são observadas em três lesões pré-malignas: neoplasias intraepiteliais pancreáticas (NIPan) que são mais frequentes e melhor caracterizadas, neoplasias intraductais papilíferas mucinosas (NIPM) e neoplasias císticas mucinosas (NCM). Estas duas últimas apresentam como característica a produção de mucina e visibilidade em exames de imagem (Maitra e Hruban, 2008; Bosman *et al.*, 2010).

Hruban e colaboradores (2000) identificaram e definiram um modelo de progressão de ADP com as alterações genéticas associadas a partir das NIPans. Segundo eles, o epitélio normal começaria a sofrer mutações em oncogenes até desenvolver pequenas lesões displásicas nos ductos pancreáticos (NIPan, estágio 1A e 1B). Outras mutações levariam à perda de função em genes supressores de tumor, causando uma displasia severa (NIPan estágio 2 e 3) e, por fim, a formação do epitélio desmoplásico e invasor, caracterizando o ADP (Hruban, Wilentz e Kern, 2000). O modelo de progressão tumoral do ADP a partir das NIPans está resumido na Figura 1.

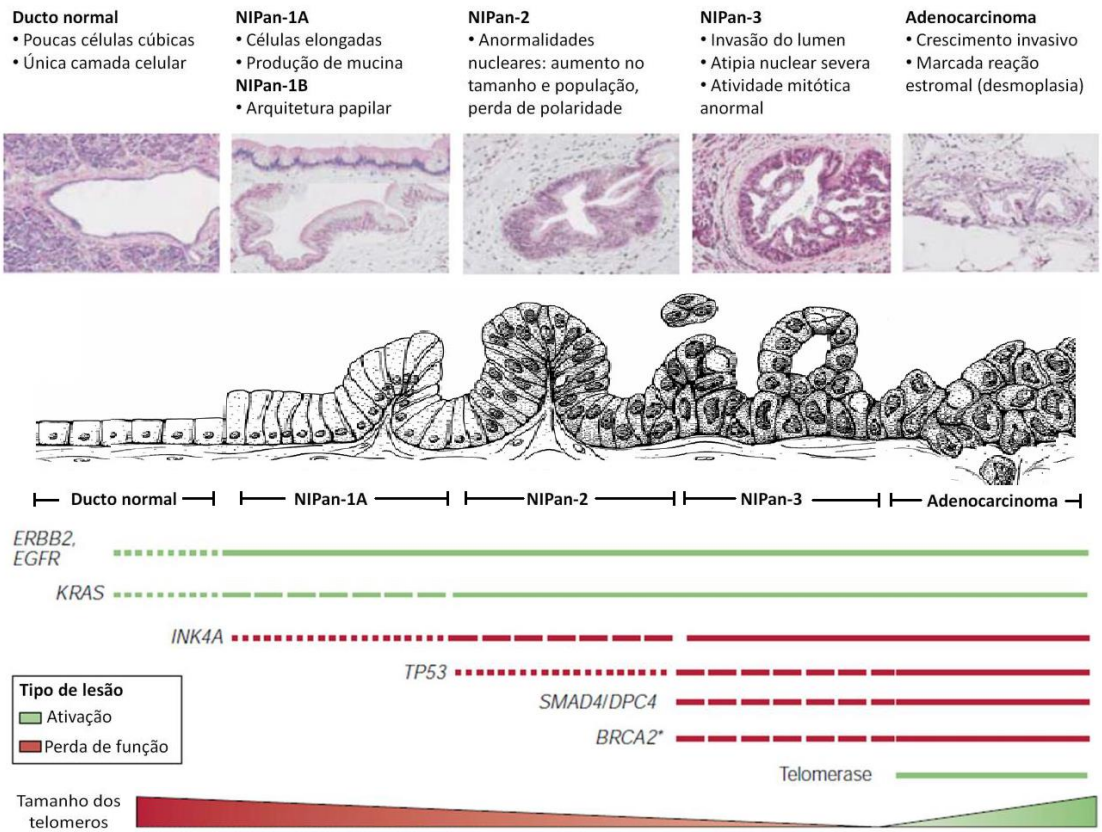


Figura 1. Modelo de progressão do câncer pancreático (adaptado de (Hruban, Wilentz e Kern, 2000; Bardeesy e Depinho, 2002).

O acúmulo de sucessivas mutações nas células pancreáticas somado a alterações epigenéticas desencadeia a formação do ADP (Omura e Goggins, 2009)**.** Entre as alterações genéticas, os genes que têm um papel crucial para a progressão e desenvolvimento tumoral são *KRAS*, *CDKN2A* (p16 INK4A)*, TP53, BRCA2* e *SMAD4/DPC4* (Bardeesy e Depinho, 2002)**.**

Nas fases iniciais da lesão, observamos a ativação de genes, em especial do gene *KRAS*, com mutações presentes em cerca de 30% das lesões iniciais e em 95% das lesões avançadas de ADP. Alterações na família *KRAS* de oncogenes induzem a proliferação, sobrevivência e invasão celular. A superexpressão do gene *ERBB2* também está presente nas NIPans 1A-1B. A telomerase é ativada no final da progressão do ADP, sendo um ponto fundamental para o processo de imortalização das células tumorais (Bardeesy e Depinho, 2002)**.**

O desenvolvimento do processo neoplásico se consolida após mutações de perda de função ocorrerem em genes supressores de tumor. A perda de

função do gene *CDKN2A* é encontrada em 80-95% dos ADPs a partir dos estágios avançados e NIPans 2-3. O gene *CDKN2A* está estritamente relacionado à transcrição dos genes supressores de tumor *INK4* e *ARF e*, desta forma, quando há alteração em sua função ocorre um distúrbio nas vias de sinalização retinoblastoma (Rb) e p53 (Liggett e Sidransky, 1998; Bardeesy e Depinho, 2002). Mutações no gene *TP53* são encontradas em 50-75% dos casos de ADP (Redston *et al.*, 1994), sendo mais pronunciadas nas NIPans 2-3. Como consequência da perda de função da proteína p53, a célula desencadeia processos de instabilidade genômica. Mutações no gene *BRCA2* são eventos mais tardios da progressão do ADP e indivíduos portadores de mutações herdadas nesse gene apresentam um risco significativo de desenvolvimento de câncer de mama, ovário e pâncreas, sendo o último menos incidente (Bardeesy e Depinho, 2002)**.** A presença de deleções ou mutações no gene *SMAD4/DPC4* pode chegar a 55% dos ADPs (Liu, 2001) e sua função está intimamente ligada à via de sinalização TGF-β, que tem um papel fundamental no bloqueio do crescimento das células epiteliais normais. Desta forma, alterações na expressão desse gene impedem o bloqueio do ciclo celular ou apoptose (Bardeesy e Depinho, 2002).

I.4. Epigenética

Segundo Feinberg, a epigenética é definida como o conjunto de modificações do genoma, transmitidas durante a divisão celular, que não envolvem uma alteração na sequência de DNA (Feinberg, 2001). Durante a embriogênese, os mecanismos epigenéticos permitem que as células progenitoras pluripotentes, que possuem o mesmo DNA genômico, obtenham a capacidade de se diferenciar em populações distintas. Estes processos regulam a expressão de redes gênicas com tempo e intensidade específicos, que por sua vez são capazes de modificar o fenótipo sem alterar a sequência do DNA (Lomberk e Urrutia, 2015). Tais processos tem influência no desenvolvimento e durante todo o tempo de vida do indivíduo e, assim, a desregulação epigenética pode estar relacionada à ativação ou inibição inapropriada de várias vias de sinalização, levando ao desenvolvimento de doenças, como o câncer (Jones e Baylin, 2002; Egger *et al.*, 2004). Existem

algumas diferenças entre a epigenética e os mecanismos genéticos convencionais, que incluem a reversibilidade e a capacidade de agir em distâncias não esperadas, maiores do que um único gene (Omura e Goggins, 2009).

Os principais mecanismos de regulação epigenética são as modificações na conformação das histonas, metilação do DNA e regulação por miRNAs (Sawan *et al.*, 2008; Lomberk e Urrutia, 2015). Os dois primeiros processos alteram a acessibilidade da cromatina, regulando a transcrição local ou global do DNA, enquanto o último regula a expressão gênica principalmente através do silenciamento de RNAs mensageiros (Lund e Van Lohuizen, 2004).

I.4.1. Modificações nas Histonas

A organização nuclear condensada nas células eucariotas é dependente do estado de agregação do nucleossoma, que por sua vez é composto por DNA e proteínas básicas denominadas histonas. Esta unidade básica da cromatina é formada pelas histonas H2A, H2B, H3 e H4, as quais formam um complexo que, por sua vez, se dimeriza a um complexo igual, formando um octâmero protéico em torno do DNA (Figura 2) (Luger *et al.*, 1997; Schneider *et al.*, 2011). Além disso, a histona H1 é uma proteína externa ao octâmero de histonas-DNA e exerce um importante papel na compactação da cromatina (Mazzio e Soliman, 2012).

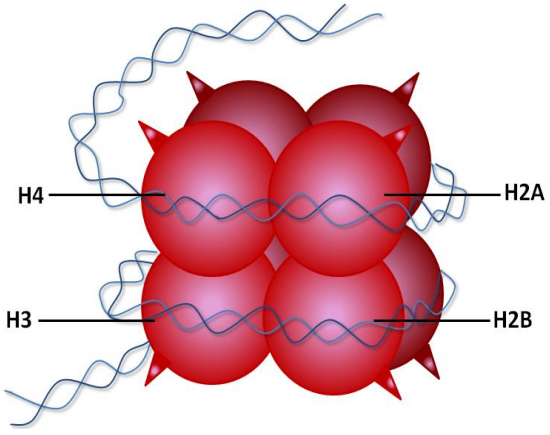


Figura 2. Estrutura do nucleossoma. Dupla hélice de DNA de 146 pb.

O domínio N-terminal das histonas pertencentes ao octâmero se estende para fora do nucleossoma, o que permite a acessibilidade de complexos reguladores epigenéticos que podem, de uma maneira geral, adicionar ou

retirar moléculas covalentes a essas proteínas. Essas modificações são capazes de alterar a conformação do nucleossoma e a acessibilidade das maquinarias de reparo, replicação e transcrição ao DNA (Lee *et al.*, 1993; Peterson e Laniel, 2004; Groth *et al.*, 2007; Mazzio e Soliman, 2012). As modificações pós-traducionais das histonas incluem: a acetilação de lisinas, a metilação de lisinas e argininas, a fosforilação de serinas e treoninas, a adenosina difosfato-ribosilação de ácido glutâmico e a ubiquitinação e sumoilação de resíduos de lisina, entre outros (Lomberk e Urrutia, 2015). As marcas epigenéticas não ocorrem isoladas nos domínios N- terminais das histonas e são capazes de exercer diferentes efeitos na expressão gênica. Strahl e Allis (2000) propuseram que uma dada alteração pode ser determinante para subsequentes modificações na mesma ou em outra histona, e esse mecanismo ficou conhecido como “código das histonas”. (Strahl e Allis, 2000; Margueron, Trojer e Reinberg, 2005). Assim, a presença de uma determinada modificação pode facilitar ou impedir que uma segunda aconteça, sendo essas alterações capazes de induzir diferentes níveis de organização da cromatina e ainda serem transmitidas para células descendentes (Lomberk e Urrutia, 2015). Atualmente, a modificação ativa no processo transcricional mais estudada é a acetilação dos resíduos de lisina das histonas 3 e 4 (H3 e H4), que levam à formação da eucromatina (Li, Carey e Workman, 2007).

O processo de modificação das histonas por acetilação é dependente de dois grupos de enzimas: as acetilases de histonas (HATs) e as desacetilases de histonas (HDACs). As HATs promovem a acetilação em resíduos de lisina nas histonas H3 e H4 e conferem a ativação da molécula de cromatina (eucromatina), permitindo que a cromatina fique acessível para a ação dos fatores de transcrição (FT). Por outro lado, a ação das HDACs consiste na remoção dos radicais acetil do aminoácido lisina nas histonas H3 e H4, o que impede o acesso dos FTs à cromatina, inativando-a (heterocromatina), como mostrado na Figura 3 (Brown *et al.*, 2000; Schneider *et al.*, 2011).

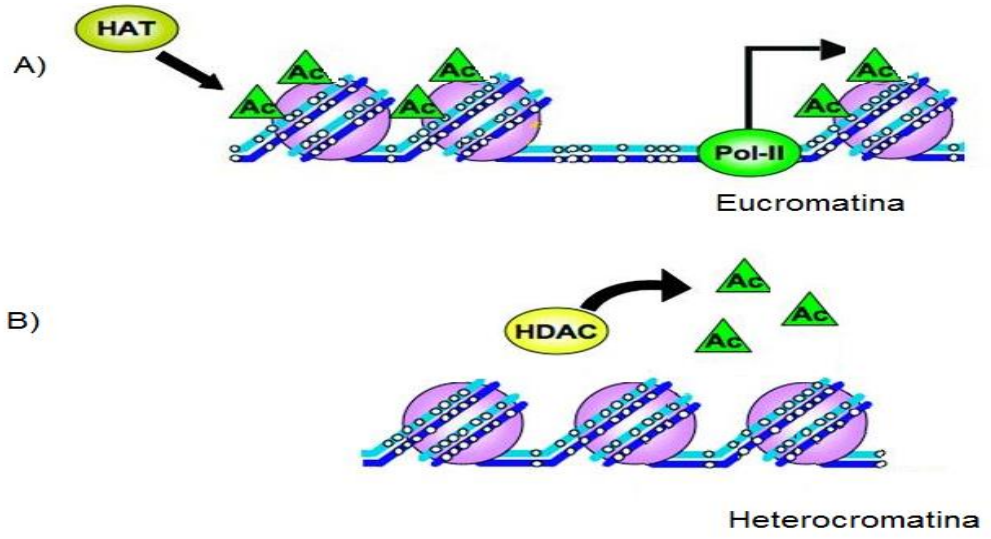


Figura 3. Modificação das histonas por acetilação (Adaptado de Sharma, Kelly e Jones, 2010). A) As acetilases de histonas (HATs) promovem a acetilação da cromatina, liberando o acesso a fatores de transcrição epermitindo a ligação da RNA Polimerase II (Pol-II). B) Com ação inversa, as desacetilases de histonas (HDACs) removem os radicais acetil das histonas, impedindo a ligação dos fatores de transcrição e envolvidas no silenciamento gênico.

A família das HDACs apresenta quatro classes que possuem algumas diferenças em relação à sua homologia no sítio catalítico, sendo então classificadas de I a IV (Figura 4). As classes I, II e IV apresentam como característica a necessidade da presença de um íon zinco para a hidrólise do grupo acetil. A classe III, também conhecida como classe das sirtuínas (SIRT), em razão da sua relação com os genes *SIRT1* e *SIR2*, é dependente da presença do cofator metabólico NAD^+ para promover a desacetilação e liberação do grupo acetil (Gregoretti, Lee e Goodson, 2004; Ekwall, 2005). A classe I possui quatro diferentes de enzimas: as HDACs 1, 2, 3 e 8. A classe II é subdividida em classe IIa, que apresenta apenas um domínio catalítico, sendo composta pelas HDACs 4, 5, 7, 9 e 10; e classe IIb, que possui dois domínios catalíticos, é composta pelas HDACs 6 e 10. A classe III é formada pelas SIRTs1, 2, 3, 4, 5, 6 e 7; e a classe IV possui apenas um representante, a HDAC11 (Grozinger e Schreiber, 2002; Yang e Seto, 2008; Schneider *et al.*, 2011).

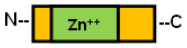
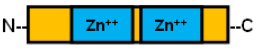
Classe	Isoforma	Subunidade Catalítica	Cofator	Localização Celular	Expressão	Domínio Catalítico
I	HDAC1	RPD3	Zn^{++}	Nuclear	Ubíqua	
	HDAC2			Nuclear		
	HDAC3			Nuclear e Citoplasmática		
	HDAC8			Nuclear		
IIa	HDAC4	HDA1	Zn^{++}	Nuclear e Citoplasmática	Específica	
	HDAC5			Nuclear e Citoplasmática		
	HDAC7			Nuclear e Citoplasmática		
	HDAC9			Nuclear e Citoplasmática		
IIb	HDAC6	HDA2	Zn^{++}	Nuclear e Citoplasmática	Específica	
	HDAC10			Nuclear e Citoplasmática		
III	SIRT1	Sir2	NAD^{+}	Nuclear	Variável	
	SIRT2			Citoplasmática		
	SIRT3			Mitocondrial		
	SIRT4			Mitocondrial		
	SIRT5			Mitocondrial		
	SIRT6			Nuclear		
	SIRT7			Nuclear		
IV	HDAC11	RPD3/HDA1	Zn^{++}	Nuclear	Ubíqua	

Figura 4. Classes de desacetilases de histonas (Adaptado de Schneider *et al.,* 2011; Shirakawa *et al*., 2013).

As HDACs também podem atuar removendo grupos acetil de proteínas não-histonas como fatores de transcrição (E2F, p53, c-Myc, NF-κB), fator indutor de hipóxia 1-alfa (HIF-1α), receptor de estrogênio, receptor andrógeno, MyoD, chaperonas (HSP90), mediadores da sinalização (Stat3, Smad7), proteínas de reparo (Ku70), α-tubulina, β-caderina, retinoblastoma (pRb) e exercendo efeitos diretos em vários processos biológicos (Figura 5) (West e Johnstone, 2014) .

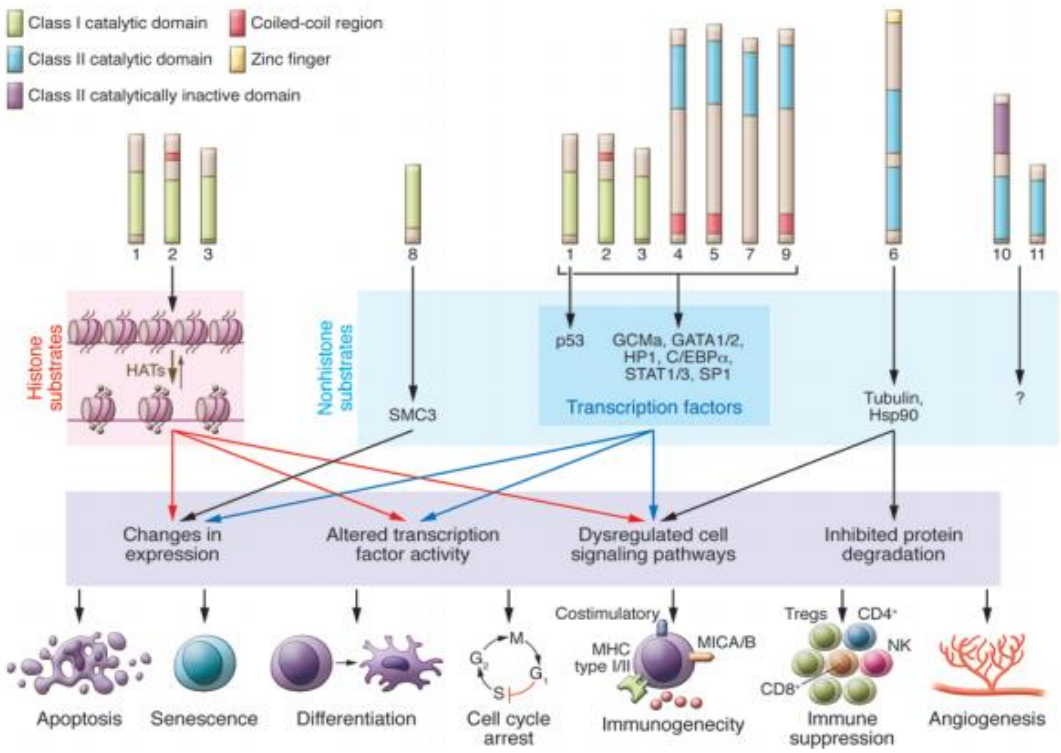


Figura 5. Consequências biológicas da desacetilação de proteínas (West e Johnstone, 2014).

I.4.1.1. Desacetilases de histonas em adenocarcinoma ductal pancreático

O papel das HDACs no desenvolvimento e controle de neoplasias tem se tornado alvo de muitos estudos, uma vez que a ação das HDACs pode resultar no impedimento da transcrição de genes regulatórios ligados a diversas vias, desempenhando um papel que favorece o aparecimento de tumores (Bi e Jiang, 2006; Haberland, Montgomery e Olson, 2009; Schneider *et al.*, 2010).

A classe I das HDACs é fortemente investigada no processo de tumorigênese do ADP, visto que muitas pesquisas mencionam a sua desregulação em amostras tumorais e linhagens celulares analisadas por imunohistoquímica (IHQ), *western blotting* e testes moleculares.

A HDAC1 foi descrita como superexpressa em tumores humanos de próstata, gástrico e de mama (Choi *et al.*, 2001; Kim *et al.*, 2004). Através de uma abordagem utilizando imunohistoquímica, foi visto que a expressão de HDAC1 está elevada em 56% dos casos de ADP e sua superexpressão está relacionada à alta atividade proliferativa, pior grau de diferenciação do tumor,

maior estadiamento TNM e pior sobrevida (n=39) (Miyake *et al.*, 2008; Wang *et al.*, 2009; Bosman *et al.*, 2010). A superexpressão de HDAC1 também foi correlacionada a características clínicas desfavoráveis tais como pior grau de diferenciação do tumor, atividade proliferativa e pior estadiamento TNM, em uma série de 54 amostras de ADP. Zhou e colaboradores (2011) observaram superexpressão de HDAC1 por IHQ em cinco casos de ADP (100% dos casos). O estudo também avaliou a expressão gênica em sete linhagens de ADP e indicou alteração em duas delas (28%) utilizando a técnica de qRT-PCR (Zhou, Liang e Yee, 2011). Lehmann e colaboradores (2009) detectaram uma expressão aumentada de HDAC1 em 32% dos casos em um estudo de IHQ (n=78) (Lehmann *et al.*, 2009).

Em relação à HDAC2, uma elevada expressão em tumores de pâncreas (63%) foi observada e relacionada a um pior grau de diferenciação do tumor (Lehmann *et al.*, 2009). A mesma característica desfavorável também foi observada por Fritsche *et al*., e o grupo observou ainda que a expressão de HDAC2 em ADP foi 2,2 vezes maior do que no tecido normal (Fritsche *et al.*, 2009).

Quanto à HDAC3, um estudo envolvendo 78 pacientes com ADP descreveu uma forte imunorreatividade nuclear para essa proteína em 79% dos casos. Adicionalmente, sua expressão foi correlacionada com a do gene *SKP2* (oncogene com papel em vias de proliferação celular) (Lehmann *et al.*, 2009). Mais recentemente, Jiao e colaboradores identificaram superexpressão de HDAC3 em um restrito número de casos de ADP (100%, n=4) por *Western Blotting.* O grupo também observou superexpressão proteica de HDAC3 em oito linhagens de ADP (100% dos casos) (Jiao *et al.*, 2014).

A classe II foi avaliada juntamente com a classe I em sete linhagens celulares de câncer de pâncreas (quatro derivadas de tumor primário e três de metástases de ADP) por *Western Blotting*. Enquanto as HDACs 4, 7 e 9 estavam superexpressas em seis linhagens (86%), a HDAC6 estava superexpressa em três (42%), e HDAC10 em apenas uma (14%) (Wang *et al.*, 2012).

A investigação proteica comparativa das HDACs em tecidos pancreático tumoral e adjacente não-tumoral está amplamente descrita na literatura, mas poucos estudos avaliaram a expressão gênica objetivando a investigação de

HDACs em tecidos de ADP. Ouaïssi *et al*., (2008) avaliaram o perfil de expressão dos quatro grupos de desacetilases de histonas em 11 pacientes com ADP, e seus resultados indicaram uma superexpressão de SIRT5 (90%) e HDAC7 (81%); para as demais desacetilases o tecido pancreático normal apresentou maior expressão (Ouaïssi *et al.*, 2008). Em 2014, o grupo ampliou a análise para 29 casos de ADP e seus resultados corroboraram a análise anterior em relação à superexpressão de HDAC7 e, adicionalmente, foi observado o mesmo perfil para a HDAC2 (Ouaïssi *et al.*, 2014). Os resultados de todos estes estudos estão resumidos na tabela 1.

Tabela 1. Principais características dos estudos de HDACs em ADP.

	Tipo de Amostra	N	Método	Resultado	Referência
HDAC1	Tecido parafinizado	39	IHQ[1]	Superexpressão em 56% dos casos	Miyake *et al.,* 2008
	Tecido parafinizado	78	IHQ	Superexpressão em 32% dos casos	Lehmenn *et al.,* 2009
	Tecido parafinizado	54	IHQ	Superexpressão	Wang *et al*., 2009
	Linhagens celulares	7 linhagens	qRT-PCR[2]	Superexpressão em duas linhagens (28%)	Zhou, Liang e Yee, 2011
	Tecido parafinizado	5	IHQ	Superexpressão em 100% dos casos	Zhou, Liang e Yee, 2011
	Linhagens celulares	7 linhagens	WB	Superexpressão em três linhagens (42%)	Wang *et al.,*2012
HDAC2	Tecido parafinizado	35	IHQ	Superexpressão	Fritsche *et al.,* 2009
	Tecido parafinizado	78	IHQ	Superexpressão em 63% dos casos	Lehmenn *et al.,* 2009
	Linhagens celulares	7 linhagens	WB[3]	Superexpressão em sete linhagens (100%)	Wang *et al*.,2012
	Tecido fresco	29	qRT-PCR	Superexpressão	Ouaïssi *et al*., 2014
HDAC3	Tecido parafinizado	78	IHQ	Superexpressão em 79% dos casos	Lehmenn *et al*., 2009
	Linhagens celulares	7 linhagens	WB	Superexpressão em seis linhagens (85%)	Wang *et al*.,2012
	Tecido parafinizado	4	WB	Superexpressão (100%)	Jiao *et al.,* 2014
	Linhagens celulares	8 linhagens	WB	Superexpressão em oito linhagens (100%)	Jiao *et al.,* 2014
HDAC7	Tecido fresco	11	qRT-PCR	Superexpressão (81%)	Ouaïssi *et al*., 2008
	Linhagens celulares	7 linhagens	WB	Superexpressão em seis linhagens (85%)	Wang *et al*.,2012
	Tecido fresco	29	qRT-PCR	Superexpressão	Ouaïssi *et al*., 2014

(1) IHQ imunohistoquímica; (2) qRT-PCR - PCR quantitativo por transcrição reversa; (3) *Western Blotting*

I.4.1.2. Uso de inibidores de desacetilases de histonas em tumores pancreáticos

Devido a dificuldades diagnósticas e a agressividade do ADP, poucos pacientes (aproximadamente 20%) são elegíveis para a cirurgia de ressecção do tumor (Lim, Chien e Earle, 2003). Para esse grupo restrito é indicada a quimioterapia adjuvante, que tem início após a cirurgia, e consiste no uso de drogas com a finalidade de eliminar células residuais locais ou circulantes, diminuir a incidência de metástases à distância e melhorar a sobrevida. O tratamento adjuvante padrão usado para o ADP é Gemcitabina, mas o 5-fluorouracil pode ser utilizado como uma alternativa em caso de intolerância. (Cid-Arregui e Juarez, 2015).

Como mencionado anteriormente, a sobrevida em casos irressecáveis é muito baixa, e por esta razão nos últimos anos surgiu uma série de drogas alvo moleculares com o objetivo de reverter esse quadro. Os inibidores de desacetilases de histonas (HDACi) são uma nova classe de fármacos anti-câncer que pertence à categoria mais ampla de "agentes modificadores da cromatina" e que tem sido utilizada para melhorar o prognóstico de doenças neoplásicas como o ADP (Lakshmaiah *et al*., 2014). Os HDACi geralmente possuem três características estruturais comuns: (1) um grupo quelante de zinco; (2) um grupo espaçador, que é geralmente hidrofóbico e (3) um grupo de ligação a enzima que confere especificidade e geralmente possui um estrutura em anel aromático, e por esta razão possui ação apenas sobre as classes I, II e IV, que apresentam o Zn^{+2} como cofator (Finnin *et al.*, 1999; Bolden, Peart e Johnstone, 2006; Marks e Xu, 2009). Os HDACi são classificados em grupos com base na sua estrutura química, incluindo ácidos hidroxâmicos (Tricostatina A, Vorinostat), ácidos carboxílicos (Valproato, Butirato), aminobenzamidas (Entinostat, Mocetinostat), peptideos cíclicos (Apicidina, Romidepsin), cetonas epóxi (Trapoxins) e moléculas híbridas (West e Johnstone, 2014).

Diversos estudos clínicos tem avaliado o uso de HDACi contra o câncer e doenças não-neoplásicas como a artrite reumatóide (Vojinovic *et al.*, 2011). Os HDACi repercutem na acetilação global da célula modificando o padrão de acetilação de proteínas histonas e não-histonas. Sua ação no contexto celular permite o maior acesso a cromatina, parada do ciclo celular, geração de

espécies reativas de oxigênio, indução da apoptose, inibição do reparo do DNA, acetilação de proteínas não-histonas, disrupção da formação de agregados proteicos e efeitos antiangiogênicos (Lakshmaiah *et al.*, 2014). Devido a sua inespecificidade e a capacidade de atuar em diversas vias biológicas, a compreensão do seu mecanismo de ação como droga antitumoral ainda não está bem definida. Contudo, a formulação desses compostos tem como principal objetivo agir especificamente nas histonas, permitindo sua acetilação e maior transcrição gênica conduzindo à inibição da proliferação, diferenciação e apoptose (Johnstone, 2002). As HDACs de classe I (HDAC1, HDAC2, e HDAC3), por exemplo, possuem subunidades catalíticas de vários complexos-chave e são recrutadas por proteínas de fusão oncogênicas, sendo assim as mais afetadas no tratamento com HDACi (West e Johnstone, 2014).

A ação de HDACi está sendo investigada por diferentes grupos (Wang *et al.*, 2012; Chien *et al.*, 2014; Iwahashi *et al.*, 2014; Xu *et al.*, 2014; Bauden, Tassidis e Ansari, 2015; Pan *et al.*, 2015; Zhao e He, 2015). As análises, em sua maioria, estão limitadas a ação da droga em linhagens celulares e os resultados apontam uma grande heterogeneidade na resposta ao tratamento. Até o momento, foram realizados poucos estudos clínicos em pacientes com ADP. O tratamento com ácido valpróico foi investigado em um estudo clínico de fase I, no qual foi avaliada a ação deste HDACi em combinação com S-1, um derivado da fluoropirimidina, em 12 pacientes com ADP irressecável. Destes, 10 pacientes permaneceram com a doença estável (tumor não apresentou diminuição nem aumento na gravidade ou extensão) (Iwahashi *et al.*, 2014; Nci, 2015).

CHR-3996 é um HDACi seletivo para a classe I que apresenta uma capacidade de inibição 3.000 a 7.000 vezes maior contra HDAC1, 2, e 3 em comparação com a HDAC6 (classe II) (Moffat *et al.*, 2010). Em 2012, pacientes com tumor avançado ou metastático que não responderam ao tratamento padrão participaram de um estudo fase I para a utilização de CHR-3996. Nove participantes com câncer de pâncreas foram incluídos no estudo, contudo apenas um paciente com tumor neuroendócrino apresentou uma resposta parcial na redução da metástase hepática. Um ponto interessante deste trabalho foi a avaliação da variação da acetilação induzida pelo HDACi (acetilação da histona H3). A análise foi realizada através de amostras do

sangue periférico (leucócitos) pelo ensaio de ELISA e do folículo capilar por microscopia confocal. O padrão de acetilação do tumor ou das desacetilases de histonas não foi avaliado (Banerji *et al.*, 2012). No mesmo ano, outro estudo fase I analisou a ação conjunta do HDACi Vorinostat e de um inibidor de proteossomo (Marizomib) em pacientes com câncer de pâncreas avançado. O grupo avaliou a ação dessas drogas em quatro pacientes com ADP e observou que as drogas apresentaram um efeito sinérgico, sendo que três pacientes apresentaram doença estável no final do estudo (Millward 2012). Em outro ensaio clínico, dez pacientes com ADP também com doença avançada foram tratados com o HDACi Romidepsin e Gemcitabina. Apesar de seis casos apresentarem doença estável, foi observada importante toxicidade hematológica em vários pacientes (Jones *et al.*, 2012).

I.4.2. MiRNAs

Outra forma de regulação epigenética pode ser conferida através dos miRNAs. Os miRNAs (também chamados de microRNAs ou miRs) consistem em uma classe de pequenos RNAs não-codificantes de fita simples (18 a 25 nucleotídeos de tamanho), envolvidos na regulação pós-transcricional da expressão gênica em todos eucariotos multicelulares (Ambros, 2004; Bartel, 2004).

I.4.2.1. Mecanismo de ação de miRNAs

Predominantemente, os miRNA agem como repressores translacionais ligando-se complementarmente a sequências na região 3' não traduzidas dos seus mRNAs alvo. A regulação da expressão gênica é realizada por meio da degradação e repressão da tradução do mRNA. Como exemplo, miRISC (complexo de silenciamento induzido por RNA) pode competir com eIF4 e impedir sua ligação à extremidade 5 ' do mRNA, evitando assim a adesão da subunidade ribossomal 60S, ou por impedir o recrutamento da subunidade 40S (Mathonnet *et al.*, 2007). Outra possibilidade é a estimulação da desadenilação da cauda do mRNA, que se torna incapaz de circularizar e a tradução é impedida (Humphreys *et al.*, 2005; Beilharz *et al.*, 2009). Uma repressão pós-iniciação da transcrição também é possível, através do desligamento prematuro do ribossomo, diminuindo ou parando o alongamento da proteína, e

aumentando a degradação do polipeptídio nascente (Nilsen, 2007). A evidência mais recente da ação de miRNAs indica que eles são capazes de servir como ativadores e repressores da expressão gênica ao se ligar a sequências reguladoras no DNA. A ligação complementar de miRNAs a regiões promotoras pode inibir a transcrição através de uma impossibilidade física do elongamento do transcrito, ou ainda ser através da ativação da transcrição ao se ligar a regiões que recrutam modificadores de histonas, conferindo a abertura da cromatina (Portnoy *et al.*, 2011; Schwarzenbach *et al.*, 2014).

I.4.3.2. MicroRNAs em câncer

Os genes de miRNAs estão frequentemente localizados em regiões genômicas associadas ao câncer e em pontos de quebra cromossômica, bem como em regiões de frequente perda de heterozigosidade, sugerindo um envolvimento dos miRNAs na iniciação e na progressão de diversos tumores humanos (Rachagani, Kumar e Batra, 2010).

Em um elegante trabalho de 2005, Lu e colaboradores demonstraram, através de experimentos de microarranjo, que os perfis de expressão de miRNAs refletiam a classificação das malignidades humanas, como o grau de diferenciação tumoral e o tipo de neoplasia (Lu *et al.*, 2005). Evidências recentes indicam que os miRNAs podem atuar como supressores de tumor ou oncogenes, sendo estes últimos chamados de "*oncomirs*". Conforme mostra a Figura 6, em tecidos normais o funcionamento adequado da transcrição, processamento e ligação dos miRNAs às sequências complementares resulta em taxas balanceadas de crescimento, proliferação, diferenciação e morte celular. Já em tecidos tumorais, duas alterações podem ser observadas em relação ao nível de expressão de miRNAs, ambas relacionadas com falhas em algum ponto da biogênese desses miRNAs: (a) a redução ou eliminação da expressão de um miRNA que atua como um supressor de tumor, processo este que favoreceria a formação de tumores devido a não-repressão da expressão de oncoproteínas; e (b) o aumento nas taxas de expressão de miRNAs que atuam como oncogenes (Esquela-Kerscher e Slack, 2006).

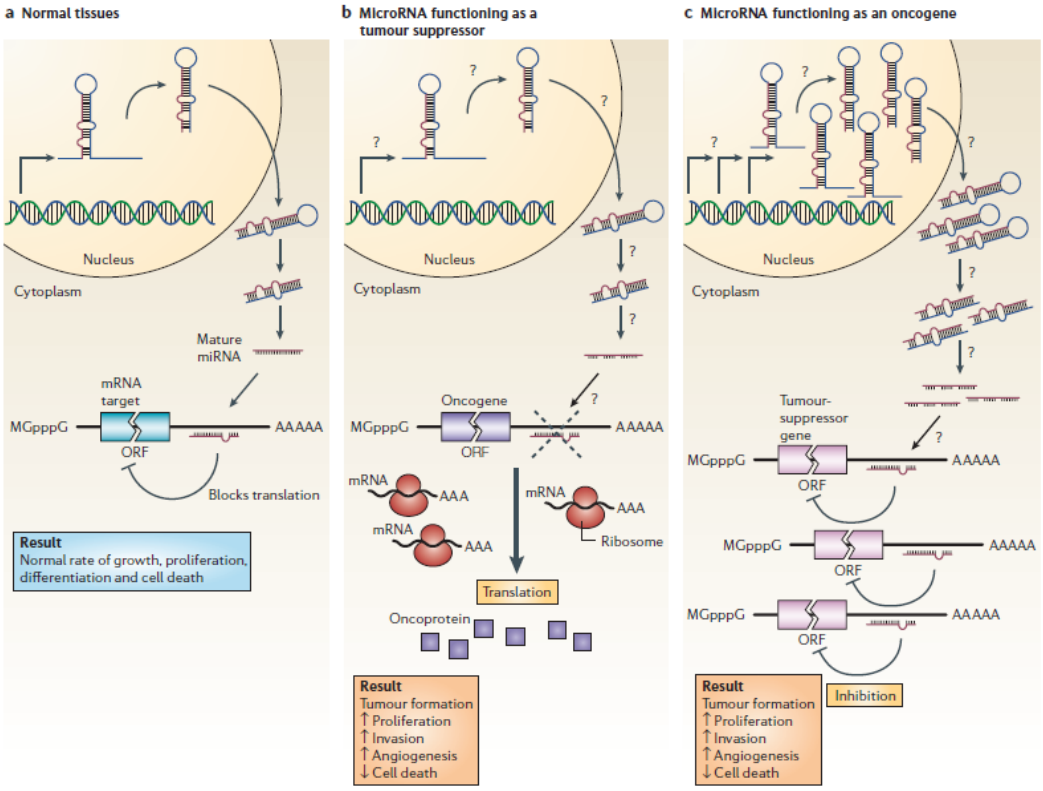


Figura 6. MicroRNAs podem atuar como oncogenes ou supressores de tumor (Esquela-Kerscher e Slack, 2006).

I.4.2.3. MicroRNAs em adenocarcinoma ductal pancreático

A pesquisa de miRNAs com perfil alterado em ADP tornou-se alvo de diversos estudos, a fim de buscar marcas moleculares no processo de carcinogênese, progressão tumoral, resposta ao tratamento e biomarcadores de diagnóstico e prognóstico (Bloomston *et al.*, 2007; Moriyama *et al.*, 2009; Zhang *et al.*, 2009; Hwang *et al.*, 2010; Alemar *et al.*, 2015). Análises envolvendo a identificação de perfis de miRNA alterados em ADP podem contribuir para definir prognóstico, intervenções terapêuticas, diagnosticar e permitir o acompanhamento da progressão da doença (Sempere e Kauppinen, 2009).

Em 2007, foi publicado o primeiro estudo utilizando qRT-PCR *array* que forneceu novas informações sobre a desregulação de miRNAs em ADP. O trabalho analisou a expressão de 201 precursores de miRNA e identificou miRNAs expressos exclusivamente em tumores pancreáticos como o miRNA-221, -376a, e -301; a análise indicou também miRNAs superexpressos em ADP

e em outros tumores (como o miR-155, -21, -221, -424, -301, e -100) (Lee *et al.*, 2007). No mesmo ano, Bloomston e colaboradores demonstraram que a alta expressão de miRNA-196a pode predizer um mal prognóstico de sobrevida (Bloomston *et al.*, 2007). Com o objetivo de buscar um padrão único de expressão dos miRNAs em ADP e alvos moleculares relevantes para novas estratégias terapêuticas e de diagnóstico, Zhang e colaboradores (2009) analisaram um painel de miRNAs em amostras de tecido tumoral de pacientes com ADP, linhagens celulares de ADP, amostras de tecido de pacientes com pancreatite crônica e pacientes saudáveis. O estudo apontou 8 miRNAs que não haviam sido relacionados ao ADP (miRNA-196a, -190, -186, -221, -222, -200b, -15b, e -95) e regulados positivamente na maioria dos tumores e linhagens celulares (Zhang *et al.*, 2009). Papaconstantinou e colaboradores (2012) agruparam os estudos mais impactantes que relataram um perfil aberrante de expressão de miRNAs em ADP (Papaconstantinou *et al.*, 2012). Uma revisão completa, publicada recentemente, sobre o papel dos miRNAs como biomarcadores em ADP é parte integrante desta dissertação, e pode ser vista no Anexo I.

CAPÍTULO II JUSTIFICATIVA

O adenocarcinoma ductal pancreático é uma neoplasia cuja incidência é quase igual à mortalidade. Dados do Instituto Nacional de Câncer (INCa) estimam que no Brasil o câncer de pâncreas representa 2% de todos os tumores e é responsável por 4% do total de mortes por câncer. As maiores taxas de mortalidade são encontradas nas regiões Sul e Sudeste, e Porto Alegre apresenta as maiores taxas (9,4 e 5,3/100.000, para homens e mulheres, respectivamente) entre as capitais. O ADP é um tumor especialmente interessante no contexto das novas pesquisas anti-câncer, visto que até o momento pouquíssimas estratégias terapêuticas tem demonstrado um efeito significativo. Embora a incidência do câncer de pâncreas seja considerada baixa, a sua alta taxa de mortalidade justifica o incentivo a estudos em diagnóstico precoce e, principalmente, novos tratamentos. Recentemente, vários estudos tem abordado o papel de alterações epigenéticas no processo de carcinogênese e na resposta a tratamentos anti-neoplásicos em diversas neoplasias, inclusive no ADP. Tendo em vista que a expressão de desacetilases de histonas tem apresentado resultados variáveis em diferentes estudos, realizados através de diferentes métodos (imunohistoquímica e qRT-PCR), questiona-se o papel biológico dessas proteínas no ADP. A expressão das HDACs pode ser influenciada por diversos fatores, como por exemplo, miRNAs. Desta forma, neste trabalho propõe-se uma análise da expressão das *HDAC1*, *2, 3* e *7* em ADP, através de uma investigação *in silico* e *in vitro* destes genes e de fatores potencialmente capazes de interferir na sua expressão.

Até o momento há poucos estudos clínicos utilizando HDACi em ADP e os mesmos apresentam resultados modestos. De fato, algumas drogas deste tipo já foram aprovadas pelo FDA (*Food and Drug Administration,* órgão regulador de alimentos e drogas dos Estados Unidos), pois apresentaram excelentes resultados no tratamento de linfoma cutâneo de células T e linfoma periférico de células T reincidente ou refratário. Contudo, essas drogas vem apresentando resultados menos significativos nas demais fases da pesquisa clínica quando se trata de tumores sólidos (Mottamal *et al.*, 2015). As razões pelas quais os inibidores de HDAC são mais eficazes em doenças hematológicas malignas do que em tumores sólidos não são bem compreendidas, mas a heterogeneidade tumoral pode ser um fator que dificulta a identificação de bons respondedores a essas terapias. Mais especificamente

em relação ao ADP, os estudos clínicos não realizaram uma análise específica da expressão dessas proteínas no tecido tumoral a fim de verificar qual grupo de pacientes teria um real benefício com a administração dessas drogas. Como já descrito anteriormente há uma certa variabilidade de expressão das HDACs em diferentes estudos. Desta forma, investigar a expressão das HDACs e seus miRNAs reguladores pode ajudar a determinar quais pacientes podem responder às terapias com HDACi.

CAPÍTULO III OBJETIVOS

III.1. Objetivo geral

Avaliar o papel das desacetilases de histonas *HDAC1*, *HDAC2, HDAC3* e *HDAC7* no adenocarcinoma ductal pancreático, através de análises de expressão e dos possíveis mecanismos de silenciamento destes genes.

III.2. Objetivos específicos

1. Avaliar o perfil de expressão dos genes *HDAC1, HDAC2, HDAC3* e *HDAC7* em adenocarcinoma ductal pancreático, em relação ao tecido não-tumoral adjacente.

2. Correlacionar os níveis de expressão dos genes *HDAC1, HDAC2, HDAC3* e *HDAC7* com características clínicas dos pacientes.

3. Comparar o perfil de expressão dos genes *HDAC1, HDAC2, HDAC3* e *HDAC7* encontrados neste estudo com os níveis de expressão descritos nos bancos de dados internacionais.

4. Identificar, através de preditores *in silico,* os miRNAs que possivelmente tem como alvo as *HDAC1, HDAC2, HDAC3* e *HDAC7.*

5. Investigar o papel dos miRNAs preditos na modulação da expressão das *HDAC1, HDAC2, HDAC3* e *HDAC7*, através de análises *in silico* utilizando dados de bancos de dados internacionais.

CAPITULO IV MANUSCRITO

Is There a Role for Histone Deacetylases as Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma?

(Formatado para submissão ao periódico PLOS ONE)

Full title: Is There a Role for Histone Deacetylases as Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma?

Short title: HDAC Expression in Pancreatic Ductal Adenocarcinoma

Cleandra Gregório[1,2*]; Bárbara Alemar[1,2]; Mariana Recamonde Mendoza[3]; Alessandro Bersch Osvaldt [5,6]; Rudinei Luis Correia[2]; Raquel Camara Rivero[4,6]; Patricia Izetti Ribeiro[2], Simone Marcia dos Santos Machado[6]; Patricia Ashton-Prolla[1,2].

[1] Programa de Pós-graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[2] Laboratório de Medicina Genômica, Centro de Pesquisa Experimental - Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Rio Grande do Sul, Brazil.

[3] Laboratório de Pesquisa Cardiovascular, Centro de Pesquisa Experimental - Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

[4] Departamento de Patologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[5] Grupo de Vias Biliares e Pâncreas – Cirurgia do Aparelho Digestivo, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

[6] Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

***Corresponding Author**

e-mail: cleandra.gregorio@gmail.com

Abstract

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal and aggressive disease. The disruption of histone acetylation through histones deacetylases (HDACs) and expression regulation by miRNAs can lead to tumor development. In this study we assessed *HDAC1, HDAC2, HDAC3* and *HDAC7* expression in PDAC and non-tumoral tissue (NT) samples using experimental and databases analysis, correlated their expression levels with clinical and pathological features in patients and performed *in silico* investigation of HDACs regulation by miRNAs.

Materials and Methods: Expression levels of HDACs were measured by qRT-PCR from 25 PDAC and 23 NT. An analysis of differential expression (DE) and correlation of HDACs and miRNAs in PDAC was performed using six GEO microarray datasets. Potential miRNA-HDACs relationships were collected from miRNA interaction databases. A $P<0.05$ was considered statistically significant.

Results: We found reduced expression in PDAC compared with NT for *HDAC1*, *HDAC2* and *HDAC3*, with $P<0.05$. Expression levels of *HDAC7* did not significantly differ between groups. However, fold-changes were very small and not biologically relevant. Only *HDAC2* and *HDAC7* were associated with age at diagnosis and no other associations between HDAC expression and clinical features were identified. DE analysis suggested significant up-regulation of *HDAC1*, *HDAC2* and *HDAC7*, and down-regulation of *HDAC3*, albeit all of them associated with small fold changes. Databases analysis identified 728 miRNAs that could be HDACs regulators. Intersections among the set of miRNAs found in differential expression analysis of GSE41369 and GSE43796 and those retrieved from target prediction identified five miRNAs targeting *HDAC1* (miR-188-5p, miR-539, miR-708, miR-4269 and miR-3616-3p) and three targeting *HDAC2* (miR-4307, miR-944 and miR-195).

Conclusions: HDACs expression is likely not a robust prognostic biomarker in PDAC since differential expression between groups is subtle. Also, this and previous studies indicate no or only very few associations between HDACs expression and clinicopathological features related to prognosis. Finally, miRNAs are probably not exerting a central role in HDAC regulation in PDAC.

Keywords: Pancreatic ductal adenocarcinoma, epigenetic, histone deacetylases, miRNA.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) corresponds to 90% of pancreatic cancer [1] and is the fourth leading cause of cancer-related deaths in the United States [2]. Its high lethality is related to difficulties in early detection, aggressive biology and absence of specific symptom in the initial stages of the disease [3]. Most PDACs occur up to the age of 60 years (80% of cases) [1] and the definitive diagnosis is usually obtained through invasive procedures [4]. Carcinogenesis of the pancreas, as of other malignancies, is a multistep process that involves the accumulation of genetic alterations and likely epigenetic changes. Among these, the remodeling of chromatin is strongly influenced by the phenomena of histone acetylation and deacetylation [5]. Histone deacetylases (HDACs) are proteins that remove the acetil radical of H3 and H4 N-terminal histone tails, repressing chromatin (heterochromatin) and leading to reduced gene expression, while histone acetylases (HATs) generate an open chromatin structure (euchromatin), enabling transcription factors to activate their target genes [6]. Proteins of the HDAC family are classified according to their homology in the catalytic site, as class I to IV enzymes. HDAC class I proteins (1, 2, 3 and 8) are the most studied while class II (4-7, 9, and 10), class III (SIRT1-7) and class IV (11) HDACs are less investigated [7, 8]. Class I, II, and IV HDACs are Zn^{2+} dependent enzymes and class III HDACs require NAD^{+} as a cofactor [9]. Histone deacetylation lead to gene silencing, decreased DNA repair and may facilitate tumor development [6]. Through overexpression, or aberrant recruitment in tumor cells, HDACs have shown to play an important role in the control of proliferation, apoptosis, differentiation, migration and angiogenesis in many cancers [5, 10]. Thus, HDACs are good candidates for diagnostic and prognostic biomarkers and also therapeutic targets. Similarly, histone deacetylase inhibitors (HDACi) are molecular targeted drugs to HDACs that have shown a promising role in the treatment of certain neoplastic diseases [11]. Therefore, it becomes essential recognize patients who have a different expression of HDACs in tumor tissue to identify groups that have a real benefit in the administration of these drugs. Several previous HDAC expression studies in PDAC have shown heterogeneous results, and therefore, the use of HDACi in PDAC is still considered questionable. Among the factors that may influence HDAC expression is the action of miRNAs.

MiRNAs are endogenous noncoding RNAs of 18-25 nucleotides in length with an important regulatory role exerted by mRNAs targeting, for cleavage or translational repression [12]. There is concrete evidence for interaction of HDACs and miRNAs in various ways during carcinogenesis [3]. In the present study, we assessed *HDAC1, HDAC2, HDAC3* and *HDAC7* expression in PDAC and non-tumoral pancreatic tissue samples using experimental and databases analyses, correlated these results with clinical and pathological features and performed an *in silico* investigation of HDAC regulation by miRNAs.

Materials and Methods

Patients

The study included 36 patients diagnosed with PDAC in a public university hospital in Southern Brazil. Inclusion criteria were recent pathology-proven diagnosis and no history of previous or current chemo- or radiotherapic treatment. Definitive tumor staging was established on the basis of surgical findings and additional exams for those patients submitted to surgical intervention. Ultrasound, computed tomography scans and/or magnetic resonance imaging data, as well as histological analyses of biopsy samples were used to determine stage in patients with inoperable tumors. Tumor stage was assigned according to UICC and WHO criteria [14]. Tumoral and adjacent non-tumoral tissue samples were obtained during surgery or diagnostic biopsy procedures from patients, and hematoxylin-eosin slides were prepared for all cases to confirm the diagnosis and assess sample quality. Samples with pancreatitis, necrosis, fibrosis and < 70% cellularity were excluded.

This study was approved by the Institutional Review Board. All participants provided written informed consent before recruitment.

Gene expression assays

Tissue samples were stored at −80°C after surgical excision until mRNA isolation. Total RNA was extracted using the mirVana PARIS kit (Ambion), and resulting mRNAs were eluted into 50 µl of pre-heated RNAse-free water, according to the manufacturer's instructions. The total RNA concentration was assessed by measuring absorbance at 260 nm using a NanoDrop

spectrophotometer (ND-1000, Thermo Scientific). Reverse transcription was performed using 30 ng of total RNA using High Capacity cDNA Archive Kit (Applied BioSystems) according to the manufacturer's recommendations. Quantitative polymerase chain reactions were performed from 2.5 µl of cDNA and run in duplicates using human TaqMan RNA Assay kits (Applied Biosystems) Hs02621185_s1 (*HDAC1*), Hs00231032_m1 (*HDAC2*), Hs00187320_m1 (*HDAC3*) and Hs00248789_m1 (*HDAC7*). Normalization was done using the Hs02758991_g1 assay (*GAPDH*). Quantitative PCR was run on a StepOne real-time PCR system (Applied Biosystems) and the cycle threshold (Ct) values were automatically calculated using the StepOne 2.2.2 Software (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative gene expression [15, 16]. Considering good practice standards, samples with Ct values same or above 36 (equivalent to no identifiable target mRNA) were excluded from the analysis [17]. Therefore, study groups do not always have the same sample size to all HDACs analyzed.

Clinical variables

Age at diagnosis, gender, TNM classification, tumor location and differentiation grade were determined from the pathology and clinical records. Clinical features were categorized as follows: differentiation grade (well differentiated/ moderate vs. moderate to poorly/ poorly differentiated); tumor location (pancreatic head vs. non-head); T stage (T1/T2 vs. T3/T4) and TNM stage (I/II vs. III/IV).

Databases analysis

Large scale expression data

Six pancreatic cancer datasets publicly available at Gene Expression Omnibus (GEO) database were used in this study. These datasets contained large-scale expression profiling of genes (GSE28735, GSE41368, GSE43795 and GSE32676) and miRNAs (GSE41369 and GSE43796) in PDAC vs. normal samples. *HDAC1*, *HDAC2*, *HDAC3*, *HDAC7* and miRNA expression profiles were analyzed for differential expression. We paired the datasets GSE41368 and GSE41369, and datasets GSE43795 and GSE43796, which provide mRNA

and miRNA expression profiling for the same patient samples. Details about the GEO accession numbers, platforms, data pre-processing and sample summary of datasets used are provided in S1 Table.

Differential expression analysis

Differential expression analysis for GEO datasets was performed with the limma R package version 3.24.5, except for dataset GSE41369, which was analyzed with the DESeq R package version 3.2 [18, 19]. Genes and miRNAs were considered differentially expressed between both groups if presenting an adjusted p-value < 0.05. To report expression distribution and fold change for HDACs analysis in cases where more than one probe mapped to the same HDAC, the expression values for each sample were reduced to a single value by taking the average among samples. The individual HDAC probe codes of each of the datasets are depicted in S2 Table.

MicroRNA-mediated regulation analysis

We investigated if HDACs 1, 2, 3 and 7 may be under influence of miRNA regulation. For this purpose, we collected experimentally validated data of miRNA target interactions from miRTarBase and starBase databases to search for known relationships between miRNAs and HDACs. For miRTarBase, we considered only interactions classified as functional, including those with weak evidence. For starBase, we required target sites to be predicted by at least two of the five softwares in addition to the supporting experiments. To complement experimental data, we performed computational target prediction using TargetScan and Diana MicroT. To restrain the large list of predicted targets, we kept only TargetScan predictions with total context score lower than -0.1 and Diana MicroT predictions with a score higher than 0.7.

Expression correlation among predicted miRNA and their target HDACs in PDAC

We performed the correlation analysis for datasets GSE41368 and GSE41369, and datasets GSE43795 and GSE43796, which provide mRNA and miRNA expression profiling for the same patient samples. We computed the Pearson correlation among expression profiles of miRNA-target pairs. Based on

the previous knowledge that miRNA and target mRNA present opposite changes in expression profiles (e.g., an upregulation of a miRNA will cause a downregulation of its targets), we removed from the list those interactions with a correlation higher than -0.25 [20].

Statistical analysis

SPSS version 18.0 (IBM) was used for data handling and statistical analyses. Considering the non-parametric characteristics of our data, the comparison between gene expression results of tumor and non-tumoral tissues was evaluated using the Mann-Whitney test. Spearman's correlation test was used to assess and compare results between genes. Mann-Whitney and Kruskal-Wallis tests were used to determine if there was a significant association between the relative tissue mRNAs levels and clinical variables. Fisher's exact and Continuity correction Chi-square tests were performed to determine if there was a significant association between the relative HDAC levels and a given clinical feature. A statistical test (t-test based on limma, or negative binomial difference test based on DESeq) was applied to identify differentially expressed genes and miRNAs, followed by p-values adjustment using the false discovery rate (FDR) method. Statistical tests were two-sided and P-values of <0.05 were considered statistically significant.

Results

Clinical data were available in all cases and are summarized in Table 1.

Table 1. Correlation between HDAC expression and clinicopathological features (n=25).

Variables	N (%)	*HDAC1* expression *P*-value	*HDAC2* expression *P*-value	*HDAC3* expression *P*-value	*HDAC7* expression *P*-value
Age (years)		*0.242*	*0.034*	*0.361*	*0.030*
≥ 65	11 (44)				
Sex		*0.469*	*1.000*	*0.257*	*0.791*
Male	12 (48)				
Differentiation grade[1]		*0.198*	*NP*	*0.752*	*0.628*
Well/ moderate	16 (64)				
Moderate to poor/ poor	09 (36)				
Location		*0.938*	*NP*	*NP*	*0.745*
Head	21 (84)				
Other (non-head)	04 (16)				
T stage[1]		*0.709*	*NP*	*0.637*	*0.467*
T1/T2	05 (20)				
T3/T4	19 (76)				
NI	01 (4)				
N stage[1]		*0.317*	*0.134*	*0.099*	*0.692*
Nx	04 (16)				
N0	06 (24)				
N1	15 (60)				
M stage[1]		*0.407*	*NP*	*0.752*	*0.914*
M0	22 (88)				
M1	03 (12)				
TNM Stage[1]		0.555	*NP*	*0.862*	*0.715*
I/II	18 (72)				
III/IV	07 (28)				

[1]According to the American Joint Committee on Cancer (AJCC) 6th edition - TNM staging system for pancreatic cancer. NI = not informed, NP= not performed (No statistically compared because all samples belong to the same group).

To assess tissue expression of HDAC, we performed quantitative RT-PCR of the 36 patients with PDAC included in the study, but only 25 PDAC tissues (PDAC) and 21 pancreatic non-tumoral tissues (NT) matched the inclusion criteria of sample quality. Although we used the gold-standard method to access gene expression, and the internal control used (GAPDH) is very established in the literature and always exhibited acceptable Ct values in our analysis, some samples showed very poor, even undetectable *HDAC 2, 3* and *7* expression. As a consequence of excluding samples who presented Ct values ≥36, the analyses were performed with different sample sizes, as shown below.

When expression levels were compared between tumor and non-tumoral samples, a statistically significant difference was observed for *HDAC1* (PDAC=24, NT=18)*, HDAC2* (PDAC=7, NT=5) and *HDAC3* (PDAC=11, NT=9) expression (*P* = 0.042; 0.012 and 0.004, respectively), with reduced expression in tumor tissue (fold-change of -0.50; -0.21; -0.21, respectively). Expression levels of *HDAC7* (PDAC=16, NT=7) did not significantly differ between PDAC and NT tissues (fold-change 1.90, *P* = 0.738). Interestingly, *HDAC7* expression in tumor tissue was the highest among all HDACs (Fig 1). All ΔCt values are shown in S1 Fig.

Next, using Spearman's rank test, we analyzed the correlation between *HDAC1*, *HDAC2, HDAC3* and *HDAC7* gene expression in tumors. Strong positive correlations were identified between *HDAC1* and *HDAC3* (r_s = 0.638; *P* = 0.002) and *HDAC2* and *HDAC3* (r_s = 0.755; *P* = 0.005). All other correlations were not significant (S2 Fig).

We investigated whether *HDAC* expression was associated with selected clinical features in this series, and results of this analysis are summarized in Table 1. As shown in the table, we found statistically significant associations between *HDAC2* and *HDAC7* expression and age (*P* = 0.034 and *P* = 0.030, respectively). There was no significant association of HDAC expression with other clinical features (sex, differentiation grade, tumor location, T, N, M and TNM stages).

Considering the lower *HDAC1*, *HDAC2* and *HDAC3* levels in tumor samples obtained from the experimental analysis, we investigated HDAC expression in the publicly available GEO database. We observed that *HDAC1*, *HDAC2* and *HDAC7* were significantly up-regulated in tumor tissues in relation

to non-tumoral tissues in the three datasets analyzed (GSE28735, GSE41368 and GSE43795). As well as seen in our results, *HDAC3* was significantly downregulated in PDAC (P< 0.001). The GSE32676 dataset did not show significant differences in expression for all *HDAC*s. When we checked the fold changes (log-scale) in datasets with significant results, we observed that all values were low (Fig 2). Next, we compared the fold change of both the experimental and databases analyses. To make this comparison with fold change obtained with the $2^{-\Delta\Delta Ct}$ method, the fold change dataset values are not in log-scale. In all experiments, differential expression between groups was subtle (Fig S3).

Finally, we investigated if some miRNA could be interfering in *HDAC* expression. First, we searched miRTarBase, starBase, TargetScan and Diana MicroT databases for evidence of miRNAs influencing HDAC expression. Based on this analysis, we identified a set of 20 experimentally validated miRNAs and a set of 717 miRNAs predicted to be regulators of *HDAC1, 2, 3* and *7*, with 9 miRNAs common to both sets. A total of 858 interactions were found among the 728 miRNAs retrieved by databases analysis and the 4 *HDACs* of interest (S3 Table). Only one predicted miRNA was a potential regulator of all *HDACs*: miR-3127-3p. Secondly, we analyzed the miRNA expression profiles from GSE41369 and GSE43796. We found 64 miRNAs to be differentially expressed in PDAC based on expression profiling from GSE43796, 11 of which were retrieved as potential regulators of *HDAC1, 2, 3* and *7*. Moreover, we analyzed GSE41369 and found 96 miRNAs to be differentially expressed, 35 of which were predicted to be regulators of *HDAC1, 2, 3* and *7* (Fig 3).

Lastly, we performed a separate analysis using matched datasets (GSE41368:GSE41369 and GSE43795:GSE43796), thus allowing correlation analysis. As a result, we identified 30 out of the 728 predicted miRNA target interactions that showed a negative correlation between gene and miRNA expression profiles between GSE41368 and GSE41369. Of these 30 miRNAs, three showed differential expression in GSE41369 and have target *HDAC1* (miR-188-5p, miR-539 and miR-708, both for *HDAC1*). Analysis of GSE43795:GSE43796 identified 95 miRNAs with negative correlation; five of these were differentially expressed in GSE43796 and target *HDAC1* and *HDAC2* (miR-4269 and miR-3616-3p for *HDAC1* and miR-4307, miR-944 and

miR-195 for *HDAC2*). A detailed analysis of the differential expression of miRNAs in the database is available in Table S4.

Discussion

Currently, there is an intensive search to identify early diagnostic and prognostic biomarkers for pancreatic cancer. Several groups reported that epigenetic alterations could play an important regulatory role in tumor biology. In this context, the expression of modifying chromatin proteins (particularly HDACs) have drawn attention for presenting differential expression in many tumors [21-24]. Although the involvement of HDACs in hematological tumors is well described, there are heterogeneous data about differential expression of these proteins in PDAC. These proteins are target by drugs known as HDAC inhibitors (HDACi) which are already in use in the clinical setting, and for this reason is important to identify eligible patients to this treatment.

Nakagawa and colleagues (2007) were the first to demonstrate HDACs expression in tissue samples of PDAC (n=20). The study was focused on the expression of HDAC1, HDAC2 and HDAC3, and indicated positivity of 85%, 90% and 100% of the samples studied, respectively. Other solid tumors (prostate, stomach, esophagus and colon) were analyzed and compared with normal adjacent tissue (normal tissue was not available in pancreatic cancer cases). The analysis showed that most of the tumors had lower or equal expression levels when compared to the matching normal tissue [24].

Since then, many groups investigated a role for HDACs in pancreatic carcinogenesis, and the effect of these proteins as therapeutic targets. The most expressive results in relation to HDACs in tissue samples from PDAC showed overexpression of HDAC1 in 56% [22]**,** HDAC2 in 63% [25]**,** HDAC3 in 79% [25] and *HDAC7* in 81% of the samples analyzed [26]. In this context, HDAC7 appears to be the most overexpressed HDAC among PDAC samples. However, in this initial study of Ouaissi *et al*., *HDAC7* expression was analyzed by RT-PCR in only a small number of cases (n = 11) [26]. Subsequently, in 2014, the analysis was extended to a larger cohort (n = 29) confirming the previous findings and also indicating that *HDAC2* was overexpressed in tumors [27]. Fritsche and colleagues (2009) also found HDAC2 overexpression by immunohistochemistry, which was more intense in undifferentiated tumors [28]. Using the same methodology, another study found overexpression of HDAC2 in

50% of PDAC cases (n=70) and correlated these results with the proliferative capacity of the tumor and clinical features, without any identifiable association [29]. In addition, HDAC3 was detected in 79% of samples (n=78) in one study [25], providing initial evidence for its investigation in the PDAC pathogenesis. Nonetheless, few posterior studies did not confirm that HDAC3 is overexpressed in PDAC. Although a more recent study tried to demonstrate again that this HDAC is overexpressed in PDAC, they only studied a limited number of cases (n=4) [30]. Lastly, HDAC1 is the most studied HDAC in PDAC. This protein was found overexpressed in 56% of PDAC samples (n=39) and its expression correlated with that of factor 1α inducible by hypoxia (HIF-1 α). In this same study, the authors explored the association of HDAC1 with clinical data, but had no significant results [22]. In contrast, other authors described an association between HDAC1 expression and tumor differentiation, increased proliferative activity and more advanced TNM stages of the disease. They also demonstrated that expression increased progressively during all stages of tumor development (precursor lesions to PDAC) [31]. However, studies with larger sample sizes (78 and 70) showed HDAC1 overexpression in less than 50% of the cases analyzed [25, 29].

In recent years, microarrays have been used in many studies to identify gene signatures associated with PDAC. To date, four groups performed comprehensive comparative analyses of the expression profile of PDAC tumor tissue vs. normal adjacent pancreatic tissue. None of them identified significant increased expression (in fold change) of *HDAC1, 2, 3* and *7* in PDAC compared to normal pancreas. Therefore, additional analyzes (such as RT-PCR or protein expression for these targets) were not performed (GSE28735, GSE41368, GSE43795 and GSE32676) [32-35].

Analysis of HDAC protein and transcript expression in pancreatic cancer cell lines have been published by several groups, nevertheless inferences about the data should be made with caution. Tissue samples have considerable intra- and intertumoral variability and this experimental limitation could be the reason of the heterogeneity in results. Similarly, each cell line pertains to a specified portion of the tumor or its metastasis. Several studies have shown differences in the pattern of clone expression as the disease progresses [36-38].

In addition to HDACs, miRNAs are also involved in the regulation of gene expression and have an established role in all stages of tumorigenesis, such as apoptosis, cell proliferation, angiogenesis, invasion and metastasis of several solid tumors [39]. To date, few studies have explored the analysis of interactions between HDACs and miRNAs. In this work we tried to explore potential associations between these epigenetic mechanisms and their contribution to the development of PDAC. In an *in silico* analysis, miR-3127-3p was identified as a regulator of the four HDACs analyzed. However, we did not identify any *in vivo* or *in vitro* experimental study investigating this interaction, nor its role in any disease. Additionally, our analysis revealed some miRNAs that may play a role in the regulation of specific HDACs in PDAC: we identified five miRNAs targeting HDAC1 (miR-188-5p, miR-539, miR-708, miR-4269, and miR-3616-3p) and three targeting HDAC2 (miR-4307, miR-944 and miR-195). Most of these miRNAs have never been studied in PDAC.

From all miRNAs pointed by *in silico* analyzes, only miR-708 was described in pancreatic samples, in a study that analyzed the expression of 846 human miRNAs in IPMNs (precursor lesion of PDAC) with low and high potential for malignancy. Among the miRNAs expressed differently, miR-708 showed 3.38x higher expression in benign lesions [40]. This same miRNA was also differentially expressed in PanIN, when compared to normal tissue [41].

Other four miRNAs (miR-188-5p, miR-539, miR-944 and miR-195) were also identified in other cancers. Low expression of miR-188-5p was described in hepatocellular carcinoma compared to non-tumoral hepatic tissue. *In silico* and *in vitro* studies indicate that this miRNA has FGF5 (Fibroblast Growth Factor) as one of its targets, a protein with significant role in the regulation of tumor growth and metastasis that is overexpressed in tumors [42]. Next, another study showed that this same miRNA has tumor suppressor functions in prostate cancer [43]. Lower expression of miR-539 was associated with a gastric MALT (Mucosa-Associated Lymphoid Tissue) lymphoma compared normal gastric mucosa [44], and also its low expression was associated with a poor prognostic in patients with osteosarcoma [45]. A subsequent study described this miRNA negatively regulating migration and invasion ability of metastatic cells of the thyroid [46]. Powrózek and colleagues (2015) evaluated the miRNA expression of 90 patients with lung cancer and 85 healthy controls. The results indicated

that miR-944 was overexpressed in individuals with cancer and had diagnostic accuracy for the detection of lung squamous cell carcinoma with potential for resection (AUC = 0.982) [47]. In other study, the miR-195 has presented strong evidence for a tumor suppressor function in some cancers (e.g. breast cancer, colorectal, hepatocellular carcinoma and osteosarcoma). In 2013, a reduced expression in the serum of this same miRNA was identified as a potential diagnosis biomarker for breast cancer cases compared to controls. The sensitivity levels of the miR-195 (73.97%) suggested that it can be used as a detection tool for this type of tumor in early stages [48]. As well as other study, significantly lower expression of this miRNA has also been proposed as a diagnostic biomarker for differentiating patients with suspected osteosarcoma ($P < 0.001$) and its expression correlated to poor prognosis [49]. Bcl-2, a key protein in the apoptosis mechanism, cell migration, invasion and metastasis [50], was described a target of miR-195 in colorectal cancer cell lines [51]. There are no reports reporting the involvement of some miRNAs identified herein, miR-4269, miR-3616-3p and miR-4307, regarding PDAC or other tumor types.

In the present study we evaluated the expression levels of four HDACs which had all been previously described as overexpressed in tumor tissues, including PDAC. Our results showed that only *HDAC7* was overexpressed in PDAC corroborating information already published, however the difference found between tumor and normal tissue was not statistically significant. The analysis of differential expression of the other HDACs was significant and showed higher expression in normal tissue, contrary to previously published studies. Importantly, the difference in expression between normal and tumor tissue was subtle for all HDACs and only *HDAC2* and *HDAC7* were associated with age at diagnosis. No other associations between HDAC expression and clinical features were identified.

The experimental results of this study must be viewed in the context of some limitations: a relatively small sample size, small number of paired samples (PDAC and normal tissue the same patient) and intratumoral heterogeneity. The analysis of public databases indicated a higher expression of HDACs in tumor tissue, except for *HDAC3*. As well as the experimental data, the difference in expression between tissues was subtle, indicating that HDAC expression is

probably not a suitable biomarker in PDAC; as a consequence, PDAC patients are likely not eligible for therapy with deacetylase inhibitors.

Finally, to evaluate miRNAs that could impact HDAC expression, we search in public miRNAs databases (GSE43796 and GSE41369) which had (a) the same sample dataset analysed for HDAC and miRNA expression, and (b) showed differential miRNA expression between tumoral and normal tissues [33, 34]. This analysis indicated eight miRNAs (miR-188-5p, miR-539, miR-708, miR-4269, miR-3616-3p, miR-4307, miR-944 and miR-1307) that can be potentially associated with HDAC1 or HDAC2 deregulation. However, based on the small difference in expression of these HDACs in tumor and normal tissue, it is likely that this silencing mechanism is not playing a central role in tumorigenesis.

We conclude that HDACs expression is likely not a robust prognostic biomarker in PDAC since differential expression between normal and tumor tissue is subtle and because this and previous studies indicate no or only very few associations between HDACs expression and clinicopathological features related to prognosis. Finally, miRNAs are probably not exerting a central role in HDAC regulation either. Additional studies should be conducted in search for diagnostic and prognostic biomarkers for PDAC.

Acknowledgments

This work was supported by CNPq/Rede Genoprot (Grant #559814/2009-7), CAPES and Fundo de Incentivo à Pesquisa - FIPE, Hospital de Clínicas de Porto Alegre (Grant #10-0162 and #11-0510). Patricia Ashton-Prolla and Alessandro Bersch Osvaldt are investigators of CNPq. We thank our colleagues from Laboratório de Biologia Molecular aplicada a Nefrologia and Unidade de Análises Moleculares e Proteínas from Hospital de Clínicas de Porto Alegre for their laboratory assistance.

References

1. Neuzillet C, Sauvanet A, Hammel P. Prognostic factors for resectable pancreatic adenocarcinoma. J Visc Surg. 2011 Sep;148(4):e232-43. PubMed PMID: 21924695.

2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013 Jan;63(1):11-30. PubMed PMID: 23335087.

3. Wray CJ, Ahmad SA, Matthews JB, Lowy AM. Surgery for pancreatic cancer: recent controversies and current practice. Gastroenterology. 2005 May;128(6):1626-41. PubMed PMID: 15887155.

4. Hidalgo M. Pancreatic cancer. N Engl J Med. 2010 Apr;362(17):1605-17. PubMed PMID: 20427809.

5. Schneider G, Krämer OH, Fritsche P, Schüler S, Schmid RM, Saur D. Targeting histone deacetylases in pancreatic ductal adenocarcinoma. J Cell Mol Med. 2010 Jun;14(6A):1255-63. PubMed PMID: 19929947.

6. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov. 2006 Jan;5(1):37-50. PubMed PMID: 16485345.

7. Grozinger CM, Schreiber SL. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol. 2002 Jan;9(1):3-16. PubMed PMID: 11841934.

8. Schneider G, Krämer OH, Schmid RM, Saur D. Acetylation as a transcriptional control mechanism-HDACs and HATs in pancreatic ductal adenocarcinoma. J Gastrointest Cancer. 2011 Jun;42(2):85-92. PubMed PMID: 21271301.

9. Yang XJ, Seto E. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol. 2008 Mar;9(3):206-18. PubMed PMID: 18292778..

10. Hagelkruys A, Sawicka A, Rennmayr M, Seiser C. The biology of HDAC in cancer: the nuclear and epigenetic components. Handb Exp Pharmacol. 2011;206:13-37. PubMed PMID: 21879444.

11. Mottamal M, Zheng S, Huang TL, Wang G. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. Molecules. 2015;20(3):3898-941. PubMed PMID: 25738536.

12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004 Jan;116(2):281-97. PubMed PMID: 14744438.

13. Swierczynski S, Klieser E, Illig R, Alinger-Scharinger B, Kiesslich T, Neureiter D. Histone deacetylation meets miRNA: epigenetics and post-transcriptional regulation in cancer and chronic diseases. Expert Opin Biol Ther. 2015 May;15(5):651-64. PubMed PMID: 25766312.

14. C SLaW. Classification of Malignant Tumors. In: Wittekind C, editor. -6th Edition ed. John Wiley & Sons-2002.

15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8. PubMed PMID: 11846609.

16. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8. PubMed PMID: 18546601.

17. Rossney AS, Herra CM, Brennan GI, Morgan PM, O'Connell B. Evaluation of the Xpert methicillin-resistant Staphylococcus aureus (MRSA) assay using the GeneXpert real-time PCR platform for rapid detection of MRSA from screening specimens. J Clin Microbiol. 2008 Oct;46(10):3285-90. PubMed PMID: 18685003.

18. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr;43(7):e47. PubMed PMID: 25605792.

19. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):R106. PubMed PMID: 20979621.

20. Li J, Zhou C, Su Z, Sang H, Jia E, Si D. Global correlation analysis for microRNA and gene expression profiles in human obesity. Pathol Res Pract. 2015 May;211(5):361-8. PubMed PMID: 25701361.

21. Jin KL, Pak JH, Park JY, Choi WH, Lee JY, Kim JH, et al. Expression profile of histone deacetylases 1, 2 and 3 in ovarian cancer tissues. J Gynecol Oncol. 2008 Sep;19(3):185-90. PubMed PMID: 19471575.

22. Miyake K, Yoshizumi T, Imura S, Sugimoto K, Batmunkh E, Kanemura H, et al. Expression of hypoxia-inducible factor-1alpha, histone deacetylase 1, and metastasis-associated protein 1 in pancreatic carcinoma: correlation with poor

prognosis with possible regulation. Pancreas. 2008 Apr;36(3):e1-9. PubMed PMID: 18362831.

23. Oehme I, Deubzer HE, Wegener D, Pickert D, Linke JP, Hero B, et al. Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin Cancer Res. 2009 Jan;15(1):91-9. PubMed PMID: 19118036.

24. Nakagawa M, Oda Y, Eguchi T, Aishima S, Yao T, Hosoi F, et al. Expression profile of class I histone deacetylases in human cancer tissues. Oncol Rep. 2007 Oct;18(4):769-74. PubMed PMID: 17786334.

25. Lehmann A, Denkert C, Budczies J, Buckendahl AC, Darb-Esfahani S, Noske A, et al. High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo. BMC Cancer. 2009;9:395. PubMed PMID: 19912635.

26. Ouaïssi M, Sielezneff I, Silvestre R, Sastre B, Bernard JP, Lafontaine JS, et al. High histone deacetylase 7 (HDAC7) expression is significantly associated with adenocarcinomas of the pancreas. Ann Surg Oncol. 2008 Aug;15(8):2318-28. PubMed PMID: 18506539.

27. Ouaïssi M, Silvy F, Loncle C, Ferraz da Silva D, Martins Abreu C, Martinez E, et al. Further characterization of HDAC and SIRT gene expression patterns in pancreatic cancer and their relation to disease outcome. PLoS One. 2014;9(9):e108520. PubMed PMID: 25275504.

28. Fritsche P, Seidler B, Schüler S, Schnieke A, Göttlicher M, Schmid RM, et al. HDAC2 mediates therapeutic resistance of pancreatic cancer cells via the BH3-only protein NOXA. Gut. 2009 Oct;58(10):1399-409. PubMed PMID: 19528037.

29. Giaginis C, Damaskos C, Koutsounas I, Zizi-Serbetzoglou A, Tsoukalas N, Patsouris E, et al. Histone deacetylase (HDAC)-1, -2, -4 and -6 expression in human pancreatic adenocarcinoma: associations with clinicopathological parameters, tumor proliferative capacity and patients' survival. BMC Gastroenterol. 2015;15:148. PubMed PMID: 26502922.

30. Jiao F, Hu H, Yuan C, Jin Z, Guo Z, Wang L. Histone deacetylase 3 promotes pancreatic cancer cell proliferation, invasion and increases drug-resistance through histone modification of P27, P53 and Bax. Int J Oncol. 2014 Oct;45(4):1523-30. PubMed PMID: 25070540.

31. Wang W, Gao J, Man XH, Li ZS, Gong YF. Significance of DNA methyltransferase-1 and histone deacetylase-1 in pancreatic cancer. Oncol Rep. 2009 Jun;21(6):1439-47. PubMed PMID: 19424621.

32. Zhang G, Schetter A, He P, Funamizu N, Gaedcke J, Ghadimi BM, et al. DPEP1 inhibits tumor cell invasiveness, enhances chemosensitivity and predicts clinical outcome in pancreatic ductal adenocarcinoma. PLoS One. 2012;7(2):e31507. PubMed PMID: 22363658.

33. Frampton AE, Castellano L, Colombo T, Giovannetti E, Krell J, Jacob J, et al. MicroRNAs cooperatively inhibit a network of tumor suppressor genes to promote pancreatic tumor growth and progression. Gastroenterology. 2014 Jan;146(1):268-77.e18. PubMed PMID: 24120476.

34. Park M, Kim M, Hwang D, Kim WK, Kim SK, Shin J, et al. Characterization of gene expression and activated signaling pathways in solid-pseudopapillary neoplasm of pancreas. Mod Pathol. 2014 Apr;27(4):580-93. PubMed PMID: 24072181.

35. Donahue TR, Tran LM, Hill R, Li Y, Kovochich A, Calvopina JH, et al. Integrative survival-based molecular profiling of human pancreatic cancer. Clin Cancer Res. 2012 Mar;18(5):1352-63. PubMed PMID: 22261810.

36. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta. 2010 Jan;1805(1):105-17. PubMed PMID: 19931353.

37. Zhou W, Liang IC, Yee NS. Histone deacetylase 1 is required for exocrine pancreatic epithelial proliferation in development and cancer. Cancer Biol Ther. 2011 Apr;11(7):659-70. PubMed PMID: 21301206..

38. Wang G, He J, Zhao J, Yun W, Xie C, Taub JW, et al. Class I and class II histone deacetylases are potential therapeutic targets for treating pancreatic cancer. PLoS One. 2012;7(12):e52095. PubMed PMID: 23251689.

39. Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett. 2009 Nov;285(2):116-26. PubMed PMID: 19464788.

40. Lubezky N, Loewenstein S, Ben-Haim M, Brazowski E, Marmor S, Pasmanik-Chor M, et al. MicroRNA expression signatures in intraductal papillary mucinous neoplasm of the pancreas. Surgery. 2013 May;153(5):663-72. PubMed PMID: 23305591.

41. Yu J, Li A, Hong SM, Hruban RH, Goggins M. MicroRNA alterations of pancreatic intraepithelial neoplasias. Clin Cancer Res. 2012 Feb;18(4):981-92. PubMed PMID: 22114139.

42. Fang F, Chang RM, Yu L, Lei X, Xiao S, Yang H, et al. MicroRNA-188-5p suppresses tumor cell proliferation and metastasis by directly targeting FGF5 in hepatocellular carcinoma. J Hepatol. 2015 Oct;63(4):874-85. PubMed PMID: 25998163.

43. Zhang H, Qi S, Zhang T, Wang A, Liu R, Guo J, et al. miR-188-5p inhibits tumour growth and metastasis in prostate cancer by repressing LAPTM4B expression. Oncotarget. 2015 Mar;6(8):6092-104. PubMed PMID: 25714029.

44. Thorns C, Kuba J, Bernard V, Senft A, Szymczak S, Feller AC, et al. Deregulation of a distinct set of microRNAs is associated with transformation of gastritis into MALT lymphoma. Virchows Arch. 2012 Apr;460(4):371-7. PubMed PMID: 22395483.

45. Mirghasemi A, Taheriazam A, Karbasy SH, Torkaman A, Shakeri M, Yahaghi E, et al. Down-regulation of miR-133a and miR-539 are associated with unfavorable prognosis in patients suffering from osteosarcoma. Cancer Cell Int. 2015;15:86. PubMed PMID: 26388701.

46. Gu L, Sun W. MiR-539 inhibits thyroid cancer cell migration and invasion by directly targeting CARMA1. Biochem Biophys Res Commun. 2015 Sep;464(4):1128-33. PubMed PMID: 26206083.

47. Powrózek T, Krawczyk P, Kowalski DM, Winiarczyk K, Olszyna-Serementa M, Milanowski J. Plasma circulating microRNA-944 and microRNA-3662 as potential histologic type-specific early lung cancer biomarkers. Transl Res. 2015 Oct;166(4):315-23. PubMed PMID: 26079400.

48. Zhao FL, Dou YC, Wang XF, Han DC, Lv ZG, Ge SL, et al. Serum microRNA-195 is down-regulated in breast cancer: a potential marker for the diagnosis of breast cancer. Mol Biol Rep. 2014 Sep;41(9):5913-22. PubMed PMID: 25103018.

49. Cai H, Zhao H, Tang J, Wu H. Serum miR-195 is a diagnostic and prognostic marker for osteosarcoma. J Surg Res. 2015 Apr;194(2):505-10. PubMed PMID: 25498513.

50. Um HD. Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. Oncotarget. 2015 Nov. PubMed PMID: 26621844.
51. Yang B, Tan Z, Song Y. Study on the molecular regulatory mechanism of MicroRNA-195 in the invasion and metastasis of colorectal carcinoma. Int J Clin Exp Med. 2015;8(3):3793-800. PubMed PMID: 26064276.

Figures

Fig 1. Relative fold change in expression of four HDACs in pancreatic adenocarcinoma and non-tumoral adjacent tissue. A. *HDAC1*; **B**. *HDAC2*; **C**. *HDAC3*; **D**. *HDAC7*. Each sample was run in duplicate. The upper and lower limits of the boxes and the lines across the boxes indicate the 75th and 25th percentiles and the median, respectively. Error bars indicate values above the 75th and 25th percentiles and below 1.5 interquartile ranges. Outliers (°) were defined as any value point more than 1.5 interquartile ranges below the first quartile or above the third quartile.

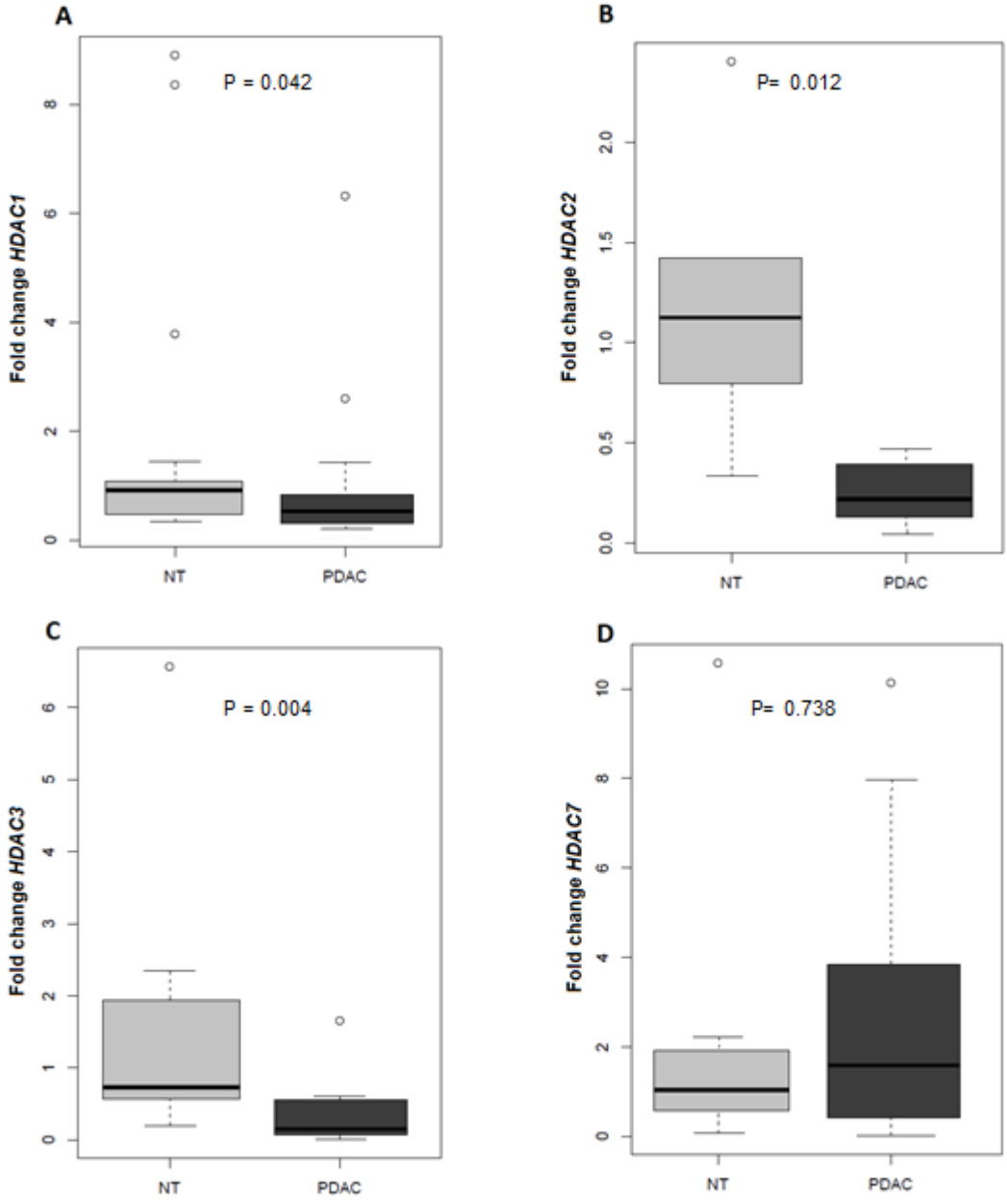


Fig 2. Relative fold change in expression of four HDACs in pancreatic adenocarcinoma and non-tumoral adjacent tissue in the GEO database.

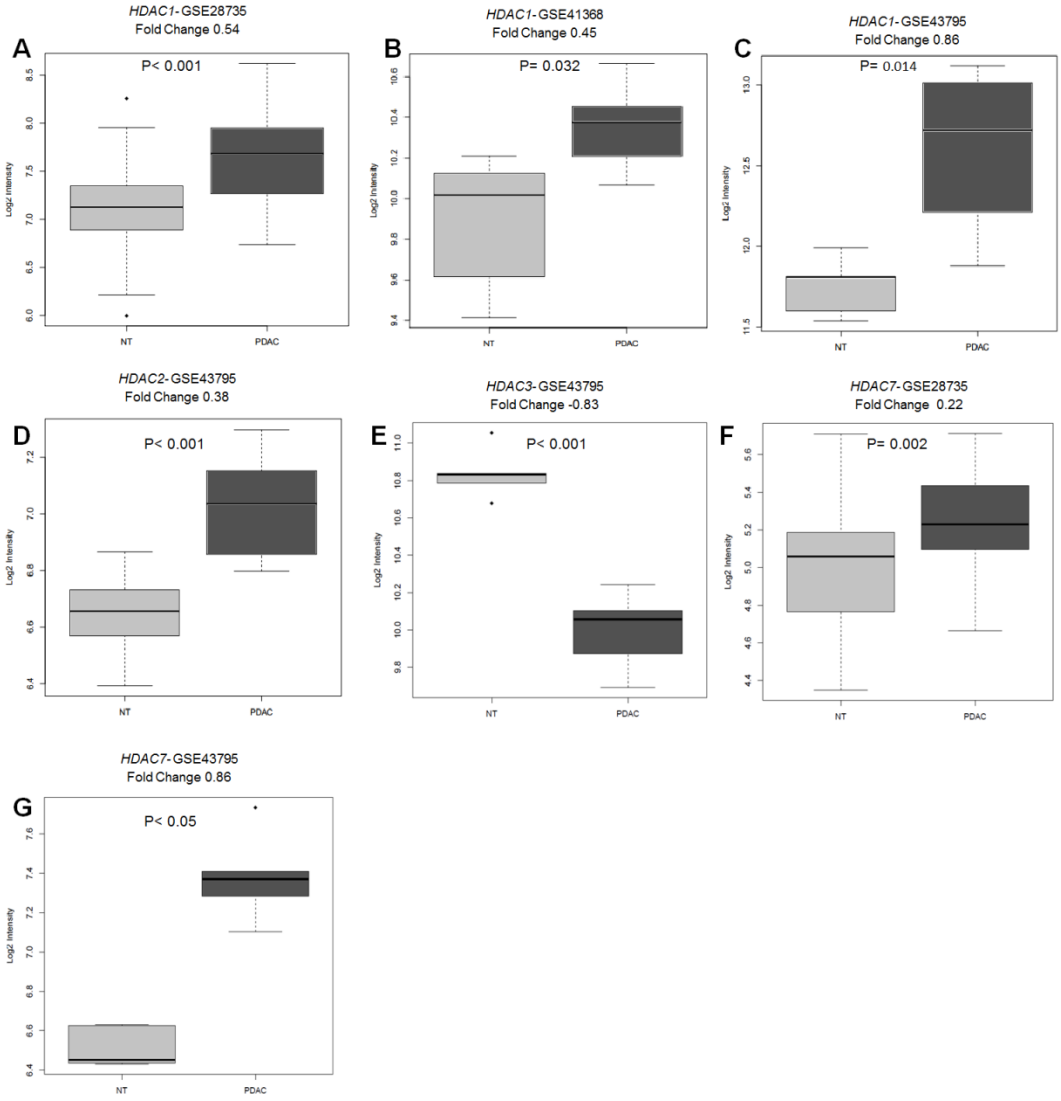
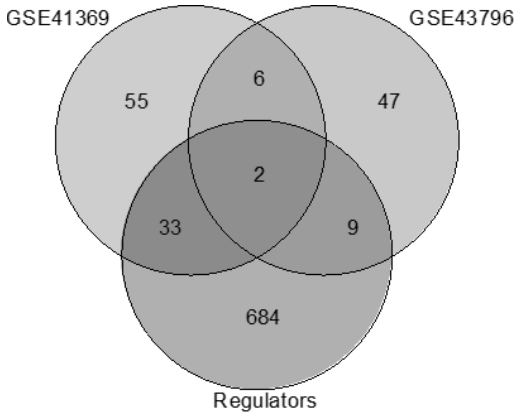
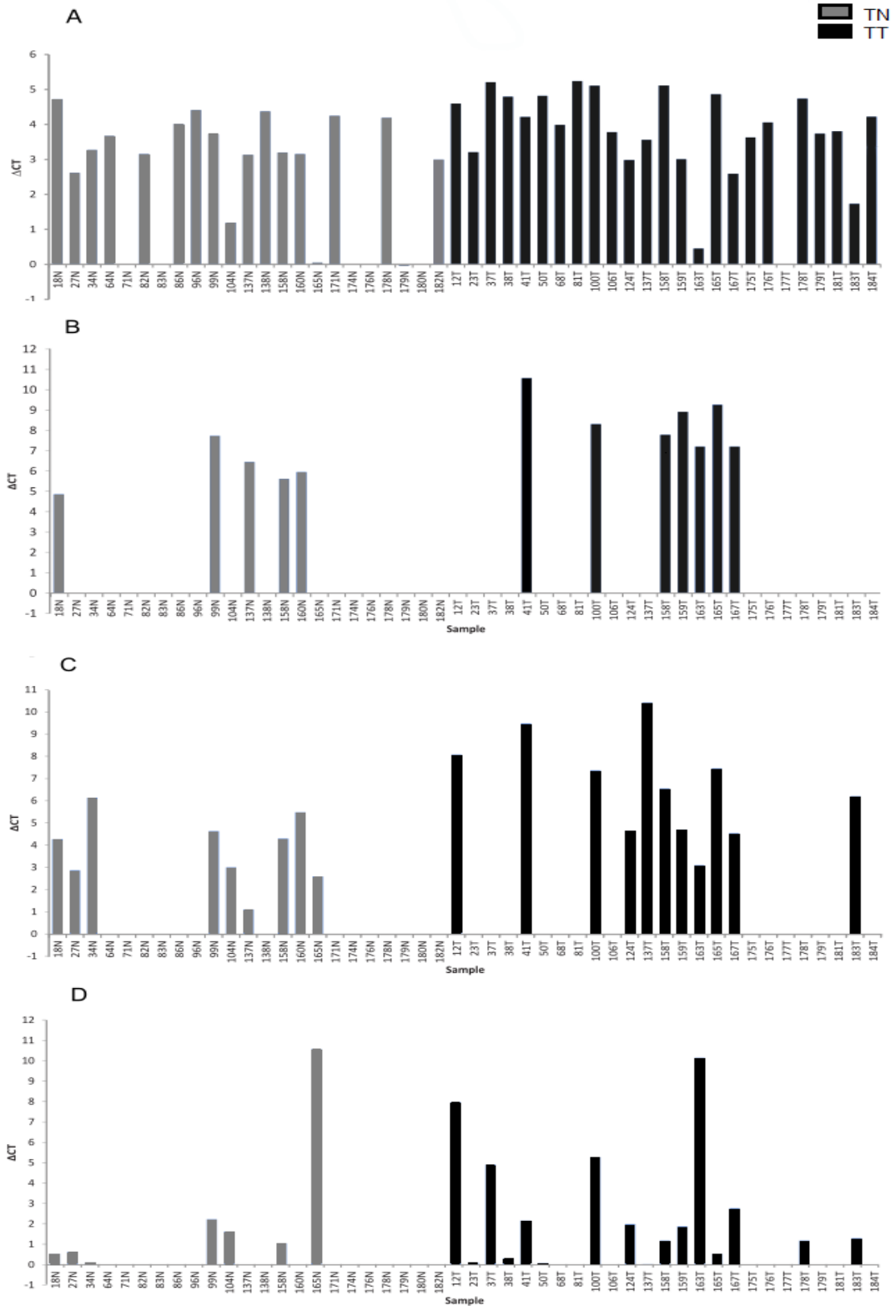


Fig 3. Intersections among the set of miRNAs found in differential expression analysis of GSE41369 and GSE43796 and those retrieved from target prediction.

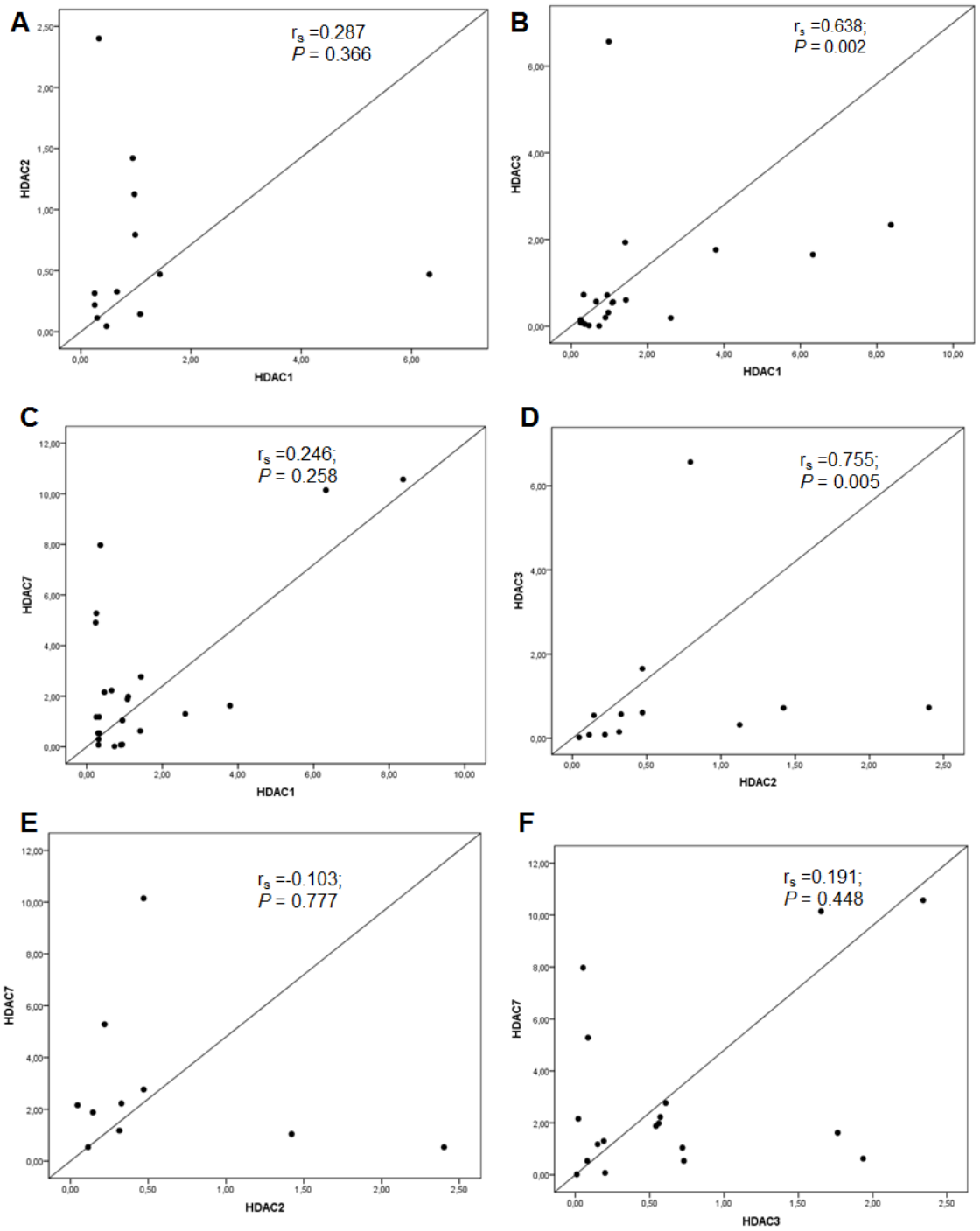


Supporting Information

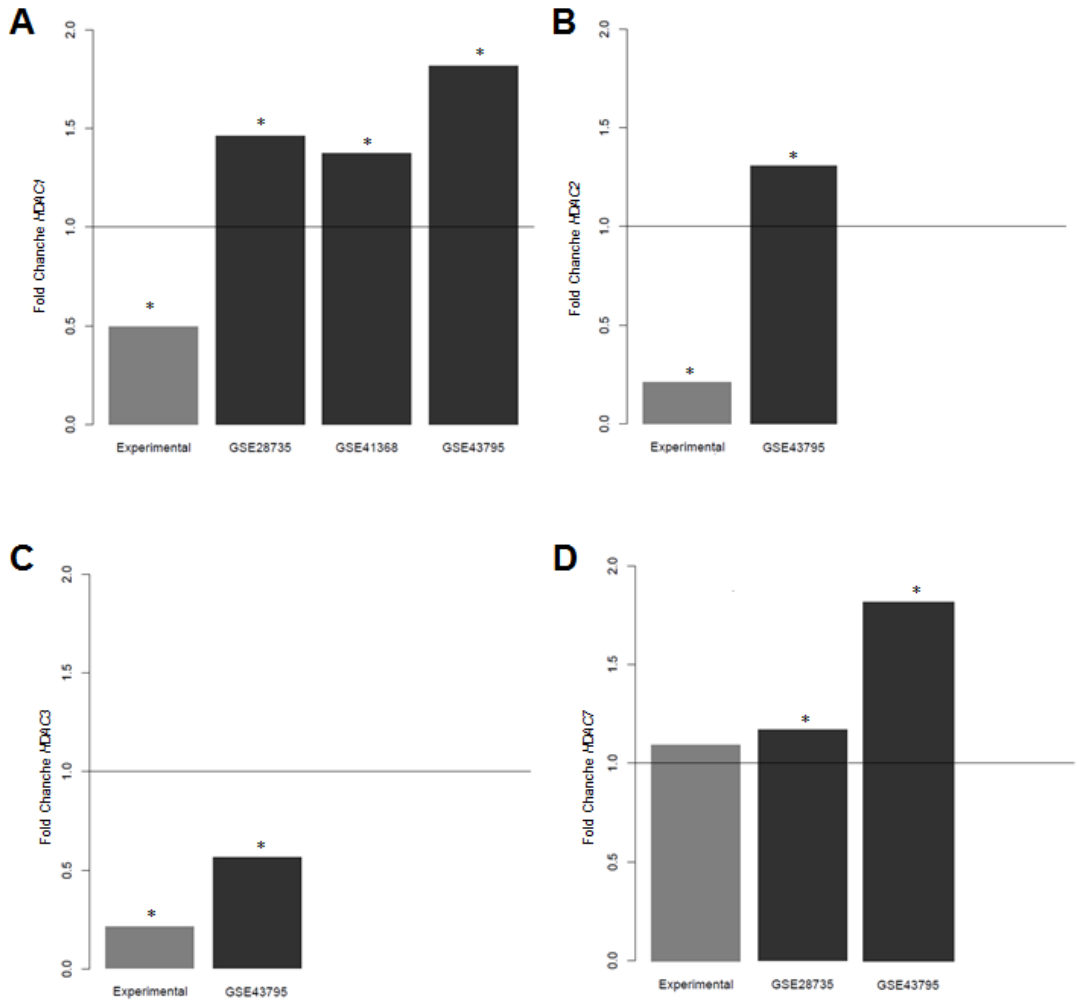
S1 Fig. ΔCt values of HDAC expression assays. A. *HDAC1,* B. *HDAC2,* C. *HDAC3,* D. *HDAC7.* NT= normal pancreas, PDAC= Pancreatic Ductal Adenocarcinoma.



S2 Fig. Spearman' correlation between expression of different HDACs. (A) *HDAC1* vs *HDAC2*; (B) *HDAC1* vs *HDAC3* and (C) *HDAC1* vs *HDAC7*; (D) *HDAC2* vs *HDAC3*; (D) *HDAC2* vs *HDAC7*; (D) *HDAC3* vs *HDAC7*.



S3 Fig. Comparison of expression fold changes of the experimental and databases analyses. *P<0.005.



S1 Table. Gene expression datasets used in this study.

Accession	Data Type	Platform	Dataset Summary
GSE28735	Expression profiling by array	Affymetrix Human Gene 1.0 ST Array [GPL6244]	45 matching pairs of pancreatic ductal adenocarcinoma tumor and adjacent non-tumor tissues. Array data were preprocessed using Robust Multiarray analysis (RMA) and log2 transformed.
GSE32676	Expression profiling by array	Affymetrix Human Genome U133 Plus 2.0 Array [GPL570]	Patient samples of normal pancreas (n=7) and pancreatic ductal adenocarcinoma (PDAC, n=25). Array data were previously normalized with the GCRMA package and log2 transformed.
GSE41368	Expression profiling by array	Affymetrix Human Gene 1.0 ST Array [GPL6244]	Patient samples of normal pancreas (n=6) and pancreatic ductal adenocarcinoma (PDAC; n=6) retrieved during surgery. Array data were preprocessed using Robust Multiarray analysis (RMA) and log2 transformed.
GSE41369	Non-coding RNA profiling by array	NanoString nCounter Human miRNA assay [GPL16142]	Patient samples of normal pancreas (n=9) and pancreatic ductal adenocarcinoma (PDAC; n=9) retrieved during surgery. Array data were quantiled normalized and log2 transformed.
GSE43795	Expression profiling by array	Illumina HumanHT-12 V4.0 expression beadchip [GPL10558]	Samples of solid-pseudopapillary neoplasms (SPN; n=14), pancreatic ductal adenocarcinoma (PDAC; n=6) and neuroendocrine tumors (NET; n=6), and nonneoplastic pancreatic tissue (n=5). Only PDAC and normal samples were used. Array data were quantiled normalized and log2 transformed.
GSE43796	Non-coding RNA profiling by array	Agilente Human miRNA microarray v16 [GPL15159]	Samples of solid-pseudopapillary neoplasms (SPN; n=14), pancreatic ductal adenocarcinoma (PDAC; n=6), neuroendocrine tumors (NET; n=6) and nonneoplastic pancreatic tissue (n=5). Only PDAC and normal samples were used. Array data were quantiled normalized and log2 transformed.

S2 Table. Gene expression datasets used in this study.

Accession	ID	Gene symbol	Adj P.Val	logFC
GSE28735	7899774	HDAC1	1.86E-06	0.548935
	7962659	HDAC7	0.002012156	0.229978
GSE32676	201209_at	HDAC1	0.189087043	0.662202
	242141_at	HDAC2	0.681331531	0.175335
	201833_at	HDAC2	1	0.032035
	216326_s_at	HDAC3	0.741263633	-0.16676
	236326_at	HDAC7	0.995995848	0.000517
	217937_s_at	HDAC7	1	-0,00744
GSE41368	7899774	HDAC1	0.032985365	0.457301
GSE43795	ILMN_1727458	HDAC1	0.014204865	0.861765
	ILMN_3251283	HDAC2	0.012873803	0.387045
	ILMN_1767747	HDAC2	0.144470876	0.326639
	ILMN_1772455	HDAC3	3.47E-07	-0.8345
	ILMN_3266186	HDAC7	3.63E-06	1.300904
	ILMN_3189715	HDAC7	0.013358771	0.424185
	ILMN_1794485	HDAC7	0.25970247	-0.16043

S3 Table. 728 miRNAs retrieved by database analysis with target in the 4 HDACs of interest.

miRNA	Target	Source
hsa-miR-1	HDAC2	Experimental
hsa-miR-149-5p	HDAC2	Experimental
hsa-miR-149-5p	HDAC7	Experimental
hsa-miR-24-3p	HDAC1	Experimental
hsa-miR-30a-5p	HDAC1	Experimental
hsa-miR-449a	HDAC1	Experimental
hsa-miR-615-3p	HDAC1	Experimental
hsa-miR-615-3p	HDAC7	Experimental
hsa-miR-671-5p	HDAC1	Experimental
hsa-miR-92a-3p	HDAC1	Experimental
hsa-miR-140-5p	HDAC7	Experimental
hsa-miR-19a-3p	HDAC7	Experimental
hsa-miR-590-3p	HDAC2	Experimental
hsa-miR-410-3p	HDAC1	Experimental
hsa-miR-1271-5p	HDAC7	Experimental
hsa-miR-143-3p	HDAC7	Experimental
hsa-miR-145-5p	HDAC2	Experimental
hsa-miR-19b-3p	HDAC7	Experimental
hsa-miR-34a-5p	HDAC1	Experimental
hsa-miR-449b-5p	HDAC1	Experimental
hsa-miR-96-5p	HDAC7	Experimental
hsa-miR-539-5p	HDAC1	Experimental
hsa-miR-548ah-3p	HDAC2	Prediction
hsa-miR-548am-3p	HDAC2	Prediction
hsa-miR-548aq-3p	HDAC2	Prediction
hsa-miR-5582-3p	HDAC2	Prediction
hsa-miR-3163	HDAC2	Prediction
hsa-miR-3613-3p	HDAC2	Prediction
hsa-miR-548ae	HDAC2	Prediction
hsa-miR-3662	HDAC2	Prediction
hsa-miR-4786-3p	HDAC2	Prediction
hsa-miR-3148	HDAC2	Prediction
hsa-miR-548f	HDAC2	Prediction
hsa-miR-193a-5p	HDAC2	Prediction
hsa-miR-548aj-3p	HDAC2	Prediction
hsa-miR-548x-3p	HDAC2	Prediction
hsa-miR-455-3p	HDAC2	Prediction
hsa-miR-4662b	HDAC2	Prediction

hsa-miR-548c-3p	HDAC2	Prediction
hsa-miR-5590-3p	HDAC2	Prediction
hsa-miR-3188	HDAC2	Prediction
hsa-miR-548z	HDAC2	Prediction
hsa-miR-548h-3p	HDAC2	Prediction
hsa-miR-548ac	HDAC2	Prediction
hsa-miR-5194	HDAC1	Prediction
hsa-miR-3977	HDAC2	Prediction
hsa-miR-4753-5p	HDAC2	Prediction
hsa-miR-548e	HDAC2	Prediction
hsa-miR-342-3p	HDAC7	Prediction
hsa-miR-4288	HDAC2	Prediction
hsa-miR-3133	HDAC2	Prediction
hsa-miR-5692a	HDAC2	Prediction
hsa-miR-5680	HDAC2	Prediction
hsa-miR-3163	HDAC1	Prediction
hsa-miR-513a-3p	HDAC2	Prediction
hsa-miR-513c-3p	HDAC2	Prediction
hsa-miR-4422	HDAC2	Prediction
hsa-miR-4698	HDAC2	Prediction
hsa-miR-1323	HDAC2	Prediction
hsa-miR-548d-3p	HDAC2	Prediction
hsa-miR-548ar-3p	HDAC2	Prediction
hsa-miR-1304-5p	HDAC2	Prediction
hsa-miR-1276	HDAC2	Prediction
hsa-miR-587	HDAC2	Prediction
hsa-miR-4284	HDAC1	Prediction
hsa-miR-4459	HDAC1	Prediction
hsa-miR-603	HDAC7	Prediction
hsa-miR-877-3p	HDAC2	Prediction
hsa-miR-3913-3p	HDAC2	Prediction
hsa-miR-5692c	HDAC2	Prediction
hsa-miR-5692b	HDAC2	Prediction
hsa-miR-4662a-3p	HDAC2	Prediction
hsa-miR-4778-3p	HDAC2	Prediction
hsa-miR-5009-5p	HDAC2	Prediction
hsa-miR-4426	HDAC2	Prediction
hsa-miR-302f	HDAC2	Prediction
hsa-miR-632	HDAC2	Prediction
hsa-miR-330-5p	HDAC3	Prediction
hsa-miR-195-3p	HDAC2	Prediction
hsa-miR-589-5p	HDAC3	Prediction
hsa-miR-5680	HDAC1	Prediction
hsa-miR-539-5p	HDAC2	Prediction

hsa-miR-889	HDAC2	Prediction
hsa-miR-3658	HDAC2	Prediction
hsa-miR-369-3p	HDAC2	Prediction
hsa-miR-548a-3p	HDAC2	Prediction
hsa-miR-656	HDAC2	Prediction
hsa-miR-4482-3p	HDAC2	Prediction
hsa-miR-16-2-3p	HDAC2	Prediction
hsa-miR-373-5p	HDAC2	Prediction
hsa-miR-548n	HDAC2	Prediction
hsa-miR-4528	HDAC2	Prediction
hsa-miR-4647	HDAC2	Prediction
hsa-miR-3156-5p	HDAC2	Prediction
hsa-miR-495	HDAC2	Prediction
hsa-miR-3688-3p	HDAC2	Prediction
hsa-miR-4668-3p	HDAC2	Prediction
hsa-miR-1261	HDAC3	Prediction
hsa-miR-584-5p	HDAC1	Prediction
hsa-miR-142-5p	HDAC2	Prediction
hsa-miR-4798-3p	HDAC2	Prediction
hsa-miR-524-5p	HDAC1	Prediction
hsa-miR-302d-5p	HDAC2	Prediction
hsa-miR-4694-3p	HDAC3	Prediction
hsa-miR-513a-5p	HDAC2	Prediction
hsa-miR-607	HDAC2	Prediction
hsa-miR-3616-5p	HDAC2	Prediction
hsa-miR-186-5p	HDAC2	Prediction
hsa-miR-616-5p	HDAC2	Prediction
hsa-miR-3121-3p	HDAC2	Prediction
hsa-miR-302b-5p	HDAC2	Prediction
hsa-miR-4768-3p	HDAC1	Prediction
hsa-miR-3922-5p	HDAC2	Prediction
hsa-miR-4725-3p	HDAC1	Prediction
hsa-miR-4252	HDAC2	Prediction
hsa-miR-130a-5p	HDAC7	Prediction
hsa-miR-5688	HDAC2	Prediction
hsa-miR-4420	HDAC2	Prediction
hsa-miR-3662	HDAC1	Prediction
hsa-miR-371b-5p	HDAC2	Prediction
hsa-miR-498	HDAC2	Prediction
hsa-miR-302c-5p	HDAC2	Prediction
hsa-miR-548g-3p	HDAC2	Prediction
hsa-miR-520d-5p	HDAC1	Prediction
hsa-miR-548at-5p	HDAC2	Prediction
hsa-miR-494	HDAC2	Prediction

hsa-miR-3121-3p	HDAC1	Prediction
hsa-miR-4795-3p	HDAC2	Prediction
hsa-miR-548l	HDAC2	Prediction
hsa-miR-3682-5p	HDAC2	Prediction
hsa-miR-548t-5p	HDAC2	Prediction
hsa-miR-548o-3p	HDAC2	Prediction
hsa-miR-543	HDAC2	Prediction
hsa-miR-34a-3p	HDAC2	Prediction
hsa-miR-4269	HDAC1	Prediction
hsa-miR-548c-3p	HDAC1	Prediction
hsa-miR-633	HDAC2	Prediction
hsa-miR-205-3p	HDAC2	Prediction
hsa-miR-466	HDAC2	Prediction
hsa-miR-7-2-3p	HDAC2	Prediction
hsa-miR-7-1-3p	HDAC2	Prediction
hsa-miR-502-5p	HDAC2	Prediction
hsa-miR-1277-5p	HDAC2	Prediction
hsa-miR-5006-5p	HDAC7	Prediction
hsa-miR-548h-5p	HDAC2	Prediction
hsa-miR-676-5p	HDAC2	Prediction
hsa-miR-129-5p	HDAC2	Prediction
hsa-miR-192-3p	HDAC2	Prediction
hsa-miR-548b-5p	HDAC2	Prediction
hsa-miR-499b-3p	HDAC2	Prediction
hsa-miR-500a-5p	HDAC2	Prediction
hsa-miR-548ar-5p	HDAC2	Prediction
hsa-miR-1202	HDAC7	Prediction
hsa-miR-499a-3p	HDAC2	Prediction
hsa-miR-548y	HDAC2	Prediction
hsa-miR-5011-5p	HDAC2	Prediction
hsa-miR-548as-5p	HDAC2	Prediction
hsa-miR-548au-5p	HDAC2	Prediction
hsa-miR-4447	HDAC7	Prediction
hsa-miR-548j	HDAC2	Prediction
hsa-miR-548c-5p	HDAC2	Prediction
hsa-miR-548am-5p	HDAC2	Prediction
hsa-miR-548ap-5p	HDAC2	Prediction
hsa-miR-548o-5p	HDAC2	Prediction
hsa-miR-4732-5p	HDAC7	Prediction
hsa-miR-4753-3p	HDAC2	Prediction
hsa-miR-548ab	HDAC2	Prediction
hsa-miR-548d-5p	HDAC2	Prediction
hsa-miR-545-5p	HDAC2	Prediction
hsa-miR-4472	HDAC7	Prediction

hsa-miR-548a-5p	HDAC2	Prediction
hsa-miR-1236	HDAC2	Prediction
hsa-miR-548i	HDAC2	Prediction
hsa-miR-373-5p	HDAC1	Prediction
hsa-miR-4441	HDAC7	Prediction
hsa-miR-522-3p	HDAC2	Prediction
hsa-miR-665	HDAC7	Prediction
hsa-miR-3671	HDAC2	Prediction
hsa-miR-216a	HDAC2	Prediction
hsa-miR-3180-5p	HDAC7	Prediction
hsa-miR-2467-5p	HDAC2	Prediction
hsa-miR-1305	HDAC2	Prediction
hsa-miR-3189-3p	HDAC3	Prediction
hsa-miR-4496	HDAC1	Prediction
hsa-miR-335-3p	HDAC2	Prediction
hsa-miR-338-5p	HDAC2	Prediction
hsa-miR-548p	HDAC2	Prediction
hsa-miR-3136-5p	HDAC2	Prediction
hsa-miR-548ak	HDAC2	Prediction
hsa-miR-888-5p	HDAC2	Prediction
hsa-miR-1279	HDAC2	Prediction
hsa-miR-3972	HDAC7	Prediction
hsa-miR-3678-3p	HDAC2	Prediction
hsa-miR-1273g-3p	HDAC2	Prediction
hsa-miR-5096	HDAC2	Prediction
hsa-miR-548w	HDAC2	Prediction
hsa-miR-559	HDAC2	Prediction
hsa-miR-5703	HDAC1	Prediction
hsa-miR-3158-5p	HDAC1	Prediction
hsa-miR-4637	HDAC2	Prediction
hsa-miR-4687-3p	HDAC7	Prediction
hsa-miR-148a-5p	HDAC2	Prediction
hsa-miR-374c-5p	HDAC2	Prediction
hsa-miR-362-5p	HDAC2	Prediction
hsa-miR-4495	HDAC2	Prediction
hsa-miR-4773	HDAC3	Prediction
hsa-miR-224-3p	HDAC2	Prediction
hsa-miR-4323	HDAC7	Prediction
hsa-miR-593-5p	HDAC7	Prediction
hsa-miR-96-3p	HDAC2	Prediction
hsa-miR-410	HDAC1	Prediction
hsa-miR-345-5p	HDAC2	Prediction
hsa-miR-3975	HDAC2	Prediction
hsa-miR-4517	HDAC2	Prediction

hsa-miR-3613-3p	HDAC3	Prediction
hsa-miR-371a-5p	HDAC2	Prediction
hsa-miR-5196-5p	HDAC1	Prediction
hsa-miR-381	HDAC2	Prediction
hsa-miR-4263	HDAC2	Prediction
hsa-miR-500b	HDAC2	Prediction
hsa-miR-4282	HDAC2	Prediction
hsa-miR-580	HDAC2	Prediction
hsa-miR-126-5p	HDAC2	Prediction
hsa-miR-5007-5p	HDAC2	Prediction
hsa-miR-5094	HDAC2	Prediction
hsa-miR-300	HDAC2	Prediction
hsa-miR-548aj-5p	HDAC2	Prediction
hsa-miR-548g-5p	HDAC2	Prediction
hsa-miR-548x-5p	HDAC2	Prediction
hsa-miR-548aq-5p	HDAC2	Prediction
hsa-miR-885-5p	HDAC2	Prediction
hsa-miR-4536-5p	HDAC2	Prediction
hsa-miR-522-3p	HDAC1	Prediction
hsa-miR-5193	HDAC7	Prediction
hsa-miR-655	HDAC2	Prediction
hsa-miR-875-3p	HDAC2	Prediction
hsa-miR-616-5p	HDAC1	Prediction
hsa-miR-575	HDAC2	Prediction
hsa-miR-490-3p	HDAC2	Prediction
hsa-miR-181a-2-3p	HDAC2	Prediction
hsa-miR-4700-5p	HDAC1	Prediction
hsa-miR-578	HDAC2	Prediction
hsa-miR-4326	HDAC2	Prediction
hsa-miR-1825	HDAC2	Prediction
hsa-miR-2054	HDAC2	Prediction
hsa-miR-4789-3p	HDAC2	Prediction
hsa-miR-4677-5p	HDAC2	Prediction
hsa-miR-942	HDAC2	Prediction
hsa-miR-4459	HDAC7	Prediction
hsa-miR-4650-5p	HDAC3	Prediction
hsa-miR-1208	HDAC2	Prediction
hsa-miR-371b-5p	HDAC1	Prediction
hsa-miR-3921	HDAC2	Prediction
hsa-miR-4531	HDAC2	Prediction
hsa-miR-4731-3p	HDAC7	Prediction
hsa-miR-3126-5p	HDAC1	Prediction
hsa-miR-548ac	HDAC3	Prediction
hsa-miR-4695-5p	HDAC1	Prediction

hsa-miR-511	HDAC2	Prediction
hsa-miR-3934	HDAC3	Prediction
hsa-miR-4477b	HDAC2	Prediction
hsa-miR-513b	HDAC2	Prediction
hsa-miR-548av-5p	HDAC2	Prediction
hsa-miR-3909	HDAC2	Prediction
hsa-miR-4662a-5p	HDAC2	Prediction
hsa-miR-130a-5p	HDAC2	Prediction
hsa-miR-3200-5p	HDAC1	Prediction
hsa-miR-4271	HDAC1	Prediction
hsa-miR-548an	HDAC2	Prediction
hsa-miR-634	HDAC2	Prediction
hsa-miR-1244	HDAC2	Prediction
hsa-miR-4659b-5p	HDAC2	Prediction
hsa-miR-218-2-3p	HDAC1	Prediction
hsa-miR-3139	HDAC1	Prediction
hsa-miR-4719	HDAC2	Prediction
hsa-miR-548k	HDAC2	Prediction
hsa-miR-4676-5p	HDAC2	Prediction
hsa-miR-182-5p	HDAC7	Prediction
hsa-miR-3679-5p	HDAC1	Prediction
hsa-miR-4305	HDAC2	Prediction
hsa-miR-4739	HDAC2	Prediction
hsa-miR-4434	HDAC1	Prediction
hsa-miR-4484	HDAC2	Prediction
hsa-miR-224-3p	HDAC1	Prediction
hsa-miR-3690	HDAC2	Prediction
hsa-miR-5047	HDAC7	Prediction
hsa-miR-625-5p	HDAC2	Prediction
hsa-miR-4270	HDAC7	Prediction
hsa-miR-515-5p	HDAC2	Prediction
hsa-miR-4638-3p	HDAC2	Prediction
hsa-miR-519e-5p	HDAC2	Prediction
hsa-miR-4789-5p	HDAC2	Prediction
hsa-miR-548av-3p	HDAC2	Prediction
hsa-miR-4685-5p	HDAC7	Prediction
hsa-miR-3673	HDAC2	Prediction
hsa-miR-4724-5p	HDAC2	Prediction
hsa-miR-1304-5p	HDAC7	Prediction
hsa-miR-1205	HDAC2	Prediction
hsa-miR-4683	HDAC2	Prediction
hsa-miR-4755-3p	HDAC7	Prediction
hsa-miR-4775	HDAC2	Prediction
hsa-miR-510	HDAC7	Prediction

hsa-miR-766-5p	HDAC7	Prediction
hsa-miR-3194-3p	HDAC2	Prediction
hsa-miR-3661	HDAC2	Prediction
hsa-miR-5009-3p	HDAC2	Prediction
hsa-miR-4690-3p	HDAC7	Prediction
hsa-miR-4275	HDAC2	Prediction
hsa-miR-4477a	HDAC2	Prediction
hsa-miR-520a-5p	HDAC7	Prediction
hsa-miR-525-5p	HDAC7	Prediction
hsa-miR-1184	HDAC7	Prediction
hsa-miR-410	HDAC2	Prediction
hsa-miR-379-3p	HDAC2	Prediction
hsa-miR-411-3p	HDAC2	Prediction
hsa-miR-488-5p	HDAC7	Prediction
hsa-miR-548z	HDAC3	Prediction
hsa-miR-548h-3p	HDAC3	Prediction
hsa-miR-3194-3p	HDAC7	Prediction
hsa-miR-518c-5p	HDAC7	Prediction
hsa-miR-3170	HDAC7	Prediction
hsa-miR-4252	HDAC7	Prediction
hsa-miR-507	HDAC2	Prediction
hsa-miR-2052	HDAC2	Prediction
hsa-miR-1270	HDAC2	Prediction
hsa-miR-4776-3p	HDAC2	Prediction
hsa-miR-3674	HDAC2	Prediction
hsa-miR-188-5p	HDAC3	Prediction
hsa-miR-3120-3p	HDAC2	Prediction
hsa-miR-4768-3p	HDAC7	Prediction
hsa-miR-33a-3p	HDAC2	Prediction
hsa-miR-362-3p	HDAC7	Prediction
hsa-miR-619	HDAC2	Prediction
hsa-miR-141-3p	HDAC2	Prediction
hsa-miR-4672	HDAC1	Prediction
hsa-miR-4742-3p	HDAC2	Prediction
hsa-miR-516a-3p	HDAC2	Prediction
hsa-miR-340-3p	HDAC2	Prediction
hsa-miR-516b-3p	HDAC2	Prediction
hsa-miR-197-3p	HDAC2	Prediction
hsa-miR-340-5p	HDAC2	Prediction
hsa-miR-4308	HDAC3	Prediction
hsa-miR-200a-3p	HDAC2	Prediction
hsa-miR-1285-3p	HDAC2	Prediction
hsa-miR-589-3p	HDAC3	Prediction
hsa-miR-4801	HDAC2	Prediction

hsa-miR-548at-3p	HDAC2	Prediction
hsa-miR-155-3p	HDAC2	Prediction
hsa-miR-329	HDAC7	Prediction
hsa-miR-4718	HDAC2	Prediction
hsa-miR-4668-3p	HDAC1	Prediction
hsa-miR-551b-5p	HDAC2	Prediction
hsa-miR-346	HDAC2	Prediction
hsa-miR-125b-2-3p	HDAC2	Prediction
hsa-miR-5195-3p	HDAC7	Prediction
hsa-miR-3160-5p	HDAC2	Prediction
hsa-miR-101-5p	HDAC2	Prediction
hsa-miR-103a-2-5p	HDAC2	Prediction
hsa-miR-4524b-5p	HDAC2	Prediction
hsa-miR-23a-3p	HDAC2	Prediction
hsa-miR-377-3p	HDAC7	Prediction
hsa-miR-548ap-3p	HDAC2	Prediction
hsa-miR-2355-5p	HDAC2	Prediction
hsa-miR-342-3p	HDAC2	Prediction
hsa-miR-618	HDAC2	Prediction
hsa-miR-4697-3p	HDAC7	Prediction
hsa-miR-367-3p	HDAC2	Prediction
hsa-miR-4524a-5p	HDAC2	Prediction
hsa-miR-488-3p	HDAC2	Prediction
hsa-miR-1301	HDAC7	Prediction
hsa-miR-23c	HDAC2	Prediction
hsa-miR-363-3p	HDAC2	Prediction
hsa-miR-5187-3p	HDAC2	Prediction
hsa-miR-3127-3p	HDAC2	Prediction
hsa-miR-548aa	HDAC2	Prediction
hsa-miR-5001-3p	HDAC2	Prediction
hsa-miR-548t-3p	HDAC2	Prediction
hsa-miR-3074-5p	HDAC2	Prediction
hsa-miR-4307	HDAC2	Prediction
hsa-miR-23b-3p	HDAC2	Prediction
hsa-miR-32-3p	HDAC2	Prediction
hsa-miR-4314	HDAC2	Prediction
hsa-miR-4439	HDAC2	Prediction
hsa-miR-4692	HDAC1	Prediction
hsa-miR-4769-3p	HDAC2	Prediction
hsa-miR-5010-3p	HDAC2	Prediction
hsa-miR-548as-3p	HDAC2	Prediction
hsa-miR-1264	HDAC2	Prediction
hsa-miR-569	HDAC2	Prediction
hsa-miR-583	HDAC1	Prediction

hsa-miR-548z	HDAC1	Prediction
hsa-miR-758	HDAC2	Prediction
hsa-miR-548h-3p	HDAC1	Prediction
hsa-miR-3926	HDAC2	Prediction
hsa-miR-3925-5p	HDAC2	Prediction
hsa-miR-483-3p	HDAC2	Prediction
hsa-miR-938	HDAC2	Prediction
hsa-miR-3184-3p	HDAC2	Prediction
hsa-miR-5590-5p	HDAC2	Prediction
hsa-miR-664-5p	HDAC2	Prediction
hsa-miR-1321	HDAC7	Prediction
hsa-miR-4709-3p	HDAC7	Prediction
hsa-miR-4747-5p	HDAC1	Prediction
hsa-miR-4802-3p	HDAC2	Prediction
hsa-miR-548b-3p	HDAC2	Prediction
hsa-miR-562	HDAC2	Prediction
hsa-miR-2467-5p	HDAC1	Prediction
hsa-miR-4639-5p	HDAC2	Prediction
hsa-miR-943	HDAC2	Prediction
hsa-miR-144-5p	HDAC1	Prediction
hsa-miR-3915	HDAC7	Prediction
hsa-miR-4761-5p	HDAC2	Prediction
hsa-miR-4480	HDAC2	Prediction
hsa-miR-370	HDAC7	Prediction
hsa-miR-5571-5p	HDAC2	Prediction
hsa-miR-1185-2-3p	HDAC2	Prediction
hsa-miR-3191-5p	HDAC3	Prediction
hsa-miR-1914-3p	HDAC1	Prediction
hsa-miR-3159	HDAC2	Prediction
hsa-miR-620	HDAC2	Prediction
hsa-miR-767-5p	HDAC7	Prediction
hsa-miR-1185-1-3p	HDAC2	Prediction
hsa-miR-4701-5p	HDAC7	Prediction
hsa-miR-4731-3p	HDAC2	Prediction
hsa-miR-577	HDAC2	Prediction
hsa-miR-518c-5p	HDAC3	Prediction
hsa-miR-1910	HDAC2	Prediction
hsa-miR-374b-5p	HDAC2	Prediction
hsa-miR-3165	HDAC2	Prediction
hsa-miR-409-3p	HDAC2	Prediction
hsa-miR-3680-5p	HDAC2	Prediction
hsa-miR-5580-3p	HDAC2	Prediction
hsa-miR-548c-3p	HDAC3	Prediction
hsa-miR-944	HDAC2	Prediction

hsa-miR-2115-3p	HDAC2	Prediction
hsa-miR-27b-3p	HDAC2	Prediction
hsa-miR-548ae	HDAC3	Prediction
hsa-miR-223-3p	HDAC2	Prediction
hsa-miR-548ah-3p	HDAC3	Prediction
hsa-miR-548am-3p	HDAC3	Prediction
hsa-miR-548aq-3p	HDAC3	Prediction
hsa-miR-1295b-5p	HDAC1	Prediction
hsa-miR-4292	HDAC1	Prediction
hsa-miR-4292	HDAC7	Prediction
hsa-miR-654-5p	HDAC7	Prediction
hsa-miR-541	HDAC7	Prediction
hsa-miR-214	HDAC1	Prediction
hsa-miR-3619-5p	HDAC1	Prediction
hsa-miR-326	HDAC3	Prediction
hsa-miR-1302	HDAC3	Prediction
hsa-miR-331-3p	HDAC2	Prediction
hsa-miR-608	HDAC7	Prediction
hsa-miR-516a-5p	HDAC7	Prediction
hsa-miR-761	HDAC1	Prediction
hsa-miR-4651	HDAC7	Prediction
hsa-miR-4298	HDAC3	Prediction
hsa-miR-4433	HDAC1	Prediction
hsa-miR-526b	HDAC2	Prediction
hsa-miR-3649	HDAC7	Prediction
hsa-miR-4723-5p	HDAC7	Prediction
hsa-miR-1910	HDAC3	Prediction
hsa-miR-2110	HDAC1	Prediction
hsa-miR-3150a-3p	HDAC7	Prediction
hsa-miR-3175	HDAC7	Prediction
hsa-miR-3168	HDAC2	Prediction
hsa-miR-4685-5p	HDAC3	Prediction
hsa-miR-646	HDAC2	Prediction
hsa-miR-4777-3p	HDAC2	Prediction
hsa-miR-2861	HDAC2	Prediction
hsa-miR-3162-5p	HDAC3	Prediction
hsa-miR-4638-5p	HDAC7	Prediction
hsa-miR-4264	HDAC3	Prediction
hsa-miR-3925-3p	HDAC7	Prediction
hsa-miR-4293	HDAC1	Prediction
hsa-miR-4690-5p	HDAC7	Prediction
hsa-miR-1266	HDAC7	Prediction
hsa-miR-574-3p	HDAC7	Prediction
hsa-miR-544b	HDAC7	Prediction

hsa-miR-188-5p	HDAC1	Prediction
hsa-miR-500b	HDAC3	Prediction
hsa-miR-1915	HDAC7	Prediction
hsa-miR-1225-3p	HDAC3	Prediction
hsa-miR-4632	HDAC7	Prediction
hsa-miR-4720-5p	HDAC2	Prediction
hsa-miR-4727-5p	HDAC2	Prediction
hsa-miR-4518	HDAC7	Prediction
hsa-miR-485-5p	HDAC7	Prediction
hsa-miR-3686	HDAC2	Prediction
hsa-miR-4690-5p	HDAC1	Prediction
hsa-miR-362-5p	HDAC3	Prediction
hsa-miR-4701-5p	HDAC2	Prediction
hsa-miR-4489	HDAC7	Prediction
hsa-miR-532-3p	HDAC7	Prediction
hsa-miR-3911	HDAC7	Prediction
hsa-miR-4799-3p	HDAC2	Prediction
hsa-miR-4488	HDAC7	Prediction
hsa-miR-4697-5p	HDAC7	Prediction
hsa-miR-4667-5p	HDAC1	Prediction
hsa-miR-4508	HDAC1	Prediction
hsa-miR-4650-3p	HDAC2	Prediction
hsa-miR-588	HDAC2	Prediction
hsa-miR-4524	HDAC2	Prediction
hsa-miR-518a-5p	HDAC2	Prediction
hsa-miR-527	HDAC2	Prediction
hsa-miR-299-3p	HDAC7	Prediction
hsa-miR-4690-3p	HDAC3	Prediction
hsa-miR-4649-3p	HDAC7	Prediction
hsa-miR-100	HDAC3	Prediction
hsa-miR-4681	HDAC1	Prediction
hsa-miR-99a	HDAC3	Prediction
hsa-miR-34a	HDAC1	Prediction
hsa-miR-3667-5p	HDAC7	Prediction
hsa-miR-3919	HDAC1	Prediction
hsa-miR-24	HDAC2	Prediction
hsa-miR-99b	HDAC3	Prediction
hsa-miR-2117	HDAC7	Prediction
hsa-miR-34c-5p	HDAC1	Prediction
hsa-miR-96	HDAC7	Prediction
hsa-miR-1178	HDAC7	Prediction
hsa-miR-3942-3p	HDAC7	Prediction
hsa-miR-3194-5p	HDAC7	Prediction
hsa-miR-3616-3p	HDAC2	Prediction

hsa-miR-3618	HDAC2	Prediction
hsa-miR-4536	HDAC2	Prediction
hsa-miR-449b	HDAC1	Prediction
hsa-miR-374c	HDAC2	Prediction
hsa-miR-1271	HDAC7	Prediction
hsa-miR-4688	HDAC3	Prediction
hsa-miR-589	HDAC3	Prediction
hsa-miR-3127-3p	HDAC1	Prediction
hsa-miR-892b	HDAC2	Prediction
hsa-miR-589	HDAC2	Prediction
hsa-miR-483-3p	HDAC3	Prediction
hsa-miR-4328	HDAC2	Prediction
hsa-miR-548ad	HDAC2	Prediction
hsa-miR-1203	HDAC7	Prediction
hsa-miR-4314	HDAC3	Prediction
hsa-miR-4509	HDAC2	Prediction
hsa-miR-584	HDAC1	Prediction
hsa-miR-3678-3p	HDAC7	Prediction
hsa-miR-3656	HDAC2	Prediction
hsa-miR-3918	HDAC7	Prediction
hsa-miR-4286	HDAC7	Prediction
hsa-miR-129-3p	HDAC2	Prediction
hsa-miR-3184	HDAC1	Prediction
hsa-miR-423-5p	HDAC1	Prediction
hsa-miR-4292	HDAC3	Prediction
hsa-miR-1285	HDAC2	Prediction
hsa-miR-4291	HDAC2	Prediction
hsa-miR-4516	HDAC1	Prediction
hsa-miR-3622a-3p	HDAC2	Prediction
hsa-miR-3622b-3p	HDAC2	Prediction
hsa-miR-1248	HDAC7	Prediction
hsa-miR-3126-3p	HDAC2	Prediction
hsa-miR-545	HDAC2	Prediction
hsa-miR-1915	HDAC3	Prediction
hsa-miR-708	HDAC1	Prediction
hsa-miR-4286	HDAC3	Prediction
hsa-miR-4450	HDAC1	Prediction
hsa-miR-4253	HDAC7	Prediction
hsa-miR-3622b-5p	HDAC7	Prediction
hsa-miR-4666-5p	HDAC2	Prediction
hsa-miR-657	HDAC2	Prediction
hsa-miR-28-5p	HDAC1	Prediction
hsa-miR-4308	HDAC1	Prediction
hsa-miR-4794	HDAC2	Prediction

hsa-miR-589	HDAC7	Prediction
hsa-miR-4483	HDAC1	Prediction
hsa-miR-4642	HDAC2	Prediction
hsa-miR-570	HDAC2	Prediction
hsa-miR-4451	HDAC7	Prediction
hsa-miR-631	HDAC1	Prediction
hsa-miR-3616-3p	HDAC1	Prediction
hsa-miR-1293	HDAC1	Prediction
hsa-miR-4436b-3p	HDAC1	Prediction
hsa-miR-4446-5p	HDAC3	Prediction
hsa-miR-4683	HDAC1	Prediction
hsa-miR-1470	HDAC3	Prediction
hsa-miR-3122	HDAC3	Prediction
hsa-miR-3913-5p	HDAC3	Prediction
hsa-miR-3689a-3p	HDAC2	Prediction
hsa-miR-3689c	HDAC2	Prediction
hsa-miR-371b-3p	HDAC7	Prediction
hsa-miR-711	HDAC7	Prediction
hsa-miR-3127-3p	HDAC3	Prediction
hsa-miR-361-3p	HDAC3	Prediction
hsa-miR-545	HDAC3	Prediction
hsa-miR-103a	HDAC2	Prediction
hsa-miR-107	HDAC2	Prediction
hsa-miR-1304	HDAC1	Prediction
hsa-miR-3135	HDAC7	Prediction
hsa-miR-4514	HDAC1	Prediction
hsa-miR-1912	HDAC1	Prediction
hsa-miR-615-5p	HDAC7	Prediction
hsa-miR-4703-3p	HDAC2	Prediction
hsa-miR-4726-5p	HDAC7	Prediction
hsa-miR-561	HDAC2	Prediction
hsa-miR-570	HDAC3	Prediction
hsa-miR-1972	HDAC3	Prediction
hsa-miR-330-3p	HDAC7	Prediction
hsa-miR-874	HDAC1	Prediction
hsa-miR-3661	HDAC1	Prediction
hsa-miR-3667-5p	HDAC1	Prediction
hsa-miR-193a-3p	HDAC3	Prediction
hsa-miR-193b	HDAC3	Prediction
hsa-miR-3126-5p	HDAC7	Prediction
hsa-miR-4308	HDAC7	Prediction
hsa-miR-3915	HDAC2	Prediction
hsa-miR-4667-3p	HDAC3	Prediction
hsa-miR-22	HDAC1	Prediction

hsa-miR-4640-3p	HDAC2	Prediction
hsa-miR-4772-5p	HDAC7	Prediction
hsa-miR-3927	HDAC3	Prediction
hsa-miR-192	HDAC2	Prediction
hsa-miR-215	HDAC2	Prediction
hsa-miR-325	HDAC2	Prediction
hsa-miR-3918	HDAC3	Prediction
hsa-miR-4640-5p	HDAC7	Prediction
hsa-miR-566	HDAC2	Prediction
hsa-miR-1268	HDAC2	Prediction
hsa-miR-1268b	HDAC2	Prediction
hsa-miR-4659a-5p	HDAC2	Prediction
hsa-miR-940	HDAC7	Prediction
hsa-miR-1911	HDAC2	Prediction
hsa-miR-1254	HDAC7	Prediction
hsa-miR-1280	HDAC7	Prediction
hsa-miR-3665	HDAC7	Prediction
hsa-miR-3937	HDAC2	Prediction
hsa-miR-4644	HDAC3	Prediction
hsa-miR-512-5p	HDAC1	Prediction
hsa-miR-935	HDAC2	Prediction
hsa-miR-920	HDAC7	Prediction
hsa-miR-1254	HDAC2	Prediction
hsa-miR-4460	HDAC7	Prediction
hsa-miR-1295	HDAC2	Prediction
hsa-miR-2682	HDAC1	Prediction
hsa-miR-365	HDAC2	Prediction
hsa-miR-449c	HDAC1	Prediction
hsa-miR-4692	HDAC7	Prediction
hsa-miR-4786-3p	HDAC1	Prediction
hsa-miR-503	HDAC2	Prediction
hsa-miR-3679-3p	HDAC7	Prediction
hsa-miR-631	HDAC2	Prediction
hsa-miR-1225-3p	HDAC7	Prediction
hsa-miR-4667-3p	HDAC7	Prediction
hsa-miR-4691-3p	HDAC1	Prediction
hsa-miR-4704-5p	HDAC2	Prediction
hsa-miR-765	HDAC3	Prediction
hsa-miR-4664-5p	HDAC7	Prediction
hsa-miR-766	HDAC3	Prediction
hsa-miR-4788	HDAC7	Prediction
hsa-miR-3150a-3p	HDAC1	Prediction
hsa-miR-3151	HDAC7	Prediction
hsa-miR-3936	HDAC3	Prediction

hsa-miR-621	HDAC2	Prediction
hsa-miR-642a	HDAC3	Prediction
hsa-miR-1233	HDAC7	Prediction
hsa-miR-1245b-5p	HDAC3	Prediction
hsa-miR-24	HDAC1	Prediction
hsa-miR-2964a-5p	HDAC2	Prediction
hsa-miR-3142	HDAC3	Prediction
hsa-miR-342-5p	HDAC7	Prediction
hsa-miR-362-5p	HDAC1	Prediction
hsa-miR-4300	HDAC7	Prediction
hsa-miR-4686	HDAC2	Prediction
hsa-miR-4755-3p	HDAC2	Prediction
hsa-miR-500b	HDAC1	Prediction
hsa-miR-1224-3p	HDAC7	Prediction
hsa-miR-1260	HDAC7	Prediction
hsa-miR-1260b	HDAC7	Prediction
hsa-miR-28-5p	HDAC3	Prediction
hsa-miR-3116	HDAC2	Prediction
hsa-miR-4514	HDAC7	Prediction
hsa-miR-708	HDAC3	Prediction
hsa-miR-4417	HDAC2	Prediction
hsa-miR-2467-3p	HDAC2	Prediction
hsa-miR-4729	HDAC7	Prediction
hsa-miR-486-5p	HDAC2	Prediction
hsa-miR-1307	HDAC7	Prediction
hsa-miR-3139	HDAC3	Prediction
hsa-miR-323b-3p	HDAC2	Prediction
hsa-miR-4480	HDAC7	Prediction
hsa-miR-1324	HDAC1	Prediction
hsa-miR-4291	HDAC1	Prediction
hsa-miR-4649-3p	HDAC1	Prediction
hsa-miR-3187-5p	HDAC2	Prediction
hsa-miR-33a	HDAC2	Prediction
hsa-miR-33b	HDAC2	Prediction
hsa-miR-4274	HDAC2	Prediction
hsa-miR-4646-5p	HDAC3	Prediction
hsa-miR-363	HDAC2	Prediction
hsa-miR-3663-3p	HDAC7	Prediction
hsa-miR-4265	HDAC2	Prediction
hsa-miR-4322	HDAC2	Prediction
hsa-miR-4667-5p	HDAC7	Prediction
hsa-miR-147	HDAC7	Prediction
hsa-miR-4319	HDAC1	Prediction
hsa-miR-4525	HDAC7	Prediction

hsa-miR-3128	HDAC2	Prediction
hsa-miR-4664-5p	HDAC3	Prediction
hsa-miR-4722-5p	HDAC2	Prediction
hsa-miR-125a-3p	HDAC3	Prediction
hsa-miR-32	HDAC2	Prediction
hsa-miR-367	HDAC2	Prediction
hsa-miR-4419a	HDAC1	Prediction
hsa-miR-4422	HDAC7	Prediction
hsa-miR-939	HDAC7	Prediction
hsa-miR-19a	HDAC7	Prediction
hsa-miR-19b	HDAC7	Prediction
hsa-miR-3199	HDAC3	Prediction
hsa-miR-3664-3p	HDAC7	Prediction
hsa-miR-4266	HDAC1	Prediction
hsa-miR-4515	HDAC3	Prediction
hsa-miR-1178	HDAC2	Prediction
hsa-miR-2053	HDAC2	Prediction
hsa-miR-342-5p	HDAC3	Prediction
hsa-miR-3691-3p	HDAC2	Prediction
hsa-miR-4278	HDAC7	Prediction
hsa-miR-4757-5p	HDAC3	Prediction
hsa-miR-491-5p	HDAC7	Prediction
hsa-miR-25	HDAC2	Prediction
hsa-miR-3192	HDAC3	Prediction
hsa-miR-3943	HDAC7	Prediction
hsa-miR-4510	HDAC1	Prediction
hsa-miR-612	HDAC2	Prediction
hsa-miR-3125	HDAC3	Prediction
hsa-miR-3916	HDAC3	Prediction
hsa-miR-4461	HDAC2	Prediction
hsa-miR-455-3p	HDAC3	Prediction
hsa-miR-4700-5p	HDAC7	Prediction
hsa-miR-670	HDAC3	Prediction
hsa-miR-146b-3p	HDAC1	Prediction
hsa-miR-185	HDAC3	Prediction
hsa-miR-4306	HDAC3	Prediction
hsa-miR-4296	HDAC2	Prediction
hsa-miR-483-5p	HDAC2	Prediction
hsa-miR-489	HDAC2	Prediction
hsa-miR-608	HDAC3	Prediction
hsa-miR-637	HDAC7	Prediction
hsa-miR-92a	HDAC2	Prediction
hsa-miR-125b	HDAC1	Prediction
hsa-miR-4512	HDAC2	Prediction

hsa-miR-3614-5p	HDAC1	Prediction
hsa-miR-539	HDAC1	Prediction
hsa-miR-4691-5p	HDAC7	Prediction
hsa-miR-4716-3p	HDAC7	Prediction
hsa-miR-4792	HDAC7	Prediction
hsa-miR-3529	HDAC2	Prediction
hsa-miR-92b	HDAC2	Prediction
hsa-miR-942	HDAC7	Prediction
hsa-miR-4739	HDAC3	Prediction
hsa-miR-764	HDAC3	Prediction
hsa-miR-1253	HDAC1	Prediction
hsa-miR-146b-5p	HDAC3	Prediction
hsa-miR-4651	HDAC3	Prediction
hsa-miR-4660	HDAC2	Prediction
hsa-miR-1229	HDAC2	Prediction
hsa-miR-204	HDAC1	Prediction
hsa-miR-211	HDAC1	Prediction
hsa-miR-223	HDAC2	Prediction
hsa-miR-3116	HDAC7	Prediction
hsa-miR-4505	HDAC2	Prediction
hsa-miR-671-5p	HDAC3	Prediction
hsa-miR-3173-5p	HDAC1	Prediction
hsa-miR-4793-3p	HDAC2	Prediction
hsa-miR-625	HDAC7	Prediction
hsa-miR-146a	HDAC3	Prediction
hsa-miR-3973	HDAC2	Prediction
hsa-miR-4650-5p	HDAC7	Prediction
hsa-miR-4728-5p	HDAC2	Prediction
hsa-miR-4762-3p	HDAC2	Prediction
hsa-miR-548m	HDAC3	Prediction
hsa-miR-146b-3p	HDAC7	Prediction
hsa-miR-16	HDAC2	Prediction
hsa-miR-195	HDAC2	Prediction
hsa-miR-326	HDAC7	Prediction
hsa-miR-3688-5p	HDAC1	Prediction
hsa-miR-4756-5p	HDAC3	Prediction
hsa-miR-4489	HDAC3	Prediction
hsa-miR-4694-5p	HDAC3	Prediction
hsa-miR-514	HDAC2	Prediction
hsa-miR-514b-3p	HDAC2	Prediction
hsa-miR-581	HDAC2	Prediction
hsa-miR-1229	HDAC7	Prediction
hsa-miR-4305	HDAC7	Prediction
hsa-miR-485-5p	HDAC2	Prediction

hsa-miR-15a	HDAC2	Prediction
hsa-miR-31	HDAC1	Prediction
hsa-miR-330-5p	HDAC7	Prediction
hsa-miR-3677-5p	HDAC7	Prediction
hsa-miR-3925-3p	HDAC3	Prediction
hsa-miR-424	HDAC2	Prediction
hsa-miR-1275	HDAC7	Prediction
hsa-miR-3975	HDAC7	Prediction
hsa-miR-4665-5p	HDAC7	Prediction
hsa-miR-4744	HDAC7	Prediction
hsa-miR-139-5p	HDAC2	Prediction
hsa-miR-23a	HDAC7	Prediction
hsa-miR-23b	HDAC7	Prediction
hsa-miR-23c	HDAC7	Prediction
hsa-miR-3188	HDAC7	Prediction
hsa-miR-3670	HDAC1	Prediction
hsa-miR-4700-3p	HDAC7	Prediction
hsa-miR-587	HDAC7	Prediction
hsa-miR-379	HDAC2	Prediction
hsa-miR-4257	HDAC3	Prediction
hsa-miR-4476	HDAC7	Prediction
hsa-miR-1321	HDAC3	Prediction
hsa-miR-15b	HDAC2	Prediction
hsa-miR-3545-5p	HDAC7	Prediction
hsa-miR-497	HDAC2	Prediction
hsa-miR-125a-5p	HDAC1	Prediction
hsa-miR-4288	HDAC3	Prediction
hsa-miR-4635	HDAC1	Prediction
hsa-miR-4639-3p	HDAC2	Prediction
hsa-miR-4792	HDAC2	Prediction
hsa-miR-133a	HDAC2	Prediction
hsa-miR-133b	HDAC2	Prediction
hsa-miR-3155	HDAC7	Prediction
hsa-miR-3155b	HDAC7	Prediction
hsa-miR-3690	HDAC7	Prediction
hsa-miR-4506	HDAC1	Prediction
hsa-miR-2110	HDAC7	Prediction
hsa-miR-520g	HDAC2	Prediction
hsa-miR-520h	HDAC2	Prediction
hsa-miR-1231	HDAC3	Prediction
hsa-miR-1253	HDAC2	Prediction
hsa-miR-155	HDAC2	Prediction
hsa-miR-4451	HDAC2	Prediction
hsa-miR-4653-5p	HDAC2	Prediction

hsa-miR-4672	HDAC7	Prediction
hsa-miR-377	HDAC7	Prediction
hsa-miR-3922-3p	HDAC3	Prediction
hsa-miR-4506	HDAC2	Prediction
hsa-miR-2115	HDAC7	Prediction
hsa-miR-3127-3p	HDAC7	Prediction
hsa-miR-4330	HDAC2	Prediction
hsa-miR-4481	HDAC7	Prediction
hsa-miR-4523	HDAC2	Prediction
hsa-miR-934	HDAC3	Prediction
hsa-miR-2467-5p	HDAC7	Prediction
hsa-miR-4536	HDAC7	Prediction
hsa-miR-4649-3p	HDAC2	Prediction
hsa-miR-4661-5p	HDAC2	Prediction
hsa-miR-4761-3p	HDAC7	Prediction
hsa-miR-608	HDAC2	Prediction
hsa-miR-632	HDAC3	Prediction
hsa-miR-3618	HDAC3	Prediction
hsa-miR-3928	HDAC2	Prediction
hsa-miR-4434	HDAC2	Prediction
hsa-miR-4516	HDAC2	Prediction
hsa-miR-548ah	HDAC3	Prediction
hsa-miR-628-5p	HDAC2	Prediction
hsa-miR-1272	HDAC2	Prediction
hsa-miR-4642	HDAC1	Prediction
hsa-miR-502-5p	HDAC3	Prediction
hsa-miR-654-3p	HDAC7	Prediction
hsa-miR-759	HDAC2	Prediction

Table S4. **MiRNAs identified in databases (a) showing differential expression between PDAC and normal pancreatic tissue and (b) having HDACs as targets.** (P<0.05).

miRNA	Target	Interaction Dataset	Log2 Fold Change of miRNA in dataset
miR-188-5p	HDAC1	GSE41368 and GSE41369	-2,03
miR-708	HDAC1	GSE41368 and GSE41369	-2,74
miR-539	HDAC1	GSE41368 and GSE41369	-1,50
miR-4269	HDAC1	GSE43795 and GSE43796	-1,41
miR-3616-3p	HDAC1	GSE43795 and GSE43796	-0,48
miR-4307	HDAC2	GSE43795 and GSE43796	-0,15
miR-944	HDAC2	GSE43795 and GSE43796	-0,13
miR-195	HDAC2	GSE43795 and GSE43796	-1,30

CAPÍTULO V DISCUSSÃO

A discussão específica referente aos resultados obtidos no presente trabalho encontra-se no manuscrito apresentado no Capítulo IV. Neste capítulo serão mencionados aspectos mais gerais referentes ao tema retomando questões não discutidas anteriormente. No entanto, alguma sobreposição de conteúdo é inevitável.

O câncer de pâncreas é um dos tumores com pior prognóstico. A dificuldade no rastreamento somado a sintomatologia tardia, rápida progressão e a baixa eficácia dos tratamentos em casos avançados são os principais fatores que levam esse tipo de tumor a possuir estatísticas tão desfavoráveis. Estudos recentes indicam que esta neoplasia será a segunda causa de morte por câncer em 2030 nos EUA (Rahib *et al.*, 2014).

Atualmente, há uma busca frequente na identificação de biomarcadores de diagnóstico precoce e prognóstico para o câncer de pâncreas. Os casos com sintomas clínicos e evidência radiológica forte de tumor podem ser avaliados através de uma biópsia tecidual por endoscopia, uma abordagem com alta especificidade. Em contrapartida, nos pacientes em que a suspeita clínica e radiológica é baixa, não há exames não invasivos suficientemente sensíveis e específicos que conduzam a uma clara definição do diagnóstico. Apesar do panorama de biomarcadores prognósticos e diagnósticos estar evoluindo constantemente com identificação de moléculas detectáveis na saliva, soro, plasma, suco pancreático, urina e fezes, até o momento não há um biomarcador validado para o ADP (Kenner *et al.*, 2015). O único marcador utilizado é o CA19-9 (antígeno carboidrato 19-9), contudo ele apresenta baixa sensibilidade (70-80%) e especificidade (68-91%). Este biomarcador está presente na superfície celular de tumores gastrointestinais, incluindo câncer de esôfago, estômago, vias biliares e pâncreas (Kannagi, 2007).

Dadas estas circunstâncias, visando a identificação de marcadores que possam auxiliar na definição do uso de terapias alvo-moleculares e, em última análise, favorecer o prognóstico de pacientes com doença avançada, diversos grupos observaram que alterações epigenéticas poderiam ter um papel regulador importante na biologia tumoral. Neste contexto, a expressão de proteínas modificadoras da cromatina (em especial as HDACs) tem chamado atenção por apresentar expressão diferenciada em diversos tumores (Nakagawa *et al.*, 2007; Jin *et al.*, 2008; Miyake *et al.*, 2008; Oehme *et al.*,

2009). O envolvimento das HDACs em neoplasias hematológicas já está bem descrito, e essas proteínas são o alvo de drogas já utilizadas na clínica (HDACi). No entanto, a expressão aberrante dessas proteínas no ADP apresenta dados heterogêneos.

O primeiro estudo que avaliou a expressão das HDACs utilizando amostras de tecido e linhagens celulares de ADP foi publicado em 2007 (Nakagawa *et al.*, 2007). Os resultados demonstraram superexpressão de algumas proteínas no tumor e sugeriram que poderiam ser promissores alvos terapêuticos. Nakagawa e colaboradores investigaram a expressão de HDAC1, HDAC2 e HADAC3 em 20 amostras de ADP e presença dessas proteínas em 85%, 90% e 100% das amostras, respectivamente. Um ponto interessante deste trabalho foi uma análise da expressão das HDACs em outros tumores (próstata, estômago, esôfago e cólon) e seus respectivos tecidos normais (tecidos normais de pâncreas não estavam disponíveis). A análise indicou que na maior parte das análises comparativas realizadas com tumores sólidos e seus tecidos normais correspondentes, os tumores apresentavam expressão menor ou igual ao tecido normal (Nakagawa *et al.*, 2007).

Desde então, muitos grupos focaram seus estudos na busca por uma relação entre HDACs e carcinogênese pancreática, bem como na identificação de um papel destas como alvo terapêutico. Os resultados mais expressivos em relação às HDACs em amostras teciduais de ADP indicaram superexpressão de HDAC1 em 56% (Miyake *et al.*, 2008)**,** HDAC2 em 63% (Lehmann *et al.*, 2009)**,** HDAC3 em 79% (Lehmann *et al.*, 2009) e *HDAC7* em 81% (Ouaïssi *et al.*, 2008) das amostras analisadas. Neste contexto, a *HDAC7* parece estar mais desregulada em ADP. Contudo, no estudo inicial publicado por Ouaïssi e colaboradores, a expressão gênica da *HDAC7* foi analisada por RT-PCR em um número pequeno de casos (N=11) (Ouaïssi *et al.*, 2008). Posteriormente, em 2014, a análise foi estendida para uma coorte maior (N= 29) que corroborou a análise anterior e ainda indicou que havia também superexpressão de *HDAC2* nos tumores (Ouaïssi *et al.*, 2014). Fritsche *et al., (*2009) também encontraram uma superexpressão de HDAC2 por imunohistoquímica e sua expressão foi mais acentuada em tumores indiferenciados (Fritsche *et al.*, 2009)**.** Usando a mesma metodologia, outro estudo encontrou superexpressão de HDAC2 em 50% dos ADP (N=70) e

correlacionou esse resultado com a capacidade proliferativa do tumor e características clínicas, entretanto não foram identificadas associações (Giaginis *et al.*, 2015)**.** Adicionalmente, a HDAC3 foi detectada em 79% das amostras (N=78) (Lehmann *et al.*, 2009), fornecendo evidências para a sua investigação mais aprofundada na patogênese do ADP. Contudo, poucos trabalhos posteriores encontraram superexpressão de HDAC3 em amostras de ADP. Apesar de o estudo mais recente ter demonstrado superexpressão da HDAC3 em ADP, a análise era composta por um número limitado de casos (n=4) (Jiao *et al.*, 2014). Por fim, a HDAC mais estudada em ADP, HDAC1, foi encontrada superexpressa em 56% dos casos (Miyake *et al.*, 2008). Esta HDAC foi correlacionada com o fator 1 α induzível por hipóxia (HIF-1 α) um fator de transcrição que ativa genes envolvidos em aspectos cruciais da biologia tumoral, como a angiogênese, metabolismo da glicose e invasão (Semenza, 2003). Miyake e colaboradores avaliaram a expressão de HDAC1 e sua possível correlação entre esta HDAC e variáveis clínicas, mas nenhuma associação significativa foi encontrada. Diferentemente, outros autores descreveram uma associação entre a expressão de HDAC1 e o grau de diferenciação do tumor, aumento da atividade proliferativa e estágios mais avançados da doença (TNM). Eles também demonstraram que a expressão aumentada progressivamente com fases posteriores do desenvolvimento do tumor (lesões precursoras para ADP) (Wang *et al.*, 2009). Entretanto, estudos com maiores tamanhos amostrais (78 e 70) identificaram superexpressão proteica em menos de 50% dos ADP analisados (Lehmann *et al.*, 2009; Giaginis *et al.*, 2015).

Recentemente, microarranjos tem sido usados em muitos estudos para identificar assinaturas gênicas associadas ao ADP e quatro grupos utilizaram esta abordagem de análise comparando amostras teciduais de ADP e pâncreas normal adjacente ao tumor. Embora a diferença entre os tecidos tenha sido estatisticamente significativa, os valores de *fold change* demonstram que a esta diferença não é relevante, no que diz respeito à expressão das *HDAC1, 2, 3 e 7.* Ainda, analises complementares como a validação por RT-PCR e expressão proteica não foram realizadas (números de acesso ao *Gene Expression Omnibus*: GSE28735, GSE41368, GSE43795 e GSE32676)

(Donahue *et al.*, 2012; Zhang *et al.*, 2012; Frampton *et al.*, 2014; Park *et al.*, 2014).

Análises de expressão proteica e de transcritos das HDACs em linhagens celulares foram publicadas por diversos grupos, mas inferências a respeito dos dados devem ser realizadas com cautela. Amostras teciduais apresentam grande variabilidade intra- e intertumoral e estas podem ser as razões da grande heterogeneidade dos resultados. Da mesma forma, cada linhagem celular corresponde a uma porção específica do tumor ou suas metástases. Diversos estudos tem demonstrado diferenças no padrão de expressão de clones à medida que a doença progride (Marusyk e Polyak, 2010). As linhagens celulares de ADP utilizadas nos estudos de tumores primários são BxPC-3, PANC-1, MIA-PaCA-2, PL-45, Panc 02.03 e HPAC e derivadas de metástases são HPAF-II, Capan-1, ASPC-1, CFPAC-1, HS-766T e SW1990. Por exemplo, em um estudo com sete linhagens celulares, Zhou e colaboradores observaram a superexpressão de *HDAC1* apenas nas linhagens BxPC-3 e PANC-1 (Zhou, Liang e Yee, 2011). Ainda para a HDAC1, sua expressão foi superior a linhagem controle de tecido pancreático não tumoral, para apenas três amostras (BxPC-3, ASPC-1 e MIA-PaCA-2) em uma análise por *Western Blotting* (Wang et al., 2012). Neste mesmo estudo a HDAC2 estava mais expressa em todas as linhagens (BxPC-3, ASPC-1, PANC-1, Capan-1, CFPAC-1, HPAC e MIA-PaCA-2), assim como HDAC3 e HDAC7 apresentaram superexpressão em seis linhagens (BxPC-3, ASPC-1, PANC-1, CFPAC-1, HPAC e MIA-PaCA-2).

Além das HDACs, os miRNAs também estão envolvidos na regulação de genes com papel crucial em todas etapas da tumorigênese, como apoptose, proliferação celular, angiogênese, invasão e metástase em diferentes tumores sólidos. (Ruan, Fang e Ouyang, 2009). Até o momento, poucos estudos exploraram a análise de interações entre HDACs e miRNAs. Neste trabalho buscamos identificar possíveis relações entre esses mecanismos epigenéticos que possam contribuir especificamente no desenvolvimento do ADP. Nas análises *in silico*, o miR-3127-3p foi identificado como um regulador das quatro HDACs analisadas. Entretanto, nós não encontramos estudos experimentais com esse regulador.

Adicionalmente, nossas análises revelaram alguns miRNAs que podem ter um papel importante na regulação específica de HDACs em ADP: foram identificados cinco miRNAs que tem como alvo a *HDAC1* (miR-188-5p, miR-539, miR-708, miR-4269 e miR-3616-3p) e três miRNAs que tem como alvo a HDAC2 (miR-4307, miR-944 e miR-195).

Somente o miR-708 foi estudado em amostras de pâncreas. Neste estudo, 846 miRNAs humanos foram avaliados em lesões precursoras do ADP (NIPMs) com o objetivo de distinguir lesões com potencial de malignidade baixo e alto. Quinze miRNAs foram diferentemente expressos entre lesões benignas e malignas de NIPM, e dentre estes o miR-708 apresentou expressão 3,38x maior em lesões benignas (Lubezky *et al.*, 2013). Este mesmo miRNA também foi identificado em outro tipo de lesão precursora do ADP, as NIPan, quando comparado ao tecido normal (Yu *et al.*, 2012)

Outros miRNAs apontados nas análises *in silico* (miR-188-5p, miR-539, miR-944 e miR-195) foram estudados em outros tipos tumorais. O miR-188-5p foi descrito recentemente por Fang e colaboradores como subexpresso em carcinoma hepatocelular em comparação ao seu tecido normal adjacente. Análises *in silico* e *in vitro* indicaram que este miRNA apresenta como um de seus alvos o FGF5 (do inglês, *Fibroblast Growth Fator*), proteína superexpressa em tumores com um importante papel na regulação do crescimento do tumor e invasão (Fang *et al.*, 2015). Em seguida, um outro estudo apontou que esse mesmo miRNA apresenta funções de supressão de tumor em câncer de próstata (Zhang *et al.*, 2015). A baixa expressão do miR-539 foi associado ao linfoma gástrico MALT (*Mucosa-Associated Lymphoid Tissue*) em comparação a mucosa gástrica normal (Thorns *et al.*, 2012), e ainda, sua baixa expressão foi associada a um pior prognóstico em pacientes com osteossarcoma (Mirghasemi *et al.*, 2015). Um estudo subsequente descreveu que esse miRNA regula negativamente a capacidade de migração e invasão de células metastáticas de tireóide (Gu e Sun, 2015).

Powrózek e colaboradores (2015) avaliaram a expressão de 90 pacientes com câncer de pulmão e 85 controles saudáveis. Os resultados indicaram que o miR-944 estava superexpresso em indivíduos com câncer e ainda apresentava acurácia diagnóstica para detecção do carcinoma de células escamosas do pulmão que apresentassem potencial para ressecção (AUC =

0,982) (Powrózek *et al.*, 2015). Em outros estudos, o miR-195 já apresentou fortes evidências para uma função supressora de tumor em alguns tipos de câncer (por exemplo, câncer de mama, colorretal, osteosarcoma e carcinoma hepatocelular). Em 2014 este mesmo miRNA foi apontado como um potencial biomarcador de diagnóstico de câncer de mama quando se observou a expressão reduzida no soro de casos com essa doença em comparação aos controles. Os níveis de sensibilidade de 73,97% sugeriram que esse miRNA pode ser usado como uma ferramenta de detecção deste tipo de tumor em estágios iniciais (Zhao *et al.*, 2014). O uso do miR-195 como biomarcador de diagnóstico também foi proposto para diferenciar pacientes com suspeita de osteossarcoma. Os níveis do miR-195 foram investigados no soro de 166 pacientes com osteossarcoma e 60 controles saudáveis e, assim como no estudo anterior, miR-195 foi significativamente menos expresso nos casos com tumor ($p < 0,001$), e os níveis de expressão foram correlacionados a um pior prognóstico (Cai *et al.*, 2015). A Bcl-2, proteína chave no mecanismo de apoptose e recentemente associada aos processos de migração celular, invasão e metástase (Um, 2015), foi descrita como alvo de miR-195 em linhagens de câncer colorretal (Yang, Tan e Song, 2015). Não há relatos na literatura relacionando o envolvimento de alguns miRNAs aqui identificados, miR-4269, miR-3616-3p e miR-4307, com o ADP ou outro tipo tumoral.

No presente estudo nós avaliamos quatro HDACs, escolhidas de acordo com dados prévios da literatura, que foram descritas como superexpressas em tecidos tumorais, incluindo o ADP. Os nossos resultados indicaram superexpressão da *HDAC7* em ADP corroborando as informações já publicadas, contudo a diferença encontrada entre a expressão em tecido tumoral e adjacente não foi estatisticamente significativa. A análise da expressão diferencial das demais HDACs foi significativa e indicou maior expressão no tecido normal. Resultados similares já foram reportados por outros estudos, onde apesar do tecido tumoral apresentar (em média) níveis superiores de expressão, diversas amostras tiveram níveis de expressão equivalentes entre os dois tecidos (normal e não-tumoral) ou ainda uma maior expressão no tecido normal. É importante destacar que a diferença de expressão entre tecido normal e tumoral foi sutil para todas as HDACs e apenas as *HDAC2* e *HDAC7* foram associadas com a idade ao diagnóstico.

Vários fatores podem explicar esses achados. O primeiro deles é o limitado tamanho amostral do estudo, que decorreu principalmente em razão de: i) a baixa prevalência do ADP (apesar do grande esforço para recrutamento de casos) e ii) as diversas perdas na seleção das amostras (por má qualidade do material demonstrado no exame anatomopatológico ou porque os casos nos quais os valores de Ct foram iguais ou superiores a 36 foram excluídos). O segundo fator limitante é o pequeno número de amostras pareadas (ADP e tecido normal do mesmo paciente) disponíveis para análise. Esta última pode ser uma limitação importante, uma vez que a exclusão de uma amostra de um paciente que apresenta uma expressão de HDAC equivalente em ambos os tecidos (tecido normal e tumoral) pode gerar desvios altos. O terceiro fator que postulamos como complicador nesse estudo é a heterogeneidade intratumoral, que também pode explicar a discordância dos resultados. A análise dos bancos de dados públicos indicou uma maior expressão das HDACs no tecido tumoral, com exceção da *HDAC3*. Assim como nos dados experimentais gerados em nosso laboratório, a diferença da expressão entre os tecidos foi sutil nas bases de dados, indicando que esta análise pode não ser suficiente para distinguir pacientes elegíveis para terapias com inibidores de desacetilases.

Por fim, para avaliar miRNAs que possam ter influência na desregulação das HDACs, investigamos em bancos de dados públicos de miRNAs (GSE43796 e GSE41369) que tinham (i) o mesmo conjunto de amostras com dados de expressão para HDACs e miRNA, e (ii) miRNAs que apresentassem expressão diferenciam entre o tecido tumoral e normal (Frampton *et al.*, 2014; Park *et al.*, 2014). A análise indicou oito miRNAs (miR-188-5p, miR-539, miR-708, miR-4269, miR-3616-3p, miR-4307, miR-944, miR-1307) que podem, potencialmente, estar associados a desregulação da *HDAC1* ou *HDAC2*. No entanto, baseado na pequena diferença de expressão das HDACs no tecido tumoral e normal, é provável que esse mecanismo de silenciamento não esteja exercendo um papel central na tumorigênese do ADP.

CAPÍTULO VI CONCLUSÕES

Considerando os resultados encontrados no presente estudo, podemos concluir que:

a. A expressão das HDACs não é um biomarcador de prognóstico robusto em APD, uma vez que a expressão diferencial entre o tecido pancreático não tumoral e tumoral foi sutil. E ainda, neste e estudos anteriores indicam nenhuma ou poucas associações entre a expressão das HDACs e características clínico-patológicas relacionadas com o prognóstico.

b. O uso de inibidores de desacetilases como terapia adjuvante no tratamento do ADP deve ser analisado com cautela, pois mesmo em estudos com uma série robusta (considerando a baixa prevalência do ADP) foi encontrada heterogeneidade na expressão proteica e de transcritos das HDACs.

c. Foi identificado expressão diferencial de alguns miRNAs que potencialmente agem sobre HDACs. No entanto, uma vez que a diferença entre a expressão HDACs foi sutil, os miRNAs, provavelmente, não estão exercendo um papel central na regulação dessas HDACs.

d. Estudos adicionais devem ser realizados em busca de biomarcadores de diagnóstico e prognóstico para PDAC.

CAPÍTULO VII PERSPECTIVAS

Como perspectivas e sugestões de análises adicionais destacamos as seguintes:

- Aumentar o tamanho amostral, tentando incluir mais casos pareados.

- Analisar a expressão proteica das HDACs em amostras teciduais de ADP e tecido pancreático normal por imunohistoquímica.

REFERÊNCIAS BIBLIOGRÁFICAS

ASCO. American Society Of Clinical Oncology. Pancreatic Cancer: Stages. 2014.

ALEMAR, B. et al. miRNA-21 and miRNA-34a Are Potential Minimally Invasive Biomarkers for the Diagnosis of Pancreatic Ductal Adenocarcinoma. Pancreas, Aug 2015. ISSN 1536-4828.

AMBROS, V. The functions of animal microRNAs. Nature, v. 431, n. 7006, p. 350-5, Sep 2004. ISSN 1476-4687.

BANERJI, U. et al. A phase I pharmacokinetic and pharmacodynamic study of CHR-3996, an oral class I selective histone deacetylase inhibitor in refractory solid tumors. Clin Cancer Res, v. 18, n. 9, p. 2687-94, May 2012. ISSN 1078-0432.

BARDEESY, N.; DEPINHO, R. A. Pancreatic cancer biology and genetics. Nat Rev Cancer, v. 2, n. 12, p. 897-909, Dec 2002. ISSN 1474-175X.

BARTEL, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, v. 116, n. 2, p. 281-97, Jan 2004. ISSN 0092-8674.

BAUDEN, M.; TASSIDIS, H.; ANSARI, D. In vitro cytotoxicity evaluation of HDAC inhibitor Apicidin in pancreatic carcinoma cells subsequent time and dose dependent treatment. Toxicol Lett, v. 236, n. 1, p. 8-15, Jul 2015. ISSN 1879-3169.

BEILHARZ, T. H. et al. microRNA-mediated messenger RNA deadenylation contributes to translational repression in mammalian cells. PLoS One, v. 4, n. 8, p. e6783, 2009. ISSN 1932-6203.

BI, G.; JIANG, G. The molecular mechanism of HDAC inhibitors in anticancer effects. Cell Mol Immunol, v. 3, n. 4, p. 285-90, Aug 2006. ISSN 1672-7681.

BLOOMSTON, M. et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA, v. 297, n. 17, p. 1901-8, May 2007. ISSN 1538-3598.

BOLDEN, J. E.; PEART, M. J.; JOHNSTONE, R. W. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov, v. 5, n. 9, p. 769-84, Sep 2006. ISSN 1474-1776.

BOSMAN, F. et al. WHO Classification of Tumours of the Digestive System. Seventh edition. Geneva, Switzerland: WHO Press, 2010.

BROWN, C. E. et al. The many HATs of transcription coactivators. Trends Biochem Sci, v. 25, n. 1, p. 15-9, Jan 2000. ISSN 0968-0004.

CAI, H. et al. Serum miR-195 is a diagnostic and prognostic marker for osteosarcoma. J Surg Res, v. 194, n. 2, p. 505-10, Apr 2015. ISSN 1095-8673..

CHIEN, W. et al. Growth inhibition of pancreatic cancer cells by histone deacetylase inhibitor belinostat through suppression of multiple pathways including HIF, NFkB, and mTOR signaling in vitro and in vivo. Mol Carcinog, v. 53, n. 9, p. 722-35, Sep 2014. ISSN 1098-2744.

CHOI, J. H. et al. Expression profile of histone deacetylase 1 in gastric cancer tissues. Jpn J Cancer Res, v. 92, n. 12, p. 1300-4, Dec 2001. ISSN 0910-5050.

CID-ARREGUI, A.; JUAREZ, V. Perspectives in the treatment of pancreatic adenocarcinoma. World J Gastroenterol, v. 21, n. 31, p. 9297-316, Aug 2015. ISSN 2219-2840.

DONAHUE, T. R. et al. Integrative survival-based molecular profiling of human pancreatic cancer. Clin Cancer Res, v. 18, n. 5, p. 1352-63, Mar 2012. ISSN 1078-0432.

EGGER, G. et al. Epigenetics in human disease and prospects for epigenetic therapy. Nature, v. 429, n. 6990, p. 457-63, May 2004. ISSN 1476-4687.

EKWALL, K. Genome-wide analysis of HDAC function. Trends Genet, v. 21, n. 11, p. 608-15, Nov 2005. ISSN 0168-9525.

ESQUELA-KERSCHER, A.; SLACK, F. J. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer, v. 6, n. 4, p. 259-69, Apr 2006. ISSN 1474-175X.

FANG, F. et al. MicroRNA-188-5p suppresses tumor cell proliferation and metastasis by directly targeting FGF5 in hepatocellular carcinoma. J Hepatol, v. 63, n. 4, p. 874-85, Oct 2015. ISSN 1600-0641.

FEINBERG, A. P. Cancer epigenetics takes center stage. Proc Natl Acad Sci U S A, v. 98, n. 2, p. 392-4, Jan 2001. ISSN 0027-8424.

FERLAY, J. et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, v. 136, n. 5, p. E359-86, Mar 2015. ISSN 1097-0215.

FINNIN, M. S. et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature, v. 401, n. 6749, p. 188-93, Sep 1999. ISSN 0028-0836.

FRAMPTON, A. E. et al. MicroRNAs cooperatively inhibit a network of tumor suppressor genes to promote pancreatic tumor growth and progression. Gastroenterology, v. 146, n. 1, p. 268-77.e18, Jan 2014. ISSN 1528-0012.

FRITSCHE, P. et al. HDAC2 mediates therapeutic resistance of pancreatic cancer cells via the BH3-only protein NOXA. Gut, v. 58, n. 10, p. 1399-409, Oct 2009. ISSN 1468-3288.

GIAGINIS, C. et al. Histone deacetylase (HDAC)-1, -2, -4 and -6 expression in human pancreatic adenocarcinoma: associations with clinicopathological parameters, tumor proliferative capacity and patients' survival. BMC Gastroenterol, v. 15, p. 148, 2015. ISSN 1471-230X.

GREGORETTI, I. V.; LEE, Y. M.; GOODSON, H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol, v. 338, n. 1, p. 17-31, Apr 2004. ISSN 0022-2836.

GROTH, A. et al. Chromatin challenges during DNA replication and repair. Cell, v. 128, n. 4, p. 721-33, Feb 2007. ISSN 0092-8674.

GROZINGER, C. M.; SCHREIBER, S. L. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol, v. 9, n. 1, p. 3-16, Jan 2002. ISSN 1074-5521.

GU, L.; SUN, W. MiR-539 inhibits thyroid cancer cell migration and invasion by directly targeting CARMA1. Biochem Biophys Res Commun, v. 464, n. 4, p. 1128-33, Sep 2015. ISSN 1090-2104.

HABERLAND, M.; MONTGOMERY, R. L.; OLSON, E. N. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet, v. 10, n. 1, p. 32-42, Jan 2009. ISSN 1471-0064.

HIDALGO, M. Pancreatic cancer. N Engl J Med, v. 362, n. 17, p. 1605-17, Apr 2010. ISSN 1533-4406.

HINGORANI, S. R. et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell, v. 7, n. 5, p. 469-83, May 2005. ISSN 1535-6108.

HRUBAN, R. H.; WILENTZ, R. E.; KERN, S. E. Genetic progression in the pancreatic ducts. Am J Pathol, v. 156, n. 6, p. 1821-5, Jun 2000. ISSN 0002-9440.

HUMPHREYS, D. T. et al. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A, v. 102, n. 47, p. 16961-6, Nov 2005. ISSN 0027-8424.

HWANG, J. H. et al. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. PLoS One, v. 5, n. 5, p. e10630, 2010. ISSN 1932-6203.

INCa. (Instituto Nacional do Câncer) Pâncreas. 2003.

INCa. Estimativa de Câncer no Brasil 2016/2017. 2015a.

INCa. (Instituto Nacional do Câncer) Estadiamento. 2015b.

IWAHASHI, S. et al. Effects of valproic acid in combination with S-1 on advanced pancreatobiliary tract cancers: clinical study phases I/II. Anticancer Res, v. 34, n. 9, p. 5187-91, Sep 2014. ISSN 1791-7530.

JIAO, F. et al. Histone deacetylase 3 promotes pancreatic cancer cell proliferation, invasion and increases drug-resistance through histone modification of P27, P53 and Bax. Int J Oncol, v. 45, n. 4, p. 1523-30, Oct 2014. ISSN 1791-2423.

JIN, K. L. et al. Expression profile of histone deacetylases 1, 2 and 3 in ovarian cancer tissues. J Gynecol Oncol, v. 19, n. 3, p. 185-90, Sep 2008. ISSN 2005-0380.

JOHNSTONE, R. W. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov, v. 1, n. 4, p. 287-99, Apr 2002. ISSN 1474-1776.

JONES, P. A.; BAYLIN, S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet, v. 3, n. 6, p. 415-28, Jun 2002. ISSN 1471-0056.

JONES, S. F. et al. Phase 1 results from a study of romidepsin in combination with gemcitabine in patients with advanced solid tumors. Cancer Invest, v. 30, n. 6, p. 481-6, Jul 2012. ISSN 1532-4192.

KANNAGI, R. Carbohydrate antigen sialyl Lewis a--its pathophysiological significance and induction mechanism in cancer progression. Chang Gung Med J, v. 30, n. 3, p. 189-209, 2007 May-Jun 2007. ISSN 2072-0939.

KENNER, B. J. et al. Early detection of sporadic pancreatic cancer: strategic map for innovation--a white paper. Pancreas, v. 44, n. 5, p. 686-92, Jul 2015. ISSN 1536-4828.

KIM, J. E. et al. Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population. J Gastroenterol Hepatol, v. 19, n. 2, p. 182-6, Feb 2004. ISSN 0815-9319.

KIM, V. M.; AHUJA, N. Early detection of pancreatic cancer. Chin J Cancer Res, v. 27, n. 4, p. 321-31, Aug 2015. ISSN 1000-9604.

LAKSHMAIAH, K. C. et al. Epigenetic therapy of cancer with histone deacetylase inhibitors. J Cancer Res Ther, v. 10, n. 3, p. 469-78, 2014 Jul-Sep 2014. ISSN 1998-4138.

LEE, D. Y. et al. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell, v. 72, n. 1, p. 73-84, Jan 1993. ISSN 0092-8674.

LEE, E. J. et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer, v. 120, n. 5, p. 1046-54, Mar 1 2007. ISSN 0020-7136

LEHMANN, A. et al. High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo. BMC Cancer, v. 9, p. 395, 2009. ISSN 1471-2407.

LI, B.; CAREY, M.; WORKMAN, J. L. The role of chromatin during transcription. Cell, v. 128, n. 4, p. 707-19, Feb 2007. ISSN 0092-8674.

LI, C. et al. Identification of pancreatic cancer stem cells. Cancer Res, v. 67, n. 3, p. 1030-7, Feb 2007. ISSN 0008-5472.

LIGGETT, W. H.; SIDRANSKY, D. Role of the p16 tumor suppressor gene in cancer. J Clin Oncol, v. 16, n. 3, p. 1197-206, Mar 1998. ISSN 0732-183X.

LIM, J. E.; CHIEN, M. W.; EARLE, C. C. Prognostic factors following curative resection for pancreatic adenocarcinoma: a population-based, linked database analysis of 396 patients. Ann Surg, v. 237, n. 1, p. 74-85, Jan 2003. ISSN 0003-4932.

LIU, F. SMAD4/DPC4 and pancreatic cancer survival. Commentary re: M. Tascilar et al., The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin. Cancer Res., 7: 4115-4121, 2001. Clin Cancer Res, v. 7, n. 12, p. 3853-6, Dec 2001. ISSN 1078-0432.

LOMBERK, G. A.; URRUTIA, R. The Triple-Code Model for Pancreatic Cancer: Cross Talk Among Genetics, Epigenetics, and Nuclear Structure. Surg Clin North Am, v. 95, n. 5, p. 935-52, Oct 2015. ISSN 1558-3171.

LU, J. et al. MicroRNA expression profiles classify human cancers. Nature, v. 435, n. 7043, p. 834-8, Jun 2005. ISSN 1476-4687.

LUBEZKY, N. et al. MicroRNA expression signatures in intraductal papillary mucinous neoplasm of the pancreas. Surgery, v. 153, n. 5, p. 663-72, May 2013. ISSN 1532-7361.

LUGER, K. et al. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature, v. 389, n. 6648, p. 251-60, Sep 1997. ISSN 0028-0836.

LUND, A. H.; VAN LOHUIZEN, M. Epigenetics and cancer. Genes Dev, v. 18, n. 19, p. 2315-35, Oct 2004. ISSN 0890-9369.

MAITRA, A.; HRUBAN, R. H. Pancreatic cancer. Annu Rev Pathol, v. 3, p. 157-88, 2008. ISSN 1553-4006.

MARGUERON, R.; TROJER, P.; REINBERG, D. The key to development: interpreting the histone code? Curr Opin Genet Dev, v. 15, n. 2, p. 163-76, Apr 2005. ISSN 0959-437X.

MARKS, P. A.; XU, W. S. Histone deacetylase inhibitors: Potential in cancer therapy. J Cell Biochem, v. 107, n. 4, p. 600-8, Jul 2009. ISSN 1097-4644..

MARUSYK, A.; POLYAK, K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta, v. 1805, n. 1, p. 105-17, Jan 2010. ISSN 0006-3002.

MATHONNET, G. et al. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science, v. 317, n. 5845, p. 1764-7, Sep 2007. ISSN 1095-9203.

MAZZIO, E. A.; SOLIMAN, K. F. Basic concepts of epigenetics: impact of environmental signals on gene expression. Epigenetics, v. 7, n. 2, p. 119-30, Feb 2012. ISSN 1559-2308.

MIRGHASEMI, A. et al. Down-regulation of miR-133a and miR-539 are associated with unfavorable prognosis in patients suffering from osteosarcoma. Cancer Cell Int, v. 15, p. 86, 2015. ISSN 1475-2867.

MIYAKE, K. et al. Expression of hypoxia-inducible factor-1alpha, histone deacetylase 1, and metastasis-associated protein 1 in pancreatic carcinoma: correlation with poor prognosis with possible regulation. Pancreas, v. 36, n. 3, p. e1-9, Apr 2008. ISSN 1536-4828.

MOFFAT, D. et al. Discovery of 2-(6-{[(6-fluoroquinolin-2-yl)methyl]amino}bicyclo[3.1.0]hex-3-yl)-N-hydroxypyrimidine-5-carboxamide (CHR-3996), a class I selective orally active histone deacetylase inhibitor. J Med Chem, v. 53, n. 24, p. 8663-78, Dec 2010. ISSN 1520-4804..

MOLIN, M.; MAITRA, A. Molecular Diagnostics of Pancreatic Cancer. In: TAN D , L. H. (Ed.). Principles of Molecular Diagnostics and Personalized Cancer Medicine. Lippincott Williams & Wilkins. Philadelphia,PA, 2012. p.399-416.

MORIYAMA, T. et al. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. Mol Cancer Ther, v. 8, n. 5, p. 1067-74, May 2009. ISSN 1538-8514.

MOTTAMAL, M. et al. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. Molecules, v. 20, n. 3, p. 3898-941, 2015. ISSN 1420-3049..

NAKAGAWA, M. et al. Expression profile of class I histone deacetylases in human cancer tissues. Oncol Rep, v. 18, n. 4, p. 769-74, Oct 2007. ISSN 1021-335X.

NCI, N. C. I. Dictionary of Cancer Terms. 2015.

NEUZILLET, C.; SAUVANET, A.; HAMMEL, P. Prognostic factors for resectable pancreatic adenocarcinoma. J Visc Surg, v. 148, n. 4, p. e232-43, Sep 2011. ISSN 1878-7886.

NILSEN, T. W. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet, v. 23, n. 5, p. 243-9, May 2007. ISSN 0168-9525.

OEHME, I. et al. Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin Cancer Res, v. 15, n. 1, p. 91-9, Jan 2009. ISSN 1078-0432.

OLIVE, K. P. et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science, v. 324, n. 5933, p. 1457-61, Jun 2009. ISSN 1095-9203.

OMURA, N.; GOGGINS, M. Epigenetics and epigenetic alterations in pancreatic cancer. Int J Clin Exp Pathol, v. 2, n. 4, p. 310-26, 2009. ISSN 1936-2625.

OUAÏSSI, M. et al. High histone deacetylase 7 (HDAC7) expression is significantly associated with adenocarcinomas of the pancreas. Ann Surg Oncol, v. 15, n. 8, p. 2318-28, Aug 2008. ISSN 1534-4681. Disponível em: <

OUAÏSSI, M. et al. Further characterization of HDAC and SIRT gene expression patterns in pancreatic cancer and their relation to disease outcome. PLoS One, v. 9, n. 9, p. e108520, 2014. ISSN 1932-6203.

PAN, Y. et al. Gd-Metallofullerenol Nanomaterial Suppresses Pancreatic Cancer Metastasis by Inhibiting the Interaction of Histone Deacetylase 1 and Metastasis-Associated Protein 1. ACS Nano, v. 9, n. 7, p. 6826-36, Jul 2015. ISSN 1936-086X.

PAPACONSTANTINOU, I. G. et al. A review on the role of microRNA in biology, diagnosis, and treatment of pancreatic adenocarcinoma. Pancreas, v. 41, n. 5, p. 671-7, Jul 2012. ISSN 1536-4828.

PARK, M. et al. Characterization of gene expression and activated signaling pathways in solid-pseudopapillary neoplasm of pancreas. Mod Pathol, v. 27, n. 4, p. 580-93, Apr 2014. ISSN 1530-0285.

PETERSON, C. L.; LANIEL, M. A. Histones and histone modifications. Curr Biol, v. 14, n. 14, p. R546-51, Jul 2004. ISSN 0960-9822..

PORTNOY, V. et al. Small RNA and transcriptional upregulation. Wiley Interdiscip Rev RNA, v. 2, n. 5, p. 748-60, 2011 Sep-Oct 2011. ISSN 1757-7012..

POWRÓZEK, T. et al. Plasma circulating microRNA-944 and microRNA-3662 as potential histologic type-specific early lung cancer biomarkers. Transl Res, v. 166, n. 4, p. 315-23, Oct 2015. ISSN 1878-1810.

RACHAGANI, S.; KUMAR, S.; BATRA, S. K. MicroRNA in pancreatic cancer: pathological, diagnostic and therapeutic implications. Cancer Lett, v. 292, n. 1, p. 8-16, Jun 2010. ISSN 1872-7980.

RAHIB, L. et al. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res, v. 74, n. 11, p. 2913-21, Jun 2014. ISSN 1538-7445.

REDSTON, M. S. et al. p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. Cancer Res, v. 54, n. 11, p. 3025-33, Jun 1994. ISSN 0008-5472.

RHIM, A. D. et al. EMT and dissemination precede pancreatic tumor formation. Cell, v. 148, n. 1-2, p. 349-61, Jan 2012. ISSN 1097-4172..

RUAN, K.; FANG, X.; OUYANG, G. MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett, v. 285, n. 2, p. 116-26, Nov 2009. ISSN 1872-7980.

SAWAN, C. et al. Epigenetic drivers and genetic passengers on the road to cancer. Mutat Res, v. 642, n. 1-2, p. 1-13, Jul 2008. ISSN 0027-5107..

SCHNEIDER, G. et al. Targeting histone deacetylases in pancreatic ductal adenocarcinoma. J Cell Mol Med, v. 14, n. 6A, p. 1255-63, Jun 2010. ISSN 1582-4934.

SCHNEIDER, G. et al. Acetylation as a transcriptional control mechanism-HDACs and HATs in pancreatic ductal adenocarcinoma. J Gastrointest Cancer, v. 42, n. 2, p. 85-92, Jun 2011. ISSN 1941-6636..

SCHWARZENBACH, H. et al. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol, v. 11, n. 3, p. 145-56, Mar 2014. ISSN 1759-4782.

SEMENZA, G. L. Targeting HIF-1 for cancer therapy. Nat Rev Cancer, v. 3, n. 10, p. 721-32, Oct 2003. ISSN 1474-175X.

SEMPERE, L. L.; KAUPPINEN, S. Translational implications of microRNAs in clinical diagnostic and therapeutics. 2nd Edition. Oxford: Academic Press: 2009.

SIEGEL, R. L.; MILLER, K. D.; JEMAL, A. Cancer statistics, 2015. CA Cancer J Clin, v. 65, n. 1, p. 5-29, 2015 Jan-Feb 2015. ISSN 1542-4863..

SIMIANU, V. V. et al. Pancreatic cancer: progress made. Acta Oncol, v. 49, n. 4, p. 407-17, May 2010. ISSN 1651-226X.

SONG, J. W.; LEE, J. H. New morphological features for grading pancreatic ductal adenocarcinomas. Biomed Res Int, v. 2013, p. 175271, 2013. ISSN 2314-6141.

STRAHL, B. D.; ALLIS, C. D. The language of covalent histone modifications. Nature, v. 403, n. 6765, p. 41-5, Jan 2000. ISSN 0028-0836.

THORNS, C. et al. Deregulation of a distinct set of microRNAs is associated with transformation of gastritis into MALT lymphoma. Virchows Arch, v. 460, n. 4, p. 371-7, Apr 2012. ISSN 1432-2307.

UM, H. D. Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. Oncotarget, Nov 2015. ISSN 1949-2553.

VINCENT, A. et al. Pancreatic cancer. Lancet, v. 378, n. 9791, p. 607-20, Aug 2011. ISSN 1474-547X.

VOJINOVIC, J. et al. Safety and efficacy of an oral histone deacetylase inhibitor in systemic-onset juvenile idiopathic arthritis. Arthritis Rheum, v. 63, n. 5, p. 1452-8, May 2011. ISSN 1529-0131.

WANG, G. et al. Class I and class II histone deacetylases are potential therapeutic targets for treating pancreatic cancer. PLoS One, v. 7, n. 12, p. e52095, 2012. ISSN 1932-6203.

WANG, W. et al. Significance of DNA methyltransferase-1 and histone deacetylase-1 in pancreatic cancer. Oncol Rep, v. 21, n. 6, p. 1439-47, Jun 2009. ISSN 1021-335X.

WEST, A. C.; JOHNSTONE, R. W. New and emerging HDAC inhibitors for cancer treatment. J Clin Invest, v. 124, n. 1, p. 30-9, Jan 2014. ISSN 1558-8238.

WRAY, C. J. et al. Surgery for pancreatic cancer: recent controversies and current practice. Gastroenterology, v. 128, n. 6, p. 1626-41, May 2005. ISSN 0016-5085.

XU, X. D. et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses vasculogenic mimicry and proliferation of highly aggressive pancreatic cancer PaTu8988 cells. BMC Cancer, v. 14, p. 373, 2014. ISSN 1471-2407.

YANG, B.; TAN, Z.; SONG, Y. Study on the molecular regulatory mechanism of MicroRNA-195 in the invasion and metastasis of colorectal carcinoma. Int J Clin Exp Med, v. 8, n. 3, p. 3793-800, 2015. ISSN 1940-5901.

YANG, X. J.; SETO, E. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol, v. 9, n. 3, p. 206-18, Mar 2008. ISSN 1471-0080.

YU, J. et al. MicroRNA alterations of pancreatic intraepithelial neoplasias. Clin Cancer Res, v. 18, n. 4, p. 981-92, Feb 2012. ISSN 1078-0432.

ZHANG, G. et al. DPEP1 inhibits tumor cell invasiveness, enhances chemosensitivity and predicts clinical outcome in pancreatic ductal adenocarcinoma. PLoS One, v. 7, n. 2, p. e31507, 2012. ISSN 1932-6203.

ZHANG, H. et al. miR-188-5p inhibits tumour growth and metastasis in prostate cancer by repressing LAPTM4B expression. Oncotarget, v. 6, n. 8, p. 6092-104, Mar 2015. ISSN 1949-2553.

ZHANG, Y. et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. World J Surg, v. 33, n. 4, p. 698-709, Apr 2009. ISSN 0364-2313.

ZHAO, B.; HE, T. Chidamide, a histone deacetylase inhibitor, functions as a tumor inhibitor by modulating the ratio of Bax/Bcl-2 and P21 in pancreatic cancer. Oncol Rep, v. 33, n. 1, p. 304-10, Jan 2015. ISSN 1791-2431.

ZHAO, F. L. et al. Serum microRNA-195 is down-regulated in breast cancer: a potential marker for the diagnosis of breast cancer. Mol Biol Rep, v. 41, n. 9, p. 5913-22, Sep 2014. ISSN 1573-4978.

ZHOU, W.; LIANG, I. C.; YEE, N. S. Histone deacetylase 1 is required for exocrine pancreatic epithelial proliferation in development and cancer. Cancer Biol Ther, v. 11, n. 7, p. 659-70, Apr 2011. ISSN 1555-8576.

ANEXOS

ANEXO I.

miRNAs As Diagnostic and Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: A Review

miRNAs As Diagnostic and Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: A Review

Bárbara Alemar[1,2], Cleandra Gregório[1,2] and Patricia Ashton-Prolla[1,2]

[1]Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. [2]Laboratório de Medicina Genômica, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

ABSTRACT: Pancreatic ductal adenocarcinoma (PDAC), a rare but lethal tumor, is difficult to diagnose without performing an invasive procedure. miRNAs are known to be deregulated in PDAC patients, and recent studies have shown that they can be used as diagnostic and prognostic of the disease. The detection of miRNAs in samples acquired through minimally or noninvasive procedures, such as serum, plasma, and saliva, can have a positive impact on the clinical management of these patients. This article is a comprehensive review of the major studies that have evaluated the expression of miRNAs as biomarkers in pancreatic cancer and its premalignant lesions.

KEYWORDS: pancreatic cancer, miRNAs, biomarkers, circulating biomarkers

CITATION: Alemar et al. miRNAs As Diagnostic and Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: A Review. *Biomarker Insights* 2015:10 113–124 doi: 10.4137/BMI.S27879.

TYPE: Review

RECEIVED: May 05, 2015. **RESUBMITTED:** August 30, 2015. **ACCEPTED FOR PUBLICATION:** September 06, 2015.

ACADEMIC EDITOR: Karen Pulford, Editor in Chief

PEER REVIEW: Seven peer reviewers contributed to the peer review report. Reviewers' reports totaled 1,159 words, excluding any confidential comments to the academic editor.

FUNDING: Patricia Ashton-Prolla is investigator for CNPq. Bárbara Alemar and Cleandra Gregório received a grant from CNPq. Funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: barbara.alemar@gmail.com

Introduction

Among all cancer types, pancreatic ductal adenocarcinoma (PDAC), although rare, is considered one of the most lethal tumors, being responsible for 3% of all new cancer cases and 7% of all cancer-related deaths. PDAC is also the fourth highest cause of cancer-related death in the United States; moreover, the National Cancer Institute estimates that in 2015 ~48,960 people will be diagnosed with pancreatic cancer and that 40,560 of them will die because of the disease.[1] Several of PDAC's associated features make it a devastating and lethal disease, for example, the early dissemination of tumor-derived cells in the blood stream,[2] substantial morbidity associated with disease progression (which often renders the patient unsuitable for surgery or even nonsurgical disease-specific treatments),[3] and widespread tumor resistance to most forms of current treatment.[4] The only curative approach for PDAC patients is surgical resection, and only patients with localized (early stage) tumors are eligible for this therapy.[5]

At present, there is no detection method for the diagnosis of early stage PDAC; indeed, pancreatic cancer is usually a silent disease that only becomes apparent after tumor invasion of the surrounding tissues or metastatic seeding of distant organs. Therefore, the discovery of new diagnostic and prognostic biomarkers in pancreatic cancer is particularly important for patient survival. The only tumor markers for pancreatic cancer currently being used in a clinical setting are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), both of which present limitations because of low sensitivity and specificity. Besides their inability to discriminate malign and benign disease, these biomarkers are not specific tumor markers; in fact, the serum levels of these markers show variation in many diseases.

miRNAs have recently emerged as promising biomarkers in PDAC because these molecules show tissue-specific expression patterns[6] and are stable in circulating samples that can be easily obtained; hence, they enable disease screening in high-risk patients (as diagnostic biomarkers) and evaluation of several disease parameters (as prognostic biomarkers).[7]

In 1993, using *Caenorhabditis elegans* as a model, Lee et al.[8] identified a gene that codes for small noncoding RNAs, and further studies have demonstrated that these small RNAs can regulate protein translation by binding to the 3′-UTR and consequently inhibit mRNAs.[9] In the following years, these small noncoding RNA genes were described in different species, including humans, and they were named microRNAs (miRNAs or miRs).[10,11] Nowadays, miRNAs are well-known cancer biomarkers; they can fulfill this role because of their deregulation in virtually all tumors and presentation of tumor-specific profiles.

The aim of the present article is to review the major studies that have evaluated the expression of miRNAs as biomarkers in pancreatic cancer and its premalignant lesions. The article is structured into five main sections. The first section, miRNAs and cancer, introduces the early studies in this field; the next three sections explore the differential expression of miRNAs in premalignant lesions and PDAC. The final section highlights the recent findings related to miRNAs as useful circulating biomarkers. The discussion of the biogenesis, mechanism of action, and editing of miRNAs is beyond the scope of this article, but excellent reviews on these topics can be found elsewhere.[12–16]

miRNAs and Cancer

An association between miRNAs and cancer was proposed for the first time in 2002, when Calin et al.[17] demonstrated that a region commonly deleted in B-cell chronic lymphocytic leukemia (B-CLL) corresponded to miR-15 and miR-16 genes. The loss of this region (13q14) is the most frequent chromosomal abnormality in CLL, and this loss also occurs in other tumors, which indicates that tumor-suppressor genes could be located in this region. Calin et al also showed that both miRNA genes are deleted or downregulated in 68% of CLL cases. In 2005, Cimmino et al.[18] demonstrated that miR-15 and miR-16 negatively regulate *BCL2*, which encodes the antiapoptotic protein Bcl2. The absence of miR-15 and miR-16 in CLL promotes Bcl2 superexpression, which inhibits apoptosis and contributes to the establishment and progression of the malignant phenotype. Two years after their study describing the relationship between miRNAs and cancer, Calin et al.[19] published a paper in which genome-wide miRNA microarray profiling was used to show, for the first time, the potential importance of miRNAs in the diagnosis and prognosis of human malignancies.

In a comprehensive study, in which bead-based flow-cytometric profiling technology was applied, the authors demonstrated that a relatively small number of miRNAs could provide a large amount of diagnostic information because the expression patterns indicated not only different human cancers types but also differentiation states. The authors also observed patterns of gene expression for each type of tumor that reflected distinct mechanisms of transformation.[6] The feasibility and utility of monitoring the expression of miRNAs in human cancer was confirmed in this study and reinforced by others, because unlike mRNAs, miRNAs remained largely intact in routinely obtained, formalin-fixed, paraffin-embedded (FFPE) tissues.[20] Following the publication of this paper, several studies have shown that miRNA expression patterns are associated with different steps of carcinogenesis, and it is now well known that miRNAs can act as onco-miRNAs or tumor-suppressor miRNAs. In the first scenario, the overexpression of an miRNA that acts as an onco-miRNA would contribute to tumor formation through the negative regulation of a tumor-suppressor mRNA. Increased levels of these mature miRNAs could occur because of miRNA gene amplifications, constitutive promoter activation, increased efficiency in miRNA processing, or increased stability of the miRNA. In the second scenario, the reduction or absence of an miRNA that acts as a tumor suppressor can lead to tumor formation because low expression levels of the miRNA promotes the overexpression of mRNAs that encode oncoproteins, and this favors the development of other features necessary for tumor progression, such as increased proliferation, invasiveness, and angiogenesis. The reduction or elimination of an miRNA can be caused by defects at any stage of miRNA biogenesis.[12] Although the correlation between miRNA and cancer was clear in 2005, the first study to show that the deregulation of a single-miRNA gene could lead to cancer was published in 2006; specifically, this study investigated a lymphoblastic leukemia/high-grade lymphoma in mice.[21]

In light of the central role that miRNAs play in carcinogenesis, they can be considered new regulators of cancer hallmarks.[22] Several examples can be used to illustrate the importance of miRNA regulation in all tumorigenic steps, such as cell proliferation, apoptosis, replicative potential, angiogenesis, immune response, tumor invasion, metastatic potential, and genomic instability. Indeed, in one elegant study, high-throughput miRNA profiling was applied to compare miRNA expression levels in premalignant stages as well as tumors and metastasis, and each stage was found to correlate with a distinct miRNA expression signature. Moreover, the wide variety of altered miRNAs was shown to reflect distinct functional roles in the acquired capabilities necessary for tumor formation and progression, the hallmarks of cancer, in a mouse model of pancreatic neuroendocrine tumors.[23,24]

According to Volinia et al.[25], despite the evident molecular differences between tumor types, some tumors present similar miRNA expression patterns, including prostate, colon, gastric, and pancreatic cancers, whereas others, such as lung and breast cancers, presented different signatures. Although some miRNAs are typical in one tumor type or tissue, other miRNAs, such as miR-21, miR-191, and miR-17-5p, are significantly overexpressed in several different tumor types. The study of Volinia et al showed that the most common miRNA alteration in solid tumors involves an increase in the expression levels, whereas loss of expression is less common and usually tissue specific. Finally, the authors' data provided further evidence to suggest that miRNAs function either in a dominant or recessive fashion by controlling the expression of protein-coding tumor suppressors and oncogenes.

Differential Expression of miRNAs in Premalignant Pancreatic Lesions: Pancreatic Intraepithelial Neoplasia

Most authors in the field propose that there are three possible precursor lesions to PDAC: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and a less prevalent precursor lesion known as mucinous cystic neoplasm (MCN). All of these alterations harbor

different types of dysplasia and involve a stepwise accumulation of genetic alterations that leads to the progression of the lesions from benign to malignant neoplasms.[26]

PanINs are found in the smaller caliber pancreatic ducts and are the most common and extensively studied of the premalignant lesions. The classic PDAC progression model is based on a sequence of molecular and histological alterations, which involves the transformation of the normal pancreatic duct in low-grade dysplasias (PanIN-IA and -IB) to high-grade dysplasias (PanIN-II and -III).[27] PanIN-IA and -IB are characterized by an increase in the ductal cells with abundant mucin production; however, in PanIN-IB, the papillary architecture replaces the flat architecture. While the lesions progress, the cells acquire moderated nuclear alterations (PanIN-II) with abnormal mitosis and lumen invasion, similar to those from in situ carcinoma (PanIN-III, when cells also present a severe nuclear alteration).[28]

The progression of premalignant lesions to invasive neoplasia is the result of successive genetic changes in a relatively predictable order in tumor-suppressor genes, oncogenes, and DNA repair genes.[27] The evaluation of miRNA expression in low- and high-grade PanINs is aimed at identifying biomarkers in the early stages of pancreatic carcinogenesis. Indeed, a PanIN progression model based on miRNA differential expression was described, showing that different miRNA expression profiles correlate with each stage of premalignant lesions.[29] In particular, the study investigated the expression profile of 735 human miRNAs in PanINs, PDAC, and normal tissues, and it included a principal component analysis demonstrating that miRNA expression profiles could be used to not only classify different PanIN stages but also correctly discriminate between PanIN and normal pancreatic (ductal) tissue. Sixty-five miRNAs were differentially expressed in precursor lesions relative to normal cells, and among these, 13 overexpressed miRNAs (miR-146a, -182, -193a-3p, -193b, -200a, -200b, -200c, -21, -29b, -425, -486-3p, -708, and -874) and one underexpressed miRNA (miR-296-5p) were selected for validation by quantitative polymerase chain reaction (qPCR) in an independent cohort (all overexpressed levels were confirmed). An interesting finding of this study was the identification of miR-196b as the most highly expressed miRNA in PanIN (compared with expression in normal cells); it was also highly expressed in PanIN-III (compared with expression in PanIN-I and -II) and expressed at even greater levels in PDAC samples. These findings led to the conclusion that miRNA expression levels can be used to discriminate patients with low-grade premalignant lesions from patients with high-grade lesions and PDAC.[29]

The same miRNA was also overexpressed in serum samples from humans and transgenic KPC mice (a mouse model of PDAC that expresses both oncogenic KRAS and mutant p53 in pancreatic cells). By analyzing the expression profiles of five miRNAs that were differentially expressed in PDAC (miR-21, -155, -196a, -196b, and -210) in KPC mice, Slater et al.[30] found that only miR-196a and -196b were significantly overexpressed. They confirmed this finding in human serum samples and found that the expression levels of miR-196a and -196b, when measured together, discriminated PanIN-II, -III, and PDAC patients from healthy controls. This panel of miRNAs reached a perfect sensitivity and specificity (100%) when used to discriminate healthy controls from high-grade PanIN patients; thus, these miRNAs are promising biomarkers for early diagnosis. In human microdissected PanIN samples, miR-155 and miR-21 were evaluated by quantitative real-time PCR (qRT-PCR): only miR-155 expression was significantly different in high-grade PanINs versus nontumoral tissues; however, no difference in miR-155 expression was observed when PanIN-I lesions were compared with nontumoral tissue.[31]

The expression levels of a panel of five miRNAs (miR-10b, -21, -148a, -196a, and -217) were analyzed in PanINs and PDAC by Xue et al.[32] The authors corroborated the results of previous studies; they found that miR-196a was overexpressed in PanIN and PDAC compared with its expression in benign pancreatic tissue. Furthermore, miR-10b was upregulated in PanIN, miR-21 was upregulated in PDAC, and miR-217 was underexpressed in PDAC samples (compared with their expression in normal tissue). miR-148a expression was inversely correlated with PDAC progression because its levels were found to be high in normal pancreatic tissue samples and low in PDAC samples [with intermediate levels observed in chronic pancreatitis (CP) and PanINs]. These findings suggested that miR-148a could be a good marker of disease progression.

Because miR-21 is a well-known oncogenic miRNA in many cancers,[33] including PDAC, its presence is common in various miRNA panels. duRieu and cols found that together with other miRNAs (eg, miR-221, -222, -200, and -205), miR-21 was found to be overexpressed in PanIN, with levels increasing as the lesion progresses and reaching its highest levels in PanIN-II and -II. In this study, the authors also identified the overexpression of let-7 (a well-characterized miRNA that inhibits cell proliferation) in PanIN-II and -III as well as its underexpression in PDAC (as expected, according to Torrisani et al.[34] and Ali et al.[35,36]), thereby characterizing this miRNA as a potential biomarker for differential diagnosis in tissue samples.[37]

Finally, in another study, the expression levels of miR-145 were also correlated with PanIN progression. PanIN-I showed high levels of miR-145, whereas PanIN-II and -III as well as PDAC tissues presented with low or absent miR-145 expression.[38]

Differential Expression of miRNA in Premalignant Pancreatic Lesions: Mucinous Lesions

In addition to PanINs, two other premalignant lesions, IPMNs and MCNs (both containing a mucinous component), can play an important role in pancreatic carcinogenesis.

IPMNs are the most common cystic precursor lesions of PDAC. These lesions are mucin-producing epithelial

neoplasms that arise within the main pancreatic duct or one of its branches. They are considered potentially malignant and, being composed of columnar cells, are macroscopically visible via imaging technology.[39] IPMNs are organized into three categories depending on the degree of cytoarchitectural atypia. Lesions with low-grade dysplasia are characterized by a single layer of well-polarized cells, small pleomorphism, and rare mitoses. Intermediate-grade dysplasia shows nuclear stratification, loss of polarity, and crowding. Finally, IPMN lesions with high-grade dysplasia show severe dysplastic epithelial changes, marked loss of polarity, and loss of differentiated cytoplasmic features.[40]

MCNs of the pancreas are single spherical lesions macroscopically visible by imaging (mean diameter: 6–10 cm); they are most often found in the pancreatic body and tail, and they do not communicate with the pancreatic ductal system.[41,42] According to the grade of intraepithelial dysplasia, the World Health Organization has classified MCNs into three categories: MCNs with low, intermediate, and high-grade dysplasia.[42]

The potential of miRNAs as biomarkers in mucinous lesions has been evaluated in several studies, given that once these lesions are diagnosed by imaging there is neither a way to evaluate the level of epithelial dysplasia nor a method to predict whether the lesion will progress to adenocarcinoma and how long this might take. The potential of miRNAs as biomarkers in mucinous lesions has been evaluated in several studies.

The first study to evaluate miRNA expression in IPMNs was published in 2009 by Habbe et al, and it included a sample set containing 13 IPMN adenomas, 31 borderline IPMNs, and 20 IPMNs with carcinoma in situ, plus 54 matched non-neoplasic pancreatic tissue samples. First, the authors conducted a pilot study in which the expression of 12 miRNAs (described in the literature as deregulated in pancreatic cancer) was compared in 15 IPMN samples and matched normal pancreatic tissues. Expressions levels for 10 of these miRNAs differed significantly between cases and controls. miR-21 and miR-155 had the highest fold-changes, 12.1 and 11.6, respectively. The expression levels of these two miRNAs were subsequently evaluated by using locked nucleic acid in situ hybridization in 64 IPMN lesions and 54 controls, which confirmed their overexpression in the IPMN samples. Expression levels of these two miRNAs were also assessed in pancreatic juice; miR-155 was overexpressed in 60% of all cases, with 20-fold or greater relative-fold expression, whereas miR-21 was also highly expressed in the cases relative to the controls, but not to the extent of miR–155. Although these were interesting results, none reached statistical significance.[43] miR-21 and miR-155, together with miR-101, were also analyzed in a multicenter study of 65 invasive IPMNs, 16 noninvasive IPMNs, and 5 normal pancreatic tissues. The expression levels of the first two miRNAs correlated with an increase in disease severity; specifically, they were overexpressed in invasive IPMN samples, expressed at lower levels in benign tissue, and almost absent in normal pancreatic cells. Similar to findings in PDAC studies, IPMN patients with high miR-21 levels had a significantly shorter overall survival (17.2 months) compared with patients who had low miR-21 levels (53.3 months). miR-101 showed an opposite expression pattern: it was equally expressed in normal pancreas tissues and benign IPMNs, but it was underexpressed in invasive IPMN samples, which suggests that miR-101 acts as a tumor suppressor.[44] Nakahara et al further investigated the role of miR-101 in pancreatic carcinogenesis.[45] With the aim of understanding whether this miRNA regulates the expression of the *EZH2* gene, which is suspected to act as an oncogene by downregulating tumor-suppressor genes,[46] the authors assessed expression of miR-101 using qPCR in five malignant tumors and five benign IPMN samples. Its expression was significantly lower in malignant IPMN than in benign samples. Thus, together with other findings, this study suggested that *EZH2* blockage by miR-101 is an important step in pancreatic carcinogenesis, and this miRNA could therefore represent a potential therapeutic target.

In a study that included several pancreatic lesions, the expression of 866 mature miRNAs was assayed in an attempt to distinguish between pancreatic tumors and lesions with low and high malignant potential. To confirm the microarray findings in this study, levels of miR-21, -126 and -16 (ie, miRNAs with known relevance to pancreatic carcinogenesis) were also measured by qRT-PCR. Expression levels of miR-21 did not differ between benign cystic tumors (comprising IPMN, MCN, and serous microcystic adenoma), PDAC, and carcinoma ex-IPMN (which is an invasive malignancy that arises from IPMN). The expression of miR-126 did not differ between IPMN, MCN, and the other groups, whereas miR-16 was overexpressed in IPMN samples relative to PDAC samples.[47] Ryu et al used a categorization strategy in their study, which involved investigating a panel of miRNAs, including two recurrently analyzed miRNAs (miR-21 and miR-155) plus miR-221, miR-17-3p, and miR-191 to discriminate mucinous (IPMN and MCN) from nonmucinous lesions [serous cystadenomas (SCAs) and other benign cysts]. Although the first four miRNAs in the aforementioned list could be used to discriminate between the groups, none of the miRNAs allowed differentiation between IPMN and MCN.[48] Using a slightly different approach in which cystic lesions were categorized into three risk categories (benign, corresponding to SCAs; premalignant, corresponding to IPMN and MCN; and malignant, corresponding to adenocarcinoma), Henry et al.[49] showed that a panel of nine miRNAs could be used to correctly classify these groups. In another study, Lee et al identified a MCN classifier, which included a panel of four miRNAs (miR-10b-5p, miR-202-3p, miR-210, and miR-375) whose expression levels could be employed to discriminate MCN from other pancreatic cystic neoplasms and PDAC with a sensitivity and specificity of 100%.[50]

Matthaei et al.[51] used a DiffPairs approach to identify novel biomarkers and analyze the expression profiles of 750 miRNAs in IPMN and aspirated cystic fluid samples, thereby identifying 26 and 37 candidate miRNAs for these respective samples. By using these candidate miRNAs, the authors were able to distinguish between high-risk lesions (high-grade IPMNs) and low-risk lesions (low-grade IPMNs and SCA). By narrowing their analysis down to a panel of nine miRNAs, they were able to discriminate both groups with a sensitivity of 89% and a specificity of 100%; furthermore, they could distinguish low-grade IPMNs/SCA from unusual cysts as cystic pancreatic neuroendocrine tumors and solid pseudopapillary tumors. Using a similar approach, Lubezky et al.[52] analyzed 846 human miRNAs in 55 specimens, including low-, moderate-, and high-grade IPMNs, PDAC, and normal pancreatic tissues. Fifteen miRNAs were differentially expressed in benign lesions (low- and moderate-grade IPMNs) compared with malignant lesions (high-grade IPMN and invasive carcinoma arising in IPMN), including overexpression of miR-155, miR-708, miR-424*, miR-21, miR-503, miR-214*, miR-150, miR-146a, and miR-21* and underexpression of miR-130b, miR-375, miR-148a, miR-216b, miR-216a, and miR-217.

More recently, studies used high-throughput methods to reveal new deregulated miRNAs in premalignant lesions, including miR-100, miR-99a, miR-99b, miR-342-3p, and miR-130a as well as miR-126. In microarray analysis, all of these miRNAs were underexpressed in high-risk IPMNs, relative to low-risk IPMNs, and this trend was confirmed by qRT-PCR in a different cohort. Moreover, an miRNA profile composed of miR-99b, miR-130a, and miR-342-3p reached an area under curve (AUC) value of 0.74 in the validation phase.[53] Wang et al.[54] employed next-generation sequencing (SOLiD platform) to identify patients with low-grade and high-grade dysplasia, and they found 13 overexpressed and 2 underexpressed miRNAs in cystic fluid samples from patients with IPMN and MCN (compared with expression in pancreatic tumor samples). However, these results were not statistically significant, perhaps because of small sample sizes. Only miR-216 was significantly overexpressed in high-risk patients; therefore, this single miRNA discriminated low-risk patients from high-risk patients.

Unfortunately, authors have used divergent nomenclature to categorize IPMN lesions in various stages, which hampers comparisons between different studies. A schematic progression model of precursor lesions (PanINs and IPMNs) and miRNAs deregulated in each stage can be seen in Figure 1.

Differential Expression of miRNAs in PDAC

More than 90% of patients diagnosed with pancreatic cancer die from the disease,[55] and accurate diagnostic and prognostic biomarkers are absent. Therefore, identification of new biomarkers that provide informative data regarding diagnosis and prognosis, as well as elucidate important aspects of tumor biology, is valuable for providing appropriate patient management.

In 2006, Lee et al.[56] used qRT-PCR in an effort to provide insights into deregulation of miRNAs in pancreatic cancer. Consequently, they published the first study comparing expression patterns of miRNA precursors in PDAC tumors, adjacent benign tissues, CP specimens, normal pancreatic tissues, and pancreatic cancer cell lines. The expression profile of 201 miRNAs showed a specific pattern for each sample type, with tumors, normal tissues (obtained from normal pancreas),

A

References	Underexpressed	Overexpressed	Normal pancreas
Yu et al., 2012; Khan et al., 2014		miR-21, miR-155, miR-145, miR-182, miR-200a, miR-200b, miR-200c,	PanIN-I
Yu et al., 2012; Ryu et al., 2010; Riau et al., 2010; Khan et al., 2014	miR-296-5p	let-7, miR-21 miR-155, miR-145, miR-182, miR-200, miR-200a, miR-200b, miR-200c, miR-205, miR-221, miR-222	PanIN-II
Yu et al., 2012; Ryu et al., 2010; Riau et al., 2010; Khan et al., 2014	miR-125b miR-126, miR-218, miR-296-5p, miR-452	let-7, miR-18a miR-21, miR-155, miR-145, miR-182, miR-183*, miR-106b, miR-200, miR-205, miR-221, miR-222, miR-338-3p, miR-486-3p	PanIN-III

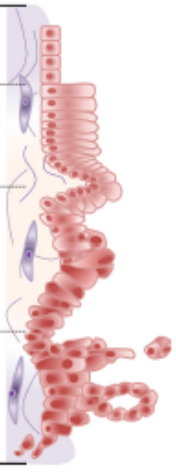


Figure 1. (Continued)

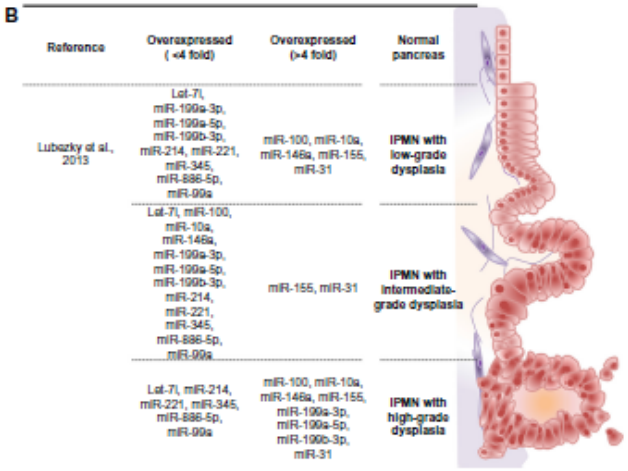


Figure 1. PanIN (A) and IPMN (B) progression models and corresponding miRNA differential expression. Normal duct cells (up) accumulate several histological and molecular abnormalities, which leads to an invasive PDAC (down).

and benign tissues (adjacent non-tumoral tissues, classified as normal tissues) profiled correctly (and differentially); a finding that reinforced the hypothesis that miRNA expression profiles may generate a unique molecular signature for each tissue. Although some miRNAs were exclusively expressed in pancreatic tumors cells (eg, miR-221, -376a, and -301), some of the most aberrantly expressed miRNAs (eg, miR-155, -21, -221) also showed abnormal expression in other cancer types. The correct categorization of different tissue types was also possible using a panel of 25 differentially expressed miRNAs (21 overexpressed and 4 underexpressed), as demonstrated by Bloomston et al.[57] These authors also showed that miRNA expression patterns could differentiate PDAC from normal pancreatic tissue and CP in 90% of all tested cases. A second panel containing 15 overexpressed and 8 underexpressed miRNAs differentiated PDAC tumors from CP with an accuracy of 93%. The authors also investigated whether absolute levels of miRNA expression could be applied to discriminate between short-term and long-term survivors with node-positive diseases, and statistical analyses revealed a subgroup of six overexpressed miRNAs in patients with long-term survival. Specifically, two miRNAs could be used to reliably predict survival: patients with increased miR196a-2 and miR-219 expression showed short-term survival (median survival of 14.3 and 13.6 months, respectively), whereas patients with low expression of these miRNAs showed long-term survival (26.5 and 23.8 months, respectively). It is interesting to note that nodal status, T stage, and histologic grade were not predictive of survival, which reinforces the specific usefulness of miRNA profiles in diagnosis and prognostic analyses. By using a set of 94 miRNAs, Szafranska et al.[58] found that the global miRNA expression in CP tissues is intermediate between normal and PDAC tissues, and they showed that expression of this panel clearly distinguished PDAC from normal and CP tissues. This study aimed to establish a pancreatic miRNome, ie, the first comprehensive miRNA expression profile in normal pancreatic tissue, CP, and PDAC. The authors found that 84 miRNAs were differentially expressed between normal pancreatic tissues and PDAC samples. Among these miRNAs, 41 were downregulated and 32 were upregulated (at least two-fold), while 11 other miRNAs were strongly enriched in PDAC.

Zhang et al.[59] analyzed expression levels of 95 miRNAs (chosen based on their involvement in cancer biology, cell development, and apoptosis) not only in PDAC and normal pancreatic tissues but also in pancreatic cell lines. They demonstrated that expression patterns in pancreatic cancer tissues and pancreatic cancer cell lines were significantly different from those observed in a normal human pancreatic ductal epithelial cell line and normal pancreatic tissues obtained from patients. They also showed that each pancreatic cancer tissue and cell type presented a unique profiling pattern, which indicated that significant intertumoral heterogeneity exists. This study identified several novel miRNAs associated with pancreatic cancer and revealed that eight miRNAs (miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95) were significantly upregulated in most PDAC tumors from patient samples and cell lines.

A meta-analysis conducted by Ding et al.[60] corroborated the findings of most published articles when it revealed that multiple-miRNA profiling assays are more accurate than single-miRNA profiling assays for diagnosing PDAC (AUC 0.92% and 0.82%, respectively).

Several recent studies have demonstrated the deregulation of miRNA expression in pancreatic cancer, which highlights their utility as diagnostic and prognostic markers. Different methodologies have been used to assess miRNA expression, including northern blotting,[61] qRT-PCR assays developed for the amplification of precursor molecules,[62] miRNA array,[63] modified Invader assays,[64] qRT-PCR developed to assay mature miRNA[65] (either by using TaqMan or SYBR Green strategies), and analysis of RNA-seq data.[66]

Regardless of the methodology used, certain miRNAs appear to have a more central role in pancreatic carcinogenesis. For example, miR-21 is consistently reported as being overexpressed in PDAC,[67,68] and studies suggest that this miRNA contributes to cell proliferation, invasion, and chemoresistance in pancreatic cancer.[69] Indeed, PDAC patients whose tumors present low miR-21 expression have a better response to adjuvant treatment,[70] and lymph node-negative patients with high (tumoral) miR-21 expression levels have shorter survival times than similar patients with low miR-21 expression levels.[67] Similar results have been reported when progression-free survival was analyzed.[68]

miR-34a is an important component of the p53 pathway and acts as a tumor suppressor; it is frequently underexpressed in PDAC cell lines,[71–73] and its expression is known to decrease according to several mechanisms.[71,74,75] In a p53-mutant pancreatic cancer cell line, restoration of miR-34a expression restored the tumor-suppressing function of p53 and sensitized the tumor cells to chemotherapy and radiation.[76] Recent studies in PDAC patients showed that patients with high miR-34a expression levels had a better survival following resection with curative intent.[77]

Overexpression of miR-155 is common in several different tumor types,[25,78,79] and this miRNA was found to be upregulated by approximately three-fold in 11 of 21 PDAC cell lines.[80] One of its targets, the p53-regulated stress-induced gene *TP53INP1*, is downregulated in PDAC and correlates with tumor progression.[81] High miR-155 expression levels are observed in the tumors of patients with worse survival curves.[82]

Several other miRNAs have been associated with pancreatic carcinogenesis and are briefly discussed here. miR-196a has been shown to be virtually absent in normal pancreatic tissue, but it increased by 14-fold in PDAC tissues and cell lines. In addition, miR-196a expression levels correlated well with survival and disease progression, with levels already altered in PanINs and in all other stages up to PDAC.[57,83] miR-200b is overexpressed in 70% of all PDAC cases; levels increased up to 43-fold in PDAC tissues relative to non-tumoral pancreatic tissues.[59] The miR-200 family of miRNAs mainly targets two transcription factors, ZEB1 and ZEB2, which negatively regulate E-cadherin; this ultimately promotes progression of the epithelial-to-mesenchymal transition (EMT), which ensures mobility to tumor cells and favors metastasis.[84,85] Khan et al.[38] showed (through an invasion assay) that miR-145 reduces the amount of invading cells and is also able to enhance the effects of gemcitabine in PDAC cell lines. miR-145, which negatively regulates the MUC13 protein (which increases tumorigenic cell signaling pathways in PDAC[86]), is virtually absent in pancreatic cancer tissues, but it is highly expressed in normal pancreatic cells. miR-125b is overexpressed in a gemcitabine-resistant pancreatic cancer cell line (BxPC3-GZR) and advanced PDAC tumor tissues, and its suppression also causes a partial reversal of the mesenchymal phenotype and increases the response to gemcitabine treatment in BxPC3-GZR and PANC-1 cell lines.[66] Finally, deregulation of another miRNA, miR-376a, in PDAC was first described in 2006 when its expression was shown to be increased by seven-fold in PDAC tissues. The exclusive expression of this miRNA in pancreatic tumor cells (it was not found in benign pancreatic acini or stromal cells) suggested that it could be a good PDAC biomarker.[56] Years later, overexpression of miR-376a was also observed in rats in a study that showed that serum levels of this miRNA reflected tumoral status.[87]

Circulating miRNAs as Biomarkers in PDAC

It is often difficult to diagnose pancreatic cancer because of limited access and the need for invasive diagnostic procedures; thus, substantial research effort has been applied to the identification of diagnostic and prognostic biomarkers in circulating samples. In 2008, two different studies showed the presence of miRNAs in serum and plasma samples, thereby highlighting the potential of miRNAs as minimally invasive biomarkers for diagnostic as well as prognostic purposes.[88,89] In addition, Mitchell et al.[7] demonstrated that miRNAs are stable in plasma because they are protected against endogenous RNase activity. In an experiment with prostate cancer xenografts, miRNAs were shown to originate from tumors and enter circulation, where their expression levels could be used to robustly distinguish xenografted mice from controls. In humans, serum levels of miR-141 distinguished patients with prostate cancer from healthy controls, and this finding led the authors to propose that measurement of tumor-derived miRNAs in serum or plasma would be a useful method for blood-based detection of human cancer. Several more recent studies have demonstrated that circulating miRNAs are included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles (up to 1 µm), and exosomes (small membrane vesicles of endocytic origin, 50–100 nm in size), or protected by Argonaute protein 2.[90–92] Therefore, they are highly stable and could be used as biomarkers for different tumor types,[93] and different sampling strategies could be employed, including minimally invasive procedures to obtain serum and plasma and noninvasive procedures to obtain samples such as saliva.[94]

In PDAC, miRNAs were reported as useful circulating biomarkers for the first time in 2009 when Wang et al demonstrated that a panel of four miRNAs (miR-21, -210, -155, and -196a) overexpressed in PDAC tissues could also be detected in the plasma of PDAC patients and that these

miRNAs clearly distinguished patients with and without cancer (sensitivity of 64% and specificity of 89%).[95] Another study measured the plasmatic levels of miR-210, (which increase in hypoxia situations[96]) and found that expression of this miRNA significantly increased (four-fold) in PDAC patients relative to controls, confirming the potential of miRNAs as diagnostic biomarkers in PDAC.[97] miR-21, miR-155, and miR-196a were also evaluated by Kong et al.[98], who investigated a larger panel of miRNAs (miR-21, -155, -196a, -181a, -181b, -221, and -222). Using serum samples, they found that miR-21, -155, and -196a could be used to correctly discriminate PDAC patients from controls. Circulating levels of miR-196a were also shown to predict prognosis: patients with unresectable PDAC (stages III and IV) had significantly higher miR-196a levels, whereas patients with low miR-196a expression levels were in the early stages of the disease (stages I and II). Finally, serum miR-196a levels were found to be a good predictor of median survival time in PDAC patients; patients with high-level miR-196a expression had a median survival of 6.1 months, whereas patients with low miR-196a levels had a median survival of 12 months. Morimura et al.[99] evaluated miRNA expression levels in the plasma and tissue of the same patients. They analyzed the expression levels of the miR-17–92 cluster in the tissue and plasma of PDAC patients and healthy controls and found that, within the cluster and considering concordant expression levels in plasma and tissue, miR-18a expression distinguished patients with and without the disease.

In a large study performed in 2012, Liu et al.[100] identified 44 overexpressed miRNAs and 19 underexpressed miRNAs in the serum of PDAC patients (where expression was compared with age- and sex-matched cancer-free controls). Surprisingly, some of the miRNAs reported as deregulated in this study were different from those described in previous studies that analyzed circulating samples from PDAC patients. Among these miRNAs, only seven (miR-20a, -21, -24, -25, -99a, -185, and -191) were expressed at significantly different levels in the serum of PDAC patients versus controls, and these could be used to distinguish cancer-affected patients from CP patients. Patients who expressed high levels of miR-21 had a lower survival rate than those with low miR-21 expression levels. In addition, a prospective study used this seven-miRNA panel to screen and follow 55 clinically suspected cases of PDAC across several months. With the pathological diagnosis considered as a standard, the seven-miRNA panel could be employed to identify PDAC patients with an accuracy of 83.6%, which was significantly higher than the accuracy of CA19-9 (56.4%), the only currently available peripheral biomarker for PDAC, and CEA (36.4%) for the same sample set. In the same year, another group also described plasmatic levels of two miRNAs (miR-196a and miR-16) as early and independent biomarkers for PDAC, more effective than CA19-9 for early diagnosis. Furthermore, the combination of miRNAs with CA19-9 significantly improved the diagnostic accuracy of the miRNA panel or CA19-9 alone.[101]

In contrast to these previous studies, Cote et al.[102] reported that miR-21 expression levels in plasma were not significantly different between PDAC patients and healthy controls. Through measurement of 10 candidate miRNAs in plasma and bile aspirates of PDAC patients, CP patients, and healthy controls, the authors identified a panel of five miRNAs (miR-10b, miR-155, miR-160b, miR-30c, and miR-212) that could distinguish cases from controls with accuracy in both sample types. When considering only plasma samples, seven miRNAs (miR-10b, -30c, -106b, -155, -181b, -196a, and -212) showed significantly different expression across all three groups and between individuals with PDAC and CP.

Although the use of miRNA panels seems to be the most effective approach to diagnosis and prognosis, some individual miRNAs also show potential. For example, circulating levels of miR-182 were shown to be increased in PDAC patients compared to those in patients with CP and healthy controls, and this trend was significantly associated with advanced clinical stages and lymph node metastases. Moreover, this single miRNA was able to predict outcome after pancreatectomy; PDAC patients with high levels of miR-182 had shorter disease-free survival and overall survival compared with those of patients with low miR-182 levels.[103] A recent study from our group[104] also revealed that the expression of two miRNAs (miR-21 and miR-34a) in serum samples could individually discriminate PDAC and healthy controls. Indeed, when both variables were considered together, only a discrete improvement was observed.

In the last few years, a growing number of researchers have employed high-throughput methodologies to analyze hundreds of miRNAs and collect data that are considered more robust. One of the first studies to achieve this was a multicenter study in which 245 samples (129 tissue and 116 blood samples) from PDAC patients, CP patients, and healthy controls were assessed for the expression of 863 miRNAs using the Geniom Biochip miRNA *Homo sapiens*. In blood samples, 87 miRNAs differentiated PDAC patients from healthy controls, whereas 18 miRNAs distinguished CP patients from controls. Interestingly, the study did not identify a single circulating miRNA that could be used to distinguish between PDAC and CP patients.[105] In the second study with a case–control design, 754 miRNAs were analyzed in the whole blood of 409 PDAC patients, 25 CP patients, and 312 healthy controls (organized into discovery, training, and validation cohorts) with the aim of finding an miRNA panel suitable for diagnosis of PDAC. Using a TaqMan human microRNA assay to access miRNA expression, two diagnostic panels (indexes I and II) were created that comprised 4 (miR-145, miR-150, miR-223, and miR-636) and 10 (miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, and miR-885-5p) miRNAs, respectively. Both panels provided satisfactory results, and when they were combined with CA19-9, index I had an AUC of 0.94 (85% sensitivity and 98% specificity) and index II had an AUC of 0.93 (85% sensitivity and 90% specificity). Furthermore, the

AUC for the combination of CA19-9 and miRNA panels was significantly higher than that for CA19-9 used alone.[106]

Finally, Lin et al.[107] analyzed differential expression of 1,711 miRNAs in the serum of six PDAC patients and six controls, and they demonstrated that 22 miRNAs were upregulated, while 23 were downregulated. Among these miRNAs, they chose the eight most deregulated (upregulated: miR-1238, miR-296–3p, miR-4290, and miR-483-5p; downregulated: miR-1280, miR-492, miR-595, and miR-663a) for validation in a larger cohort of 49 PDAC and 27 control serum samples. Receiver operating characteristic curve analysis showed that miR-492 and miR-663a yielded the largest AUCs, 0.787 and 0.870, respectively. In an analysis that was even more comprehensive, Kojima et al.[108] performed a 3D-Gene assay, ie, a sensitive microarray developed to detect 2,555 human miRNAs registered in the miRBase (release 20; http://www.mirbase.org/), using 100 serum samples from PDAC patients and 150 from healthy controls, in addition to other digestive cancers samples. Notwithstanding the finding that miR-6836-3p showed high sensitivity and specificity when used alone (89.4% and 82.3%, respectively), the authors found that a combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) had an even higher sensitivity (80.3%) and specificity (97.6%). As expected, CA19-9 and CEA showed lower sensitivities (65.6% and 40%, respectively) and specificities (92.9% and 88.6%, respectively) in the same sample cohort. These results suggest that the assessment of miRNA expression levels, when miRNAs are used alone or organized into different panel types, is clinically valuable for identifying patients with pancreatic tumors who could benefit from surgical intervention, chemotherapy, or radiotherapy.

Besides plasma and serum biomarkers, some authors have focused on salivary biomarkers because saliva is composed of a complex combination of enzymes, hormones, antibodies, etc., which makes saliva samples as informative as blood in certain clinical situations.[109] Moreover, obtaining a saliva sample is noninvasive and involves low cost, and several studies have shown that salivary molecules can be useful as cancer biomarkers.[110–113] In 2010, Zhang et al.[114] demonstrated that a panel of four salivary mRNAs (KRAS, MBD3L2, ACRV1, and DPM1) distinguished PDAC patients from CP patients and healthy controls with 90% sensitivity and 95% specificity. More recent studies have shown that miRNAs can be detected in saliva samples and used as biomarkers.[94,115] Indeed, Xie et al.[116] performed a pilot study using the saliva samples of eight PDAC patients and eight healthy controls, which were analyzed with the human miRNA microarray (Agilent; capable of profiling the expression of 2,006 miRNAs). Considering certain criteria, the authors chose the 10 most deregulated miRNAs and tested them by using qPCR in the same cohort. Among the miRNAs, miR-3679-5p and miR-940 showed significant differential expression between the two groups, and when these were assayed in a larger cohort, miR-3679-5p was found to be downregulated and miR-940 upregulated in PDAC patients compared with expression levels in healthy controls. Both miRNAs reached satisfactory sensitivities (82.5% and 90%, respectively) but not specificities (45% and 40%, respectively). In a recent study, miRNA expression was assayed in saliva samples and similar issues with specificity were not observed; specifically, miR-21, miR-23a, miR-23b, and miR-29c were significantly upregulated in PDAC patients compared with controls, and their specificity was 100%, while their sensitivities were variable (71.4%, 85.7%, 85,7%, and 57%, respectively).[117] Another pilot study using salivary samples did not obtain statistically significant results and found (using qRT-PCR) high Ct values, indicating an almost undetectable expression of the miRNAs assayed.[104]

Conclusions

Usually, early stage pancreatic cancer is a silent disease; it becomes apparent only after tumor invasion of surrounding tissues or metastatic seeding of distant organs. Suspected PDAC cases are primarily identified through imaging (eg, ultrasonography, computed tomography, and magnetic resonance), but this approach only identifies a pancreatic mass, which could be related to nonmalignant pancreatic disorders. Thus, imaging is usually insufficient for providing the final diagnosis of malignancy, CP, or other benign pancreatic lesions. Definitive diagnosis is usually obtained through invasive procedures, such as biopsies, which are usually accomplished by endoscopic ultrasonography, with diagnostic confirmation only possible in some cases after a laparotomy; thus, the definitive diagnosis of PDAC or pancreatic precursor lesions is almost impossible when using only noninvasive procedures.[118,119]

The biomarkers most commonly used in clinical settings, CEA and CA19-9, are not sufficiently accurate for the detection of pancreatic cancer, but they can be useful in follow-ups to assess disease progression in patients who have been already diagnosed. Therefore, the identification of highly sensitive and specific diagnostic and/or prognostic biomarkers is important for avoiding multiple surgical procedures and helping to distinguish, without invasive procedures, patients with and without cancer as well as patients with different premalignant lesions.

Several different approaches have revealed a number of miRNAs that could be used as biomarkers in PDAC, but it is important to also highlight some limitations. Only a few miRNAs have been appropriately validated in independent cohorts or investigated for their function in carcinogenesis. The wide variety of analytical methods employed in different studies is a barrier to comparing existing published results, a fact that reinforces the need to confirm the differential expression of miRNAs encountered in distinct populations. In some studies, sample size is also an issue, and the low prevalence of pancreatic cancer and its precursor lesions should stimulate efforts to conduct more collaborative projects. Furthermore, the use of circulating miRNAs as biomarkers is also limited by their heterogeneous origin. Blood and endothelial cells are

Table 1. Circulating miRNAs deregulated in PDAC patients.

ORIGIN	miRNA	REGULATION	REFERENCE
Serum	miR-125a-3p, miR-492, miR-595, miR-663a, miR-1280, miR-4294, miR-4476, miR-4530, miR-6880-5p	Down	Lin et al, 2014; Kojima et al, 2015
	miR-21, miR-155, miR-196a, miR-296-3p, miR-483-5p, miR-1238, miR-4290, miR-6075	Up	Kong et al, 2011; Lin et al, 2014; Kojima et al, 2015
Plasma	miR-10b, miR-16, miR-18a, miR-20a, miR-21, miR-24, miR-25, miR-30c, miR-99a, miR-106b, miR-155, miR-181b, miR-182, miR-185, miR-191, miR-196a, miR-210, miR-212	Up	Wang et al, 2007; Ho et al, 2010; Morimura et al, 2011; Liu R et al, 2012; Liu J et al, 2012; Cote et al, 2014; Chen et al, 2014
Whole Blood	miR-31, miR-31*, miR-93, miR-126*, miR-150, miR-663a, miR-935,	Down	Schultz et al, 2014
	miR-34a, miR-122, miR-145, miR-199b-5p, miR-582-3p, miR-769-5p, miR-885-5p	Up	Schultz et al, 2014
Saliva	miR3679-5p	Down	Xie et al, 2015
	miR-21, miR-23a, miR-23b, miR-29c, miR-940	Up	Xie et al, 2015; Humeau et al, 2015

the origin of most of the cell-free miRNAs in the blood, and this could be a confounding factor in the search for tumor-specific miRNAs. Indeed, circulating miRNAs are only stable (ie, protected against RNase activity) because of their association with other structures (eg, apoptotic bodies, exosomes, high-density lipoprotein vesicles, and mainly proteins from the Argonaute family[90–92,120]). This primordial interaction adds another bias if we consider that differences in miRNA expression levels could be because of differential degradation in different environments. The future perspectives in the miRNA field should focus on overcoming these limitations to find a robust panel of miRNAs for clinical use.

As shown in this review, miRNAs, considered either individually or in panels, are key players in a wide variety of applications. Although making robust comparisons is difficult because of the different analytical approaches used to date, some miRNAs have been consistently reported as biomarkers, either for diagnostic or prognostic purposes. For example, miR-21, miR-155, and miR-196 can be used to distinguish between normal, premalignant, and PDAC tissues in a tissue-approach, and they discriminated healthy controls from PDAC patients in serum and plasma samples in more than one independent study. Other deregulated miRNAs in different sample types are shown in Table 1. Despite the aforementioned limitations, substantial progress has been made toward the discovery of novel diagnostic and prognostic PDAC biomarkers, and miRNAs show particular promise in this field.

Author Contributions

Conceived and designed the experiments: BA. Analyzed the data: BA, CG. Wrote the first draft of the manuscript: BA, CG. Contributed to the writing of the manuscript: BA, CG, PAP. Agree with manuscript results and conclusions: BA, CG, PAP. Jointly developed the structure and arguments for the paper: BA, CG, PAP. Made critical revisions and approved final version: BA, CG, PAP. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015;65(1):5–29.
2. Rhim AD, Mirek ET, Aiello NM, et al. EMT and dissemination precede pancreatic tumor formation. *Cell.* 2012;148(1–2):349–61.
3. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet.* 2004;363(9414):1049–57.
4. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science.* 2009;324(5933):1457–61.
5. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med.* 2014;371(22):2140–1.
6. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature.* 2005;435(7043):834–8.
7. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A.* 2008;105(30):10513–8.
8. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell.* 1993;75(5):843–54.
9. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell.* 1993;75(5):855–62.
10. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science.* 2001;294(5543):853–8.
11. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science.* 2001;294(5543):862–4.
12. Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer.* 2006;6(4):259–69.
13. Nilsen TW. Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet.* 2007;23(5):243–9.
14. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116(2):281–97.
15. Wahlstedt M, Liechti A, Halbert J, Valloton D, Kaessmann H. Conserved microRNA editing in mammalian evolution, development and disease. *Genome Biol.* 2014;15(6):R83.
16. Gong J, Wu Y, Zhang X, et al. Comprehensive analysis of human small RNA sequencing data provides insights into expression profiles and miRNA editing. *RNA Biol.* 2014;11(11):1375–85.
17. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* 2002;99(24):15524–9.
18. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A.* 2005;102(39):13944–9.

19. Calin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci U S A.* 2004;101(32):11755–60.
20. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA. 2007;13(10):1668–74.
21. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A.* 2006;103(18):7024–9.
22. Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. *Cancer Lett.* 2009;285(2):116–26.
23. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100(1):57–70.
24. Olson P, Lu J, Zhang H, et al. MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer. *Genes Dev.* 2009;23(18):2152–65.
25. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006;103(7):2257–61.
26. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol.* 2008;3:157–88.
27. Hruban RH, Wilentz RE, Kern SE. Genetic progression in the pancreatic ducts. *Am J Pathol.* 2000;156(6):1821–5.
28. Schneider G, Siveke JT, Eckel F, Schmid RM. Pancreatic cancer: basic and clinical aspects. *Gastroenterology.* 2005;128(6):1606–25.
29. Yu J, Li A, Hong SM, Hruban RH, Goggins M. MicroRNA alterations of pancreatic intraepithelial neoplasias. *Clin Cancer Res.* 2012;18(4):981–92.
30. Slater EP, Strauch K, Rospleszcz S, et al. MicroRNA-196a and -196b as potential biomarkers for the early detection of familial pancreatic cancer. *Transl Oncol.* 2014;7(4):464–71.
31. Ryu JK, Hong SM, Karikari CA, Hruban RH, Goggins MG, Maitra A. Aberrant microRNA-155 expression is an early event in the multistep progression of pancreatic adenocarcinoma. *Pancreatology.* 2010;10(1):66–73.
32. Xue Y, Abou Tayoun AN, Abo KM, et al. MicroRNAs as diagnostic markers for pancreatic ductal adenocarcinoma and its precursor, pancreatic intraepithelial neoplasm. *Cancer Genet.* 2013;206(6):217–21.
33. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. *J Cell Mol Med.* 2009;13(1):39–53.
34. Torrisani J, Bournet B, du Rieu MC, et al. Let-7 microRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression. *Hum Gene Ther.* 2009;20(8):831–44.
35. Ali S, Almhanna K, Chen W, Philip PA, Sarkar FH. Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. *Am J Transl Res.* 2010;3(1):28–47.
36. Ali S, Saleh H, Sethi S, Sarkar FH, Philip PA. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. *Br J Cancer.* 2012;107(8):1354–60.
37. du Rieu MC, Torrisani J, Selves J, et al. MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. *Clin Chem.* 2010;56(4):603–12.
38. Khan S, Ebeling MC, Zaman MS, et al. MicroRNA-145 targets MUC13 and suppresses growth and invasion of pancreatic cancer. *Oncotarget.* 2014;5(17):7599–609.
39. Hruban RH, Takaori K, Klimstra DS, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am J Surg Pathol.* 2004;28(8):977–87.
40. Adsay NV, Fukushima N, Furukawa T, et al. Intraductal neoplasm of the pancreas. Bosman FT, Carneiro F, Hruban RH, Theise ND, Editors. *WHO Classification of Tumors of the Digestive System.* 4th ed. Lyon: WHO Press; 2010:304–13.
41. Fukushima N, Fukayama M. Mucinous cystic neoplasms of the pancreas: pathology and molecular genetics. *J Hepatobiliary Pancreat Surg.* 2007;14(3):238–42.
42. Zamboni G, Fukushima N, Hruban RH, Kloppel G. Mucinous cystic neoplasms. Bosman FT, Carneiro F, Hruban RH, Theise ND, Editors. *WHO Classification of Tumors of the Digestive System.* 4th ed. Lyon: WHO Press; 2010:300–3.
43. Habbe N, Koorstra JB, Mendell JT, et al. MicroRNA miR-155 is a biomarker of early pancreatic neoplasia. *Cancer Biol Ther.* 2009;8(4):340–6.
44. Caponi S, Funel N, Frampton AE, et al. The good, the bad and the ugly: a tale of miR-101, miR-21 and miR-155 in pancreatic intraductal papillary mucinous neoplasms. *Ann Oncol.* 2013;24(3):734–41.
45. Nakahata O, Takamori H, Iwatsuki M, et al. Carcinogenesis of intraductal papillary mucinous neoplasm of the pancreas: loss of microRNA-101 promotes overexpression of histone methyltransferase EZH2. *Ann Surg Oncol.* 2012;19(suppl 3):S565–71.
46. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J.* 2003;22(20):5323–35.
47. Jiao LR, Frampton AE, Jacob J, et al. MicroRNAs targeting oncogenes are down-regulated in pancreatic malignant transformation from benign tumors. *PLoS One.* 2012;7(2):e32068.
48. Ryu JK, Matthaei H, Dal Molin M, et al. Elevated microRNA miR-21 levels in pancreatic cyst fluid are predictive of mucinous precursor lesions of ductal adenocarcinoma. *Pancreatology.* 2011;11(3):343–50.
49. Henry JC, Bassi C, Giovinazzo F, Bloomston M. MicroRNA from pancreatic duct aspirate differentiates cystic lesions of the pancreas. *Ann Surg Oncol.* 2013;20(suppl 3):S661–6.
50. Lee LS, Szafranska-Schwarzbach AE, Wylie D, et al. Investigating microRNA expression profiles in pancreatic cystic neoplasms. *Clin Transl Gastroenterol.* 2014;5:e47.
51. Matthaei H, Wylie D, Lloyd MB, et al. miRNA biomarkers in cyst fluid augment the diagnosis and management of pancreatic cysts. *Clin Cancer Res.* 2012;18(17):4713–24.
52. Lubezky N, Loewenstein S, Ben-Haim M, et al. MicroRNA expression signatures in intraductal papillary mucinous neoplasm of the pancreas. *Surgery.* 2013;153(5):663–72.
53. Permuth-Wey J, Chen YA, Fisher K, et al. A genome-wide investigation of microRNA expression identifies biologically-meaningful microRNAs that distinguish between high-risk and low-risk intraductal papillary mucinous neoplasms of the pancreas. *PLoS One.* 2015;10(1):e0116869.
54. Wang J, Paris PL, Chen J, et al. Next generation sequencing of pancreatic cyst fluid microRNAs from low grade-benign and high grade-invasive lesions. *Cancer Lett.* 2015;356(2 pt B):404–9.
55. Neuzillet C, Sauvanet A, Hammel P. Prognostic factors for resectable pancreatic adenocarcinoma. *J Visc Surg.* 2011;148(4):e232–43.
56. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer.* 2007;120(5):1046–54.
57. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA.* 2007;297(17):1901–8.
58. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene.* 2007;26(30):4442–52.
59. Zhang Y, Li M, Wang H, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg.* 2009;33(4):698–709.
60. Ding Z, Wu H, Zhang J, Huang G, Ji D. MicroRNAs as novel biomarkers for pancreatic cancer diagnosis: a meta-analysis based on 18 articles. *Tumour Biol.* 2014;35(9):8837–48.
61. Wang X, Zhao J, Huang J, Tang H, Yu S, Chen Y. The regulatory roles of miRNA and methylation on oncogene and tumor suppressor gene expression in pancreatic cancer cells. *Biochem Biophys Res Commun.* 2012;425(1):51–7.
62. Schmittgen TD, Jiang J, Liu Q, Yang L. A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res.* 2004;32(4):e43.
63. Babak T, Zhang W, Morris Q, Blencowe BJ, Hughes TR. Probing microRNAs with microarrays: tissue specificity and functional inference. *RNA.* 2004;10(11):1813–9.
64. Allawi HT, Dahlberg JE, Olson S, et al. Quantitation of microRNAs using a modified invader assay. *RNA.* 2004;10(7):1153–61.
65. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res.* 2005;33(17):5394–403.
66. Bera A, VenkataSubbaRao K, Manoharan MS, Hill P, Freeman JW. A miRNA signature of chemoresistant mesenchymal phenotype identifies novel molecular targets associated with advanced pancreatic cancer. *PLoS One.* 2014;9(9):e106343.
67. Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg.* 2008;12(12):2171–6.
68. Giovannetti E, Funel N, Peters GJ, et al. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res.* 2010;70(11):4528–38.
69. Moriyama T, Ohuchida K, Mizumoto K, et al. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. *Mol Cancer Ther.* 2009;8(5):1067–74.
70. Hwang JH, Voortman J, Giovannetti E, et al. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS One.* 2010;5(5):e10630.
71. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell.* 2007;26(5):745–52.
72. Tarasov V, Jung P, Verdoodt B, et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle.* 2007;6(13):1586–93.
73. He-meking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ.* 2010;17(2):193–9.
74. Calhoun ES, Hucl T, Gallmeier E, et al. Identifying allelic loss and homozygous deletions in pancreatic cancer without matched normals using high-density single-nucleotide polymorphism arrays. *Cancer Res.* 2006;66(16):7920–8.
75. Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle.* 2008;7(16):2591–600.

76. Ji Q, Hao X, Zhang M, et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One*. 2009;4(8):e6816.
77. Jamieson NB, Morran DC, Morton JP, et al. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. *Clin Cancer Res*. 2012;18(2):534–45.
78. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102(52):19075–80.
79. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A*. 2005;102(10):3627–32.
80. Kent OA, Mullendore M, Wentzel EA, et al. A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells. *Cancer Biol Ther*. 2009;8(21):2013–24.
81. Gironella M, Seux M, Xie MJ, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A*. 2007;104(41):16170–5.
82. Greither T, Grochola LF, Udelnow A, Lautenschläger C, Würl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. *Int J Cancer*. 2010;126(1):73–80.
83. Szafranska AE, Doleshal M, Edmunds HS, et al. Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. *Clin Chem*. 2008;54(10):1716–24.
84. Li Y, VandenBoom TG 2nd, Kong D, et al. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res*. 2009;69(16):6704–12.
85. Koepal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem*. 2008; 283(22):14910–4.
86. Chauhan SC, Ebeling MC, Maher DM, et al. MUC13 mucin augments pancreatic tumorigenesis. *Mol Cancer Ther*. 2012;11(1):24–33.
87. Yabushita S, Fukamachi K, Tanaka H, et al. Circulating microRNAs in serum of human K-ras oncogene transgenic rats with pancreatic ductal adenocarcinomas. *Pancreas*. 2012;41(7):1013–8.
88. Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008;141(5):672–5.
89. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18(10):997–1006.
90. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One*. 2012;7(3):e30679.
91. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*. 2010;101(10):2087–92.
92. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011;108(12):5003–8.
93. Albulescu R, Neagu M, Albulescu L, Tanase C. Tissular and soluble miRNAs for diagnostic and therapy improvement in digestive tract cancers. *Expert Rev Mol Diagn*. 2011;11(1):101–20.
94. Patel RS, Jakymiw A, Yao B, et al. High resolution of microRNA signatures in human whole saliva. *Arch Oral Biol*. 2011;56(12):1506–13.
95. Wang J, Chen J, Chang P, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)*. 2009;2(9):807–13.
96. Kulshreshtha R, Ferracin M, Wojcik SE, et al. A microRNA signature of hypoxia. *Mol Cell Biol*. 2007;27(5):1859–67.
97. Ho AS, Huang X, Cao H, et al. Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. *Transl Oncol*. 2010;3(2):109–13.
98. Kong X, Du Y, Wang G, et al. Detection of differentially expressed microRNAs in serum of pancreatic ductal adenocarcinoma patients: miR-196a could be a potential marker for poor prognosis. *Dig Dis Sci*. 2011;56(2):602–9.
99. Morimura R, Komatsu S, Ichikawa D, et al. Novel diagnostic value of circulating miR-18a in plasma of patients with pancreatic cancer. *Br J Cancer*. 2011;105(11):1733–40.
100. Liu R, Chen X, Du Y, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem*. 2012;58(3):610–8.
101. Liu J, Gao J, Du Y, et al. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer*. 2012;131(3):683–91.
102. Cote GA, Gore AJ, McElyea SD, et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. *Am J Gastroenterol*. 2014;109(12):1942–52.
103. Chen Q, Yang L, Xiao Y, Zhu J, Li Z. Circulating microRNA-182 in plasma and its potential diagnostic and prognostic value for pancreatic cancer. *Med Oncol*. 2014;31(11):225.
104. Alemar B, Izetti P, Gregório C, et al. miRNA-21 and miRNA-34a are potential minimally invasive biomarkers for the diagnosis of pancreatic ductal adenocarcinoma. *Pancreas*. 2015.
105. Bauer AS, Keller A, Costello E, et al. Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One*. 2012;7(4):e34151.
106. Schultz NA, Dehlendorff C, Jensen BV, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA*. 2014;311(4):392–404.
107. Lin MS, Chen WC, Huang JX, Gao HJ, Sheng HH. Aberrant expression of microRNAs in serum may identify individuals with pancreatic cancer. *Int J Clin Exp Med*. 2014;7(12):5226–34.
108. Kojima M, Sudo H, Kawauchi J, et al. MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. *PLoS One*. 2015;10(2):e0118220.
109. Lee YH, Wong DT. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent*. 2009;22(4):241–8.
110. Franzmann EJ, Reategui EP, Carraway KL, Hamilton KL, Weed DT, Goodwin WJ. Salivary soluble CD44: a potential molecular marker for head and neck cancer. *Cancer Epidemiol Biomarkers Prev*. 2005;14(3):735–9.
111. Li Y, St John MA, Zhou X, et al. Salivary transcriptome diagnostics for oral cancer detection. *Clin Cancer Res*. 2004;10(24):8442–50.
112. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res*. 2000;6(6):2363–70.
113. Brooks MN, Wang J, Li Y, Zhang R, Elashoff D, Wong DT. Salivary protein factors are elevated in breast cancer patients. *Mol Med Rep*. 2008;1(3):375–8.
114. Zhang L, Farrell JJ, Zhou H, et al. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology*. 2010;138(3):e941–7.
115. Park NJ, Zhou H, Elashoff D, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res*. 2009;15(17):5473–7.
116. Xie Z, Yin X, Gong B, et al. Salivary microRNAs show potential as a noninvasive biomarker for detecting resectable pancreatic cancer. *Cancer Prev Res (Phila)*. 2015;8(2):165–73.
117. Humeau M, Vignolle-Vidoni A, Sicard F, et al. Salivary microRNA in pancreatic cancer patients. *PLoS One*. 2015;10(6):e0130996.
118. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362(17):1605–17.
119. Mayo SC, Austin DF, Sheppard BC, Mori M, Shipley DK, Billingsley KG. Evolving preoperative evaluation of patients with pancreatic cancer: does laparoscopy have a role in the current era? *J Am Coll Surg*. 2009;208(1):87–95.
120. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13(4):423–33.

ANEXO II

Termo de Consentimento Livre e Esclarecido (TCLE)

TCLE projeto 10-0162

ANEXO I

Termo de Consentimento Livre e Esclarecido - Casos

BIOMARCADORES EM CÂNCER DE PÂNCREAS: ESTUDOS GENÔMICOS E PROTEÔMICOS EM MATERIAL BIOLÓGICO

Pesquisadores: Patricia Izetti, Bárbara Alemar, Alessandro Bersch Osvaldt, Gabriel Macedo, Cleandra Gregório Silva, Úrsula Matte, Patrícia Ashton-Prolla

Consentimento Informado para Armazenamento de Material Biológico e Estudos Moleculares em Pacientes com Doenças do Pâncreas

O Grupo de Oncogenética e Grupo de Vias Biliares e Pâncreas do Hospital de Clínicas de Porto Alegre estão realizando um estudo para procurar novas alternativas para o diagnóstico de doenças do pâncreas. Nesse estudo serão avaliados materiais biológicos de pessoas com e sem doenças pancreáticas. Por meio deste estudo, convidamos pessoas com alterações no pâncreas a doar um ou mais dos seguintes materiais: sangue, tecido pancreático (uma pequena parte do pâncreas que poderá ser retirada durante a cirurgia) e saliva dos quais serão extraídos DNA, RNA e proteínas (material genético e proteômico). A partir do estudo deste material, pretendemos correlacionar os achados moleculares a fim de identificar marcadores associados ao desenvolvimento de doenças do pâncreas. Isso poderá contribuir para o estabelecimento de estratégias que visem a detecção e o manejo precoce dessas doenças.

Este material será estocado no Hospital de Clínicas de Porto Alegre e poderá ser utilizado para futuros estudos sobre fatores genéticos associados a diversas doenças do pâncreas. Os dados pessoais e clínicos constantes do prontuário dos participantes serão digitados em um banco de dados e mantidos sob sigilo. Todas as análises laboratoriais serão realizadas sem identificação nominal das pessoas. A quantidade de tecido pancreático e sangue doado para estes estudos não irão prejudicar sua saúde.

Rubrica do paciente

Se você desejar participar do estudo, quaisquer perguntas que você tiver em relação às coletas e aos procedimentos que serão feitos no futuro serão respondidas por membros da equipe de cirurgia que está lhe acompanhando ou por membros da equipe de pesquisa. Se você não desejar participar do estudo, a sua decisão não afetará seu acompanhamento médico ou cirúrgico no Hospital de Clínicas de Porto Alegre. Se você deseja participar e concorda em doar estes materiais para pesquisa, por favor, responda às perguntas a seguir:

Rubrica do pesquisador

Comitê de Ética em Pesquisa
GPPG/HCPA
VERSÃO APROVADA
29 / 03 / 2012
nº 100162

1. Você concorda que as suas amostras obtidas neste estudo sejam armazenadas e utilizadas neste estudo sobre doenças de pâncreas?

☐ sim ☐ não

2. Você quer ser informado sobre os resultados destes estudos?

☐ sim ☐ não

Se você respondeu "sim" a esta pergunta, será avisado sobre qualquer informação obtida neste estudo.

3. Se você respondeu "sim" à pergunta anterior, e por alguma razão estiver impossibilitado de receber estes resultados, você deseja que estes sejam transmitidos a alguma pessoa próxima a você?

☐ sim (*neste caso, indicar nome e telefone*_____)

☐ não

4. Você concorda que suas amostras sejam armazenadas para uso futuro em outras pesquisas não relacionadas a doenças do pâncreas?

☐ sim ☐ não

Se você respondeu "sim" a esta questão, será contatado para conceder ou não sua autorização para o uso do material biológico doado na realização de novos estudos, bem como o será para receber os resultados dos mesmos, caso assim deseje.

5. Você concorda que suas amostras sejam enviadas para centros de pesquisa no exterior, para realização de análises complementares?

☐ sim ☐ não

As amostras serão enviadas sem identificação, e os resultados obtidos não trarão benefício direto para você.

O armazenamento da amostra não implica em qualquer custo. O período de armazenamento é de 5 anos, mas seu consentimento pode ser retirado a qualquer momento se você mudar de idéia, e neste caso as suas amostras serão destruídas. Este documento não torna obrigatória a realização de nenhum dos procedimentos acima mencionados. Em nenhuma hipótese haverá quebra de sigilo quanto aos seus dados pessoais ou a liberação de amostras identificadas ou resultados para terceiros sem a sua autorização por escrito. Se publicados em revistas científicas, os resultados desse estudo serão apresentados de forma anônima, sem identificação dos participantes.

Rubrica do paciente

Rubrica do pesquisador

Comitê de Ética em Pesquisa
GPPG/HCPA
VERSÃO APROVADA
29 / 03 / 2012
nº 10A162

Para que se cumpram os efeitos legais, o presente documento composto de três páginas foi elaborado em duas vias de igual teor, ambas firmadas e rubricadas pelos envolvidos (paciente e pesquisador) abaixo identificados.

Declaro que li e compreendi as informações acima, e recebi uma via assinada e rubricada deste documento. Declaro, ainda, que tive minhas dúvidas esclarecidas por

_____ _____

Nome do entrevistador Assinatura do entrevistador

Ass. _____

Assinatura do paciente

Nome completo: _____

Data de nascimento: _____ Data: _____

Testemunha: _____

Nome e assinatura

Data: _____

Pesquisadores responsáveis: Em caso de dúvida os pesquisadores e o comitê de ética poderão fornecer esclarecimentos. Telefones para contato: Pesquisadores → Dr. Alessandro Osvaldt, Dra Patricia Ashton-Prolla, Dra. Patrícia Izetti e mestranda Bárbara Alemar: (51) 3359-7661

Comitê de Ética HCPA (51) 3359-8304.

Rubrica do paciente

Rubrica do pesquisador

Comitê de Ética em Pesquise
GPPG/HCPA
VERSÃO APROVADA
29 / 03 / 2012
002.100162

ANEXO III.

TCLE projeto 14-0526

Termo de Consentimento Livre e Esclarecido – Casos

CARACTERIZAÇÃO DA HETEROGENEIDADE GENÔMICA E EPIGENÔMICA DOS TUMORES DE PÂNCREAS E SUAS LESÕES PRECURSORAS

Pesquisadores: Alessandro Bersch Osvaldt, Bárbara Alemar, Cleandra Gregório Silva, Patrícia Ashton-Prolla

Consentimento Informado para Armazenamento de Material Biológico e Estudos Moleculares em Pacientes com Doenças do Pâncreas

O Laboratório de Medicina Genômica e Grupo de Vias Biliares e Pâncreas do Hospital de Clínicas de Porto Alegre estão realizando um estudo para melhorar o entendimento atual sobre as lesões pancreáticas e assim tentar encontrar novas alternativas para o diagnóstico e prognóstico de doenças do pâncreas. Nesse estudo serão avaliados materiais biológicos de pessoas com e sem doenças pancreáticas. Por meio deste estudo, convidamos pessoas com alterações no pâncreas a doar um ou mais dos seguintes materiais: sangue e tecido pancreático (uma pequena parte do pâncreas que poderá ser retirada durante a cirurgia, e outra fração do tecido, que fica emblocada em parafina no Departamento de Patologia deste Hospital), dos quais serão extraídos DNA, RNA e proteínas, que serão investigados através de análises moleculares. A partir do estudo deste material, pretendemos correlacionar os achados moleculares a fim de identificar marcadores associados ao desenvolvimento de doenças do pâncreas. Isso poderá contribuir para o estabelecimento de estratégias que visem a detecção e o manejo precoce dessas doenças.

Rubrica do paciente

Rubrica do pesquisador

Este material será estocado no Hospital de Clínicas de Porto Alegre e poderá ser utilizado para futuros estudos sobre fatores genéticos associados a diversas doenças do pâncreas. Os dados pessoais e clínicos constantes do prontuário dos participantes serão digitados em um banco de dados e mantidos sob sigilo. Todas as análises laboratoriais serão realizadas sem identificação nominal das pessoas. A quantidade de tecido pancreático e sangue doado para estes estudos não irão prejudicar sua saúde.

Se você desejar participar do estudo, quaisquer perguntas que você tiver em relação às coletas e aos procedimentos que serão feitos no futuro serão respondidas por membros da equipe de cirurgia que está lhe acompanhando ou por

membros da equipe de pesquisa. Se você não desejar participar do estudo, a sua decisão não afetará seu acompanhamento médico ou cirúrgico no Hospital de Clínicas de Porto Alegre. Se você deseja participar e concorda em doar estes materiais o a pesquisa, por favor, responda às perguntas a seguir:

1. Você concorda que as suas amostras obtidas neste estudo sejam armazenadas e utilizadas neste estudo sobre doenças de pâncreas?

☐ sim ☐ não

2. Você quer ser informado sobre os resultados destes estudos?

☐ sim ☐ não

Se você respondeu "sim" a esta pergunta, será avisado sobre qualquer informação obtida neste estudo.

3. Se você respondeu "sim" à pergunta anterior, e por alguma razão estiver impossibilitado de receber estes resultados, você deseja que estes sejam transmitidos a alguma pessoa próxima a você?

☐ sim (*neste caso, indicar nome e telefone*_____)

☐ não

4. Você concorda que suas amostras sejam armazenadas para uso futuro em outras pesquisas ?

☐ sim ☐ não

Se você respondeu "sim" a esta questão, será contatado para conceder ou não sua autorização para o uso do material biológico cedido na realização de novos estudos, bem como o será para receber os resultados dos mesmos, caso assim deseje. Caso não seja possível contatá-lo, este fato será resolvido junto ao Comitê de Ética em Pesquisa (CEP) do HCPA.Toda nova pesquisa a ser desenvolvida com o material armazenado será submetida para aprovação do CEP - HCPA e, quando for o caso, da Comissão Nacional de Ética em Pesquisa.

Rubrica do paciente

Rubrica do pesquisador

5. Você concorda que suas amostras sejam enviadas para centros de pesquisa no exterior, para realização de análises complementares?

☐ sim ☐ não

Você pode aceitar participar das demais partes do estudo sem, contudo, ter suas amostras retiradas do país. Ressaltamos que as amostras serão enviadas sem identificação, e os resultados obtidos não trarão benefício direto para você.

O armazenamento da amostra não implica em qualquer custo. O período de armazenamento é de 5 anos, mas seu consentimento pode ser retirado a qualquer momento se você mudar de idéia, e neste caso as suas amostras serão destruídas. Este documento não torna obrigatória a realização de nenhum dos procedimentos acima mencionados. Em nenhuma hipótese haverá quebra de sigilo quanto aos seus dados pessoais ou a liberação de amostras identificadas ou resultados para terceiros sem a sua autorização por escrito. Se publicados em revistas científicas, os resultados desse estudo serão apresentados de forma anônima, sem identificação dos participantes.

Para que se cumpram os efeitos legais, o presente documento composto de três páginas foi elaborado em duas vias de igual teor, ambas firmadas e rubricadas pelos envolvidos (paciente e pesquisador) abaixo identificados.

Declaro que li e compreendi as informações acima, e recebi uma via assinada e rubricada deste documento. Declaro ainda que tive minhas dúvidas esclarecidas por

_____ _____

Nome do entrevistador Assinatura do entrevistador

Ass. _____

Assinatura do paciente

Nome completo: _____

Data de nascimento: _____ Data: _____

Pesquisadores responsáveis: Em caso de dúvida os pesquisadores e o comitê de ética poderão fornecer esclarecimentos. Telefones para contato: Pesquisadores →Alessandro Osvaldt, Bárbara Alemar, Cleandra Gregório, Patricia Ashton-Prolla,: (51) 3359-7661

Comitê de Ética HCPA (51) 3359-8304.

ANEXO IV.

Cartas de aprovação do projeto – CEP HCPA

Porto Alegre, 21 de janeiro de 2013.

Ao GPPG

Exmo. Coordenador

Prof. Flávio Kapczinski

RE: Emenda ao Projeto GPPG nº 10-0162 - "BIOMARCADORES EM CÂNCER DE PÂNCREAS: ESTUDOS GENÔMICOS E PROTEÔMICOS EM MATERIAL BIOLÓGICO"

Caro Prof. Flávio,

Venho, por intermédio desta, solicitar emenda ao projeto GPPG 10-0162. Considerando o objetivo principal do referido projeto de pesquisa, que inclui a busca por biomarcadores de risco e prognóstico em câncer de pâncreas, os autores do projeto propõem ampliação dos objetivos específicos incluindo:

Avaliação da expressão dos genes HDAC1, HDAC2 e HDAC3 e análise de seu papel como possíveis fatores prognósticos em Adenocarcinoma Ductal Pancreático.

Para atingir este objetivo propomos observar a expressão de *HDAC1*, *HDAC2* e *HDAC3* em tecidos tumorais de pacientes (já recrutados) com Adenocarcinoma Ductal Pancreático correlacionando com a expressão no tecido não-tumoral adjacente ao tumor em tecido igualmente já armazenado no projeto por PCR em tempo real. Da mesma forma, seria feita a correlação dos níveis de expressão dos genes *HDAC1*, *HDAC2* e *HDAC3* com fatores

prognósticos clínicos, resposta ao tratamento, sobrevida livre de progressão e sobrevida global.

Este objetivo está sendo proposto em seguimento aos achados do próprio projeto 10-0162, bem como em sequência aos resultados do projeto 10-0418, relacionado ao projeto 10-0162 e intitulado "AVALIAÇÃO DA MODIFICAÇÃO DAS HISTONAS POR TÉCNICA IMUNO-HISTOQUÍMICA COMO BIOMARCADORES PREDITIVOS E NOVOS ALVOS-TERAPÊUTICOS EM ADENOCARCINOMA DE PÂNCREAS" que identificou expressão diferencial e possível papel regulatório de algumas histonas, o qual será investigado em maior detalhe com a nova abordagem aqui proposta.

Este objetivo será cumprido como parte de um trabalho de conclusão de curso da acadêmica Cleandra Gregório Silva, aluna do curso de Biomedicina da UFCSPA que já participa do projeto de pesquisa 10-0162 desde janeiro de 2012. Os recursos financeiros para realização dos experimentos necessários para cumprir este objetivo estão disponíveis.

Permaneço a disposição para esclarecimentos adicionais.

Atenciosamente,

Profa. Patricia Ashton-Prolla

Pesquisadora responsável pelo projeto 10-0162

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós-Graduação

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB00000921) analisaram o projeto:

Projeto: 100162

Pesquisador Responsável

PATRICIA ASHTON PROLLA

Título: BIOMARCADORES EM CÂNCER DE PÂNCREAS: ESTUDOS GENÔMICOS E PROTEÔMICOS EM MATERIAL BIOLÓGICO

Data da Versão:

EMENDA VI AO PROJETO 22/01/2013

Este documento referente ao projeto acima foi APROVADO em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, 28 de janeiro de 2013.

Prof. Flávio Kapczinski
Coordenador GPPG/HCPA

Porto Alegre, 17 de março de 2015.

Ao Comitê de Ética em Pesquisa
Exmo. Coòrdenador
Prof. José Roberto Goldim

RE: Emenda ao Projeto GPPG n° 10-0162 – "BIOMARCADORES EM CÂNCER DE PÂNCREAS: ESTUDOS GENÔMICOS E PROTEÔMICOS EM MATERIAL BIOLÓGICO"

Caro Prof. Goldim,

Venho, por intermédio desta, solicitar emenda ao projeto GPPG 10-0162.

Na data de 22/01/2013 foi encaminhada ao Comitê uma emenda com objetivo de avaliar a expressão dos genes *HDAC1, HDAC2 e HDAC3 e HDAC3* e analisar de seu papel como possíveis fatores prognósticos em Adenocarcinoma Ductal Pancreático utilizando a técnica de por PCR em Tempo Real, a qual foi aprovada na data de 28 de janeiro de 2013. Os resultados desta análise estão prontos e estamos compilando os mesmos nesse momento. No entanto, para fins de publicação, será necessária a análise dos produtos destes mesmos genes por imuno-histoquímica, para complementar os achados da expressão gênica, bem como análise complementar de uma outra desacetilase de histona, a HDAC7, que apresenta uma relevância importante nesse tipo tumoral demonstrada recentemente na literatura.

Cabe salientar que não há necessidade de apoio financeiro complementar, pois já dispomos do recurso para estas análises adicionais.

Permaneço a disposição para esclarecimentos adicionais. Atenciosamente,

Profa. Patrícia Ashton-Prolla
Pesquisadora responsável pelo projeto 10-0162