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CARACTERIZAÇÃO DE ALTERAÇÕES MOLECULARES EM TUMORES DO PÂNCREAS E REGIÃO PERIAMPULAR

Tese 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 Doutora em Genética e Biologia Molecular.

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SUMÁRIO

ABREVIATURAS

ADP: Adenocarcinoma Ductal Pancreático Pâncreas

AD: Adenocarcinoma Duodenal

CAV: Carcinoma de Ampola De Vater

CCd: Colangiocarcinoma Distal

CP: Carcinoma Periampular

DNMT: *DNA methyltransferase*

FT: Fator de Transcrição

GIST: *Gastrointestinal Tumors*

HDACs: *Histone deacetylases*

HATs: *Histone acetyltransferases*

IMC: Índice de Massa Corporal

LINEs: *Long interspersed nuclear elements*

NIPan: Neoplasia Intraepitelial Pancreática

NIPM: Neoplasia Mucinosas Papilares Intraductais

NCM: Neoplasis Císticas Mucinosas

OMS: Organização Mundial da Saúde

PanN: Tecido Pancreático Normal Adjacentes ao Tumor

SINEs: *Short Interspersed Nuclear Elements*

TCGA: *The Cancer Genome Atlas*

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RESUMO

A ampola de Vater é uma área de convergência do ducto biliar comum, do ducto pancreático e do duodeno. A região periampular dista em torno de 2cm da ampola de Vater e neoplasias que se originam nesta região são denominadas carcinomas periampulares (CPs). Os CPs incluem quatro grupos tumorais originados da cabeça do pâncreas, da ampola de Vater, ducto biliar distal ou do duodeno e correspondem a 0,5% de todas as neoplasias digestivas. Tumores originados no pâncreas são mais frequentes entre os CPs e estes têm as maiores taxas de mortalidade sendo representados majoritariamente pelo adenocarcinoma ductal pancreático (ADP), subtipo histológico mais frequente e agressivo. Tumores mais raros como os outros CPs também apresentam altas taxas de mortalidade, mas são menos estudados quanto às suas alterações genéticas e epigenéticas, biologia tumoral e influência de fatores genéticos no prognóstico. Neste contexto, o objetivo deste trabalho foi realizar uma caracterização molecular do adenocarcinoma ductal pancreático e de tumores periampulares com o objetivo de contribuir para melhor compreensão do processo de carcinogênese e identificar marcadores prognósticos relacionados a esses tumores.

Inicialmente, avaliamos o perfil de metilação do DNA no ADP em busca de potenciais alvos terapêuticos nas vias moleculares associadas à carcinogênese. Verificamos que o ADP apresenta genes diferencialmente metilados em relação ao tecido normal adjacente e identificamos diversos genes da via de sinalização do cálcio diferencialmente metilados, e muitos destes são compartilhados com outras vias-chave da carcinogênese pancreática, como as vias Ras e Hippo. Os genes *ADCY8*, *CACNA1A*, *CACNA1B*, *CACNA1H*, e *RYR3,* que controlam o influxo de Ca^{+2} da membrana plasmática no retículo endoplasmático rugoso, estavam mais frequentemente hipermetilados e, mediante análise *in silico* de dados do TCGA, observamos que a redução da expressão desses genes se mostrou associada à redução da sobrevida global nos pacientes. Este achado é relevante, pois pode indicar potenciais marcadores prognósticos e alvos terapêuticos para casos selecionados de ADP. Adicionalmente, os resultados obtidos nesta tese indicam que a via de sinalização

do cálcio parece ter um papel importante desde as etapas muito iniciais da carcinogênese pancreática.

Quanto aos outros CPs, pouco se sabe a respeito da modulação epigenética regulada pelas desacetilases de histonas (HDACs). Assim, caracterizamos o perfil de expressão das HDAC1, HDAC2, HDAC3 e HDAC7 nestas neoplasias, bem como investigamos o possível papel das mesmas no desenvolvimento dos carcinomas de ampola de Vater (CAVs). Utilizamos bancos de dados de expressão para avaliar o perfil das HDACs e segundo nosso conhecimento, este foi o primeiro estudo a avaliar a sua expressão no adenocarcinoma duodenal. Os CAVs e os adenocarcinomas duodenais apresentaram um perfil de expressão semelhante paras *HDAC1* e *HDAC2*. Esse trabalho ainda avaliou, pela primeira vez, a expressão proteica das HDACs em amostras de adenocarcinoma de ampola de Vater (o subtipo histológico mais frequente) e tecidos normais adjacentes (pancreático, ampular e duodenal). Encontramos um perfil de expressão semelhante entre todos os tecidos, sugerindo que estas HDACs não estão diretamente envolvidas na carcinogênese do CAVs, embora possam ter um papel auxíliando o fenótipo tumoral, modulando genes envolvidos proliferação celular, regulação do ciclo celular e apoptose.

Por fim, descrevemos um caso clínico atípico e mais grave de Neurofibromatose 1 com múltiplos tumores periampulares onde a presença de duas variantes germinativas patogênicas em genes distintos (*NF1* e *CFTR*) relacionados a doenças pâncreáticas podem ter agido sinergicamente. Em especial, a variante germinativa patogênica do gene *CFTR* associada ao pâncreas *divisum* pode ter contribuído para a ocorrência de múltiplos tumores na paciente portadora de Neurofibromatose tipo 1. Este relato de caso reforça a importância de uma análise molecular mais abrangente, incluindo avaliação de múltiplos genes relacionados ao fenótipo em casos atípicos ou com fenótipo mais grave que o habitual. A informação obtida no estudo de caso será também muito relevante para os familiares do caso índice.

Os resultados desta tese contribuem para o melhor entendimento do perfil genético e epigenético dos adenocarcinomas ductais pancreáticos e de carcinomas

periampulares. Os resultados também indicam importantes linhas de investigação para explorar novas vias de sinalização que possam trazer informações relevantes para o desenvolvimento de novas estratégias terapêuticas.

ABSTRACT

Ampulla of Vater is a convergence area of the common bile duct, pancreatic duct, and duodenum. The periampullary region is located within about 2 cm from the ampulla of Vater and neoplasms originated in this region are called periampullary carcinomas (PCs). PCs include four tumoral groups that arise from the pancreatic head, Ampulla of Vater, distal biliary duct or duodenum and correspond to 0.5% of all digestive neoplasms. Tumors originated in the pancreas are the most frequent among PCs and they have the highest mortality rates, being represented mainly by the pancreatic ductal adenocarcinoma (PDAC), the most frequent and aggressive histological subtype. Other PCs are rarer and also have high mortality rates, however, their genetic and epigenetic alterations, tumor biology, and genetic influence in prognostic factors are less studied. In this context, the objective of this study was to perform a molecular characterization of pancreatic ductal adenocarcinoma and periampullary tumors in order to contribute to a better understanding of the carcinogenesis process and to identify prognostic markers related to these tumors.

First, we evaluated the DNA methylation profile in PDAC samples, searching for potential therapeutic targets in the molecular pathways associated with PDAC carcinogenesis. PDAC showed differentially methylated genes compared to adjacent normal tissue and we identified several differentially methylated genes in the Calcium Signaling Pathway. Many of these are shared with other key pancreatic carcinogenesis pathways, such as the Ras and Hippo pathways. The *ADCY8*, *CACNA1A*, *CACNA1B*, *CACNA1H*, and *RYR3* genes, which control Ca^{2+} influx into the rough plasma membrane or endoplasmic reticulum, were most often hypermethylated. Using *in silico* analysis of TCGA data, we observed that reduced expression of these genes was associated with reduced overall survival in PDAC patients. This finding is relevant as may indicate potential prognostic markers and therapeutic targets for selected PDAC cases. Additionally, the results obtained in this thesis indicate that the calcium signaling pathway seems to play an important role since the very early stages of pancreatic carcinogenesis.

In relation to other PCs, there is little knowledge about epigenetic modulation regulated by histone deacetylases (HDACs). Thus, we characterized the expression profile of HDAC1, HDAC2, HDAC3 and HDAC7 in these neoplasms, as well as investigated their possible role in the development of ampulla of Vater carcinomas (AVCs). We used expression databases to evaluate the profile of HDACs, and to our knowledge, this was the first study to evaluate their expression in duodenal adenocarcinoma. AVCs and duodenal adenocarcinomas showed a similar expression profile for *HDAC1* and *HDAC2*. This work also evaluated, for the first time, the protein expression of HDACs in AVCs samples (the most common histological subtype) and adjacent normal tissues (pancreatic, ampullary and duodenal). We found a similar expression profile among all tissues, suggesting that these HDACs are not directly involved in the AVCs carcinogenesis. Nonetheless, they may play a role supporting the tumor phenotype by modulating genes involved in cell proliferation, cell cycle regulation and apoptosis.

Finally, we described an atypical and more severe case of Neurofibromatosis 1 with multiple periampullary tumors, where the presence of two pathogenic germline variants in different genes (*NF1* and *CFTR*) related to pancreatic diseases may have acted synergistically. In particular, the *CFTR* pathogenic germline variant associated with pancreas *divisum* may have contributed to the occurrence of multiple tumors in the Neurofibromatosis type 1 patient. This case report reinforces the importance of a broader molecular analysis, including evaluation of multiple genes related to phenotype, in atypical cases or with more severe phenotype than usual. The information obtained from the case report will also be very relevant to the case index relatives.

The thesis results contribute to a better understanding of the genetic and epigenetic profile of pancreatic ductal adenocarcinomas and periampullary carcinomas. The results also indicate important research topics to be explored, including new signaling pathways that may provide relevant information for the development of new therapeutic strategies.

CAPÍTULO I: INTRODUÇÃO

1. Câncer e epidemiologia do câncer de pâncreas

O câncer é a segunda principal causa de morte no mundo, sendo responsável por cerca de 9,6 milhões de mortes no ano de 2018 (Iarc, 2018). Segundo a Organização Mundial da Saúde (OMS) estima-se 14 milhões de óbitos relacionados a esta doença devam ocorrer no mundo em 2035 (Rahib *et al.*, 2014), reforçando o impacto do câncer como um problema de saúde pública. A crescente incidência e mortalidade vinculada ao câncer podem ter múltiplas explicações, contudo o reflexo do envelhecimento populacional combinado a maior exposição a fatores de risco são aspectos frequentemente reportados (Bray *et al.*, 2018).

Embora o conhecimento científico tenha melhorado a sobrevivência de muitos pacientes oncológicos, os dados relacionados às neoplasias de pâncreas permanecem alarmantes. O câncer de pâncreas é uma das neoplasias mais agressivas, cuja incidência é quase igual à mortalidade, ocupando o 14º lugar no mundo em frequência, mas a sétima posição entre as causas de morte relacionadas ao câncer. A sobrevida global em 5 anos é inferior a 8% e o tempo médio de sobrevida é de 13 a 20 meses após a cirurgia de ressecção do tumor (Neuzillet *et al.*, 2011; Collisson and Maitra, 2017). No Brasil, 10.754 mortes foram relacionadas ao câncer de pâncreas em 2017, correspondendo a 4% do total de óbitos relacionados ao câncer. Nos Estados Unidos, estima-se que o câncer de pâncreas se torne a segunda causa de morte por câncer em 2030 (Inca, 2014; Rahib *et al.*, 2014; Inca, 2018).

1.1. Fatores de risco para câncer de pâncreas

Alguns fatores de risco para ocorrência de tumores pancreáticos estão claramente estabelecidos e incluem idade avançada, sexo masculino, etnia afro-americana, diabetes, obesidade, história pessoal de pancreatite crônica, história familiar de câncer de pâncreas, tabagismo e alcoolismo (Neuzillet *et al.*, 2011; Mcguigan *et al.*, 2018; Tsai and Chang, 2019). Mais recentemente, a pancreatite aguda (Kirkegård *et al.*, 2018) e o tipo de microbiota intestinal (Tsai and Chang, 2019; Wang *et al.*, 2019) foram propostas como fatores de risco importantes para a doença.

Neoplasias pancreáticas são comumente associadas a idade mais avançada, sendo que cerca de 90% dos casos são diagnosticados em pacientes com idade superior a 55 anos (Midha *et al.*, 2016) e a doença atinge seu ápice entre os 60 e os 80 anos de idade (Rawla *et al.*, 2019). Neoplasias pancreáticas são mais incidentes em homens (5,5 por 100.000 para homens, em comparação a 4,0 para 100.000 mulheres) (Bray *et al.*, 2018) possivelmente devido a exposição a fatores ambientais, bem como estilo de vida (maior uso de tabaco e álcool por homens) (Mcguigan *et al.*, 2018).

A associação positiva entre diabetes tipo I e II e o risco de câncer de pâncreas tem sido relatada ao longo dos anos (Mcauliffe and Christein, 2013; Pezzilli and Pagano, 2013; Batabyal *et al.*, 2014; Maisonneuve and Lowenfels, 2015) . Um estudo epidemiológico da população italiana revelou que cerca de 9,7% dos tumores pancreáticos são devidos à diabetes (Rosato *et al.*, 2015). Alguns estudos mostram que ter diabetes pode aumentar o risco de desenvolver câncer de pâncreas em 1,8 vezes, particularmente em homens asiáticos e hispânicos, em comparação com brancos e negros (Li *et al.*, 2011; Liao *et al.*, 2012).

A pancreatite é uma inflamação do pâncreas que induz dano pancreático pela ativação precoce de enzimas digestivas, ainda no parênquima pancreático onde são produzidas e antes de sua liberação no intestino delgado(Rawla *et al.*, 2019). Alguns estudos indicam que a pancreatite crônica pode elevar o risco relativo de desenvolver câncer de pâncreas em 5-16 vezes (Raimondi *et al.*, 2010; Kirkegard *et al.*, 2017). A principal causa de pancreatite crônica é o consumo de álcool (70-80%), mas outros fatores de risco foram identificados como tabagismo, doenças auto-imunes, distúrbios metabólicos, obstrução ductal, anomalias anatômicas como o pâncreas *divisum* e fatores hereditários (Elsherif *et al.*, 2019). Variantes patogênicas germinativas nos genes *PRSS1, SPINK1* ou *CFTR* associadas ao pancreas *divisum* conferem maior predisposição à pancreatite e em muitos indivíduos os episódios de pancreatite são precoces e recorrentes, consequentemente aumentando o risco de câncer de pâncreas (Zhan *et al.*, 2018; Lee and Papachristou, 2019) . O papel da pancreatite aguda como fator predisponente ao câncer de pâncreas foi muito discutido e permaneceu

controverso por anos, mas estudos recentes sugerem que a doença pode conferir maior risco para o desenvolvimento de neoplasias pancreáticas (Chung *et al.*, 2012; Munigala *et al.*, 2014; Kirkegård *et al.*, 2018) . O que deve ser ressaltado é que o risco parece estar limitado a pacientes com pancreatite aguda que evoluem para pancreatite crônica (Rijkers *et al.*, 2017; Lee and Papachristou, 2019).

Obesidade é outro fator de risco potencial que vem chamado atenção de muitos pesquisadores. Li et al., associou o índice de massa corporal (IMC) relacionado ao sobrepeso e obesidade (25,0 - 29,9 kg / m^2 e ≥ 30 kg / m^2) com um maior risco de desenvolver câncer de pâncreas, durante o início da idade adulta. Além disso, a obesidade em uma idade mais avançada (30 - 79 anos) foi associada a menor sobrevida global (Li *et al.*, 2009). Um estudo que avaliou 14 coortes com mais de 2.000 casos de câncer de pâncreas constatou que os obesos apresentavam risco 54% maior de desenvolver câncer de pâncreas (Genkinger *et al.*, 2011)**.**

Em relação aos fatores ambientais, o tabagismo é o fator de risco mais importante e consistente. Aproximadamente 20 a 25% dos tumores pancreáticos podem ser atribuídos ao tabagismo (Raimondi *et al.*, 2009) e o risco é quase duas vezes maior em fumantes do que em não fumantes e aumenta com a duração e o número de cigarros consumidos diariamente (Kuzmickiene *et al.*, 2013; Mizuno *et al.*, 2014). O risco pode persistir por 10-15 anos após a cessação do tabagismo (Iodice *et al.*, 2008; Lynch *et al.*, 2009). Outro fator que tem sido associado ao câncer de pâncreas, porém de forma menos consistente, é o alcoolismo. Os estudos são controversos a respeito do seu impacto no desenvolvimento desta neoplasia, contudo um risco aumentado de 15% foi relacionado ao alto consumo de álcool, não havendo associação de consumo baixo ou moderado com a doença (Wang *et al.*, 2016). Por fim, um grande estudo caso-controle em 2010 não encontrou associação geral significativa entre a ingestão total de álcool e o risco de câncer de pâncreas, embora para consumo ≥ 45g de álcool por dia, o risco tende a aumentar em homens (Pelucchi *et al.*; Michaud *et al.*, 2010) . Ainda que a relação causal entre álcool e câncer de pâncreas seja controversa, sabe-se que o

consumo excessivo de álcool também é a principal causa de pancreatite crônica, que é um fator de risco estabelecido para câncer de pâncreas (Samokhvalov *et al.*, 2015).

Estima-se que cerca de 5 a 10% dos pacientes com câncer de pâncreas tenham história familiar da doença (Greer *et al.*, 2007; Shi *et al.*, 2009). O câncer de pâncreas familiar é definido pela presença de dois ou mais familiares de primeiro ou segundo grau acometidos por esse tumor. Familiares de primeiro grau de pacientes com câncer pancreático têm um risco nove vezes maior de desenvolver esse diagnóstico quando comparados à população em geral (Klein *et al.*, 2004), e quando há dois familiares de primeiro grau diagnosticados este risco duplica, podendo chegar a um 32 vezes maior para pessoas com mais de três familiares de primeiro grau afetados (Greer *et al.*, 2007; Vincent, Herman*, et al.*, 2011). Estes dados epidemiológicos atestam a importância de fatores hereditários/genéticos na etiologia da doença. Ademais, o câncer de pâncreas é parte do espectro tumoral de algumas síndromes de predisposição hereditária ao câncer incluindo a Síndrome de Peutz-Jeghers, Síndrome do Melanoma Familiar, Síndrome de câncer de mama e ovário hereditários, câncer colorretal hereditário não-polipomatoso, polipose adenomatosa familiar e Síndrome de Li-Fraumeni, entre outras (Zhan *et al.*, 2018). Especificamente para neoplasias neuroendócrinas, a Neoplasia endócrina múltipla tipo 1, Síndrome de von Hippel-Lindau, Neurofibromatose tipo 1, Esclerose tuberosa são síndromes bem estabelecidas e associadas a esses tumores. Em geral, tumores neuroendócrinos associados às síndromes hereditárias possuem uma apresentação multifocal multifocal (Guilmette and Nosé, 2019) (Tabela1).

Quadro 1. Mecanismo de doença, função, genes e Síndromes hereditárias associadas ao câncer de pâncreas (adaptado de Zhan et al., 2018; Guilmette and Nosé, 2019).

Mecanismos de doença	**Função**	**Genes**	**Síndrome associada**
Dano celular (suscetibilidade à pancreatite)	Protease, a ativação prematura induz a pancreatite.	*PRSS1*	Pancreatite hereditária
	Inibidor de tripsina.	*SPINK1*	Pancreatite hereditária
	Metabolismo da glutationa, defesa antioxidante.	*GGT1*	Risco para câncer de pâncreas
	Degradação prematura da tripsina ativada.	*CTRC*	Pancreatite hereditária
	Canal iônico necessário para secreção / absorção de células epiteliais.	*CFTR*	Fibrose cística, pancreatite hereditária
	Regulação e parada do ciclo celular após dano ao DNA, apoptose.	*TP53*	Síndrome de Li-Fraumeni
	Supressor de tumor, inibição de crescimento.	*SMAD4*	Telangiectasia hemorrágica hereditária, síndrome da polipose juvenil, síndrome de Myhre
Crescimento celular / controle do ciclo	Resposta a danos no DNA a quebras de fita dupla.	*ATM*	Ataxia Telangiectasia
	Regulador do ponto de verificação do ciclo celular e resposta a danos no DNA a quebras de fita dupla.	*CHEK2*	Síndrome de Li-Fraumeni
	Parada celular nos postos de controle G1 e G2, apoptose.	*CDKN2A*	Síndrome do Melanoma Múltiplo Familiar Atípico
	Regula o crescimento celular, proliferação e resposta a danos no DNA.	*STK11*	Síndrome de Peutz-Jeghers
	Regula o ciclo celular, a sobrevivência e o metabolismo.	*PTEN*	Síndrome do tumor PTEN-hamartom

Quadro 1. Continuação.

Mecanismos de doença	Função	Genes	Síndrome associada
Reparo do DNA	Reparo de quebra dupla no DNA.	*BRCA1, BRCA2 BARD1*	Síndrome hereditária do câncer de mama e ovário
	Reparo de quebra dupla no DNA.	*PALB2, FANCA, FANCC, FANCG FANCM*	Anemia de Fanconi
	Reparo de bases mal pareadas.	*MLH1, MSH2, MSH6, PMS2 e EPCAM **	Síndrome de Lynch
	Reparo de danos ao DNA.	*NBN*	Síndrome de quebras de Nijmegen
Adesão celular	Regula a migração e adesão celular, antagonista da via Wnt.	*APC*	Polipose adenomatosa familiar
Apoptose	Regulador transcricional, sendo componente essencial do complexo MLL/SET1 .	*MEN1*	Neoplasia endócrina múltipla tipo 1
	Regulada ubiquitinação e subsequente degradação proteassomal de proteínas.	*VHL*	Síndrome de von Hippel-Lindau
	Regula atividade GTPase da oncoproteína Ras.	*NF1*	Neurofibromatose tipo 1
	Reguladores da via mTOR.	*TSC1 TSC2*	Esclerose tuberosa

*Deleções nos últimos éxons do *EPCAM* inativam *MSH2* e podem causar indiretamente defeitos no reparo de bases mal pareadas.

1.2. Classificação histopatológica dos tumores do pâncreas

As neoplasias do pâncreas compreendem um amplo conjunto de tumores e são geralmente classificadas de acordo com sua diferenciação histológica e comportamento biológico. As neoplasias do pâncreas endócrino são representadas pelos tumores originários e não-originários das ilhotas pancreáticas e embora sejam relativamente raras (incidência de 0,43 por 100.000 pessoas ao ano nos Estados Unidos) estão associadas a melhor prognóstico em relação aos tumores exócrinos do pâncreas (Fernández Pérez *et al.*, 2018) (Tabela 2). Os tumores do pâncreas exócrino, que constituem 90% dos tumores de pâncreas, são na grande maioria adenocarcinomas ductais pancreáticos (ADP) ou variantes destes (Neuzillet *et al.*, 2011).

Tabela 1. Taxas de sobrevida de acordo com o estágio clínico do câncer de pâncreas, comparando tumores do pâncreas exócrino com tumores do pâncreas endócrino (adaptado de Rawla et al., 2019).

Estágio clínico	Sobrevida em cinco anos (%)	
	Câncer de pâncreas exócrino	Câncer de pâncreas endócrino (PanNET) tratado com cirurgia
I A	14	61
IB	12	61
II	7	52
III	3	41
IV	1	16

1.2.1. Adenocarcinoma ductal pancreático

O ADP é uma neoplasia sólida de caráter infiltrante e intensa reação não-neoplásica no tecido adjacente, composta por fibroblastos, linfócitos e matriz extracelular (reação desmoplásica). Microscopicamente, observam-se glândulas malignas atípicas, irregulares, pequenas e geralmente revestidas por células epiteliais cubóides a colunares anaplásicas e formação de estruturas tubulares ou agregados celulares (Haeberle and Esposito, 2019). (Maitra and Hruban, 2008) As lesões precursoras do ADP podem ser divididas em precursores microscópicos e macroscópicos. Os precursores microscópicos incluem a neoplasia intra-intrapitelial pancreática (NIPan), que são mais frequentes e melhor caracterizadas; e as lesões

planas atípicas. NIPans 1A, 1B e 2 são classificadas como lesões de baixo grau de malignidade, enquanto NIPan 3 é classificada como lesão de alto grau. As lesões precursoras macroscópicas são caracterizadas pela visibilidade em exames de imagem, sendo representadas pelas neoplasias mucinosas papilares intraductais (NMPI), neoplasias císticas mucinosas (NCM) e neoplasias tubulopapilares intraductais. As duas primeiras são caracterizadas pela produção de mucina (Maitra and Hruban, 2008; Bosman *et al.*, 2010), ao contrário das neoplasias tubulopapilares intraductais que raramente produzem essas glicoproteínas, portanto, geralmente não se apresentam como lesões císticas (Yamaguchi *et al.*, 2013).

1.2.1.1. Diagnóstico, agressividade e tratamento do adenocarcinoma ductal pancreático

O ADP em estágios iniciais é geralmente clinicamente silencioso, e a maioria dos pacientes que apresentam sintomas atribuíveis a neoplasia possuem tumores localmente avançados e/ou metástases (80-90% dos casos), que contraindicam ou dificultam a ressecção cirúrgica (Neuzillet *et al.*, 2011; Vincent, Herman*, et al.*, 2011; Rawla *et al.*, 2019). Diversos fatores contribuem para esse panorama, como sinais e sintomas inespecíficos da doença, limitações metodológicas relacionadas aos exames de diagnóstico, e biologia tumoral agressiva.

A sintomatologia do ADP geralmente é inespecífica e depende em grande parte da localização do tumor e estágio da doença: em cerca de 65% dos casos o tumor está localizado na cabeça do pâncreas, em 15% no corpo/cauda e em 20% dos casos o tumor envolve o órgão de uma forma difusa. A obstrução da via biliar frequentemente é relacionada a tumores localizados na cabeça do pâncreas, causando colestase, que se expressa por icterícia, colúria e acolia. Além disso, dor abdominal, glicemia alterada, astenia, anorexia e perda de peso são manifestações recorrentes que podem ser relacionadas a um grande número de doenças como colangite, colecistite, colelitíase, coledocolitíase, cistos de colédoco, pancreatite, entre outras (Hidalgo, 2010; Simianu *et al.*, 2010; Rawla *et al.*, 2019).

Atualmente, as ferramentas de diagnóstico / estadiamento pré-operatórias são a ultrassonografia abdominal, tomografia computadorizada com protocolo pancreático trifásico (padrão ouro para o diagnóstico e estadiamento) (Wong and Lu, 2008; Klauss *et al.*, 2009), ressonância magnética e aspiração por agulha fina guiada por ultrassonografia endoscópica. Embora os três primeiros exames sejam fundamentais para a conduta terapêutica e determinação do estadiamento, a detecção de lesões pequenas (<2 cm), com ausência de dilatação biliar, e envolvimento vascular são difíceis de serem visualizadas. Nessas situações, a aspiração por agulha fina guiada por ultrassonografia endoscópica tem sensibilidade relatada em cerca de 80% dos casos (Harewood and Wiersema, 2002; Guarneri *et al.*, 2019), contudo é um exame de alto custo e não disponível em todos os serviços de saúde, mesmo para indivíduos de alto risco. Além disso, em pacientes sintomáticos, a avaliação do antígeno de superfície CA19-9 pode ajudar a confirmar o diagnóstico e prever prognóstico e recorrência após a ressecção (Tempero *et al.*, 2017). No entanto, o CA19-9 possui limitações importantes, como a não-especificidade para câncer de pâncreas ou sua ausência em pacientes que são negativos para o antígeno de Lewis a ou b (5 a 10% da população caucasiana). Pacientes com o antígeno de Lewis a ou b podem possuir resultados falsos negativos para os níveis séricos de CA 19-9 mesmo na presença de câncer pancreático avançado (Scarà *et al.*, 2015).

Uma característica peculiar do ADP é a existência de um extenso estroma desmoplásico associado a células mielóides infiltrantes (Dougan, 2017). A hipóxia é outra característica importante do microambiente tumoral que, ligada à desmoplasia, atua como barreira à infiltração de células T (Ene-Obong *et al.*, 2013; Özdemir *et al.*, 2014; Daniel *et al.*, 2019). Além disso, dentre as células mielóides, os macrófagos associados ao tumor são uma das células mais abundantes e promovem, no tumor, eventos mitóticos e angiogênicos, e inibição da apoptose (Zhu *et al.*, 2017). Outra particularidade do ADP é sua progressão precoce para doença metastática (Kleeff *et al.*, 2016). Os pacientes comumente apresentam metástases hepáticas, na cavidade peritoneal, pulmões e outros órgãos gastrointestinais (Poruk *et al.*, 2013) em estágios

avançados, mas estas já podem aparecer em estágios iniciais. De fato, foi observado por Rhim e colaboradores que o desenvolvimento de metástases pode acontecer concomitantemente ao aparecimento da lesão intraepitelial pancreática 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).

O único tratamento do ADP com potencial curativo é a ressecção cirúrgica, contudo, devido às características mencionadas acima, apenas 20% dos pacientes diagnosticados são elegíveis a cirurgia. Em relação ao tratamento não-cirúrgico, uma característica marcante do ADP é o seu alto grau de resistência a praticamente qualquer tipo de terapias disponíveis (Amrutkar and Gladhaug, 2017; Grasso *et al.*, 2017; Morrison *et al.*, 2018). Os agentes quimioterápicos usados atualmente são os do esquema FOLFIRINOX (5-fluouracil, oxaliplatina e irinotecano) ou gemcitabina combinada com nab-paclitaxel (Orth *et al.*, 2019).

1.2.1.2. Biologia molecular do adenocarcinoma ductal pancreático

Entre os grandes desafios das pesquisas em câncer está a elucidação das alterações genéticas responsáveis pela iniciação e progressão tumoral de cada tipo de câncer e o desenvolvimento de terapias específicas para cada uma dessas etapas. Nesse contexto, a genômica tem contribuído para a identificação de biomarcadores que possam auxiliar na compreensão dos mecanismos envolvidos na carcinogênese e de alvos moleculares potenciais.

Hruban e colaboradores definiram um modelo de progressão genética para o desenvolvimento do ADP, no qual são descritas alterações genéticas e epigenéticas nas lesões pré-malignas do ADP (Hruban *et al.*, 2000; Omura and Goggins, 2009) (Figura 1). 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 RAS 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 and Depinho, 2002).

O desenvolvimento do processo neoplásico se consolida com a ocorrência de mutações de perda de função em genes supressores de tumor. A perda de função do gene *CDKN2A* é encontrada em 80-95% das NIPans 2-3 e ADPs em estágios avançados. 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 do retinoblastoma (Rb) e de p53 (Liggett and Sidransky, 1998; Bardeesy and 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, se observa 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, menos frequentemente, pâncreas (Bardeesy and Depinho, 2002). Deleções ou mutações no gene *SMAD4/DPC4* podem estar presentes em até 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 and Depinho, 2002).

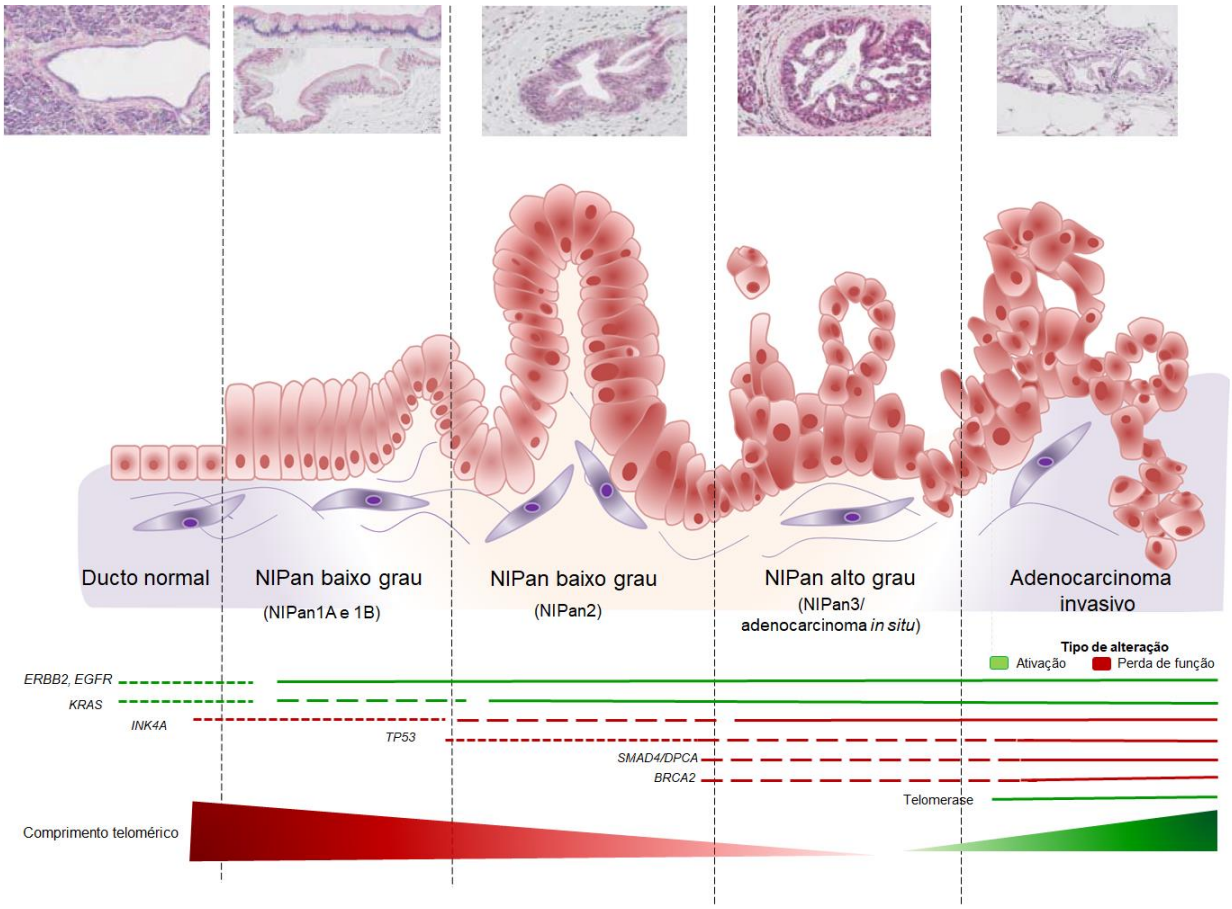


Figura 1. Modelo de progressão do câncer pancreático (adaptado de Hruban et al., 2000; Bardeesy and Depinho, 2002; Alemar et al., 2015). NIPan= neoplasia intra-intrapitelial pancreática.

Outra alteração genética importante na carcinogênese do ADP é a ocorrência de variações no número de cópias (do inglês, *copy number variation*, CNV) definidas por variações estruturais de segmentos iguais ou maiores que 1 kilobase (deleções ou duplicações). As CNVs podem representar uma importante fonte de variabilidade genética e fenotípica, mas também são responsáveis por muitas doenças complexas como o câncer (Fanciulli *et al.*, 2010). CNVs correspondem a uma fração importante da variabilidade genética importante, e podem modular a expressão gênica (Iafrate *et al.*, 2004; Sebat *et al.*, 2004; Levy *et al.*, 2007). Nas lesões precursoras do ADP em pacientes com história familiar, CNVs ocorrem com relativa frequência, sendo que em

um estudo, CNVs foram identificadas em 7/37 amostras, inclusive com ocorrência de CNVs em mais de um *locus* em algumas lesões. Nos cromossomos com perfil alterado, contudo, não foi identificada nenhuma deleção em genes supressores de tumor ou amplificação de oncogenes (Hong *et al.*, 2012). Em uma tentativa recente de correlacionar CNVs a predisposição genética ao desenvolvimento do ADP, Willis et al., utilizaram amostras de DNA genômico (sangue ou saliva) de 263 pacientes, mas não observaram uma contribuição substancial destas alterações para a etiologia da doença (Willis *et al.*, 2014).

2. Região periampular e carcinomas periampulares

A ampola de Vater é uma área de convergência do ducto biliar comum, do ducto pancreático e do duodeno (Kunath and Hommerding, 1981; Kim *et al.*, 2002). A estrutura é anatomicamente complexa e heterogênea, sendo formada por fibras musculares e elementos neuronais especiais que regulam, através da papila duodenal (ou esfíncter de Oddi), o fluxo da bile e suco pancreático para o trato digestivo (Kunath and Hommerding, 1981; Ishibashi *et al.*, 2000) (Figura 2)**.** Do ponto de vista histológico, e corroborado por análises morfológicas e imuno-histoquímicas, a Ampola de Vater apresenta dois revestimentos epiteliais distintos, o pancreatobiliar e o intestinal. A região periampular dista em torno de 2cm da ampola de Vater e compreende as seguintes estruturas: pâncreas (ao nível da cabeça do órgão), Ampola de Vater, ducto biliar distal e duodeno (Haddad, 2009; Gaspar *et al.*, 2013; He *et al.*, 2014).

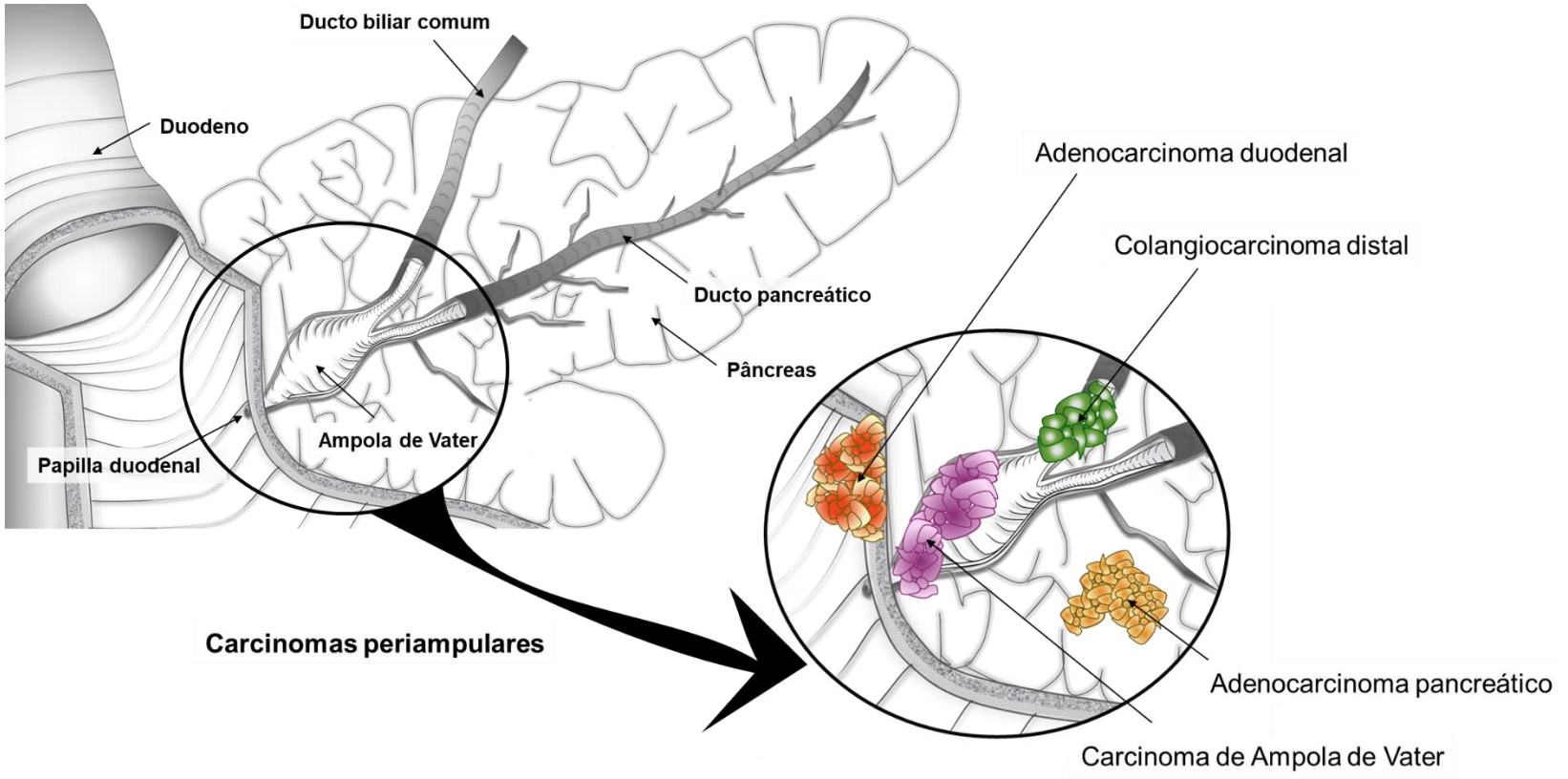


Figura 2. Localização anatômica da região ampular e carcinomas periampulares (figura elaborada pela autora).

Neoplasias que se originam dentro dessa região são denominadas carcinomas periampulares (CP). A precisa classificação diagnóstica do local primário desses tumores é difícil mesmo com a existência de uma avaliação histopatológica padronizada. Em geral, os CPs envolvem mais de um potencial local de origem destroem

a anatomia periampular normal e apresentam displasia epitelial em mais de uma porção da região, dificultando o diagnóstico (Monson *et al.*, 1991; Lüttges *et al.*, 1999; Fisher and Bakey, 2007).

Os CPs compreendem 0,5% de todas as neoplasias digestivas (Albores-Saavedra *et al.*, 2009) e suas taxas de incidência são inferiores a 1/100.000 casos por ano na população mundial. Dados da região sudeste do Brasil indicam uma taxa de 0,7/100.000 casos na população oriunda de São Paulo-Brasil (Randi *et al.*, 2009; Gaspar *et al.*, 2013). Entre todos os CPs, tumores pancreáticos são os mais frequentes, seguidos dos tumores da ampola de Vater, ducto biliar distal e duodeno(Kamarajah, 2018) (Tabela 3).

Tabela 2. Características dos pacientes submetidos à cirurgia de ressecção dos carcinomas periampulares (n= 9877) (adaptado de Kamarajah, 2018).

	Local de origem			
	Pâncreas	**Ampola de Vater**	**Ducto biliar distal**	**Duodeno**
Histologia predominante	Adenocarcinoma pancreático (ADP)	Adenocarcinoma de ampola de Valer (CAV)	Colangiocarcinoma distal (CCd)	Adenocarcinoma Duodenal (AD)
Frequência	79%	11%	6%	4%
Sobrevida em 5 anos	19%	47%	32%	42%

A taxa de sobrevida em 5 anos de pacientes submetidos a rececção cirúrgica com CAV, CCd e DA varia de 32 a 47% e são significativamente melhores do que as observadas em pacientes com ADP (Kamarajah, 2018). Esses dados sugerem que as diferenças na biologia do tumor também podem ser uma explicação para a sobrevida relativamente mais favorável dos pacientes com estas doenças.

2.1. Carcinoma periampular originado do pâncreas

Segundo a classificação dos carcinomas periampulares, os tumores pancreáticos podem ser considerados periampulares quando estiverem localizados na cabeça do pâncreas distando de 2cm da ampola de Vater. O ADP, subtipo histológico

mais prevalente das neoplasias pancreáticas, foi extensamente discutido e caracterizado nas sessões anteriores e não será abordado neste tópico.

2.2. Carcinoma periampular originado da ampola de Vater

Em virtude da Ampola de Vater possuir os revestimentos epiteliais pancreatobiliar e intestinal, os tumores originados dessas estruturas apresentam a histologia de adenocarcinoma pancreatobiliar ou intestinal (carcinomas de ampola de Vater, CAV). Em situações nas quais há a coexistência de aspectos de ambos os subtipos, o tumor é denominado adenocarcinoma misto (Kimura *et al.*, 1994; Chang *et al.*, 2013; Yachida *et al.*, 2016; Who *et al.*, 2019)**.** O subtipo misto é o subgrupo predominante de CAVs, representando até 40% dos casos (Gingras *et al.*, 2016; Xue *et al.*, 2017; Mafficini *et al.*, 2018).

O subtipo intestinal é frequentemente associado a um componente não invasivo (adenoma duodenal). A análise histopatológica deste subtipo aponta carcinomas compostos de glândulas tubulares bem formadas, com áreas cribriformes complexas em forma de ninhos sólidos, que são indistinguíveis do adenocarcinoma colorretal. As células tumorais são colunares, altas e pseudoestratificadas, frequentemente contendo mucina, com núcleos ovais ou em forma de “charuto” localizados basalmente (Kimura *et al.*, 1994; Kimura *et al.*, 2004; Kumari *et al.*, 2013). Ainda, o tumor está relacionado com uma melhor sobrevida quando comparado ao subtipo pancreatobiliar (Carter *et al.*, 2008; Westgaard *et al.*, 2008; Kohler *et al.*, 2011), pois os fatores patológicos relacionados à agressividade, como invasão linfovascular e perineural, não são tão frequentes (Howe *et al.*, 1998; Zhou *et al.*, 2004; Westgaard *et al.*, 2008; Lee *et al.*, 2010) . O subtipo pancreatobiliar é morfologicamente semelhante ao ADP. Entre suas características morfológicas destacam-se presença de glândulas simples ou ramificadas e pequenos ninhos de células circundados por um denso estroma desmoplásico. As células tumorais são cubóides ou colunares baixas, dispostas em uma única camada, sem pseudoestratificação. Os núcleos são arredondados apresentando marcado pleomorfismo (Kimura *et al.*, 1994; Kimura *et al.*, 2004; Kumari *et al.*, 2013) .

2.2.1. Etiologia dos carcinomas de ampola de Vater

A maioria dos CAVs não apresentam etiologia evidente (Ohike *et al.*, 2010; Adsay *et al.*, 2012), embora algumas doenças inflamatórias (Doença de Crohn e Doença celíaca) e procedimentos cirúrgicos prévios tenham sido reportados como causas (WHO, 2019). Condições hereditárias como a Polipose adenomatosa familiar, Síndrome de Lynch e Síndrome de Peutz-Jeghers foram associadas a maior risco de adenocarcinomas na região enquanto a Neurofibromatose tipo 1 está associada a presença de tumores neuroendócrinos da ampola (Relles *et al.*, 2010).

2.2.2. Alterações moleculares dos carcinomas de ampola de Vater

A heterogeneidade morfológica marcante dos CAVs e a falta de confiabilidade prognóstica da classificação histológica (mesmo com o auxílio de painéis imunohistoquímicos) levaram à investigação de alterações moleculares a fim de melhor definir o prognóstico e o tratamento dessas neoplasias.

Atualmente, alguns grupos de pesquisa têm investigado características moleculares em CAV, contudo mais estudos são necessários para o entendimento completo da carcinogênese nesta neoplasia rara. Alterações no gene *KRAS* são mais frequentemente relatadas em pacientes com CAV (Schönleben *et al.*, 2009; Guo *et al.*, 2014; Sandhu *et al.*, 2015; Valsangkar *et al.*, 2015) , contudo não foi encontrada prevalência significativa de mutações ativadoras nos oncogenes *BRAF* e *PIK3CA* (Overman et al., 2013). Em um estudo, a expressão proteica de p53, p21 e Bcl2 (proteínas ligadas à supressão tumoral) em 92 casos de CAV foram avaliados, e os achados imunohistoquímicos indicaram superexpressão de p53 e p21 e subexpressão de Bcl2 no tumor em comparação com tecido não tumoral (Guo *et al.*, 2014). Adicionalmente, assinaturas moleculares de mRNA de CAV foram avaliadas em dois estudos. No primeiro Sandhu et al., observaram a superexpressão de *WNT3A, TGFB1, HDAC, BDNF* e *ERBB4* no subtipo pancreatobiliar, enquanto *CDKN2A, RB1* e *PPARA* tinham o mesmo perfil de expressão no subtipo intestinal (Sandhu *et al.*, 2015). No segundo estudo, Overman et al., não conseguiram identificar diferenças significativas

entre os dois subtipos histológicos, provavelmente em função do pequeno tamanho amostral (n=14). Entretanto conseguiram identificar claramente que o CAV apresenta um perfil de expressão gênica diferente do perfil de ADP (Overman *et al.*, 2013). Por fim, Gingras et al., realizaram sequenciamento exômico e analisaram a variação do número de cópias em 160 amostras de tumores da região ampular, sendo este o estudo mais robusto de CAV até o presente momento. Como resultado, 98 amostras foram classificadas como CAV e apresentaram alta frequência de mutações de inativação do gene *ELF3*, instabilidade de microssatélite e deleções e amplificações focais comuns; sugerindo que essas alterações são “marcas” frequentes na patogênese molecular destes tumores (Gingras *et al.*, 2016).

2.3. Carcinoma periampular originado do ducto biliar distal

O colangiocarcinoma é uma neoplasia epitelial, que pode se originar em diversos locais dentro da árvore biliar. A classificação dos tumores é baseada na sua localização anatômica e inclui os colangiocarcinomas intra-hepático e extra-hepático (peri-hilar e distal) (Razumilava and Gores, 2014). O colangiocarcinoma distal (CCd) está localizado entre a origem do ducto cístico e a ampola de Vater, e apenas essa classificação é considerada um tipo de carcinoma periampular, correspondendo a cerca de 40% dos colangiocarcinomas (Deoliveira *et al.*, 2007) (Figura 3).

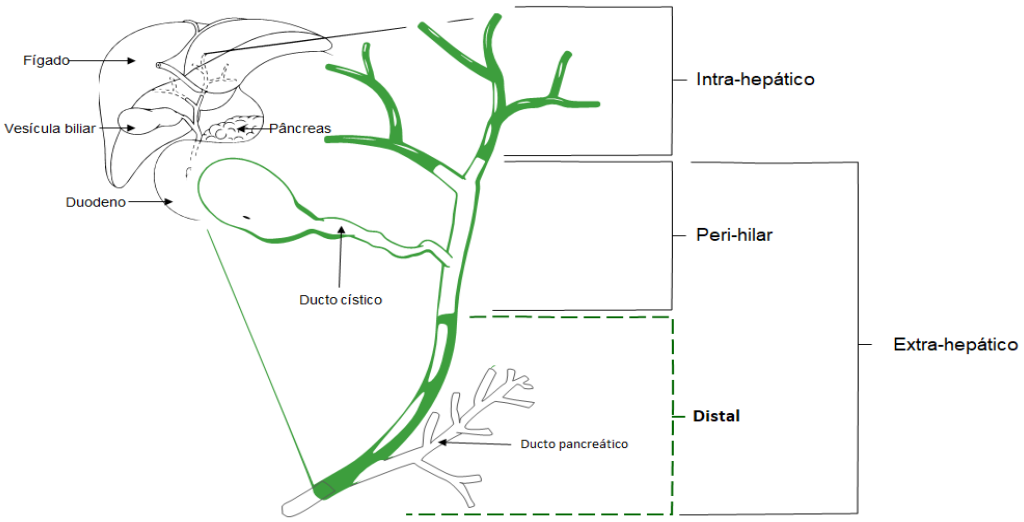


Figura 3. Classificação do colangiocarcinoma baseado na localização da árvore biliar (adaptado de Fundation, 2020).

2.4. Carcinoma periampular originado do duodeno

O adenocarcinoma duodenal representa menos de 1% de todos os tumores gastrointestinais (Overman *et al.*, 2010; Overman *et al.*, 2012) e 4% dos carcinomas periampulares (Kamarajah, 2018). De acordo com estudos epidemiológicos, adenocarcinomas e tumores neuroendócrinos são as neoplasias mais frequentes do duodeno seguidas por tumores estromais gastrointestinais (do inglês *gastrointestinal stromal tumor*, GIST) e maligno (Terada, 2012).

A maior parte dos GISTs é assintomática (Beltran and Cruces, 2007) e geralmente são achados incidentais em tomografias de abdômen, durante endoscopia ou em procedimentos cirúrgicos para outras manifestações abdominais (Parab *et al.*, 2019). Cerca de 75-80% dos GISTs apresentam mutação no gene *KIT* e 10% no gene *PDGFRA*. Embora a maior parte dos tumores seja esporádico, os GISTs foram descritos em algumas síndromes de predisposição hereditária ao câncer destacando-se entre estas a neurofibromatose tipo1 (NF1). Entre os tumores observados em pessoas com NF1, cerca de 34% são GISTs (Gutmann *et al.*, 1997; Relles *et al.*, 2010).

3. 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). Alterações epigenéticas podem resultar na ativação ou inibição inapropriada de várias vias de sinalização, levando ao desenvolvimento de diversas doenças, inclusive câncer (Jones and Baylin, 2002; Egger *et al.*, 2004) . Uma peculiaridade da regulação epigenética é a característica de ser um processo reversível e que pode agir sobre uma área extensa, incluindo mais do que um único gene (Omura and Goggins, 2009).

Os principais mecanismos de regulação epigenética são as modificações na conformação das histonas, metilação do DNA (Sawan *et al.*, 2008; Lomberk and Urrutia, 2015), que alteram a acessibilidade da cromatina, regulando a transcrição local ou global (Lund and Van Lohuizen, 2004). Além disso, os miRNAs podem atuar como moduladores epigenéticos, controlando os níveis das principais enzimas responsáveis por reações epigenéticas, como as metiltransferases de DNA (do inglês *DNA methyltransferases*, DNMTs), desacetilases de histonas (do inglês *histone deacetylases*, HDACs) entre vários outros reguladores epigenéticos (Yao *et al.*, 2019).

3.1. Metilação do DNA

Entre as modificações epigenéticas, a metilação do DNA tem um papel relevante em diversos processos biológicos, incluindo a embriogênese, envelhecimento e carcinogênese. A metilação consiste em uma modificação covalente do DNA, na qual um grupamento metil (CH_3) é transferido da S-adenosilmetionina (SAM) para o carbono 5 de uma citosina, que geralmente precede uma guanina, pela ação das DNA-metiltransferases (do inglês *DNA methyltransferases*, DNMTs)(Figura 4A) (Razin, 1998).

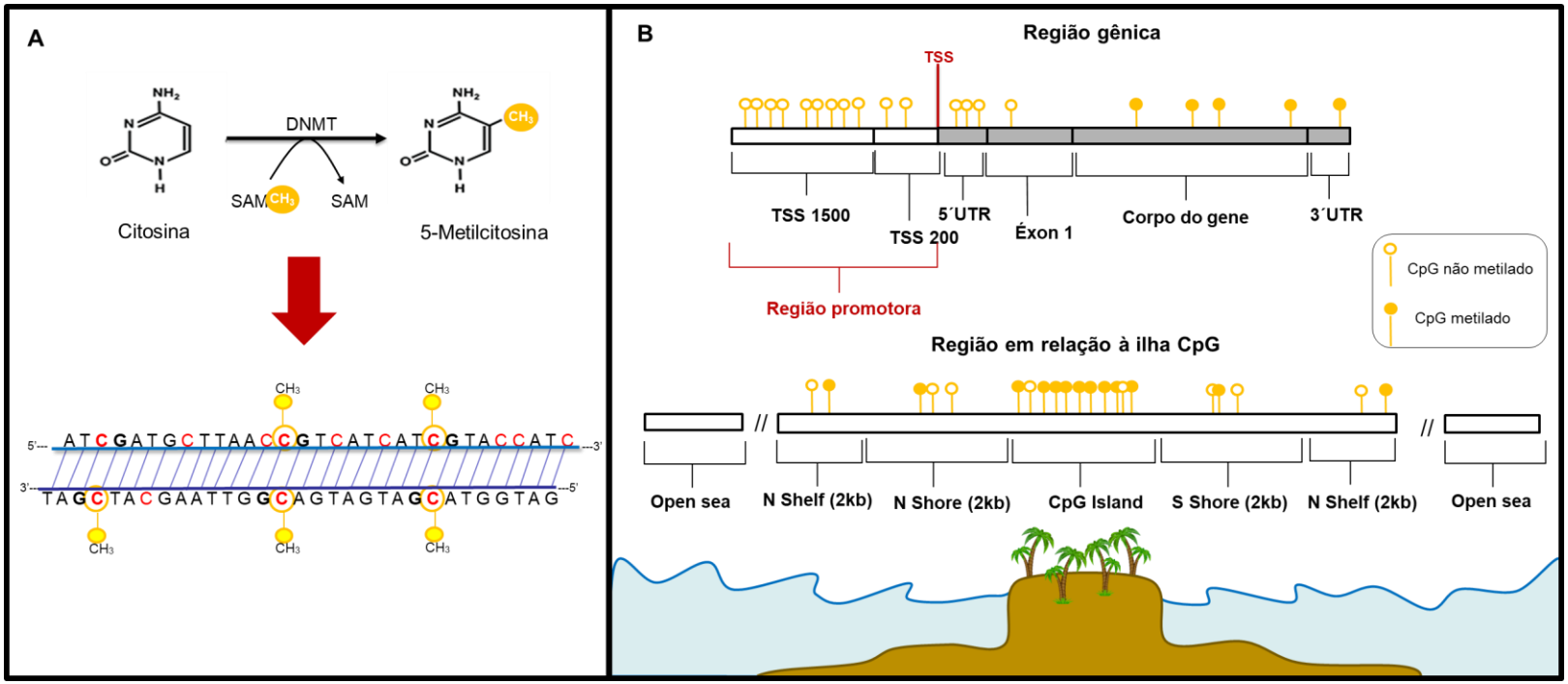


Figura 4. Metilação do DNA e diagrama esquemático da distribuição genômica da metilação do DNA. A, Representação da ligação do grupo CH_3 no carbono 5 da citosina gerando a 5-metilcitosina, o processo mediado pelas enzimas DNA metiltransferases**. B**, Distribuição dos dinucleotídeos CpG em relação ao gene e em relação às ilhas (figura elaborada pela autora).

A metilação do DNA ocorre principalmente em regiões ricas em conteúdo CG, chamadas ilhas CpG, frequentemente encontradas em regiões promotoras ou próximas a estas. As ilhas são definidas como regiões genômicas com mais de 500 pares de bases de comprimento, com um grande número de dinucleotídeos G e C (≥55%) e uma razão na qual os dinucleotídeos CpG observado / esperado é de pelo menos 60%. No genoma margeando as ilhas CpGs existem as *shores* e as *shelves.* As duas regiões possuem uma baixa densidade de CpG, sendo que a primeira é localizada a 2 kb de uma ilha de CpG e a segunda 4kb. Outras terminologias relacionadas à metilação são *open sea* (regiões com poucos ou nenhum dinucleotídeo CpG) e corpo do gene (local do início ao fim da transcrição) (Figura 4B) (Asmar *et al.*, 2015) . Os dinucleotídeos CpG correspondem a uma pequena porção do genoma (2-5%) e não são distribuídos de forma aleatória (Lopez *et al.*, 2009)**.** CpGs são encontrados principalmente em sequências repetitivas, como nos SINEs e LINEs (do inglês *short interspersed nuclear elements* e *long interspersed nuclear elements*), nas regiões promotoras e no primeiro éxon dos genes (Cohen *et al.*; Lopez *et al.*, 2009). A adição do radical metil à citosina impede a ligação de fatores de transcrição, silenciando a transcrição gênica, que é um mecanismo natural de regulação da transcrição.

Em células normais, SINE e LINE são hipermetiladas enquanto a região promotora de genes fundamentais para o funcionamento celular são hipometilados. Durante a carcinogênese, ocorrem mudanças características no perfil de metilação do DNA como hipometilação genômica global e a hipermetilação de promotores (Herman and Baylin, 2003; Jiang *et al.*, 2013). Células malignas possuem de 20 a 60% menos 5-metilcitosina do que células normais, essa condição promove instabilidade genômica, causando desregulação dos cromossomos durante a divisão celular e a ativação indesejada de elementos transponíveis no genoma, levando a mais danos genéticos (Locke *et al.*, 2019). A hipermetilação dos promotores de genes supressores de tumor é um evento precoce na formação do tumor, aumentando progressivamente ao longo do processo carcinogênico e contribuindo para a aquisição do fenótipo maligno (Baylin and Herman, 2000)**.** A metilação de um único supressor de tumor pode

ser ocasionalmente encontrada em lesões benignas, contudo a metilação de múltiplos genes supressores de tumor é um marcador confiável de malignidade (Pfeifer, 2018). Ainda que certos padrões de metilação sejam compartilhados por alguns tumores, a hipermetilação das ilhas CpG varia de acordo com cada tipo e estádio tumoral, de forma que cada subtipo apresenta uma assinatura, que pode ser exclusiva da neoplasia, como acontece com marcadores genéticos e citogenéticos (Pfeifer, 2018)**.**

Adicionalmente, a hipermetilação no corpo do gene pode possuir um efeito modulador importante na regulação transcricional. A metilação no corpo do gene promove o silenciamento de promotores alternativos, conferindo mudanças na expressão de isoformas do mesmo e confere níveis mais altos de expressão gênica (Pfeifer, 2018)**.**

3.1.1. Metilação do DNA em carcinomas periampulares

A exceção do ADP, as informações acerca do *status* de metilação dos carcinomas periampulares são muito limitadas, tendo como o foco um ou poucos genes relacionados à carcinogênese.

Kim et al., (2003) realizou uma análise comparativa do perfil de metilação em uma pequena série de casos de CPs comparado ao tecido duodenal e biliar adjacente (carcinoma de ampola de Vater= 9, colangiocarcinoma distal= 18 e adenocarcinoma duodenal=12) em 13 genes associados ao câncer. No estudo; os genes *p16*, *p14*, *MLH1*, *MGMT*, *MINT1, MINT25, MINT27* e *ER* foram metilados com maior frequência em adenocarcinomas duodenais em comparação com a mucosa duodenal não neoplásica. O perfil de metilação dos colangiocarcinoma distal e carcinoma de ampola de Vater foram comparados com o tecido biliar adjacente. Níveis aumentados de metilação foram encontrados para o gene *p16* nas duas neoplasias, enquanto que *RARβ* e *ER* foram hipermetilados exclusivamente nos colangiocarcinomas distais e carcinomas de ampola de Vater, respectivamente (Kim *et al.*, 2003). A hipermetilação de genes envolvidos na carcinogênese gastrointestinal (*hMLH1, HPP1, p14, p16* e *APC*) são frequentes no adenocarcinoma duodenal (Brücher *et al.*, 2006), bem como a metilação do gene *MGMT,* um dos componentes

do sistema de reparo do DNA. A metilação de *MGMT* foi associada a um mal prognóstico em pacientes com adenocarcinoma duodenal em estágio avançado da doença (Fu *et al.*, 2016)

Especificamente para os carcinomas de ampola de Vater, há uma grande dificuldade de comparar os níveis de metilação do tumor, visto que quando o mesmo se estabelece na pequena região da ampola de Vater, o epitélio normal raramente é preservado. Usando amostras de tecido ampular normal obtidos de autópsia Tozawa et al. (2004) identificou hipermetilação nos genes *CHFR, DAPK1, CDH1, p16, RASSF1A* e *RUNX3* nos tumores (Tozawa *et al.*, 2004) .

Em tumores pancreáticos, a alteração do perfil de metilação do DNA é notoriamente marcante em comparação com células pancreáticas normais (Delpu *et al.*, 2011). A hipometilação global do DNA e a hipermetilação de ilhas CpG de vários promotores gênicos que ocorre nesse tumor, também é característico a vários tumores como mama, esôfago e gástrico (Ehrlich, 2009). Muitas dessas alterações ocorrem desde o início da carcinogênese pancreática, nas NIPans, como a inativação por metilação do gene supressor de tumor *CDKN2A* (p16) e do gene *ppENK,* relacionado ao crescimento celular na carcinogênese pancreática (Fukushima *et al.*, 2002; Tang *et al.*, 2015). Os níveis de metilação de *ppENK* aumentam progressivamente conforme a progressão das lesões precursoras (Fukushima *et al.*, 2002). Alguns outros genes, como *SPARC*, *TSLC1, TFPI-2*, *BRCA1*, *APC* , *CDKN2A* e *TIMP3* já foram identificados hipermetilados em carcinomas pancreáticos, sendo alguns deles metilados em até 90% dos casos analisados (Peng *et al.*, 2006; Brune *et al.*, 2008) . O avanço tecnológico de análises genômicas ampliou o conhecimento a respeito da correlação entre os níveis de metilação e expressão gênica. Um dos primeiros estudos com esse enfoque identificou que alguns oncogenes (*MYB, JUNB* e *FOS*) e modificadores cromatina (*SET8, KDM6A* e *EP400*) eram modulados por metilação no ADP (Vincent, Omura*, et al.*, 2011)**.** Genes relacionados a agressividade tumoral como *MYB* (responsável pelo crescimento celular, invasão, metástase, e modificação e *splicing* do RNA) (Vincent, Omura*, et al.*, 2011), relacionados a adesão celular (*PCDH1, PCDH10, CDH2 e CDH4*), sinalização WNT (*SOX1, APC2 e WNT5A*) e

pluripotência (*BMP3, FOXD3 e BMI1*) foram reportados ao longo dos anos como diferentemente metilados nas neoplasias pancreáticas (Vincent, Omura*, et al.*, 2011). Um estudo com 167 amostras de ADP identificou que a metilação afeta vias-chave da carcinogênese pancreática, tais como TGF-β, Wnt, e sinalização de orientação do axônio (Nones *et al.*, 2014).

3.2. Modificações nas Histonas

O estado de agregação do nucleossoma, estrutura composta por DNA e proteínas básicas denominadas histonas, é o que regula a organização nuclear nas células eucariotas. O nucleossoma também pode ser denominado unidade básica da cromatina e é formado 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 proteico em torno do DNA (Figura 5) (Luger *et al.*, 1997; Schneider, G. *et al.*, 2011).

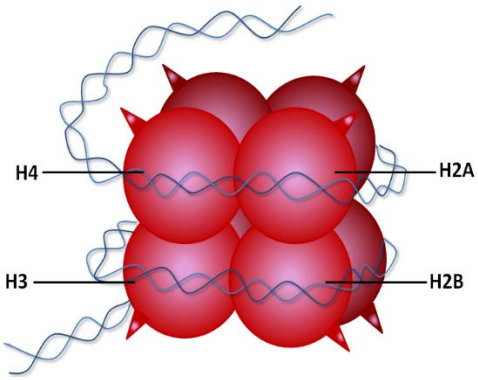


Figura 5. Estrutura do nucleossoma. Dupla hélice de DNA de 146 pb envolve esta estrutura (figura elaborada pela autora).

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 adicionar ou retirar moléculas covalentes a essas proteínas. A alteração da conformação do nucleossoma é mediada por essas alterações e tem como resultado final a modificação da acessibilidade das maquinarias de reparo, replicação e transcrição (Lee *et al.*, 1993; Peterson and Laniel, 2004; Groth *et al.*, 2007; Mazzio and 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 and 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 *et al.*, 2007).

O processo de modificação das histonas por acetilação é dependente de dois grupos de enzimas: as acetilases de histonas (do inglês *histone acetyltransferases*, HATs) e as HDACs. 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 6 (Brown *et al.*, 2000; Schneider, G. *et al.*, 2011).

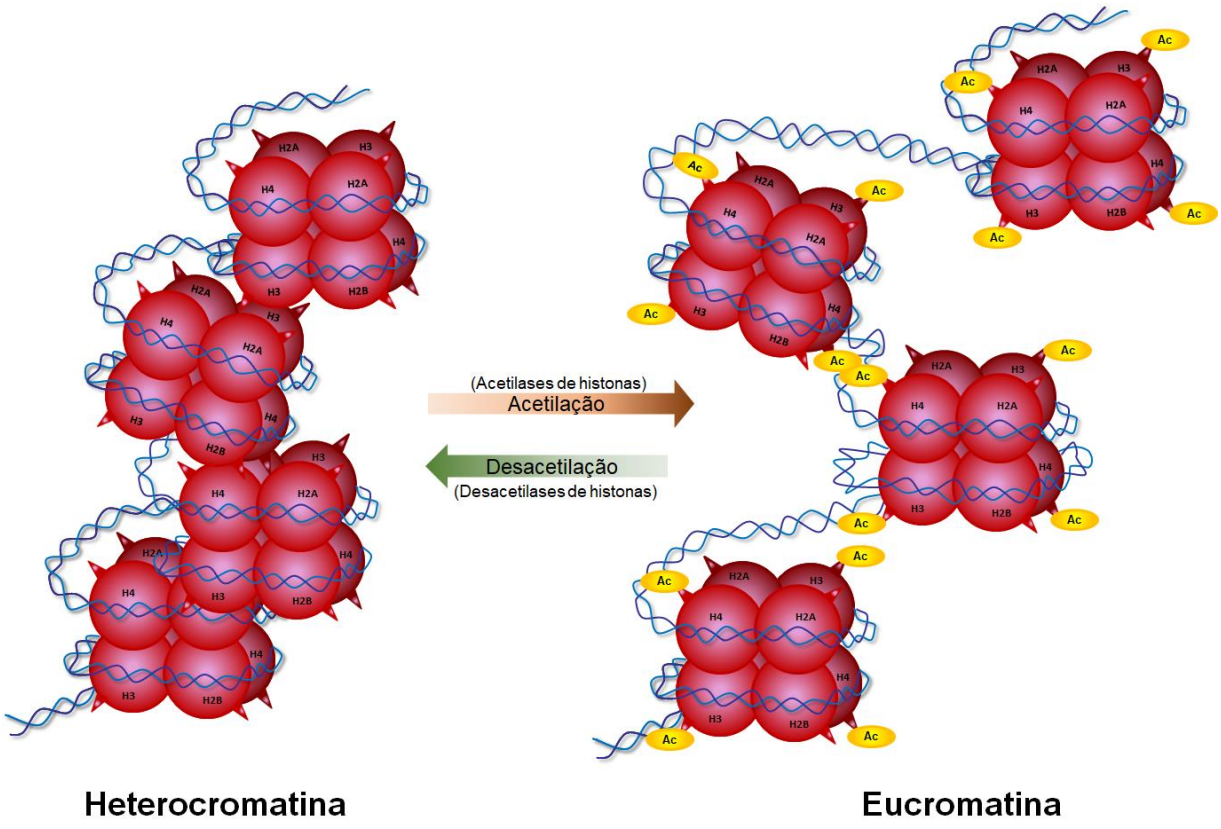


Figura 6. Transição entre eucromatina e heterocromatina mediada pelas acetilases de histonas (HATs) e desacetilases de histonas (HDACs). HATs acetilam os resíduos H3 e H4 das histonas modificando a afinidade destas com o DNA e permitindo maior acessibilidade de proteínas ao DNA (eucromatina). A remoção dos grupos acetil mediada pelas HDACs restaura a afinidade basal das estruturas (heterocromatina)(figura elaborada pela autora).

A família das HDACs apresenta quatro classes que diferem em relação à sua homologia no sítio catalítico, sendo classificadas de I a IV (Figura 7). As classes I, II e IV usam o íon zinco para a hidrólise do grupo acetil, enquanto a classe III (ou sirtuínas, SIRT), é dependente da presença do cofator metabólico NAD^+ para promover a desacetilação e liberação do grupo acetil (Gregoretti *et al.*, 2004; Ekwall, 2005). A classe I é composta pelas HDACs 1, 2, 3 e 8 e a classe II é subdividida em classe IIa composta pelas HDACs 4, 5, 7, 9 e 10 (que possuem apenas um domínio catalítico); e classe IIb composta pelas HDACs 6 e 10 (que possui dois domínios catalíticos). A classe III é representada pelas SIRTs1, 2, 3, 4, 5, 6 e 7; e a classe IV possui apenas

um representante, a HDAC11 (Grozinger and Schreiber, 2002; Yang and Seto, 2008; Schneider, G. *et al.*, 2011).

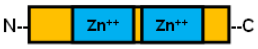
Classe	Isoforma	Subunidade Catalítica	Cofator	Localização Celular	Expressão	Domínio Catalítico
I	HDAC1	RPD3	Zn^{++}	Nuclear	Ubíqua	
	HDAC2			Nuclear		
	HDAC3			Nuclear e Citoplasmática		
	HDAC8			Nuclear		
IIa	HDAC4	HDA1	Zn^{++}	Nuclear e Citoplasmática	Específica	
	HDAC5			Nuclear e Citoplasmática		
	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 7. Classes de desacetilases de histonas (adaptado de Schneider, A. *et al.*, 2011; Shirakawa *et al.*, 2013)**.**

As HDACs ainda atuam removendo grupos acetil de proteínas não-histonas como fatores de transcrição (E2F, p53, c-Myc, NF-κB), chaperonas (HSP90), mediadores da sinalização (Stat3, Smad7), proteínas de reparo (Ku70), α-tubulina, β-caderina, retinoblastoma (pRb), 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), exercendo efeitos diretos em vários processos biológicos (Figura 8) (West and Johnstone, 2014) .

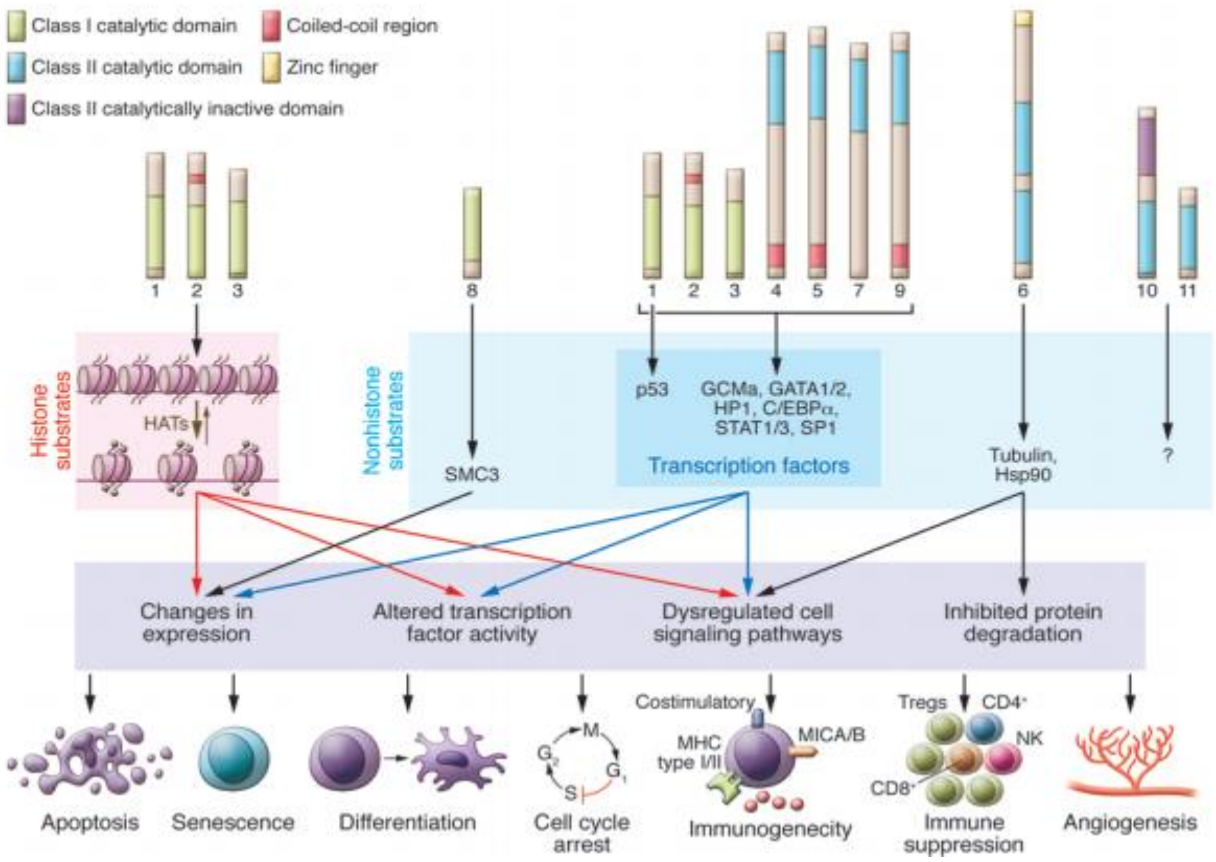


Figura 8. Consequências biológicas da desacetilação de proteínas (West and Johnstone, 2014).

3.2.1. Desacetilases de histonas em carcinomas periampulares

O papel das HDACs no desenvolvimento e controle de neoplasias tem chamado à atenção de muitos estudos, uma vez que a superexpressão das mesmas pode resultar no impedimento da transcrição de genes ligados a vias fundamentais para a carcinogênese (Bi and Jiang, 2006; Haberland *et al.*, 2009; Schneider *et al.*, 2010; West and Johnstone, 2014). A expressão aumentada das HDACs é frequentemente observada em várias neoplasias sólidas e hematológicas, como câncer de mama, ovário, colorretal e pâncreas (Sanaei and Kavoosi, 2019).

A proliferação e manutenção do ADP envolvem HDACs de classes I e II. A classe I desempenha um papel predominante na carcinogênese (Singh *et al.*, 2016). Várias HDACs, como HDAC1, HDAC2, HDAC3 e HDAC7, foram superexpressas nas

linhagens celulares e amostras teciduais do ADP (Fritsche *et al.*, 2009; Lehmann *et al.*, 2009; Wang *et al.*, 2012; Ouaïssi *et al.*, 2014) . Entre os mecanismos caracterizados envolvendo as HDACs no ADP destacam-se: 1) Controle da proliferação celular, HDACs controlam a expressão de p21, p27 e ciclina B1 modulando a fase G1 / S ou G2 / M ou o ciclo celular; 2) Inibição a apoptose, HDACs contribuem para a expressão desequilibrada dos genes anti-apoptótico (*BCL-W, MCL1, BCL-XL* e *c-FLIP*) e pró-apoptótico (*BIM, BAX* e *NOXA*); e 3) Metástase, HDAC1 e 2 reprimem a expressão de E-caderina, favorecendo a transição epitélio- mesenquimal (Sanaei and Kavoosi, 2019). A tabela 4 apresenta um resumo das principais características dessas proteínas nos tumores pancreáticos.

Quadro 2. Expressão e consequência biológica das HDACs no ADP.

HDAC	Expressão	Consequência	Referência
HDAC1	Superexpressão	Alta atividade proliferativa, pior sobrevida	Miyake *et al.*, 2008; Wang *et al.*, 2009; Bosman *et al.*, 2010; Sanaei and Kavoosi, 2019
HDAC2	Superexpressão	Correlação com pior grau de diferenciação do tumor, modula a resistência à apoptose	Fritsche *et al.*, 2009; Lehmann *et al.*, 2009
HDAC3	Superexpressão	Envolvida no controle da transição epitélio mesenquimal, metástase, proliferação celular	Lehmann *et al.*, 2009; Jiao *et al.*, 2014; Sanaei and Kavoosi, 2019
HDAC6	Superexpressão	Protege as células tumorais da apoptose, ajudando a reduzir a quantidade intercelular de proteínas mal dobradas	Klieser *et al.*, 2015; Sanaei and Kavoosi, 2019
HDAC7	Superexpressão	Media negativamente atividades antiproliferativas e condução da apoptose	Ouaïssi *et al.*, 2008; Ouaïssi *et al.*, 2014; Cai *et al.*, 2018

Atualmente não há relatos da expressão das HDACs no adenocarcinoma duodenal e para os outros carcinomas periampulares as informações acerca de sua expressão são muito limitadas. As HDACs foram melhores caracterizadas nos colangiocarcinomas, contudo a maior parte dos estudos aborda os colangiocarcinomas intra-hepáticos ou não especifica a classificação tumoral do colangiocarcinoma estudada estudada (Yamaguchi *et al.*, 2010; Morine *et al.*, 2012;

Sriraksa and Limpaiboon, 2013; He *et al.*, 2016; Jung *et al.*, 2017). He et al., avaliou a expressão gênica da classe I e II das HDACs por qRT-PCR em 26 colangiocarcinomas (de origem não especificada) comparados com tecidos não tumorais adjacentes. No estudo, os autores encontraram superexpressão das *HDAC1*, *HDAC2*, *HDAC3*, *HDAC8* e *HDAC9* nos tumores e investigação proteica confirmou a superexpressão das HDAC2, HDAC3 e HDAC8. Adicionalmente, a alta expressão das HDAC2 e HDAC3 foi correlacionada com comprometimento de linfonodos, pior estadiamento e grau de diferenciação (He *et al.*, 2016). O carcinoma neuroendócrino de células grandes é um carcinoma neuroendócrino de alto grau, originalmente descrito no pulmão. O tumor raramente ocorre em locais extrapulmonares como o trato gastrointestinal, e apenas alguns exemplos foram descritos na ampola de Vater. A expressão das HDAC1, HDAC2 e HDAC3 foi investigada nesse subtipo histológico raro de carcinoma da ampola de Vater em combinação com uma série de marcadores imunohistoquímicos para a caracterização do tumor e avaliação da agressividade. Os níveis de expressão foram cerca de 60% para todas as HDAC analisadas (Stojsic *et al.*, 2010).

CAPÍTULO II: JUSTIFICATIVA

Carcinomas periampulares são neoplasias raras que podem ter quatro origens teciduais distintas, com perfil de agressividade variado. Tumores pancreáticos são os mais frequentes entre os carcinomas periampulares e estes têm as piores taxas de mortalidade. Especificamente para o ADP, até o momento, pouquíssimas estratégias terapêuticas tem demonstrado um efeito significativo na sobrevida em virtude de fatores intrínsecos da neoplasia, da sintomatologia inespecífica da doença, e de limitações metodológicas relacionadas aos exames de diagnóstico. Desta forma, a descoberta de novos biomarcadores para diagnóstico precoce, desenvolvimento de novas terapias direcionadas a alvo e definição de prognóstico são necessidades urgentes.

Em relação aos outros tumores periampulares, as informações genéticas e epigenéticas ainda são limitadas, tanto a respeito da sua biologia tumoral como seu prognóstico. Ao que nos consta, o perfil de desacetilases de histonas nunca foi investigado em adenocarcinomas duodenais e nos carcinomas de ampola de Vater informações são muito escassas. Considerando que vários estudos têm abordado o papel de alterações epigenéticas no processo de carcinogênese e na resposta a tratamentos anti-neoplásicos de outros tumores, a caracterização de mecanismos epigenéticos dos carcinomas periampulares pode fornecer embasamento para essas abordagens terapêuticas. Considerando que alguns aspectos moleculares importantes desses tumores ainda precisam ser esclarecidos, essa tese propôs, entre outros objetivos, sanar dúvidas relevantes sobre características epigenômicas e genômicas dos tumores periampulares, contribuindo para um melhor entendimento da carcinogênese destes tumores e contribuindo com informações para identificação de novos marcadores prognósticos.

CAPÍTULO III: OBJETIVOS

Objetivo geral

Realizar a caracterização molecular do adenocarcinoma ductal pancreático e de tumores periampulares com o objetivo de contribuir para melhor compreensão do processo de carcinogênese e identificar marcadores prognósticos relacionados a esses tumores.

Objetivos específicos

1. Caracterizar o perfil de metilação do DNA do adenocarcinoma ductal pancreático em busca de potenciais alvos terapêuticos nas vias moleculares associadas à carcinogênese destes tumores.
2. Avaliar a correlação entre o perfil de metilação do DNA e o perfil de expressão gênica do adenocarcinoma ductal pancreático e investigar possíveis biomarcadores prognósticos.
3. Caracterizar o perfil de expressão das desacetilases de histonas *HDAC1*, *HDAC2*, *HDAC3* e *HDAC7* em tumores periampulares em especial no adenocarcinoma de ampola de Vater.
4. Descrever uma apresentação incomum de múltiplos tumores periampulares em um paciente com neurofibromatose tipo 1 e as potenciais correlações genótipo-fenótipo existentes.

CAPÍTULO IV: Manuscrito 1

"Pancreatic Ductal Adenocarcinoma methylome analysis identifies differentially methylated genes in the Calcium signaling pathway and early methylation alterations"

Manuscrito a ser submetido à revista *Cancers* (Fator de impacto: 6,1).

Pancreatic Ductal Adenocarcinoma methylome analysis identifies differentially methylated genes in the Calcium signaling pathway and early methylation alterations.

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with high mortality rates. PDAC initiation and progression are promoted by genetic and epigenetic dysregulation. Here, we aimed to characterize the PDAC DNA methylome in search of novel altered pathways associated with tumor development. We examined the genome-wide DNA methylation profile of PDAC in an exploratory cohort including comparative analyses of tumoral and non-tumoral pancreatic (PT) tissues. Pathway enrichment analysis was used to select differentially methylated (DM) CpGs with potential biological relevance. Additional samples were used in a validation cohort. DNA methylation impact on gene expression and gene expression association with overall survival (OS) was investigated from PDAC TCGA data. Pathway analysis revealed DM genes in the Calcium signaling pathway, which linked to key pathways in pancreatic carcinogenesis. DNA methylation was frequently correlated with expression and a subgroup of Calcium signaling genes was associated with OS, reinforcing its probable phenotypic effect. Cluster analysis of PT samples revealed that some of the methylation alterations observed in the Calcium signaling pathway seem to occur early in the carcinogenesis process, a finding that may open new insights about PDAC tumor biology.

Keywords: Pancreatic Ductal Adenocarcinoma, Epigenetics, Calcium signaling pathway, Early methylation alterations**.**

1. Introduction

Pancreatic cancer is a very aggressive disease, with 5-year survival rates below 8% and a strong ability to metastasize even before the primary tumor is clinically detected [1; 2]. Tumor aggressiveness, lack of specific symptoms in early stages, and resistance to cytotoxic drugs, all contribute to the high mortality rates[3]. In fact, current estimates predict that pancreatic cancer will be the second most lethal tumor by 2030[4]. Considering all pancreatic malignancies, pancreatic ductal adenocarcinoma (PDAC) is the most prevalent type, accounting for more than 90% of the cases[5]. PDAC initiation and progression are promoted by the interaction between driver mutations and the disruption of epigenetic regulatory circuits such as DNA methylation[6].

DNA methylation, which is most often associated with gene expression regulation, is one of the best understood epigenetic mechanisms of transcriptional regulation. In cancer, the DNA methylation landscape often involves global hypomethylation mainly described at CpG sites located in intergenic regions, including repetitive elements [7]. On the other hand, studies using RefSeq gene analysis (such as the 450K BeadChip Array platform) show that most CpGs are hypermethylated in cancer, affecting mainly tumor suppressor genes [8; 9]. Although different studies have investigated the PDAC methylation profile, only few analyzed wide DNA methylation patterns[10; 11].

The discovery of new biomarkers including DNA methylation and others biologic process for development of novel target-driven therapies, and definition of prognosis in PDAC is an urgent need. In this study, we aimed to characterize the PDAC DNA methylome in search of novel altered pathways associated with tumor development through a comparative analysis of tumoral and non-tumoral pancreatic tissues. An important new finding resulting from this analysis was the identification of several differentially methylated genes of the Calcium (Ca^{2+}) signaling pathway, which linked to key pathways in pancreatic carcinogenesis. In addition, some of the methylation alterations observed in this pathway seem to occur early in the carcinogenesis process, a finding that may open new insights about PDAC tumor biology.

2. Results and Discussion

2.1. Patients and experimental design

Differentially methylated genes were selected of an exploratory cohort (six PDAC and nine adjacent pancreatic non-tumoral tissue samples), and validation was performed by pyrosequencing in a biological and technical validation cohort comprising a different set including 43 PDAC and 24 PT samples. Clinical features are shown in **Table S1**, and the overall experimental design is summarized in **Figure 1**.

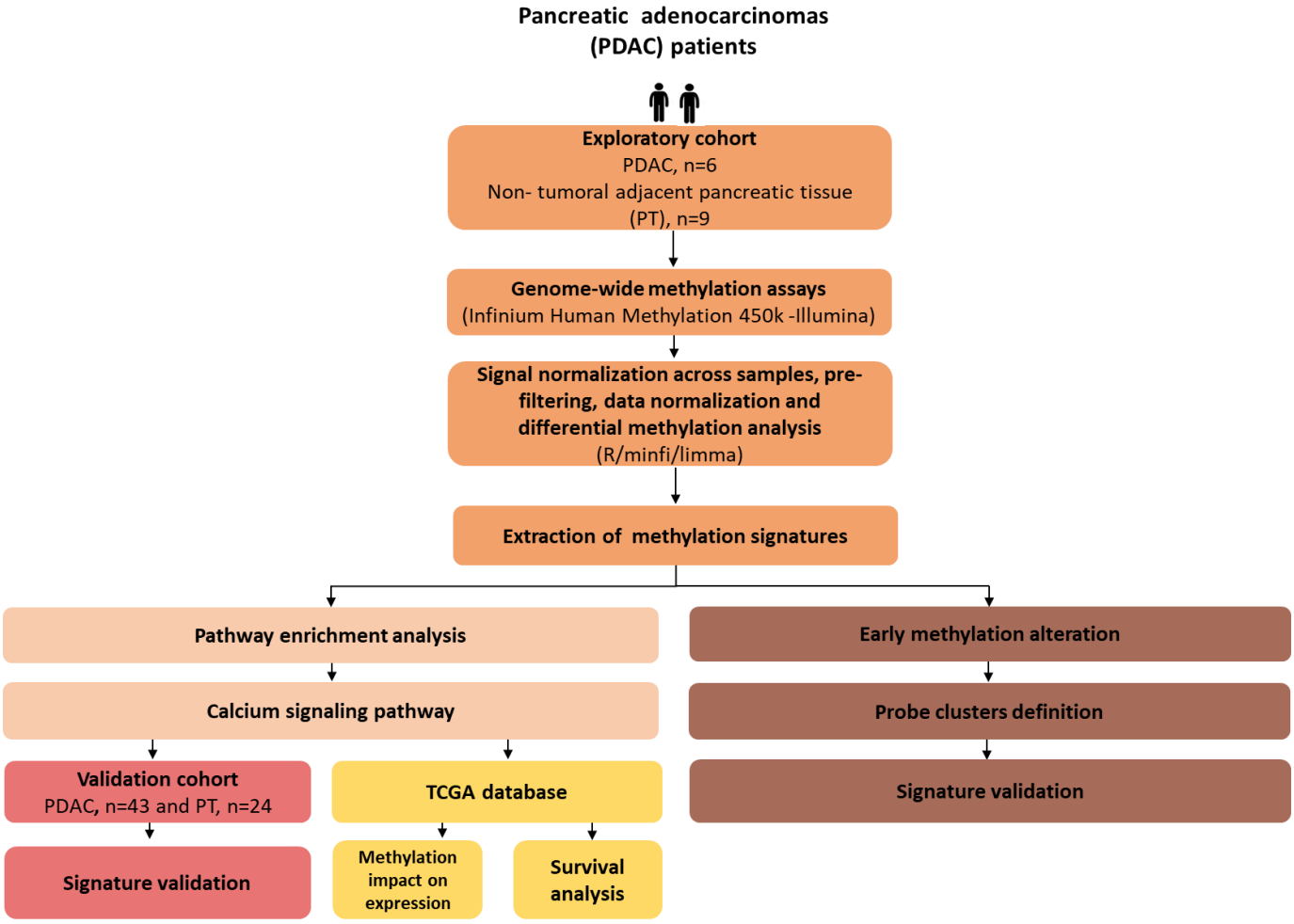

Figure 1. Flow chart illustrating the overall design of the study.

2.2. Genome-wide DNA methylation profile in pancreatic adenocarcinoma

The genome-wide DNA methylation profile of PDAC and PT samples was determined in an exploratory cohort using Infinium 450K beadchips. The unsupervised hierarchical clustering analysis of the 10,361 differentially methylated probes (DMPs, adjusted p-value < 0.01 and $\Delta\beta \geq 0.2$) exhibited clear separation between PDAC and PT. As shown in **Figure 2A**, two major clusters emerged: the first comprised PT samples and was predominantly hypomethylated, while PDAC samples formed the second cluster, which showed an overall hypermethylated profile (75.48% of the DMPs). PDAC hypermethylated probes were most frequently annotated to promoter regions (40.23%), while most hypomethylated probes were mapped to gene bodies (37.76%). Stratification by distance to CpG islands revealed that hypomethylated CpG sites most commonly encompassed open sea regions (68.66%) and hypermethylation was most common at CpG islands (72.68%) (**Figure 2B, 2C, 2D**).

The 450K BeadChip Array, used here, was previously employed to build two PDAC methylome databases and previous analyses are reported in the literature. Nones *et. al.* and Mishra *et. al.* have also shown that the majority of DMPs in PDAC were hypermethylated and located in promoter regions, 54.21% and 56.99%, respectively[10; 11]. CpG island methylation in promoters is frequently associated with gene silencing during tumorigenesis, providing an alternative mechanism to mutations by which tumor suppressor genes may be inactivated within a cancer cell[12; 13]. In our exploratory cohort, as well as in other studies[14; 15; 16; 17; 18; 19], the tumor suppressor genes *NPTX2, CDO1, TFPI2, SFRP1, SFRP2, PENK* and *FOXE1* had a remarkable hypermethylation pattern in the PDAC group[14; 15; 16; 17; 18; 19].

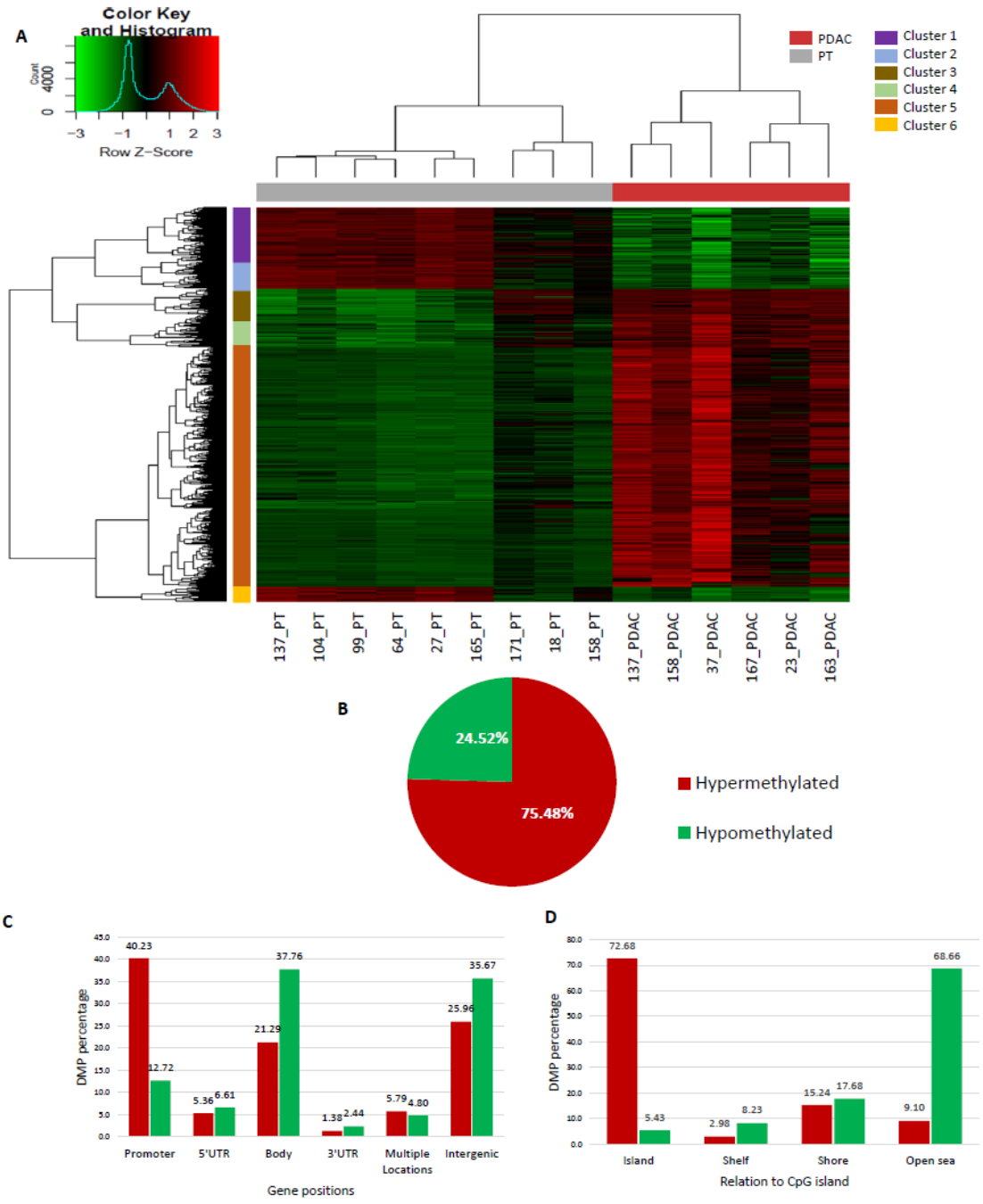

Figure 2. DNA methylation profile of pancreatic adenocarcinoma. Heatmap showing the unsupervised hierarchical clustering of pancreatic adenocarcinoma (PDAC) and non-tumoral adjacent pancreatic tissue (PT) with a minimum 80% cellularity according to the methylation profile of the 10,361 probes found to be differentially methylated between groups (adjusted p-value < 0.01 and $\Delta\beta \geq 0.2$). Hyper- and hypomethylation

are represented in red and green, respectively. Colored bars on the left side of the heatmap represent probe clusters defined by their methylation similarities (A); Pie chart showing the percentage of hypomethylated and hypermethylated probes in PDAC samples relative to non-tumoral adjacent tissues (B); Overall distribution of hypo- and hypermethylated probes according to their gene position (C); Overall distribution of hypo- and hypermethylated probes according to their relation to CpG islands (D).

2.3. Pathway enrichment analysis

The next step was to analyze the potential biological relevance of the differentially methylated CpG sites identified in the supervised comparison between PDAC and PT samples. The DMPs mapped to 2,715 genes, among which 1,766 are hypermethylated and 1,100 are hypomethylated, with an overlap of 151 genes among both sets. From these, 611 hypermethylated and 386 hypomethylated genes were annotated in Kyoto Encyclopedia of Genes and Genomes (KEGG) database and were used as query gene sets to assess functional enrichment of DMPs. Hypermethylated and hypomethylated genes were significantly associated with the enrichment of 36 and 25 cellular pathways, respectively (**Figure S1A, Figure S1B, Table S2 and 3**). Subsequently, we merged both analyses in order to compare which biological pathways were frequently deregulated in PDAC tumors by both hyper and hypomethylation. As shown in **Figure 3A**, several pathways well-known to be involved in cancer development were identified, such as the MAPK signaling pathway, which is engaged in multiple proliferative cellular processes (cell differentiation, proliferation, and apoptosis) [20], and the focal adhesion pathway which may play a role in the development and progression of cancer[21]. Additionally, we observed that the Ca^{2+} signaling pathway had a high number of genes both significantly hypo- and hypermethylated. This pathway is intrinsic to multiple aspects of cancer biology, such as tumor initiation, metastasis, and drug resistance[22]. Although Ca^{2+} pathway methylation in PDAC is still poorly explored, many of its genes have already been described as differentially methylated in other solid tumors, including gastric[8], prostate[8; 23], and breast cancer [8; 23]. The overlap between the differentially methylated genes of the Ca^{2+} signaling pathway and other cellular pathways was investigated. This analysis revealed that several genes of the Ca^{2+} signaling pathway

are shared with other significantly enriched pathways, such as the Hippo and Ras signaling pathways (**Figure 3B**).

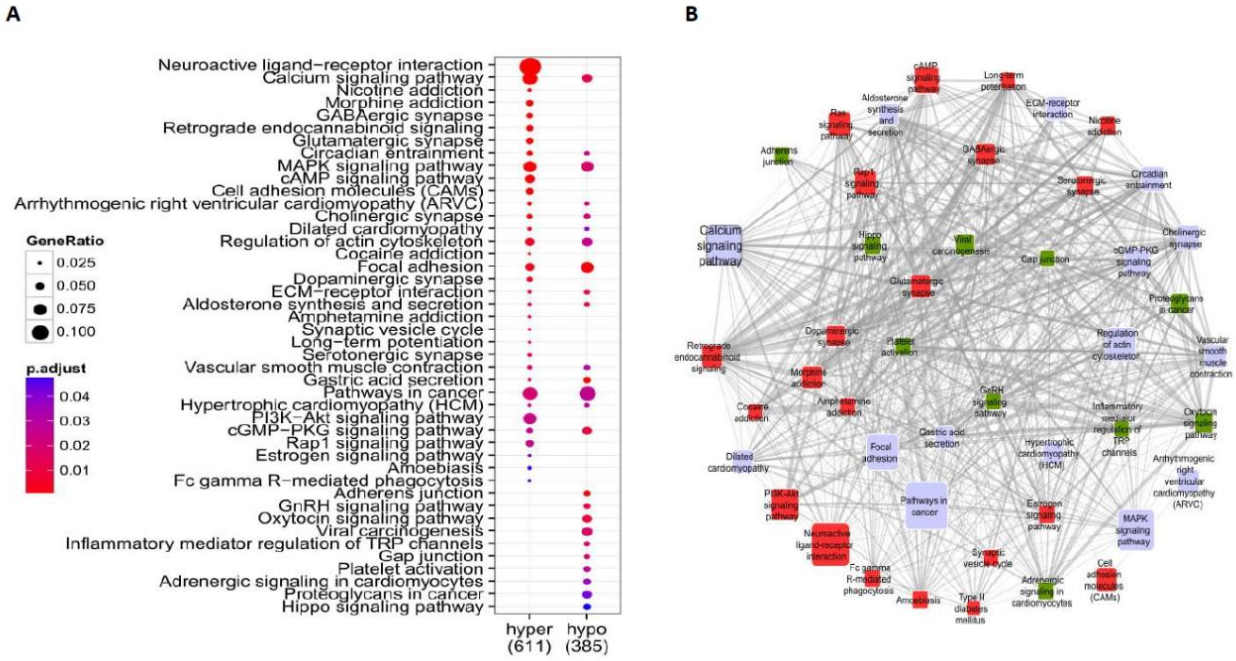

Figure 3. Pathway enrichment analysis of differentially methylated genes (n=996) in pancreatic adenocarcinoma. Functional enrichment of hypermethylated (n=611) and hypomethylated genes (n=385) annotated from DMPs (A); Interactions between enriched pathways evidencing the number of shared differentially methylated genes. Pathways in red and green are those enriched for hyper and hypomethylated genes, respectively, and those in lilac are enriched for both types of genes (B).

2.4. Validation cohort

We performed an in-depth analysis of the Ca^{2+} signaling pathway and the methylation profile of five genes that were assessed in an independent validation cohort. Genes of the functional enrichment that were exclusively hypo- or hypermethylated were selected according to the following criteria: genes with a previously described role in cancer and containing DMPs associated with a high $|\Delta\beta|$. One hypermethylated promoter (*RYR3*) and 4 hypomethylated gene body probes

(*EGFR, ITPR2, CAMK2A, and CALM2*) were chosen. Methylation levels at the CpG sites interrogated by the Infinium probe as well as surrounding CpG sites were evaluated, and the β-values obtained by pyrosequencing were in agreement and strongly correlated with the Infinium assay (**Figure S2 and Figure S3**).

2. 5. The Cancer Genome Atlas (TCGA) analysis

2.5.1. Methylation impact on expression

Considering the unavailability of RNA samples from our cohort, we used the information available in TCGA about PDAC to explore the impact of DNA methylation on the expression of Ca^{2+} signaling pathway genes[24]. A total of 173 probes were analyzed, and 112 (64.73%) showed a significant correlation with gene expression (**Table S4**). **Figure 4A** depicts the methylation profile of these probes as well as the correlation with expression. Methylation levels of probes annotated to promoters were significantly and inversely correlated with expression in 63.9% of the cases. On the other hand, the methylation profile of probes located in gene bodies showed a correlation with expression in 59.6% of the cases, 64.3% inverse and 35.7% direct.

In **Figure 4B**, genes for which a significant correlation between methylation and expression was observed are highlighted showing the differential methylation in promoters and gene bodies identified in the TCGA dataset. Genes that control intracellular Ca^{2+} storage, such as ryanodine receptors (*RYR2* and *RYR3*), were hypermethylated (promoters and gene bodies), while inositol 1,4,5- trisphosphate receptor (*ITPR1*) showed a heterogeneous DNA methylation profile in the body region.

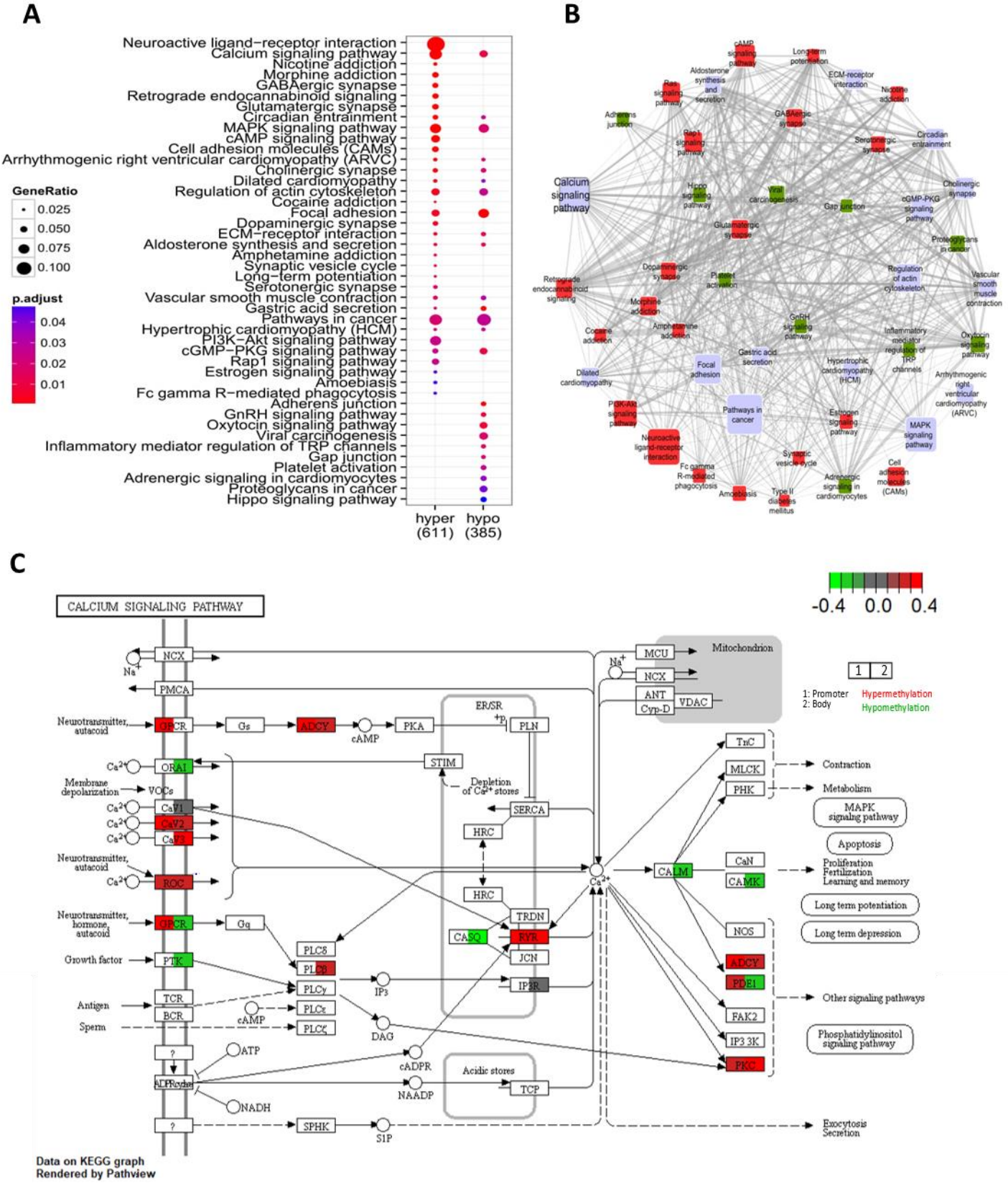
A
Neuroactive ligand−receptor interaction
Calcium signaling pathway
Nicotine addiction
Morphine addiction
GABAergic synapse
Retrograde endocannabinoid signaling
Glutamatergic synapse
Circadian entrainment
MAPK signaling pathway
cAMP signaling pathway
Cell adhesion molecules (CAMs)
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Cholinergic synapse
Dilated cardiomyopathy
Regulation of actin cytoskeleton
Cocaine addiction
Focal adhesion
Dopaminergic synapse
ECM−receptor interaction
Aldosterone synthesis and secretion
Amphetamine addiction
Synaptic vesicle cycle
Long−term potentiation
Serotonergic synapse
Vascular smooth muscle contraction
Gastric acid secretion
Pathways in cancer
Hypertrophic cardiomyopathy (HCM)
PI3K−Akt signaling pathway
cGMP−PKG signaling pathway
Rap1 signaling pathway
Estrogen signaling pathway
Amoebiasis
Fc gamma R−mediated phagocytosis
Adherens junction
GnRH signaling pathway
Oxytocin signaling pathway
Viral carcinogenesis
Inflammatory mediator regulation of TRP channels
Gap junction
Platelet activation
Adrenergic signaling in cardiomyocytes
Proteoglycans in cancer
Hippo signaling pathway
hyper (611)
hypo (385)
GeneRatio
0.025
0.050
0.075
0.100
p.adjust
0.04
0.03
0.02
0.01
B
Calcium signaling pathway
C
CALCIUM SIGNALING PATHWAY
-0.4 0.0 0.4
1: Promoter Hypermethylation
2: Body Hypomethylation
Mitochondrion
Contraction
Metabolism
MAPK signaling pathway
Apoptosis
Proliferation
Fertilization
Learning and memory
Long term potentiation
Long term depression
Other signaling pathways
Phosphatidylinositol signaling pathway
Exocytosis
Secretion
Data on KEGG graph
Rendered by Pathview

Figure 4. Calcium (Ca^{2+}) signaling pathway analysis. **A**. Heatmap showing the methylation profile of probes (n= 112 probes) annotated to genes involved in the Ca^{2+} signaling pathway for which a significant correlation (false discovery rate (FDR) - adjusted p-value < 0.05) with gene expression was observed in TCGA dataset (n= 141). The bars located on the left of heatmap indicate the correlation coefficient per gene region affected by differential methylation. **B.** Schematic diagram of the Ca^{2+} signaling pathway. The network was built based on the KEGG pathway map (KEGG: hsa04020). Genes for which a significant correlation between methylation and expression was observed are highlighted showing the differential methylation in promoters and gene bodies, as well as copy number alterations identified in TCGA dataset (N=141). Red squares indicate hypermethylated genes while green squares represent hypomethylated genes.

2.5.2. Survival analysis (TCGA)

Ca^{2+} pathway genes that displayed significant correlations between methylation and expression in TCGA pancreatic cancer samples (**Table S4**) were selected for survival analysis. We evaluated the expression impact of thirty-two genes on PDAC patient´s overall survival (OS), including two genes that had already been investigated in the validation cohort (*CALM2*, and *RYR3*).

PDAC patients with low *ADCY8*, *CACNA1A*, *CACNA1B*, *CACNA1D*, *CACNA1H*, *ORAI2*, *PDE1C*, *PLCB1*, and *RYR3* expression showed decreased OS when compared to those with high gene expression. On the other hand, decreased *HRH1* expression was associated with increased OS (**Figure S4 and Table S5**).

2.6. Early methylation alterations

We investigated the overall methylation profile of three PT samples (18PT, 158PT, and 171PT) with intermediate methylation levels at DMPs between PDAC and PT (**Figure 2A**). These samples will be named PT-like from now on. To validate this discordant methylation profile, first a multidimensional scaling plot was used, showing that PT-like samples were more closely related to PDAC than PT samples, when considering principal component 1 (**Figure 4A and Figure S5A**). This finding may be associated with molecular field cancerization, since these samples showed a high

percentage of normal ducts (>80%), small focal fibrosis regions and no evidence of neoplastic cell contamination (**Figure S5B-D**).

Field cancerization is defined by a set of genetic and epigenetic alterations that indicate that a specific tissue area is undergoing a transformation process or has a predisposition to initiate such process, and this may occur without overt morphological changes [25]. Considering that this process occurred in PT-like samples, we performed a deep analysis of DMP clusters comparing the major similarities between PDAC and PT-like. Three clusters derived from the previous unsupervised hierarchical clustering analysis were selected for further investigation, namely, clusters 2, 3, and 6. After a new unsupervised hierarchical clustering analysis using only DMPs of these clusters, PT-like samples grouped with PDAC samples (**Figure 5-A**). Then, we performed the pathway enrichment analysis with the corresponding annotated genes and observed that 23 differentially methylated genes were involved in the Ca^{2+} signaling pathway: Twelve genes from cluster 2, 8 from cluster 3 and 6 from cluster 6; and 3 genes appeared in more than one cluster (**Figure 5B-D**). The stepwise methylation profile in PT, PT-like and PDAC samples was observed in two previously validated genes (*CALM2* and *CAMK2A,* **Figure 5E-F**).

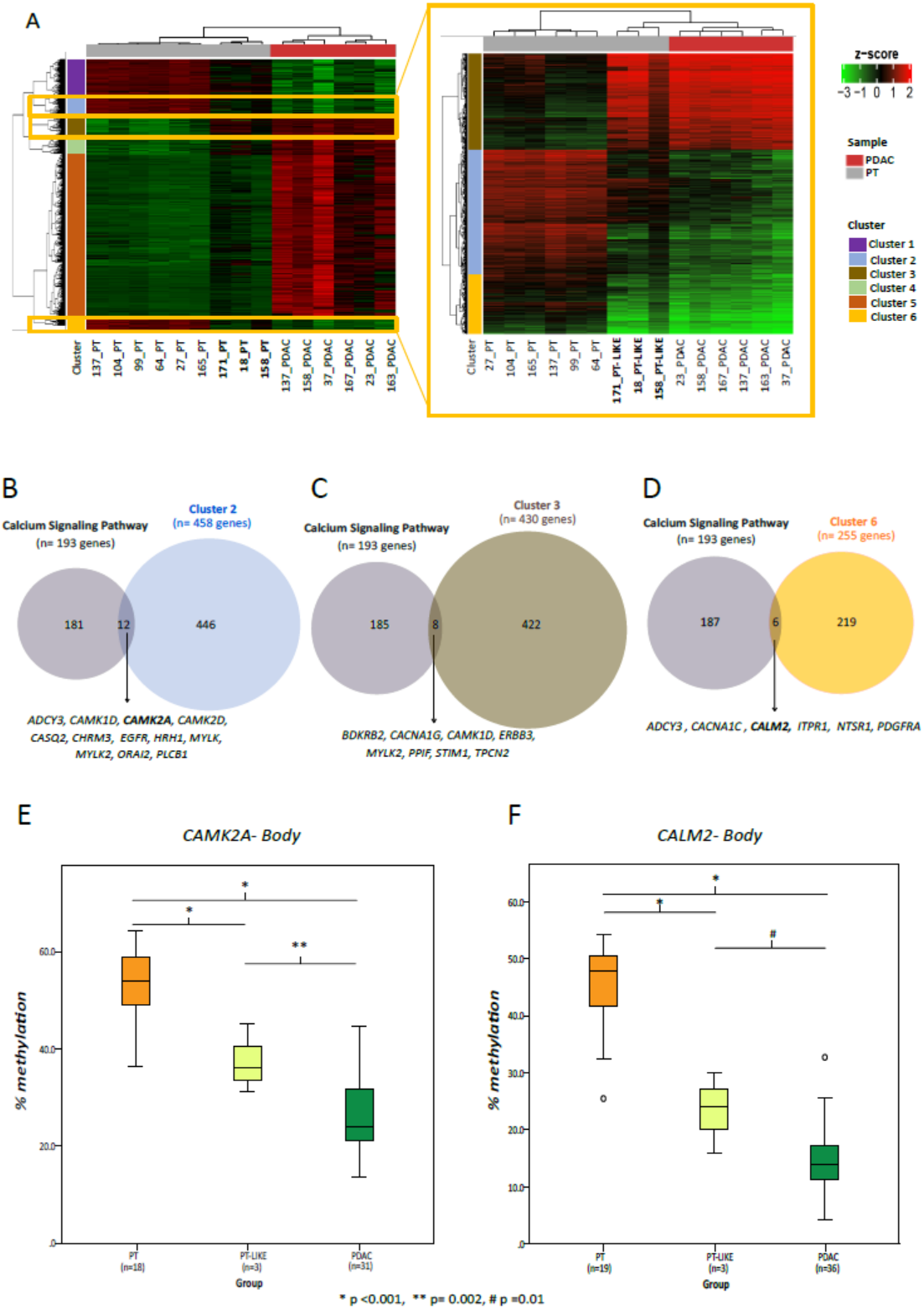
A
z-score
-3 -1 0 1 2
Sample
PDAC
PT
Cluster
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 137_PT 104_PT 99_PT 64_PT 27_PT 165_PT 171_PT 18_PT 158_PT 137_PDAC 158_PDAC 37_PDAC 167_PDAC 23_PDAC 163_PDAC
Cluster 27_PT 104_PT 165_PT 137_PT 99_PT 64_PT 171_PT-LIKE 18_PT-LIKE 158_PT-LIKE 23_PDAC 158_PDAC 167_PDAC 137_PDAC 163_PDAC 37_PDAC
B
Calcium Signaling Pathway
(n= 193 genes)
Cluster 2
(n= 458 genes)
181
12
446
ADCY3, CAMK1D, CAMK2A, CAMK2D, CASQ2, CHRM3, EGFR, HRH1, MYLK, MYLK2, ORAI2, PLCB1
C
Calcium Signaling Pathway
(n= 193 genes)
Cluster 3
(n= 430 genes)
185
8
422
BDKRB2, CACNA1G, CAMK1D, ERBB3, MYLK2, PPIF, STIM1, TPCN2
D
Calcium Signaling Pathway
(n= 193 genes)
Cluster 6
(n= 255 genes)
187
6
219
ADCY3 , CACNA1C , CALM2, ITPR1, NTSR1, PDGFRA
E
CAMK2A- Body
% methylation
PT (n=18)
PT-LIKE (n=3)
PDAC (n=31)
Group
F
CALM2- Body
% methylation
PT (n=19)
PT-LIKE (n=3)
PDAC (n=36)
Group
* p <0.001, ** p= 0.002, # p =0.01

Figure 5. Comparative DNA methylation analysis in PT, PT-like and PDAC samples with ≥ 20% cellularity. Heatmaps highlighting the methylation profile of probe clusters supposed to be altered in early stages of PDAC development. Heatmap on the left shows the 10,361 DMPs between PDAC and PT. Clusters 2, 3 and 6 were selected for a detailed analysis (heatmap on the right) since their probes showed intermediate methylation levels (between PDAC and PT) in PT-like samples (A); Overlap between genes involved in the calcium signaling pathway and genes belonging to each selected probe cluster (B, cluster 2; C, cluster 3; and D, cluster 6)(B- D). **E- F**. Boxplots representing the overall methylation level of *CALM2* (E) and *CAMK2A* (F) in PT, PT-like and PDAC samples. P-values were calculated using the Generalized Estimating Equations.

Ca^{2+} is a ubiquitous intracellular messenger that controls diverse processes in cellular physiology, such as gene transcription, cell progression, cell motility, and apoptosis[32]. Resting cytosolic free Ca^{2+} is maintained at lower levels than those of extracellular space, and its equilibrium dynamics is carefully regulated by the plasmatic membrane, endoplasmic reticulum (ER), and mitochondria[33], using a “toolkit” of channels, pumps, and cytosolic buffers to control Ca^{2+} cell homeostasis [34]. The spatial and temporal dynamics of Ca^{2+} signaling results in specific cellular responses mediated by the activation of a subset of Ca^{2+}-dependent effectors[35].

During carcinogenesis, several cellular metabolic functions become deregulated, including those related to Ca^{2+} signaling[27; 28] and therefore it is not surprising that changes in the expression or function of Ca^{2+} handling proteins impact tumorigenesis. In fact, previous studies have reported altered expression or mutations in genes involved in Ca^{2+} signaling in different tumors, such as colorectal[36], breast[37], and pancreatic cancer[38]. However, little information is available regarding methylation-related expression deregulation of genes involved in the Ca^{2+} signaling pathway in PDAC. In one of the few studies, the methylation profile of *S100A4*, a Ca^{2+}-binding protein previously implicated in metastasis[39], was evaluated in PDAC samples and cell lines. Hypomethylation was detected in tumors, whereas all normal pancreatic tissue

samples analyzed were hypermethylated in the same region. Moreover, gene and protein expression patterns correlated with the methylation profile were associated with poor tumor differentiation[40]. In addition, methylation patterns of *PCDH10*, a member of the non-clustered protocadherin family which plays an important role in Ca^{2+}-dependent cell-cell signal transduction and adhesion, was investigated in PDAC cell lines (Capan-1, Panc-1, AsPC-1 and BxPC-3). *PCDH10* promoter methylation was observed in 50% of the cell lines studied and resulted in marked reduction of expression[41]. Later on, this tumor suppressor gene was investigated in other PDAC cell lines and as expected, *PCDH10* promoter methylation again correlated with reduced protein expression. The study also showed high levels of methylation in clinical samples (n=23) and presence of this methylation pattern was associated with reduced progression-free survival [42]. Using TCGA data, Mishra *et. al.* analyzed a genome-wide DNA methylation profile, and although genes of the Ca^{2+} signaling pathway occupied the fourth position on functional enrichment of differentially methylated probes, this finding was not further explored [11]. Also using TCGA data, another study combined methylation and expression data of twelve solid tumors (no PDAC included) in order to identify common patterns of methylation. Alterations in the Ca^{2+} signaling pathway were observed in nine cancer types and *AGTR1*, *GRIN2A*, *ITPKB*, and *SLC8A3* were repressed by hypermethylation in six of them[8].

Cytoplasmic Ca^{2+} concentrations rise in response to a variety of stimuli, which activate Ca^{2+} channels in the plasma membrane (as ORAI), or by release from intracellular stores through inositol 1,4,5- trisphosphate receptors (IP_3R) or ryanodine receptors (RyRs). Growth factors binding to tyrosine kinase receptors (e.g. epidermal growth factor receptor, EGFR) are one way to control the intracellular Ca^{2+}. After binding, phospholipase C is activated and promotes the generation of inositol-1,4,5-trisphosphate (IP_3), which lead to the release of Ca^{2+} from the ER into the cytosol by IP_3R channels (such as *ITPR2*) [43]. As well as *ITPR2, EGFR* is frequently mutated or overexpressed in various cancer types[44; 45], and in PDAC particularly *EGFR* is essential for *KRAS*-driven pancreatic carcinogenesis[46; 47; 48]. It is known that activating *KRAS* mutations are early events in PDAC carcinogenesis and occur in ~90% of the cases [49].

RAS proteins can also active phospholipase C and generate IP3, leading to Ca^{2+} influx, so the most frequently mutated gene in PDAC is directly linked to Ca^{2+} signaling.

RyRs represent another way to control Ca^{2+} ER store. These receptors are regulated by Ca^{2+} voltage channels and by various ions, molecules and proteins, e.g. Ca^{2+}, Mg^{2+}, calmodulin (CALM), Ca^{2+} /calmodulin- dependent protein kinase II (CAMK2), and nicotine [50; 51]. Once Ca^{2+} levels rise in the cytoplasm, they are strictly controlled by CALM. Ca^{2+} binding dramatically changes the conformation of CALM and increases its affinity for a large number of CALM-binding proteins, including the multifunctional CALM kinases such as CAMK2[52]. CAMK2 phosphorylates nearly 40 different proteins, including enzymes, ion channels, kinases, and transcription factors[53] and it is overexpressed in digestive cancers, such as in colorectal cancer[54; 55]. Epithelial-mesenchymal transition, one of the cancer hallmarks, is also controlled by Ca^{2+} levels through CAMK2A. Focal adhesion kinase (FAK), which increases the turnover of cell–cell contacts, is phosphorylated by CAMK2 and consequently upregulated [56]. It is important to mention that FAK pathway was differentially methylated in our analysis.

3. Materials and methods

3.1. Patients and sample collection

A prospective database and biological sample biobank of patients diagnosed with PDAC was established in a public university hospital in Southern Brazil in 2012. In the present study, patients recruited for this database and biobank between 2012 and 2018 were included. Inclusion criteria were: pathology-proven diagnosis of PDAC and no history of previous or current chemo- or radiation therapy. PDAC and PT samples were obtained during surgery or diagnostic biopsy procedures, and hematoxylin-eosin slides were prepared for all cases to confirm the diagnosis and assess sample quality. Samples with pancreatitis, necrosis, fibrosis and less than 20% cellularity were excluded [10]. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hospital de Clínicas de

Porto Alegre (Project Number: 2014-0526). All subjects gave their informed consent for inclusion before they participated in the study. Information about age at diagnosis, gender, TNM classification, tumor location, and differentiation grade were obtained from the patient's electronic medical record. Tumor location was categorized as pancreatic head vs. non-head.

We performed a genome-wide DNA methylation profiling in frozen PDAC samples (n=6) and adjacent pancreatic non-tumoral tissue samples (PT, n=9) in an exploratory cohort using the 450K BeadChip Array platform (Illumina Infinium Human Methylation). Differentially methylated genes were selected, and validation was performed by pyrosequencing in a biological and technical validation cohort comprising a different set including 43 PDAC and 24 PT samples.

3.2. DNA isolation and bisulfite conversion

The PureLink Genomic DNA Kit (Thermo Fisher Scientific) was used to isolate DNA from tissues according to the manufacturer's protocol and eluted DNA was quantified using Qubit V2.0 (Invitrogen, Carlsbad, USA). DNA from each sample was bisulfite converted using the EZ-DNA methylation kit (Zymo Research Corporation, California) according to the manufacturers' protocol.

3.3. Human Methylation 450K array and data preprocessing

PDAC and PT tissues were used to establish an exploratory cohort. Illumina Infinium Human Methylation450k (HM450K) Bead-Chips (Illumina, San Diego, CA) were used for investigating genome-wide DNA methylation profile. Raw data were subjected to quality control, prefiltering, signal normalization across samples, and normalization using the funnorm function (R packages minfi) [63; 64]. The Infinium data generated in this study were deposited in Gene Expression Omnibus (GEO) database, and are openly available under accession number (waiting number).

3.4. Differential methylation analysis

Differential methylation analysis was performed with *limma* [65] R/Bioconductor package, using the M-values matrix as input[65]. Correction for multiple testing was conducted with the Benjamini–Hochberg FDR procedure. Probes were annotated for approved Gene Symbols using annotation files provided by the manufacturer, and all probes with ambiguous annotations were removed from further analyses. Identification of DMPs in PDAC and PT samples was performed as previously described [66]. DMPs were identified by using a cut-off of FDR-corrected p-values < 0.01 and an absolute difference between the means of the β values ($\Delta\beta$) ≥ 0.2. To verify the methylation patterns in cases and controls, a hierarchical clustering analysis was applied to the M-values from DMPs using the complete linkage method and Euclidean distance, as implemented by the *heatmap.2* function from *gplots* R package.

3.5. Functional enrichment analysis

DMPs were subject to functional enrichment analysis using pathway annotations from the KEGG [67; 68] and the *clusterProfiler* package [69] in R/Bioconductor environment. For this purpose, gene symbol annotation of hyper- and hypo-methylated probes were analyzed separately. Only KEGG pathways with a minimum size of 30 annotated genes were considered for further analyses. Statistical significance for enrichment of KEGG pathways was estimated with a hypergeometric test and adjusted to account for multiple hypotheses testing using the FDR procedure. Pathways with a FDR-corrected p-value < 0.05 were highlighted as potentially enriched for hypo- or hyper-methylated genes. Results were visualized using Cytoscape v3.4.0.

3.6. Technical and biologic Validation

In order to biologically and technically validate the array data, we performed pyrosequencing (PyroMark Q96 ID- Qiagen) of selected Ca^{2+} pathway genes. Genes were chosen According to the following selection criteria: only genes with DMP which were exclusively hypo- or hypermethylated; genes associated with a high $|\Delta\beta|$; and/or genes with previously described roles in carcinogenesis. After applying this filter, five

genes were selected: *RYR3* (hypermethylated) and *CALM2*, *CAMK2A*, *ITPR2* and *EGFR* (all of them hypomethylated).

3.7. *TCGA analysis*

RNA-seq and methylation microarray (HM450K) data were downloaded using the GDC Data Transfer Tool. Only patients who had both methylation and expression data for all target genes were included. The unsupervised hierarchical clustering (heatmap with dendograms) and KEGG pathway figures were built with the Complex Heatmap v.2.0 and KEGG graph v.1.44.0 packages, respectively, and the correlations (p-adjust <0.05 by FDR) were performed with the psych v.1.8.12 package. Promoter category includes probes located in genomic region TSS1500, TSS200 and 1stExon. We used the Kaplan–Meier Plotter database (http://kmplot.com/analysis) to analyze mRNA expression prognostic values of selected Ca^{2+} pathway genes in PDCA samples. mRNA expression of selected genes was divided by tertiles and the lower and higher tertiles were used to classify cases into low and high expression, respectively. P-Values were calculated by a log rank test.

4. Conclusions

In summary, our results show that DNA methylation alterations are involved in the deregulation of important tumorigenesis cellular pathways in PDAC. Among them, Ca^{2+} signaling attracted our attention not only for the high number of affected genes, but also it is intrinsic to multiple aspects of cancer biology. Furthermore, DNA methylation was frequently correlated with expression, reinforcing a likely phenotypic effect, which was associated with overall survival. It is also noteworthy that, although the study was not designed with this purpose, we identified aberrant DNA methylation patterns in morphologically normal pancreatic tissues, suggesting these alterations might occur early in pancreatic cell transformation. Future studies should be performed to validate our findings, but data presented here indicates a significant role of epigenetic alterations in the calcium signaling pathway as drivers in pancreatic carcinogenesis.

5. Author contributions

Conceptualization CG, SCSL, and BA; methodology CG, SCSL, BA, RCR, SMSM, and ABO; software SCSL, MRM, and DC; validation CG, SCSL, and BA; formal analysis CG, SCSL, and BA; writing—original draft preparation CG, SCSL, BA, MRM, DC, and PAP; writing—review and editing CG, SCSL, BA, DC, and PAP; supervision LFRP. All authors read and contributed, with critical revision of the paper, and to the final version of the manuscript.

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8. Conflicts of interest

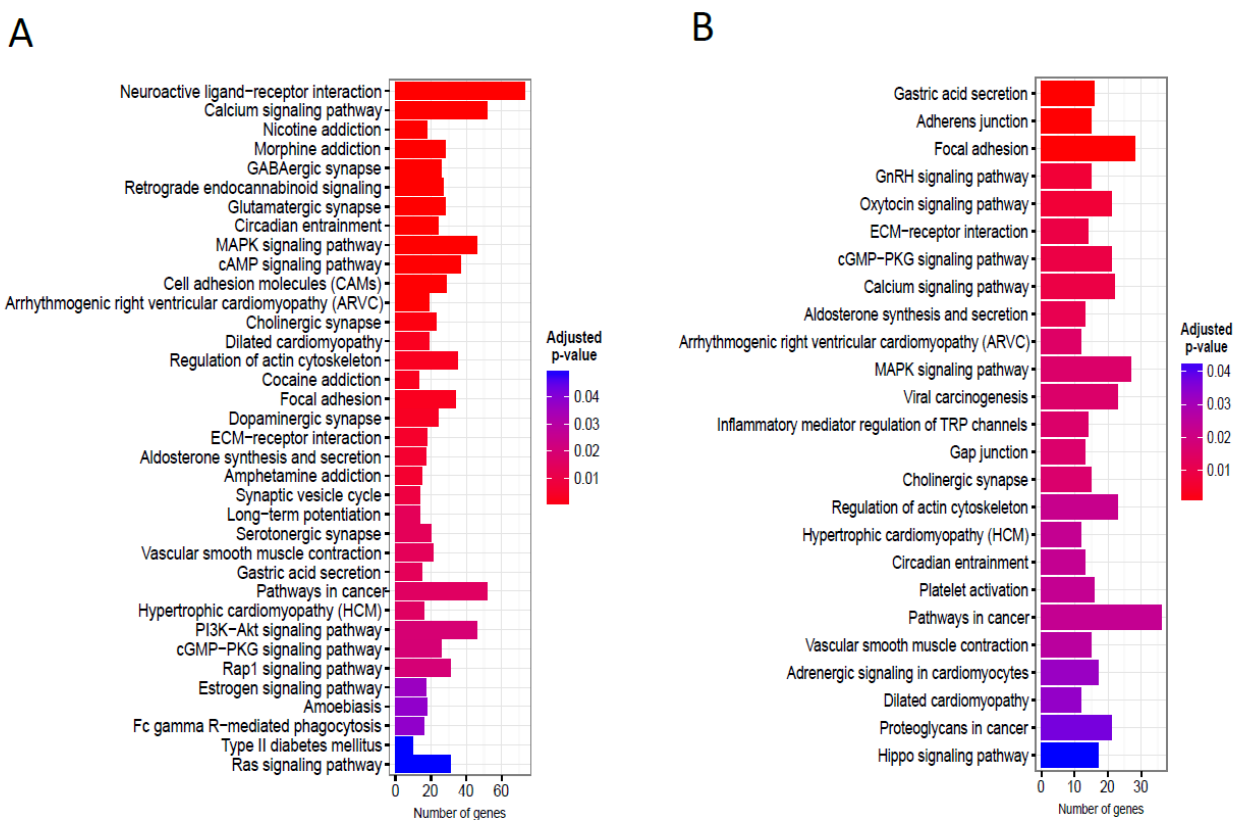
All authors declare no potential financial or ethical conflicts of interest regarding the contents of this submission.

Abbreviations

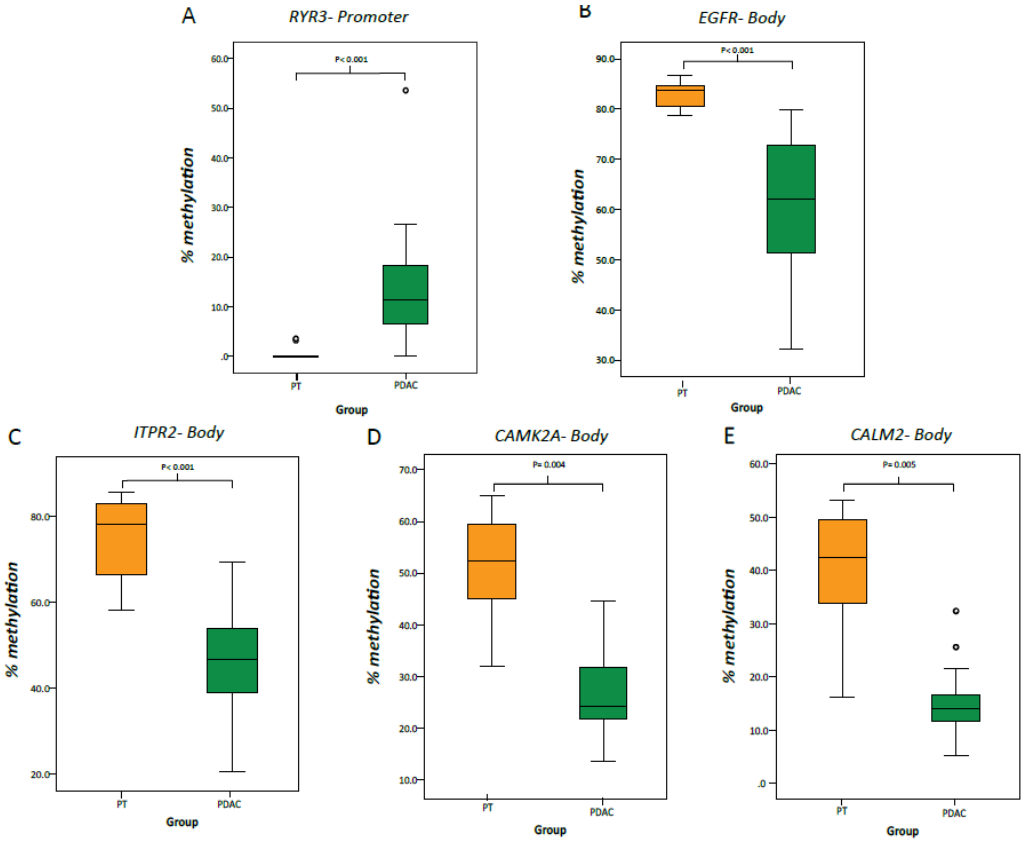
ADCY8	Adenylate Cyclase 8
Ca^{2+}	Calcium
CACNA1A	Calcium Voltage-Gated Channel Subunit Alpha1 A
CACNA1B	Calcium Voltage-Gated Channel Subunit Alpha1 B
CACNA1D	Calcium Voltage-Gated Channel Subunit Alpha1 D
CACNA1H	Calcium Voltage-Gated Channel Subunit Alpha1 H
CALM2	Calmodulin 2

CAMK2A	Calcium/Calmodulin Dependent Protein Kinase II Alpha
CAMKK1	Calcium/Calmodulin Dependent Protein Kinase Kinase 1
DMPs	Differentially Methylated Probes
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
FAK	Focal Adhesion Kinase
FDR	False Discovery Rate
HM450K	Human Methylation 450k
IP_3	Inositol-1,4,5-trisphosphate
IP_3Rs	Inositol 1,4,5- trisphosphate Receptors
ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1
ITPR2	Inositol 1,4,5-Trisphosphate Receptor Type 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRAS	KRAS Proto-Oncogene, GTPase
Mg^{2+}	Magnesium
ORAI2	ORAI Calcium Release-Activated Calcium Modulator 2
OS	Overall Survival
PDAC	Pancreatic Ductal Adenocarcinoma
PDE1C	Phosphodiesterase 1C
PLCB1	Phospholipase C Beta 1
PT	Pancreatic non-tumoral tissue
PT-LIKE	Pancreatic non-tumoral-like tissue
RYR2	Ryanodine Receptor 2
RYR3	Ryanodine Receptor 3
RyRs	Ryanodine Receptors
SERCA	Sarcoendoplasmic Reticulum Ca^{2+} ATPases
TCGA	Cancer Genome Atlas

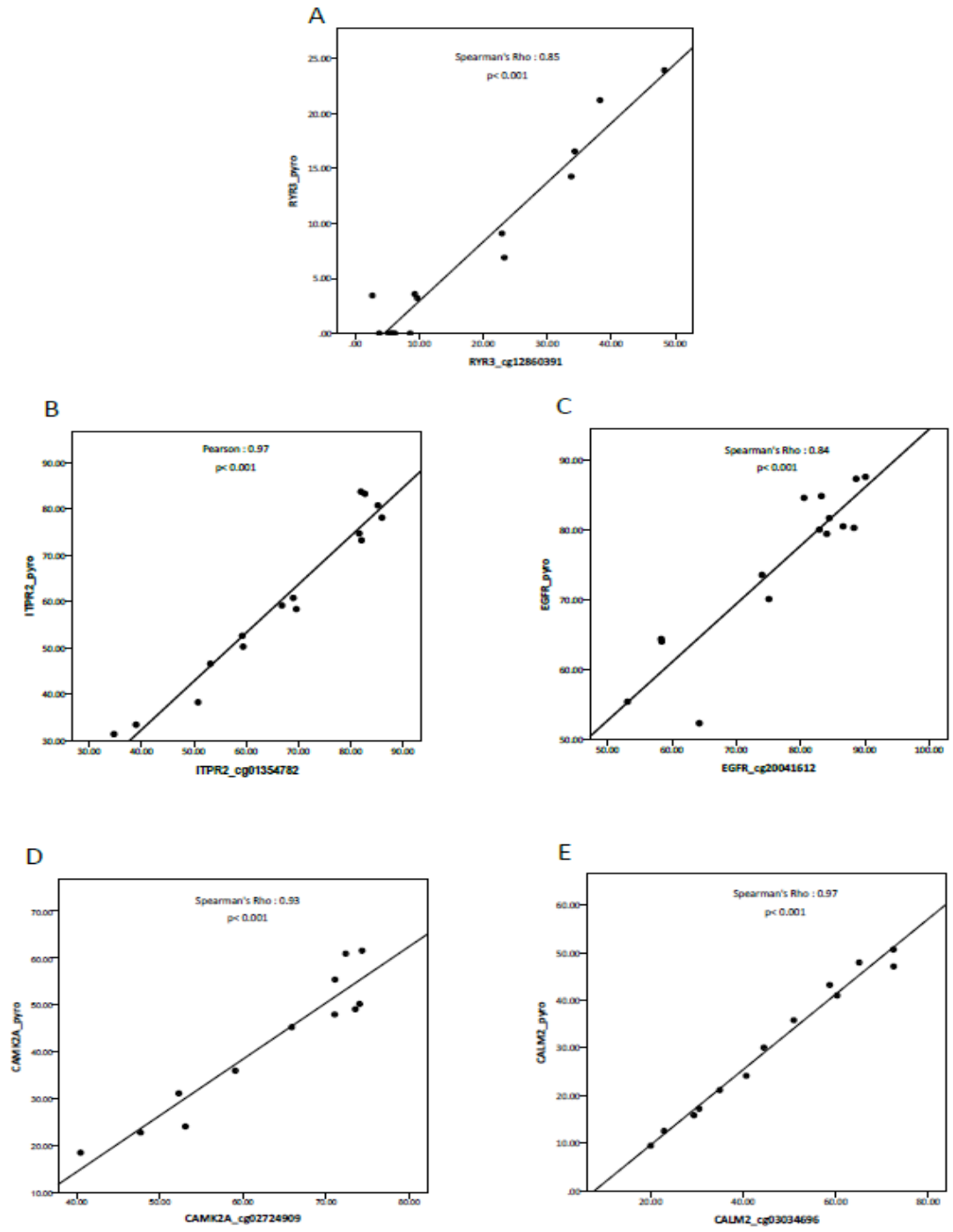
Supplementary information



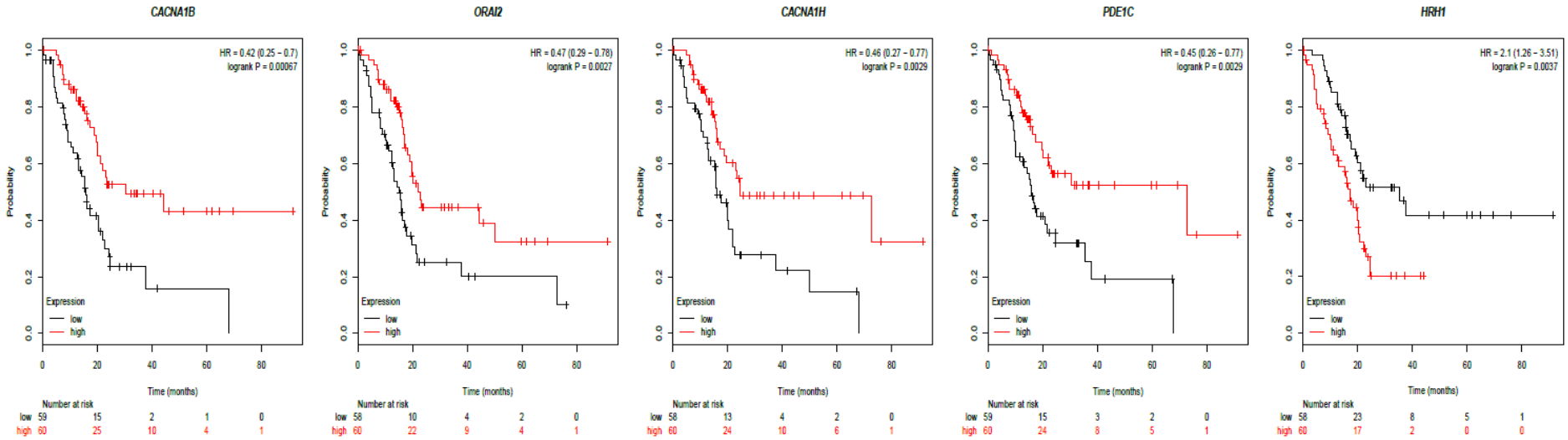
Supplementary Figure 1. Functional enrichment of differentially methylated genes in pancreatic adenocarcinoma. **(a)** Pathways affected by gene hypermethylation (total of 611 functionally annotated genes); **(b)** Pathways affected by gene hypomethylation (total of 385 functionally annotated genes).



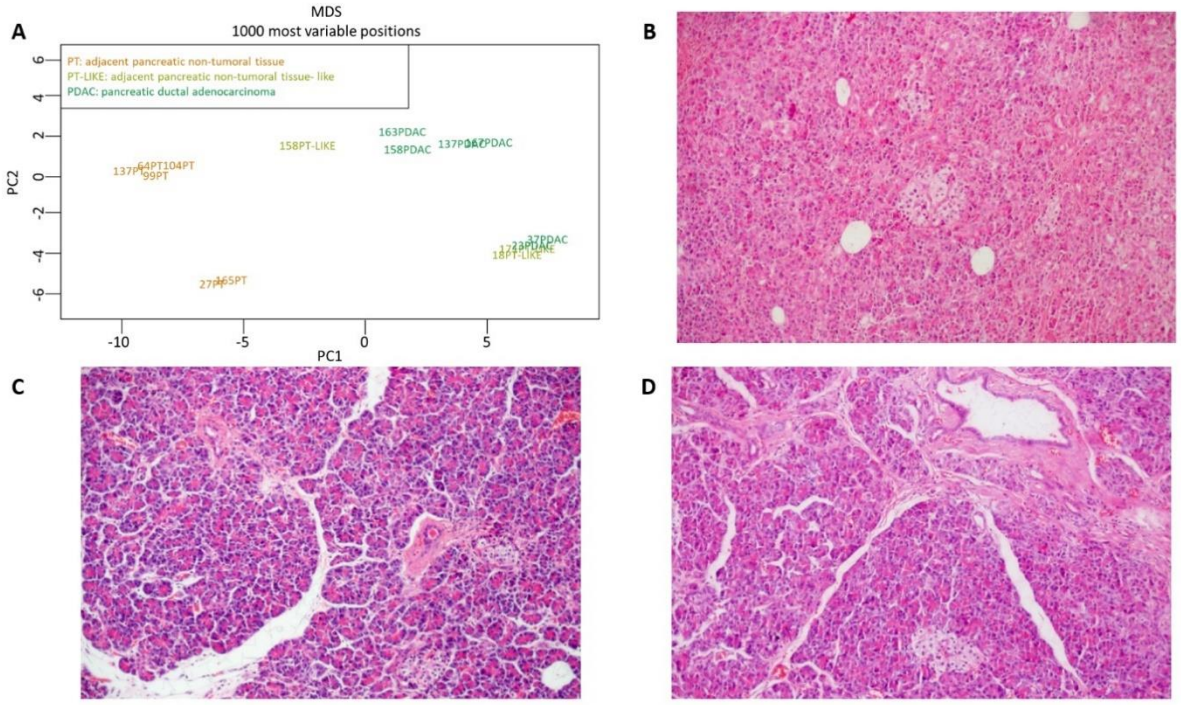
Supplementary Figure 2. Validation of the genome-wide DNA methylation results with pyrosequencing. Boxplots represent the overall methylation of pancreatic adenocarcinoma (PDAC, n= 43) and non-tumoral adjacent pancreatic tissue (PT, n= 24) samples in the following genes: **(a)** *RYR3* (promoter)*;* **(b)** *EGFR* (gene body); **(c)** *ITPR2* (gene body), **(d)** *CAMK2A* (gene body); **(e)** *CALM2* (gene body).



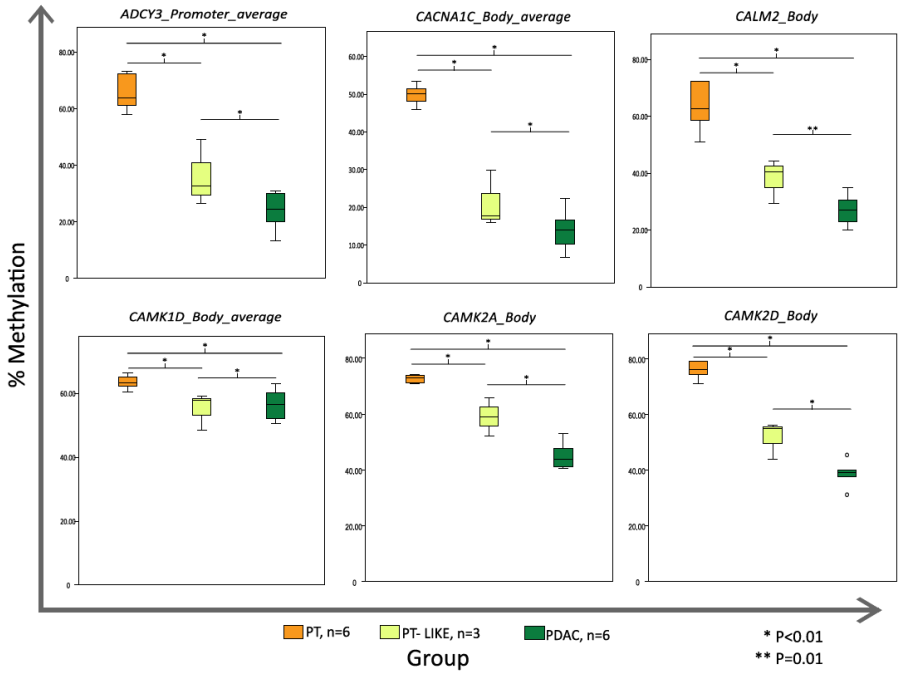
Supplementary Figure 3. Correlation between methodologies. Plots display the correlation between the methylation levels of each DMP determined by microarray (X-axis) and pyrosequencing (Y-axis) in the following genes: *RYR3* (A), *ITPR2* (B), *EGFR* (C), *CAMK2A* (D) and *CALM2* (E). The corresponding correlation coefficients as well as p-values are displayed in each plot.

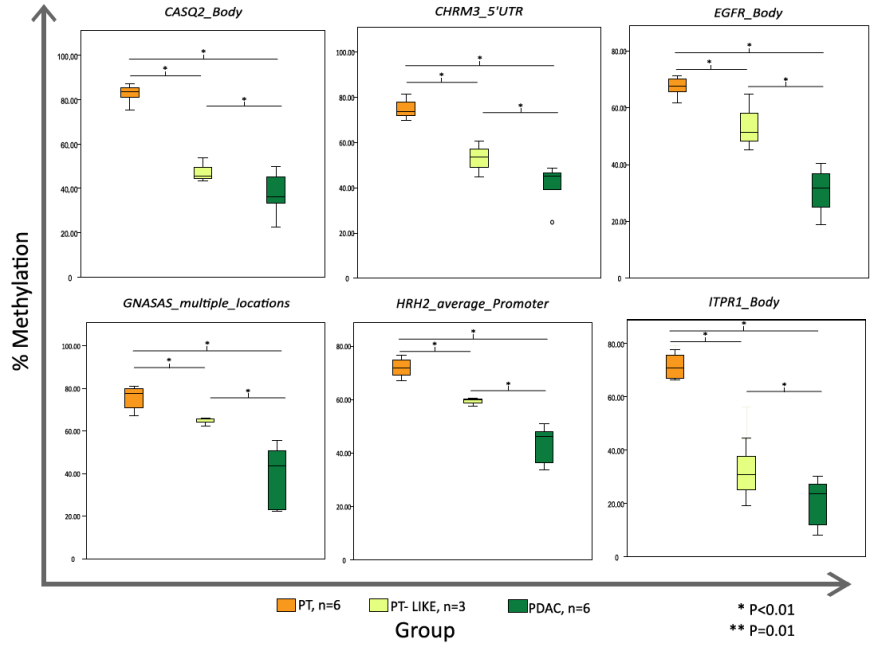

CACNA1B
HR = 0.42 (0.25 − 0.7)
logrank P = 0.00067
Probability
Expression
low
high
Time (months)
Number at risk
low 59 15 2 1 0
high 60 25 10 4 1
ORAI2
HR = 0.47 (0.29 − 0.78)
logrank P = 0.0027
Probability
Expression
low
high
Time (months)
Number at risk
low 58 10 4 2 0
high 60 22 9 4 1
CACNA1H
HR = 0.46 (0.27 − 0.77)
logrank P = 0.0029
Probability
Expression
low
high
Time (months)
Number at risk
low 58 13 4 2 0
high 60 24 10 6 1
PDE1C
HR = 0.45 (0.26 − 0.77)
logrank P = 0.0029
Probability
Expression
low
high
Time (months)
Number at risk
low 59 15 3 2 0
high 60 24 8 5 1
HRH1
HR = 2.1 (1.26 − 3.51)
logrank P = 0.0037
Probability
Expression
low
high
Time (months)
Number at risk
low 58 23 8 5 1
high 60 17 2 0 0
RYR3
HR = 0.5 (0.3 − 0.86)
logrank P = 0.011
Probability
Expression
low
high
Time (months)
Number at risk
low 58 16 3 2 0
high 57 19 8 5 1
CACNA1D
HR = 0.52 (0.31 − 0.87)
logrank P = 0.011
Probability
Expression
low
high
Time (months)
Number at risk
low 58 13 3 1 0
high 59 26 7 4 1
CACNA1A
HR = 0.56 (0.34 − 0.92)
logrank P = 0.02
Probability
Expression
low
high
Time (months)
Number at risk
low 58 17 3 2 0
high 60 21 11 5 1
PLCB1
HR = 0.55 (0.33 − 0.92)
logrank P = 0.022
Probability
Expression
low
high
Time (months)
Number at risk
low 58 18 4 0
high 60 18 8 4
ADCY8
HR = 0.54 (0.31 − 0.95)
logrank P = 0.03
Probability
Expression
low
high
Time (months)
Number at risk
low 66 43 21 8 5 3 2 0
high 45 31 16 12 8 4 3 1

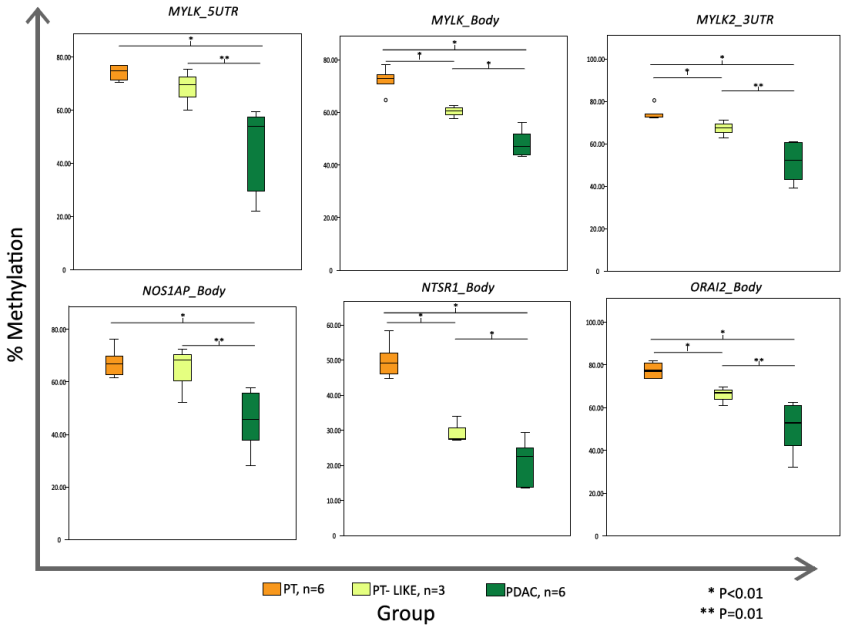
Supplementary Figure 4: Survival analysis. Kaplan–Meier curves depict the impact of gene expression on PDAC patients' overall survival. Individual genes are depicted on the top of each plot. Expression cut-offs were defined by tumor expression tertiles for each gene. High expression (red line) corresponds to the third tertile while low expression (black line) corresponds to the first tertile. Log-rank p values and hazard ratios (HRs; 95 % confidence interval in parentheses) are shown.

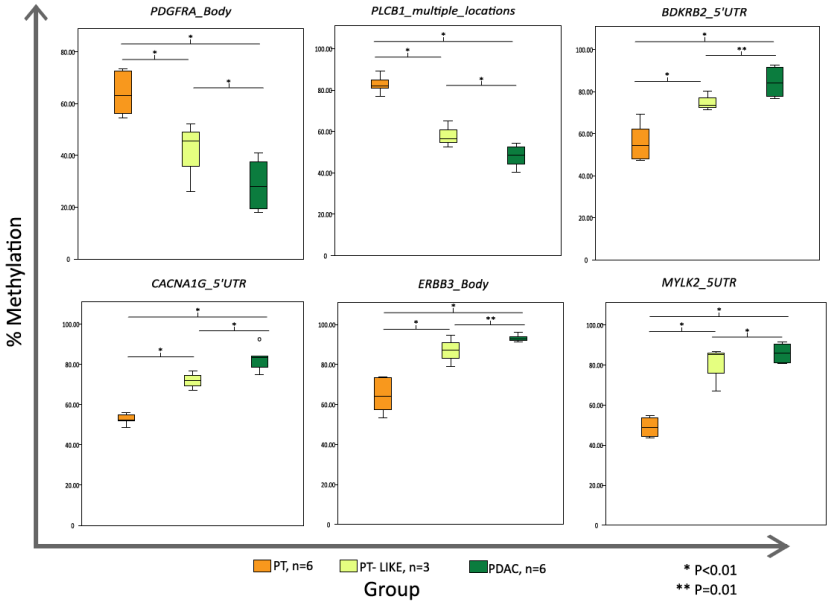


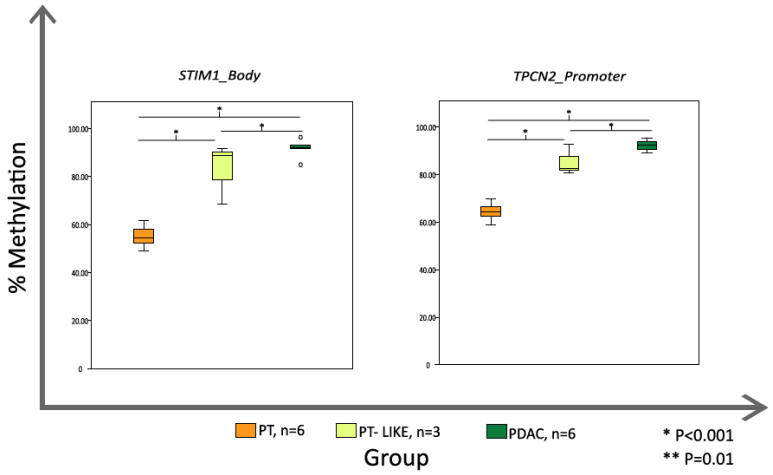
Supplementary Figure 5. PT-like tissue analysis. Multidimensional scaling (MDS) plot showing sample clustering (A), Hematoxylin and eosin staining (100x) of pancreatic tissue sections: PT samples 18, 171 and 158, respectively (B-D). PC, principal component.


ADCY3_Promoter_average
CACNA1C_Body_average
CALM2_Body
CAMK1D_Body_average
CAMK2A_Body
CAMK2D_Body
% Methylation
PT, n=6
PT- LIKE, n=3
PDAC, n=6
Group
* P<0.01
** P=0.01


CASQ2_Body
CHRM3_5'UTR
EGFR_Body
GNASAS_multiple_locations
HRH2_average_Promoter
ITPR1_Body
% Methylation
PT, n=6
PT- LIKE, n=3
PDAC, n=6
Group
* P<0.01
** P=0.01


MYLK_5UTR
MYLK_Body
MYLK2_3UTR
NOS1AP_Body
NTSR1_Body
ORAI2_Body
% Methylation
PT, n=6
PT- LIKE, n=3
PDAC, n=6
Group
* P<0.01
** P=0.01


PDGFRA_Body
PLCB1_multiple_locations
BDKRB2_5'UTR
CACNA1G_5'UTR
ERBB3_Body
MYLK2_5UTR
% Methylation
PT, n=6
PT- LIKE, n=3
PDAC, n=6
Group
* P<0.01
** P=0.01



Supplementary Figure 6. Intermediate methylation profile PT-like samples compared with PT and PDAC samples. Boxplots represent the overall methylation percentage of PT, PT-like and PDAC of 26 differentially methylated probes of the Calcium signaling pathway from cluster analysis. Individual genes are depicted on top of each plot. P-value was calculated using the Generalized Estimating Equations.

Supplementary Table 1. PDAC clinicopathological features (N=43).

Variables	N	%
Age (years)		
≥ 65	19	44.2
Sex		
Male	22	51.2
Differentiation grade[1]		
Well	0	0
Moderate	28	65.1
Poor	11	25.6
NI	4	9.3
Localization		
Head	35	81.4
Other (non-head)	8	18.6
T stage[1]		
Tx	5	11.6
T1	4	9.3
T2	18	41.9
T3	10	23.3
T4	6	14
N stage[1]		
Nx	8	18.6
N0	15	34.9
N1	16	37.2
N2	4	9.3
M stage[1]		
Mx	3	7
M0	35	81.4
M1	5	11.6
TNM Stage[1]		
IA	4	9.3
IB	5	11.6
IIA	2	4.7
IIB	14	32.5
III	11	25.6
IV	5	11.6
NI	2	4.7
Smokers[2]		
No	23	53.5
Yes	17	39.5
NI	3	7

[1]According to the American Joint Committee on Cancer (AJCC) 8th edition - TNM staging system for pancreatic cancer. NI = not informed, [2] Smokers group include smokers and former smokers. NI= non-informed.

Supplementary Table 2. Enriched KEGG terms for Hypermethylated Genes

ID	Description	GeneRatio	BgRatio	P-value	Adjusted P-value (FDR)	geneID	geneCount
hsa04080	Neuroactive ligand-receptor interaction	73/611	277/6963	9.84E-19	2.18E-16	*CHRM2/CHRNA3/CHRNA4/CRHR1/CRHR2/ADRA1D/ADRA1A/ADRB3/DRD1/DRD2/DRD5/AGTR1/EDNRB/P2RX2/GABBR1/GABRA5/GABRB2/GABRB3/GABRG2/GABRG3/GABRP/GALR1/GHR/GHSR/GLRA1/GLRB/NPBWR1/PRLHR/MLNR/GRIA2/GRIA4/GRIK3/GRIK5/GRIN1/GRIN2A/GRIN2C/GRIN2D/GRM1/GRM5/GRM6/GRM7/HRH1/HRH2/HTR1A/HTR2C/HTR4/HTR5A/LEP/LEPR/LHCGR/MTNR1B/NMBR/NPY1R/NPY2R/NPY5R/NTSR1/OPRK1/OPRM1/RXFP3/AVPR1A/PTGDR/PTGER3/PTGFR/PTH2R/BDKRB2/SSTR4/TACR1/TACR3/VIPR2/MCHR2/GALR2/GPR50/GABBR2*	73
hsa04020	Calcium signaling pathway	52/611	179/6963	1.70E-15	1.89E-13	*PPIF/ADCY1/CHRM2/ADCY8/ADRA1D/ADRA1A/ADRB3/DRD1/DRD5/AGTR1/EDNRB/ERBB3/P2RX2/PLCB1/GNAL/GRIN1/GRIN2A/GRIN2C/GRIN2D/GRM1/GRM5/HRH1/HRH2/HTR2C/HTR4/HTR5A/MYLK4/ITPR1/LHCGR/NTSR1/PDE1C/AVPR1A/PRKCB/PRKCG/PTGER3/PTGFR/BDKRB2/RYR2/RYR3/SLC8A2/SLC8A1/STIM1/TACR1/TACR3/CACNA1A/CACNA1B/CACNA1C/CACNA1E/MYLK2/CACNA1I/CACNA1H/CACNA1G*	52
hsa05033	Nicotine addiction	18/611	40/6963	1.34E-09	8.67E-08	*CHRNA4/SLC32A1/GABRA5/GABRB2/GABRB3/GABRG2/GABRG3/GABRP/GRIA2/GRIA4/GRIN1/GRIN2A/GRIN2C/GRIN2D/SLC17A7/SLC17A6/CACNA1A/CACNA1B*	18
hsa05032	Morphine addiction	28/611	91/6963	1.56E-09	8.67E-08	*ADCY1/PDE10A/ADCY5/ADCY8/SLC32A1/DRD1/GABBR1/GABRA5/GABRB2/GABRB3/GABRG2/GABRG3/GA*	28

							BRP/GNG4/GRK5/OPRM1/PDE1C/PDE3A/PDE4B/PDE4C/PDE4D/PRKCB/PRKCG/GNG12/GNB4/CACNA1A/CACNA1B/GABBR2	
hsa04727	GABAergic synapse	26/611	88/6963	1.57E-08	6.95E-07		*ADCY1/ADCY5/ADCY8/SLC32A1/ABAT/GABBR1/GABRA5/GABRB2/GABRB3/GABRG2/GABRG3/GABRP/GAD1/GAD2/GNG4/PRKCB/PRKCG/GNG12/SLC12A5/GNB4/SLC6A1/SLC6A11/CACNA1A/CACNA1B/CACNA1C/GABBR2*	26
hsa04723	Retrograde endocannabinoid signaling	27/611	101/6963	8.55E-08	3.16E-06		*ADCY1/ADCY5/ADCY8/SLC32A1/RIMS1/PLCB1/GABRA5/GABRB2/GABRB3/GABRG2/GABRG3/GABRP/GNG4/GRIA2/GRIA4/GRM1/GRM5/ITPR1/PRKCB/PRKCG/GNG12/SLC17A7/SLC17A6/GNB4/CACNA1A/CACNA1B/CACNA1C*	27
hsa04724	Glutamatergic synapse	28/611	114/6963	3.43E-07	1.09E-05		*ADCY1/ADCY5/ADCY8/PLCB1/GNG4/GRIA2/GRIA4/GRIK3/GRIK5/GRIN1/GRIN2A/GRIN2C/GRIN2D/GRM1/GRM5/GRM6/GRM7/ITPR1/SHANK1/PLA2G4A/PRKCB/PRKCG/GNG12/SLC17A7/SLC17A6/GNB4/CACNA1A/CACNA1C*	28
hsa04713	Circadian entrainment	24/611	95/6963	1.38E-06	3.45E-05		*ADCY1/ADCY5/ADCY8/PLCB1/GNG4/GRIA2/GRIA4/GRIN1/GRIN2A/GRIN2C/GRIN2D/GUCY1A2/ITPR1/MTNR1B/PRKCB/PRKCG/GNG12/GNB4/RYR2/RYR3/CACNA1C/CACNA1I/CACNA1H/CACNA1G*	24
hsa04010	MAPK signaling pathway	46/611	255/6963	1.40E-06	3.45E-05		*CACNG3/DUSP10/AKT1/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/FLNC/IL1R1/MAP3K1/MAP3K3/MAP3K5/MOS/NTF3/NTRK2/NLK/PLA2G4A/PRKCB/PRKCG/GNG12/RASGRF1/RASGRF2/CACNG8/CACNG7/CACNG6/BDNF/CACNA1A/CACNA1B/CACNA1C/CACNA1E/CA*	46

						CNB2/DUSP16/PTPN5/MKNK1/CACNA1I/CACNA1H/CACNA1G/MAP3K6/TAOK2/MAP4K4/MAPK8IP1	
hsa04024	cAMP signaling pathway	37/611	199/6963	7.53E-06	0.000167209	*VAV3/ADCY1/ADCY5/CHRM2/ADCY8/CNGA3/DRD1/DRD2/DRD5/AKT1/PIK3R5/GABBR1/GHSR/GLI3/GRIA2/GRIA4/GRIN1/GRIN2A/GRIN2C/GRIN2D/HTR1A/HTR4/NPY/NPY1R/PDE3A/PDE4B/PDE4C/PDE4D/PTGER3/RYR2/BDNF/HHIP/TIAM1/VIPR2/CACNA1C/GABBR2/CREB5*	37
hsa04514	Cell adhesion molecules (CAMs)	29/611	142/6963	1.16E-05	0.000234857	*CDH2/CDH4/CNTN1/VCAN/NTNG1/NLGN1/NFASC/CLDN14/CNTNAP2/HLA-DMB/HLA-G/ITGA6/ITGA4/NCAM1/NCAM2/CLDN11/NLGN4X/CADM3/PTPRF/PVRL1/SELPLG/MADCAM1/NTNG2/ITGA8/CD8A/NRXN3/NRXN1/NRXN2/LRRC4B*	29
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	19/611	74/6963	1.35E-05	0.000249448	*CDH2/CACNG3/CTNNA2/DES/DMD/ITGA11/ITGA6/ITGA4/LAMA2/CACNG8/CACNG7/CACNG6/RYR2/SLC8A1/CACNA1C/CACNB2/ITGA8/ACTN2/ACTN3*	19
hsa04725	Cholinergic synapse	23/611	111/6963	7.34E-05	0.001252788	*ADCY1/CHAT/ADCY5/CHRM2/CHRNA3/CHRNA4/ADCY8/AKT1/PLCB1/PIK3R5/GNG4/ITPR1/KCNQ1/KCNQ2/PRKCB/PRKCG/GNG12/KCNQ5/GNB4/CACNA1A/CACNA1B/CACNA1C/CREB5*	23
hsa05414	Dilated cardiomyopathy	19/611	89/6963	0.000203484	0.003017821	*CACNG3/ADCY1/ADCY5/ADCY8/DES/DMD/ITGA11/ITGA6/ITGA4/LAMA2/CACNG8/CACNG7/CACNG6/RYR2/SLC8A1/TPM4/CACNA1C/CACNB2/ITGA8*	19
hsa04810	Regulation of actin cytoskeleton	35/611	214/6963	0.000205441	0.003017821	*VAV3/BAIAP2/IQGAP2/CHRM2/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/ITGA11/PIK3R5/CYFIP2/MYLK4/PFN3/ITGA6/ITGA4/*	35

						MOS/MSN/ARHGEF4/GNG12/BDKRB2/TIAM1/VCL/ITGA8/MYLK2/ACTN2/ARHGEF7/ACTN3/WASF1/WASL/BCAR1	
hsa05030	Cocaine addiction	13/611	49/6963	0.000217501	0.003017821	*ADCY5/DRD1/DRD2/FOSB/GRIA2/GRIN1/GRIN2A/GRIN2C/GRIN2D/BDNF/SLC6A3/SLC18A2/CREB5*	13
hsa04510	Focal adhesion	34/611	207/6963	0.000232338	0.003034066	*LAMC3/VAV3/COL2A1/COL5A1/COMP/AKT1/ITGA11/FLNC/FLT1/FLT4/PIK3R5/COL24A1/LAMA1/MYLK4/ITGA6/ITGA4/KDR/LAMA2/COL5A3/PRKCB/PRKCG/RELN/RASGRF1/THBS3/THBS4/TNR/TNXB/VCL/ITGA8/MYLK2/ACTN2/ACTN3/CCND2/BCAR1*	34
hsa04728	Dopaminergic synapse	24/611	129/6963	0.000297814	0.003673041	*ADCY5/DRD1/DRD2/DRD5/AKT1/PLCB1/GNAL/GNG4/GRIA2/GRIA4/GRIN2A/ITPR1/KIF5C/ARNTL/PRKCB/PRKCG/GNG12/GNB4/SLC6A3/SLC18A2/CACNA1A/CACNA1B/CACNA1C/CREB5*	24
hsa04512	ECM-receptor interaction	18/611	87/6963	0.000446122	0.005212579	*LAMC3/COL2A1/COL5A1/COMP/ITGA11/SV2C/COL24A1/LAMA1/ITGA6/ITGA4/LAMA2/COL5A3/RELN/THBS3/THBS4/TNR/TNXB/ITGA8*	18
hsa04925	Aldosterone synthesis and secretion	17/611	81/6963	0.000536079	0.005799262	*ADCY1/ADCY5/ADCY8/AGTR1/PLCB1/ITPR1/KCNK3/KCNK9/PRKCB/PRKCE/PRKCG/CAMK1D/CACNA1C/CACNA1I/CACNA1H/CACNA1G/CREB5*	17
hsa05031	Amphetamine addiction	15/611	67/6963	0.000548579	0.005799262	*ADCY5/DRD1/FOSB/GRIA2/GRIA4/GRIN1/GRIN2A/GRIN2C/GRIN2D/PRKCB/PRKCG/SLC6A3/SLC18A2/CACNA1C/CREB5*	15
hsa04721	Synaptic vesicle cycle	14/611	63/6963	0.000900692	0.009088798	*TCIRG1/CPLX2/CPLX1/SLC32A1/AP2A2/RIMS1/DNM3/ATP6V0C/SLC17A7/SLC17A6/SLC18A2/SYT1/CACNA1A/CACNA1B*	14
hsa04720	Long-term potentiation	14/611	66/6963	0.001458485	0.013889223	*ADCY1/ADCY8/PLCB1/GRIA2/GRIN1/GRIN2A/GRIN2C/GRIN2D/GRM1/G*	14

						RM5/ITPR1/PRKCB/PRKCG/CACNA1C	
hsa04726	Serotonergic synapse	20/611	112/6963	0.001573729	0.013889223	*ADCY5/PLCB1/GABRB2/GABRB3/GNG4/HTR1A/HTR2C/HTR4/HTR5A/ITPR1/KCNN2/PLA2G4A/PRKCB/PRKCG/GNG12/GNB4/SLC18A2/CACNA1A/CACNA1B/CACNA1C*	20
hsa04270	Vascular smooth muscle contraction	21/611	120/6963	0.001581563	0.013889223	*RAMP3/ADCY1/ADCY5/ADCY8/ADRA1D/ADRA1A/AGTR1/PLCB1/GUCY1A2/MYLK4/ITPR1/KCNMA1/PLA2G4A/AVPR1A/PRKCB/PRKCD/PRKCE/PRKCG/CACNA1C/MYLK2/PPP1R14A*	21
hsa04971	Gastric acid secretion	15/611	74/6963	0.001626666	0.013889223	*ADCY1/ADCY5/ADCY8/PLCB1/HRH2/MYLK4/ITPR1/KCNJ1/KCNQ1/ATP4A/KCNK10/PRKCB/PRKCG/SST/MYLK2*	15
hsa05200	Pathways in cancer	52/611	397/6963	0.001911632	0.015717862	*CDK6/LAMC3/ADCY1/ADCY5/FZD10/ADCY8/CTBP1/CTNNA2/AGTR1/EDNRB/AKT1/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/FOXO1/FLT3/PLCB1/PIK3R5/GLI2/GLI3/GNG4/LAMA1/ITGA6/LAMA2/MMP2/MMP9/EGLN1/PRKCB/PRKCG/GNG12/LPAR5/PTGER3/GNB4/RET/BDKRB2/HHIP/SHH/BMP4/HSP90B1/TRAF5/WNT2/WNT6/AXIN1/CASP9/CCNA1/WNT3A*	52
hsa05410	Hypertrophic cardiomyopathy (HCM)	16/611	83/6963	0.002004281	0.015891083	*CACNG3/DES/DMD/ITGA11/ITGA6/ITGA4/LAMA2/CACNG8/CACNG7/CACNG6/RYR2/SLC8A1/TPM4/CACNA1C/CACNB2/ITGA8*	16
hsa04151	PI3K-Akt signaling pathway	46/611	346/6963	0.002565706	0.019640925	*CDK6/LAMC3/CHRM2/COL2A1/COL5A1/COMP/EFNA2/EIF4EBP1/AKT1/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/ITGA11/FLT1/FLT4/PIK3R5/COL24A1/GHR/GNG4/LAMA1/ITGA6/ITGA4/KDR/LAMA2/NGFR/COL5A3/GNG12/RELN/LPA*	46

						R5/GNB4/SGK1/THBS3/THBS4/TNR/TNXB/HSP90B1/CASP9/ITGA8/CCND2/CREB5	
hsa04022	cGMP-PKG signaling pathway	26/611	167/6963	0.002692612	0.01992533	*PPIF/ADCY1/ADCY5/ADCY8/ADRA1D/ADRA1A/ADRB3/AGTR1/EDNRB/AKT1/PLCB1/PIK3R5/GUCY1A2/MYLK4/ITPR1/KCNJ8/KCNMA1/PDE3A/PRKCE/BDKRB2/SLC8A2/SLC8A1/TRPC6/CACNA1C/MYLK2/CREB5*	26
hsa04015	Rap1 signaling pathway	31/611	211/6963	0.002816472	0.020169573	*ADCY1/ADCY5/ADCY8/DRD2/EFNA2/AKT1/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/FLT1/PLCB1/FLT4/PIK3R5/GRIN1/GRIN2A/PFN3/KDR/NGFR/APBB1IP/PRKCB/PRKCG/LPAR5/TIAM1/BCAR1/RAPGEF5*	31
hsa04915	Estrogen signaling pathway	17/611	99/6963	0.005208406	0.036133318	*ADCY1/ADCY5/ADCY8/AKT1/ESR1/PLCB1/PIK3R5/GABBR1/GRM1/ITPR1/MMP2/MMP9/OPRM1/PRKCD/HSP90B1/GABBR2/CREB5*	17
hsa05146	Amoebiasis	18/611	108/6963	0.005673151	0.038095673	*LAMC3/ADCY1/COL2A1/COL5A1/PLCB1/PIK3R5/COL24A1/GNAL/LAMA1/IL1R1/LAMA2/COL5A3/PRKCB/PRKCG/VCL/ACTN2/ACTN3/CD1D*	18
hsa04666	Fc gamma R-mediated phagocytosis	16/611	92/6963	0.005834472	0.038095673	*VAV3/WASF3/DOCK2/AKT1/PIK3R5/AMPH/HCK/MYO10/PLA2G4A/PRKCB/PRKCD/PRKCE/PRKCG/PPAP2B/WASF1/WASL*	16
hsa04930	Type II diabetes mellitus	10/611	48/6963	0.007697446	0.048610379	*PIK3R5/MAFA/PRKCD/PRKCE/ABCC8/CACNA1A/CACNA1B/CACNA1C/CACNA1E/CACNA1G*	10
hsa04014	Ras signaling pathway	31/611	226/6963	0.007882764	0.048610379	*EFNA2/AKT1/FGF3/FGF4/FGF5/FGF8/FGF10/FGF12/FGF13/FGF14/FGFR2/RASA3/FLT1/FLT4/PIK3R5/GNG4/GRIN1/GRIN2A/KDR/NGFR/PLA2G4A/PRKCB/PRKCG/GNG12/RASGRF1/RASGRF2/GNB4/TIAM1/RASAL1/KSR1/RAPGEF5*	31

Supplementary Table 3. Enriched KEGG terms for Hypomethylated Genes.

ID	Description	GeneRatio	BgRatio	P-value	Adjusted P-value (FDR)	geneID	geneCount
hsa04971	Gastric acid secretion	16/385	74/6963	1.96E-06	0.000426491	ADCY3/CHRM3/PLCB1/MYLK4/ITPR1/ITPR2/KCNJ15/KCNQ1/SLC9A4/MYLK/ACTB/SLC9A1/CALM2/CAMK2A/CAMK2D/MYLK2	16
hsa04520	Adherens junction	15/385	73/6963	8.00E-06	0.000616679	BAIAP2/SORBS1/CTNNA2/EGFR/IGF1R/LMO7/PARD3/RAC1/ACTB/SRC/TCF7L2/VCL/TCF7L1/ACTN1/IQGAP1	15
hsa04510	Focal adhesion	28/385	207/6963	8.49E-06	0.000616679	VAV3/COL3A1/COL4A1/COL4A2/EGFR/FLNA/MYLK4/IGF1R/ITGA3/ITGB3/ITGB4/LAMA4/LAMA5/LAMB1/MYLK/PDGFRA/PIK3CD/PDGFC/RAC1/ACTB/SRC/TNR/TNXB/VCL/CAPN2/TLN2/MYLK2/ACTN1	28
hsa04912	GnRH signaling pathway	15/385	91/6963	0.000120829	0.006485182	ADCY3/EGFR/PLCB1/ITPR1/ITPR2/MAP3K1/MAP3K4/PLD1/MAP2K6/SRC/CACNA1C/CACNA1D/CALM2/CAMK2A/CAMK2D	15
hsa04921	Oxytocin signaling pathway	21/385	158/6963	0.000148743	0.006485182	ADCY3/EGFR/PLCB1/GUCY1A2/MYLK4/ITPR1/ITPR2/MYLK/PRKAG2/PIK3CD/CAMK1D/ACTB/SRC/CACNA1C/CACNA1D/CACNB4/CALM2/CAMK2A/CAMK2D/MYLK2/CACNA2D4	21
hsa04512	ECM-receptor interaction	14/385	87/6963	0.000263092	0.008895587	COL3A1/COL4A1/COL4A2/ITGA3/ITGB3/ITGB4/LAMA4/LAMA5/LAMB1/TNR/TNXB/CD36/CD44/SV2B	14
hsa04022	cGMP-PKG signaling pathway	21/385	167/6963	0.000324796	0.008895587	ADCY3/PLCB1/GNA12/GTF2I/GUCY1A2/MYLK4/IRS1/ITPR1/ITPR2/KCNMA1/MYLK/PIK3CD/PRKCE/PRKG1/VDAC3/CACNA1C/CACNA1D/CALM2/MYLK2/CREB3L1/GTF2IRD1	21
hsa04020	Calcium signaling pathway	22/385	179/6963	0.000326444	0.008895587	ADCY3/CHRM3/EGFR/PLCB1/GRM5/HRH1/MYLK4/ITPR1/ITPR2/MYLK/NTSR1/PDE1A/PDGFRA/SPHK2/	22

hsa04925	Aldosterone synthesis and secretion	13/385	81/6963	0.000445435	0.010789434	VDAC3/CACNA1C/CACNA1D/ORAI2/CALM2/CAMK2A/CAMK2D/MYLK2	13
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	12/385	74/6963	0.000666782	0.014535849	ADCY3/PLCB1/ITPR1/ITPR2/PRKCE/CAMK1D/CACNA1C/CACNA1D/CALM2/CAMK2A/CAMK2D/CREB3L1/SCARB1	13
hsa04010	MAPK signaling pathway	27/385	255/6963	0.000806386	0.015284571	CTNNA2/ITGA3/ITGB3/ITGB4/ACTB/TCF7L2/CACNA1C/CACNA1D/CACNB4/TCF7L1/ACTN1/CACNA2D4	12
hsa05203	Viral carcinogenesis	23/385	205/6963	0.000887917	0.015284571	DUSP1/EGFR/FGF1/FLNA/GNA12/IL1B/STMN1/MAP3K1/MAP3K4/MAP3K5/NF1/PDGFRA/ZAK/GNG12/MAP2K6/RAC1/RELB/RPS6KA2/TNFRSF1A/TRAF2/TRAF6/CACNA1C/CACNA1D/CACNB4/IL1R2/CACNA2D4/CDC25B	27
hsa04750	Inflammatory mediator regulation of TRP channels	14/385	98/6963	0.000911465	0.015284571	HDAC5/CDK6/UBR4/GTF2E1/GTF2H3/HLA-A/HPN/IL6ST/IRF7/LYN/HDAC7/PIK3CD/VAC14/RAC1/SRC/C3/TRAF2/VDAC3/MAD1L1/ACTN1/CREB3L1/HDAC9/HDAC4	23
hsa04540	Gap junction	13/385	88/6963	0.001002581	0.015611613	ADCY3/PLCB1/HRH1/IL1B/ITPR1/ITPR2/TRPV2/PIK3CD/PRKCE/MAP2K6/SRC/CALM2/CAMK2A/CAMK2D	14
hsa04725	Cholinergic synapse	15/385	111/6963	0.001089002	0.015826824	ADCY3/EGFR/TUBB/PLCB1/GRM5/GUCY1A2/ITPR1/ITPR2/PDGFRA/PRKG1/PDGFC/SRC/TUBB1	13
hsa04810	Regulation of actin cytoskeleton	23/385	214/6963	0.001591431	0.02168325	ADCY3/CHRM3/PLCB1/GNG7/ITPR1/ITPR2/KCNQ1/PIK3CD/GNG12/CACNA1C/CACNA1D/CAMK2A/CAMK2D/CHRNA6/CREB3L1	15
						VAV3/BAIAP2/CHRM3/EGFR/FGF1/GNA12/MYLK4/ITGA3/ITGB3/ITGB4/MYLK/PDGFRA/PIK3CD/GNG12/	23

hsa05410	Hypertrophic cardiomyopathy (HCM)	12/385	83/6963	0.001878158	0.022547351	PDGFC/RAC1/ACTB/SLC9A1/SRC/VCL/MYLK2/ACTN1/IQGAP1 ITGA3/ITGB3/ITGB4/MYBPC3/PRKAG2/ACTB/TPM2/TPM4/CACNA1C/CACNA1D/CACNB4/CACNA2D4	12
hsa04713	Circadian entrainment	13/385	95/6963	0.002052343	0.022547351	ADCY3/PLCB1/GNG7/GUCY1A2/ITPR1/PRKG1/GNG12/CACNA1C/CACNA1D/CALM2/CAMK2A/CAMK2D/NOS1AP	13
hsa04611	Platelet activation	16/385	130/6963	0.002054244	0.022547351	ADCY3/COL3A1/PLCB1/GUCY1A2/MYLK4/ITGB3/ITPR1/ITPR2/LYN/MYLK/PIK3CD/PRKG1/ACTB/SRC/TLN2/MYLK2	16
hsa05200	Pathways in cancer	36/385	397/6963	0.002068564	0.022547351	CDK6/ADCY3/COL4A1/COL4A2/CSF1R/CTNNA2/EGFR/ETS1/FGF1/PLCB1/CBLC/ABL1/GLI2/GNA12/GNG7/IGF1R/ITGA3/LAMA4/LAMA5/LAMB1/MITF/PDGFRA/PIK3CD/PLD1/GNG12/RAC1/SLC2A1/TCEB1/TCF7L2/TRAF2/TRAF6/WNT9B/AXIN2/TCF7L1/RUNX1/ARNT2	36
hsa04270	Vascular smooth muscle contraction	15/385	120/6963	0.002407219	0.024989229	ADCY3/PLCB1/GNA12/GUCY1A2/MYLK4/ITPR1/ITPR2/KCNMA1/MYLK/PRKCE/PRKG1/CACNA1C/CACNA1D/CALM2/MYLK2	15
hsa04261	Adrenergic signaling in cardiomyocytes	17/385	148/6963	0.003171833	0.03142998	ADCY3/RAPGEF4/PLCB1/KCNQ1/PIK3CD/PPP2R3A/SLC9A1/TPM2/TPM4/CACNA1C/CACNA1D/CACNB4/CALM2/CAMK2A/CAMK2D/CREB3L1/CACNA2D4	17
hsa05414	Dilated cardiomyopathy	12/385	89/6963	0.003415299	0.032371095	ADCY3/ITGA3/ITGB3/ITGB4/MYBPC3/ACTB/TPM2/TPM4/CACNA1C/CACNA1D/CACNB4/CACNA2D4	12
hsa05205	Proteoglycans in cancer	21/385	203/6963	0.003966959	0.036033213	EGFR/FLNA/CBLC/ANK1/ANK3/IGF1R/ITGB3/ITPR1/ITPR2/MIR21/PIK3CD/PLAUR/RAC1/ACTB/SLC9A1/SRC/WNT9B/CAMK2A/CAMK2D/IQGAP1/CD44	21

hsa04390	Hippo signaling pathway	17/385	154/6963	0.004784005	0.041716526	YAP1/FRMD6/CTNNA2/FGF1/BBC3/GLI2/CRB2/PARD3/ACTB/BMP6/TCF7L2/TEAD1/TEAD3/TP73/WNT9B/AXIN2/TCF7L1	17

Supplemantary Table 4. Correlation between probe methylation and gene expression.

ProbeID	Gene	r	FDR-adjusted p-value	Δbeta	UCSCGenomicRegion
cg07960450	ADCY1	-0.36355	2.79338E-05	0.22	1stExon
cg16848624	ADCY1	-0.36065	2.79338E-05	0.23	1stExon
cg02914422	PDE1C	-0.5396	1.01017E-11	0.22	5'UTR
cg03437186	ADCY1	-0.32817	0.000118439	0.26	Body
cg07651242	ADCY1	-0.32029	0.000135012	0.22	1stExon
cg24801123	ADCY1	-0.29823	0.000328372	0.22	Body
cg05229355	ADCY8	-0.23284	0.049157202	0.2	Promoter
cg10811502	MYLK2	-0.48711	1.81283E-09	0.27	5'UTR
cg23008606	ADRA1A	-0.28073	0.001595309	0.2	Promoter
cg20303399	ADRA1A	-0.27728	0.001595309	0.22	Promoter
cg26730369	ADRA1A	-0.27015	0.001595309	0.21	Promoter
cg09557462	ADRA1A	-0.18174	0.031021689	0.25	1stExon
cg12215340	ADRA1D	-0.20135	0.016655571	0.23	Promoter
cg04807594	AGTR1	-0.383	5.53422E-06	0.27	Promoter

cg26727693	AVPR1A	-0.34826	4.63549E-05	0.3	1stExon
cg09208611	AVPR1A	-0.29582	0.000368857	0.21	1stExon
cg17509220	CACNA1A	-0.29569	0.001484251	0.25	1stExon
cg00465319	MYLK	-0.45275	6.93079E-08	-0.23	MultipleLocations
cg22491927	CACNA1A	-0.25394	0.004754249	0.32	Promoter
cg17509967	CACNA1A	-0.23111	0.007769955	0.23	Promoter
cg04842426	CHRM2	-0.43578	5.30296E-07	0.22	5'UTR
cg22187630	CACNA1A	-0.16842	0.045896158	0.21	1stExon
cg05863502	CACNA1B	-0.29431	0.001189499	0.3	Promoter
cg17218713	PTGER3	-0.42623	1.36587E-07	0.26	MultipleLocations
cg11827453	CACNA1B	-0.27265	0.001339111	0.23	Body
cg13610307	CACNA1B	-0.26756	0.001339111	0.25	Body
cg19128261	CACNA1D	0.345396	2.73262E-05	-0.21	Body
cg09874822	CACNA1H	-0.31918	0.000228863	0.35	Body
cg10455938	CACNA1I	-0.25252	0.005371259	0.26	Body
cg12511214	CACNA1I	-0.24377	0.005371259	0.29	Body
cg03034696	CALM2	-0.41705	2.68304E-07	-0.28	Body
cg12273284	CAMK1D	0.272439	0.001082011	-0.37	Body
cg14985891	CASQ2	0.179318	0.033369303	-0.34	Body
cg07664198	CHRM2	-0.41972	8.8364E-07	0.27	Promoter

cg08323651	CHRM2	-0.41216	1.01647E-06	0.21	Promoter
cg24575234	CHRM2	-0.38252	5.71053E-06	0.3	Promoter
cg24228819	CHRM2	-0.37452	7.6086E-06	0.23	Promoter
cg25632105	CHRM2	-0.34198	3.79124E-05	0.21	Promoter
cg18190187	DRD1	-0.5414	4.15626E-12	0.29	Promoter
cg09191036	DRD5	-0.25114	0.011506701	0.25	Promoter
cg00939495	DRD5	-0.24926	0.011506701	0.29	Promoter
cg06469345	DRD5	-0.23522	0.013302972	0.24	Promoter
cg23847712	DRD5	-0.19037	0.045061003	0.27	Promoter
cg03045635	DRD5	-0.1849	0.045061003	0.3	Promoter
cg26622320	EDNRB	-0.51919	5.06575E-10	0.22	Promoter
cg10068300	GNAL	-0.36652	2.34679E-05	0.21	MultipleLocations
cg04532057	CACNA1G	0.073954	0.383461216	0.24	3'UTR
cg04878152	AGTR1	-0.36618	7.98863E-06	0.25	5'UTR
cg04390523	EDNRB	-0.49957	1.28605E-09	0.21	Promoter
cg06971129	EDNRB	-0.49833	1.28605E-09	0.23	Promoter
cg19630629	CHRM2	-0.35999	1.55026E-05	0.23	5'UTR
cg16571983	EDNRB	-0.41923	6.8662E-07	0.24	Promoter
cg19742055	EDNRB	-0.41072	8.91594E-07	0.23	Promoter
cg23766591	EDNRB	-0.40995	8.91594E-07	0.23	Promoter

cg01910869	EDNRB	-0.40512	9.62351E-07	0.21	Promoter
cg23316360	EDNRB	-0.40476	9.62351E-07	0.25	Promoter
cg08875948	GRM1	-0.32507	0.000419395	0.23	MultipleLocations
cg05327864	CHRM2	-0.32395	8.90563E-05	0.26	5'UTR
cg25958283	GNAL	-0.32346	0.00013711	0.32	MultipleLocations
cg03307465	SLC8A1	-0.32035	0.000107671	0.26	5'UTR
cg12847373	EDNRB	-0.39105	2.17514E-06	0.24	Promoter
cg21675115	EDNRB	-0.38905	2.23512E-06	0.23	Promoter
cg10904109	GRM1	-0.30778	0.000512626	0.27	MultipleLocations
cg19916212	EDNRB	-0.36698	8.2928E-06	0.23	Promoter
cg13818654	EDNRB	-0.3531	1.74931E-05	0.2	Promoter
cg03058660	GRIN2D	0.234612	0.005106178	0.25	Body
cg10161743	GRIN2D	0.302132	0.00036189	0.22	Body
cg23444265	GRIN2D	0.493065	1.05032E-09	0.23	Body
cg24924936	PTGFR	-0.28513	0.000610315	0.23	5'UTR
cg25781595	GRIN2D	0.513271	3.04654E-10	0.21	Body
cg27642554	GRM1	-0.27883	0.001355809	0.26	MultipleLocations
cg16929739	HRH1	-0.48872	3.06259E-09	-0.24	Promoter
cg20717123	P2RX2	-0.27341	0.003109689	0.32	MultipleLocations
cg27092248	HRH1	-0.48127	3.06259E-09	-0.24	Promoter

cg06457736	HRH1	-0.46963	5.64785E-09	-0.31	Promoter
cg17660833	HRH1	-0.46055	9.11844E-09	-0.3	Promoter
cg22123464	SLC8A2	-0.2682	0.001302513	0.24	5'UTR
cg10901633	ITPR1	-0.19368	0.021380337	0.28	Body
cg23662097	ITPR1	0.277842	0.00170142	-0.37	Body
cg12351433	LHCGR	-0.20965	0.03625775	0.26	Promoter
cg19403014	LHCGR	-0.19878	0.03625775	0.22	Promoter
cg00254133	NTSR1	-0.27299	0.002111939	0.28	Promoter
cg24235518	NTSR1	-0.2354	0.004955187	-0.22	Body
cg22402224	ORAI2	0.287225	0.000553743	-0.23	Body
cg12825070	HTR4	-0.19282	0.065939115	0.39	Promoter
cg00058329	P2RX2	-0.24416	0.005288868	0.24	Promoter
cg22368664	P2RX2	-0.23252	0.005527824	0.23	Promoter
cg01245966	ADCY8	-0.18358	0.069816122	0.21	Promoter
cg08958294	GRM1	-0.23328	0.006713311	0.27	5'UTR
cg00470341	PDE1A	0.246838	0.003169998	-0.2	Body
cg22131691	PDE1C	-0.38538	2.36957E-06	0.24	Promoter
cg23731805	ERBB3	0.040023	0.637493214	0.21	MultipleLocations
cg13570585	PLCB1	-0.17902	0.033670365	0.24	Body
cg03217795	PRKCB	-0.57578	4.03634E-13	0.22	1stExon

cg03306374	PRKCB	-0.48396	3.0091E-09	0.42	Promoter
cg06922606	GRIN2A	-0.00588	0.944810577	0.2	5'UTR
cg04279973	PRKCB	-0.38957	2.99892E-06	0.23	Promoter
cg21370856	PRKCB	-0.30235	0.00033565	0.33	Body
cg03949391	PTGFR	-0.43298	1.64247E-07	0.26	Promoter
cg26956874	RYR2	-0.47044	2.76408E-08	0.26	Body
cg03422911	RYR2	-0.44896	8.23456E-08	0.29	Promoter
cg18375860	RYR2	-0.43975	1.13742E-07	0.35	Promoter
cg06522054	GNAL	-0.17318	0.040006403	0.2	MultipleLocations
cg07790615	RYR2	-0.40006	1.5532E-06	0.22	Promoter
cg07557260	ADCY8	-0.18295	0.069816122	0.26	Promoter
cg19609923	MYLK2	0.170945	0.042689862	-0.21	3'UTR
cg04625338	EGFR	-0.17725	0.053240679	-0.32	Body
cg07914084	RYR2	-0.39642	1.5925E-06	0.25	Body
cg17987176	CACNA1E	-0.17558	0.078586817	0.26	Promoter
cg03120091	ADCY8	-0.17471	0.069816122	0.21	Promoter
cg26332560	ADCY8	-0.17424	0.069816122	0.23	Promoter
cg14688342	EGFR	-0.19692	0.053240679	-0.24	Body
cg04902729	CACNA1E	-0.1713	0.078586817	0.22	Promoter
cg08622198	CHRM3	0.201477	0.016587245	-0.26	5'UTR

cg09936561	DRD5	-0.16703	0.063653499	0.24	Promoter
cg15742412	CACNA1E	-0.16587	0.078586817	0.23	Promoter
cg02974928	CACNA1E	-0.16372	0.078586817	0.26	Promoter
cg18445088	CACNA1I	-0.15738	0.062355323	0.22	Body
cg20041612	EGFR	-0.15636	0.0640988	-0.22	Body
cg07312654	ADCY8	-0.1614	0.080166122	0.25	Promoter
cg09687738	HTR2C	0.159125	0.118938534	0.22	Promoter
cg26033710	TACR1	-0.15825	0.060898038	0.24	Promoter
cg17910564	VDAC3	-0.15519	0.066123668	-0.32	Promoter
cg11657808	RYR2	-0.38756	2.39662E-06	0.31	Body
cg07621385	MYLK	-0.09321	0.543227208	-0.26	5'UTR
cg03873049	CAMK2A	-0.17513	0.075586256	-0.22	Body
cg20669834	MYLK	-0.00614	0.942404616	-0.22	MultipleLocations
cg27178677	PLCB1	0.238456	0.008812773	-0.26	MultipleLocations
cg19764418	RYR2	-0.37553	4.4632E-06	0.32	Promoter
cg22762091	ADCY8	-0.15483	0.080166122	0.27	Promoter
cg02842496	ADCY8	-0.15238	0.080166122	0.27	Promoter
cg22834542	GRM5	-0.10841	0.20069535	-0.28	Body
cg09576209	CACNA1C	0.167152	0.237894234	0.26	Body
cg26296488	DRD5	-0.14759	0.092263276	0.29	Promoter

cg12860391	RYR3	-0.48108	9.80063E-09	0.27	Promoter
cg26351229	ADCY8	-0.14263	0.091564242	0.26	Promoter
cg02273436	PDGFRA	0.095732	0.258801101	-0.28	Body
cg24632756	RYR3	-0.47324	9.80063E-09	0.23	Promoter
cg18533386	CACNA1E	-0.14087	0.114813845	0.22	Promoter
cg05579652	CACNA1C	-0.10914	0.362839204	-0.27	Body
cg24033471	CACNA1C	0.104439	0.362839204	0.22	Body
cg02186782	RYR3	-0.46972	9.80063E-09	0.26	Promoter
cg26361533	CACNA1C	0.089678	0.362839204	-0.26	Body
cg00609966	CACNA1H	-0.13682	0.105702689	0.27	Promoter
cg20156659	LHCGR	-0.13511	0.146916787	0.24	Promoter
cg00735962	PRKCB	-0.13161	0.11978971	0.24	Promoter
cg25513776	MYLK4	0.122253	0.148690967	0.21	Promoter
cg02849693	PRKCG	0.068834	0.417337784	0.21	Body
cg10635895	CACNA1C	0.058723	0.48913717	0.33	Body
cg11789740	RYR3	-0.45433	2.66712E-08	0.2	Promoter
cg04597433	DRD5	-0.12215	0.149023729	0.26	Promoter
cg10421002	RYR3	-0.43098	1.33877E-07	0.22	Body
cg00209038	LHCGR	-0.11898	0.159950582	0.23	1stExon
cg09258813	ADRB3	-0.10879	0.199097574	0.28	1stExon

cg02724909	CAMK2A	-0.05383	0.526140997	-0.23	Body
cg04159077	PPIF	0.049775	0.557774358	0.21	Body
cg00840960	HTR4	-0.10721	0.308598308	0.23	Promoter
cg16888658	ADCY3	0.098639	0.452123452	-0.38	Promoter
cg26177041	CAMK2D	-0.04707	0.57944011	-0.29	Body
cg21725954	GRM1	-0.08888	0.294624104	0.22	Promoter
cg09096555	GRIN2C	0.021615	0.799181535	0.2	Body
cg19611163	MYLK	-0.027	0.942404616	-0.2	Body
cg17644208	ADCY3	0.087641	0.452123452	-0.26	Promoter
cg18538958	TACR3	-0.082	0.333724827	0.23	1stExon
cg02918903	HTR2C	-0.00152	0.985754365	0.27	Body
cg24106020	CACNA1E	-0.06277	0.459649107	0.21	Promoter
cg04181150	RYR3	-0.372	6.49924E-06	0.25	Body
cg15428578	RYR3	-0.34296	3.13958E-05	0.25	Body
cg17865555	SPHK2	-0.10981	0.1949025	-0.23	3'UTR
cg21519787	SPHK2	-0.12906	0.1949025	-0.24	3'UTR
cg26752663	ADCY3	0.033413	0.694069586	-0.31	Promoter
cg07102705	HTR4	-0.02751	0.746062037	0.3	Promoter
cg13450708	HTR5A	-0.01075	0.899367825	0.2	Promoter

Supplementary Table 5. Log-rank test to determine prognostic factors of overall survival of 32 selected genes.

Gene	Reference category	Basal time- Tercil 1 (n)	Basal Time - Tercil 3 (n)	HR (95% CI)	Logrank P
ADCY1	High	59	59	0.62 (0.37-1.02)	0.0563
ADCY8	High	66	45	0.54 (0.31-0.95)	0.0296
ADRA1A	High	58	54	0.64 (0.37-1.09)	0.0954
ADRA1D	High	61	58	0.77 (0.47-1.26)	0.2992
AGTR1	High	60	60	0.62 (0.37-1.05)	0.0726
AVPR1A	Low	59	60	1.14 (0.68-1.9)	0.6167
CACNA1A	High	58	60	0.56 (0.34-0.92)	0.0199
CACNA1B	High	59	60	0.42 (0.25-0.7)	0.0007
CACNA1D	High	58	59	0.52 (0.31-0.87)	0.0109
CACNA1H	High	58	60	0.46 (0.27-0.77)	0.0029
CACNA1I	High	61	60	0.73 (0.44-1.21)	0.2197
CALM2	Low	58	59	1.64 (0.98-2.77)	0.0591
CAMK1D	High	58	60	1.03 (0.62-1.71)	0.8983
CASQ2	High	60	60	0.79 (0.48-1.4)	0.3517
CHRM2	High	88	56	0.97 (0.6-1.55)	0.8846
DRD1	Low	60	57	1.13 (0.67-1.91)	0.6534
DRD5	High	72	39	0.76 (0.43-1.33)	0.3307
EDNRB	High	58	60	0.74 (0.44-1.25)	0.2611
GRIN2D	Low	58	60	1.21 (0.73-2.01)	0.4521
HRH1	High	58	60	2.1 (1.26-3.51)	0.0037
ITPR1	High	59	60	0.62 (0.37-1.02)	0.0571
LHCGR	High	99	17	0.89 (0.4-1.99)	0.7774
NTSR1	Low	59	60	1.39 (0.84-2.26)	0.2
ORAI2	High	58	60	0.47 (0.29-0.78)	0.0027
P2RX2	High	61	56	0.62 (0.36-1.04)	0.0691
PDE1A	Low	58	60	1.04 (0.63-1.72)	0.8863
PDE1C	High	59	60	0.45 (0.26-0.77)	0.0029

PLCB1	High	58	60	0.55 (0.33-0.92)	0.0219
PRKCB	High	58	60	0.86 (0.52-1.43)	0.5582
PTGFR	High	58	59	0.99 (0.59-1.64)	0.9656
RYR2	High	58	60	0.93 (0.57-1.53)	0.7796
RYR3	High	58	57	0.5 (0.3-0.86)	0.0107

High and low expression groups of patients were established according to tumor expression tertile value (tertile1 X tertile3).

References

1 COLLISSON, E. A.; MAITRA, A. Pancreatic Cancer Genomics 2.0: Profiling Metastases. **Cancer Cell,** v. 31, n. 3, p. 309-310, 03 2017. ISSN 1878-3686.

2 SIEGEL, R. L.; MILLER, K. D.; JEMAL, A. Cancer statistics, 2018. **CA Cancer J Clin,** v. 68, n. 1, p. 7-30, Jan 2018. ISSN 1542-4863.

3 CHAKRABORTY, S. et al. Current status of molecular markers for early detection of sporadic pancreatic cancer. **Biochim Biophys Acta,** v. 1815, n. 1, p. 44-64, Jan 2011. ISSN 0006-3002.

4 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.

5 KLEEFF, J. et al. Pancreatic cancer. **Nat Rev Dis Primers,** v. 2, p. 16022, 04 2016. ISSN 2056-676X.

6 ORTH, M. et al. Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. **Radiat Oncol,** v. 14, n. 1, p. 141, Aug 2019. ISSN 1748-717X.

7 EHRLICH, M. DNA hypomethylation in cancer cells. **Epigenomics,** v. 1, n. 2, p. 239-59, Dec 2009. ISSN 1750-192X.

8 WANG, X. X. et al. Large-scale DNA methylation expression analysis across 12 solid cancers reveals hypermethylation in the calcium-signaling pathway. **Oncotarget,** v. 8, n. 7, p. 11868-11876, Feb 2017. ISSN 1949-2553.

9 AHUJA, N.; SHARMA, A. R.; BAYLIN, S. B. Epigenetic Therapeutics: A New Weapon in the War Against Cancer. **Annu Rev Med,** v. 67, p. 73-89, 2016. ISSN 1545-326X.

10 NONES, K. et al. Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling. **Int J Cancer,** v. 135, n. 5, p. 1110-8, Sep 2014. ISSN 1097-0215.

11 MISHRA, N. K.; GUDA, C. Genome-wide DNA methylation analysis reveals molecular subtypes of pancreatic cancer. **Oncotarget,** v. 8, n. 17, p. 28990-29012, Apr 2017. ISSN 1949-2553.

12 BAYLIN, S. B.; JONES, P. A. A decade of exploring the cancer epigenome - biological and translational implications. **Nat Rev Cancer,** v. 11, n. 10, p. 726-34, Sep 2011. ISSN 1474-1768.

[13] YAMASHITA, K. et al. Epigenetic biomarkers of promoter DNA methylation in the new era of cancer treatment. **Cancer Sci,** v. 109, n. 12, p. 3695-3706, Dec 2018. ISSN 1349-7006.

[14] NISHIZAWA, N. et al. Diagnostic potential of hypermethylation of the cysteine dioxygenase 1 gene (CDO1) promoter DNA in pancreatic cancer. **Cancer Sci**, Jul 2019. ISSN 1349-7006.

[15] KINUGAWA, Y. et al. Methylation of Tumor Suppressor Genes in Autoimmune Pancreatitis. **Pancreas,** v. 46, n. 5, p. 614-618, 2017 May/Jun 2017. ISSN 1536-4828.

[16] MATSUBAYASHI, H. et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. **Cancer Res,** v. 66, n. 2, p. 1208-17, Jan 2006. ISSN 0008-5472.

[17] YANG, L. et al. ppENK Gene Methylation Status in the Development of Pancreatic Carcinoma. **Gastroenterol Res Pract,** v. 2013, p. 130927, 2013. ISSN 1687-6121.

[18] BRUNE, K. et al. Genetic and epigenetic alterations of familial pancreatic cancers. **Cancer Epidemiol Biomarkers Prev,** v. 17, n. 12, p. 3536-42, Dec 2008. ISSN 1055-9965.

[19] SATO, N. et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. **Cancer Res,** v. 63, n. 13, p. 3735-42, Jul 1 2003. ISSN 0008-5472 (Print) 0008-5472.

[20] BOUTROS, T.; CHEVET, E.; METRAKOS, P. Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. **Pharmacol Rev,** v. 60, n. 3, p. 261-310, Sep 2008. ISSN 1521-0081.

[21] MAZIVEYI, M.; ALAHARI, S. K. Cell matrix adhesions in cancer: The proteins that form the glue. **Oncotarget,** v. 8, n. 29, p. 48471-48487, Jul 2017. ISSN 1949-2553.

[22] XU, M. et al. A temporal examination of calcium signaling in cancer- from tumorigenesis, to immune evasion, and metastasis. **Cell Biosci,** v. 8, p. 25, 2018. ISSN 2045-3701.

[23] LI, H. et al. Bioinformatics-Based Identification of Methylated-Differentially Expressed Genes and Related Pathways in Gastric Cancer. **Dig Dis Sci,** v. 62, n. 11, p. 3029-3039, 11 2017. ISSN 1573-2568.

[24] ANDREW_AGUIRRE@DFCI.HARVARD.EDU, C. G. A. R. N. E. A.; NETWORK, C. G. A. R. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. **Cancer Cell,** v. 32, n. 2, p. 185-203.e13, 08 2017. ISSN 1878-3686.

[25] CURTIUS, K.; WRIGHT, N. A.; GRAHAM, T. A. An evolutionary perspective on field cancerization. **Nat Rev Cancer,** v. 18, n. 1, p. 19-32, Jan 2018. ISSN 1474-1768.

[26] NETWORK, C. G. A. Comprehensive molecular portraits of human breast tumours. **Nature,** v. 490, n. 7418, p. 61-70, Oct 2012. ISSN 1476-4687.

[27] FEBER, A. et al. Amplification and overexpression of E2F3 in human bladder cancer. **Oncogene,** v. 23, n. 8, p. 1627-30, Feb 2004. ISSN 0950-9232.

[28] HOLCOMB, I. N. et al. Comparative analyses of chromosome alterations in soft-tissue metastases within and across patients with castration-resistant prostate cancer. **Cancer Res,** v. 69, n. 19, p. 7793-802, Oct 2009. ISSN 1538-7445.

[29] LUCITO, R. et al. Copy-number variants in patients with a strong family history of pancreatic cancer. **Cancer Biol Ther,** v. 6, n. 10, p. 1592-9, Oct 2007. ISSN 1555-8576

[30] AL-SUKHNI, W. et al. Identification of germline genomic copy number variation in familial pancreatic cancer. **Hum Genet,** v. 131, n. 9, p. 1481-94, Sep 2012. ISSN 1432-1203.

[31] WILLIS, J. A. et al. Genome-wide analysis of the role of copy-number variation in pancreatic cancer risk. **Front Genet,** v. 5, p. 29, 2014. ISSN 1664-8021.

[32] BERRIDGE, M. J.; LIPP, P.; BOOTMAN, M. D. The versatility and universality of calcium signalling. **Nat Rev Mol Cell Biol,** v. 1, n. 1, p. 11-21, Oct 2000. ISSN 1471-0072.

[33] PEDRIALI, G. et al. Regulation of Endoplasmic Reticulum-Mitochondria Ca. **Front Oncol,** v. 7, p. 180, 2017. ISSN 2234-943X.

[34] BOOTMAN, M. D. Calcium signaling. **Cold Spring Harb Perspect Biol,** v. 4, n. 7, p. a011171, Jul 2012. ISSN 1943-0264.

[35] EICHBERG, J. J.; MZHU, I. X. **Calcium Entry Channels in Non-Excitable Cells**. Gewerbestrasse 11, 6330 Cham, Switzerland: 2017. 645 ISBN 978-3-319-57732-6.

[36] IBRAHIM, S. et al. Expression Profiling of Calcium Channels and Calcium-Activated Potassium Channels in Colorectal Cancer. **Cancers (Basel),** v. 11, n. 4, Apr 2019. ISSN 2072-6694.

[37] MCANDREW, D. et al. ORAI1-mediated calcium influx in lactation and in breast cancer. **Mol Cancer Ther,** v. 10, n. 3, p. 448-60, Mar 2011. ISSN 1538-8514.

[38] CROTTÈS, D. et al. TMEM16A controls EGF-induced calcium signaling implicated in pancreatic cancer prognosis. **Proc Natl Acad Sci U S A,** v. 116, n. 26, p. 13026-13035, Jun 2019. ISSN 1091-6490.

[39] BOYE, K.; MAELANDSMO, G. M. S100A4 and metastasis: a small actor playing many roles. **Am J Pathol,** v. 176, n. 2, p. 528-35, Feb 2010. ISSN 1525-2191.

[40] ROSTY, C. et al. Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. **Am J Pathol,** v. 160, n. 1, p. 45-50, Jan 2002. ISSN 0002-9440.

[41] QIU, C.; BU, X.; JIANG, Z. Protocadherin-10 acts as a tumor suppressor gene, and is frequently downregulated by promoter methylation in pancreatic cancer cells. **Oncol Rep,** v. 36, n. 1, p. 383-9, Jul 2016. ISSN 1791-2431.

[42] CURIA, M. C. et al. High methylation levels of PCDH10 predict poor prognosis in patients with pancreatic ductal adenocarcinoma. **BMC Cancer,** v. 19, n. 1, p. 452, May 2019. ISSN 1471-2407.

[43] RODERICK, H. L.; COOK, S. J. Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. **Nat Rev Cancer,** v. 8, n. 5, p. 361-75, May 2008. ISSN 1474-1768.

[44] YARDEN, Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. **Eur J Cancer,** v. 37 Suppl 4, p. S3-8, Sep 2001. ISSN 0959-8049.

[45] CUI, C. et al. Targeting calcium signaling in cancer therapy. **Acta Pharm Sin B,** v. 7, n. 1, p. 3-17, Jan 2017. ISSN 2211-3835.

[46] NAVAS, C. et al. EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma. **Cancer Cell,** v. 22, n. 3, p. 318-30, Sep 2012. ISSN 1878-3686.

[47] BALSANO, R.; TOMMASI, C.; GARAJOVA, I. State of the Art for Metastatic Pancreatic Cancer Treatment: Where Are We Now? **Anticancer Res,** v. 39, n. 7, p. 3405-3412, Jul 2019. ISSN 1791-7530.

[48] SHI, J. L.; FU, L.; WANG, W. D. High expression of inositol 1,4,5-trisphosphate receptor, type 2 (ITPR2) as a novel biomarker for worse prognosis in cytogenetically normal acute myeloid leukemia. **Oncotarget,** v. 6, n. 7, p. 5299-309, Mar 2015. ISSN 1949-2553.

[49] PRIOR, I. A.; LEWIS, P. D.; MATTOS, C. A comprehensive survey of Ras mutations in cancer. **Cancer Res,** v. 72, n. 10, p. 2457-67, May 2012. ISSN 1538-7445.

[50] SCHAAL, C.; PADMANABHAN, J.; CHELLAPPAN, S. The Role of nAChR and Calcium Signaling in Pancreatic Cancer Initiation and Progression. **Cancers (Basel),** v. 7, n. 3, p. 1447-71, Jul 2015. ISSN 2072-6694.

[51] LANNER, J. T. et al. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. **Cold Spring Harb Perspect Biol,** v. 2, n. 11, p. a003996, Nov 2010. ISSN 1943-0264.

[52] BRZOZOWSKI, J. S.; SKELDING, K. A. The Multi-Functional Calcium/Calmodulin Stimulated Protein Kinase (CaMK) Family: Emerging Targets for Anti-Cancer Therapeutic Intervention. **Pharmaceuticals (Basel),** v. 12, n. 1, Jan 2019. ISSN 1424-8247.

[53] WANG, Y. Y.; ZHAO, R.; ZHE, H. The emerging role of CaMKII in cancer. **Oncotarget,** v. 6, n. 14, p. 11725-34, May 2015. ISSN 1949-2553.

[54] CHEN, W. et al. Ca. **World J Gastroenterol,** v. 23, n. 33, p. 6111-6118, Sep 2017. ISSN 2219-2840.

[55] TAI, Y. L.; CHEN, L. C.; SHEN, T. L. Emerging roles of focal adhesion kinase in cancer. **Biomed Res Int,** v. 2015, p. 690690, 2015. ISSN 2314-6141.

[56] BOUCHARD, V. et al. Fak/Src signaling in human intestinal epithelial cell survival and anoikis: differentiation state-specific uncoupling with the PI3-K/Akt-1 and MEK/Erk pathways. **J Cell Physiol,** v. 212, n. 3, p. 717-28, Sep 2007. ISSN 0021-9541.

[57] VANDECAETSBEEK, I. et al. The Ca2+ pumps of the endoplasmic reticulum and Golgi apparatus. **Cold Spring Harb Perspect Biol,** v. 3, n. 5, May 2011. ISSN 1943-0264.

[58] KOROSEC, B. et al. ATP2A3 gene is involved in cancer susceptibility. **Cancer Genet Cytogenet,** v. 188, n. 2, p. 88-94, Jan 2009. ISSN 1873-4456.

[59] XU, X. Y. et al. Aberrant SERCA3 expression is closely linked to pathogenesis, invasion, metastasis, and prognosis of gastric carcinomas. **Tumour Biol,** v. 33, n. 6, p. 1845-54, Dec 2012. ISSN 1423-0380.

[60] NORTH, R. A. Molecular physiology of P2X receptors. **Physiol Rev,** v. 82, n. 4, p. 1013-67, Oct 2002. ISSN 0031-9333.

[61] BIRNBAUM, D. J. et al. Expression of Genes with Copy Number Alterations and Survival of Patients with Pancreatic Adenocarcinoma. **Cancer Genomics Proteomics,** v. 13, n. 3, p. 191-200, 2016 May-Jun 2016. ISSN 1790-6245.

[62] GUTIÉRREZ, M. L. et al. Association between genetic subgroups of pancreatic ductal adenocarcinoma defined by high density 500 K SNP-arrays and tumor histopathology. **PLoS One,** v. 6, n. 7, p. e22315, 2011. ISSN 1932-6203.

[63] ARYEE, M. J. et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. **Bioinformatics,** v. 30, n. 10, p. 1363-9, May 2014. ISSN 1367-4811.

[64] FORTIN, J. P. et al. Functional normalization of 450k methylation array data improves replication in large cancer studies. **Genome Biol,** v. 15, n. 12, p. 503, Dec 2014. ISSN 1474-760X.

[65] DU, P. et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. **BMC Bioinformatics,** v. 11, p. 587, Nov 2010. ISSN 1471-2105.

[66] MARTIN, M. et al. Dynamic imbalance between cancer cell subpopulations induced by transforming growth factor beta (TGF-β) is associated with a DNA methylome switch. **BMC Genomics,** v. 15, p. 435, Jun 2014. ISSN 1471-2164.

[67] KANEHISA, M.; GOTO, S. KEGG: kyoto encyclopedia of genes and genomes. **Nucleic Acids Res,** v. 28, n. 1, p. 27-30, Jan 2000. ISSN 0305-1048.

[68] KANEHISA, M. et al. KEGG as a reference resource for gene and protein annotation. **Nucleic Acids Res,** v. 44, n. D1, p. D457-62, Jan 2016. ISSN 1362-4962.

[69] YU, G. et al. clusterProfiler: an R package for comparing biological themes among gene clusters. **OMICS,** v. 16, n. 5, p. 284-7, May 2012. ISSN 1557-8100.

[70] MORRIS, T. J. et al. ChAMP: 450k Chip Analysis Methylation Pipeline. **Bioinformatics,** v. 30, n. 3, p. 428-30, Feb 2014. ISSN 1367-4811.

CAPÍTULO V: Manuscrito 2

"Histone deacetylase expression profile in periampullary carcinomas: a preliminary study based on public datasets and clinical samples"

Manuscrito submetido à revista *Molecular Biology Reports* (Fator de impacto: 2,1).

Title: Histone deacetylase expression profile in periampullary carcinomas: a preliminary study based on public datasets and clinical samples.

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Abstract:

Background: Periampullary carcinomas (PACs) represent 0.5% of gastrointestinal malignancies and include pancreatic ductal adenocarcinoma (PDAC), ampulla of Vater carcinoma (AVC), distal cholangiocarcinoma (dCCA), and duodenal adenocarcinoma (DAC). Histone acetylation alteration through histones deacetylases (HDACs) can play a crucial role in cancer development; however, their expression is not entirely characterized in PACs. Hence, we aimed to evaluate cancer-related HDACs gene expression among PACs and surrounding non-tumoral tissue (NT) using public datasets. Furthermore, we investigate HDACs immunostaining profile in AVC and NT samples.

Methods and Results: *HDAC1*, *HDAC2*, *HDAC3* and *HDAC7* differential expression analysis were performed from two microarrays reported in Gene Expression Omnibus. All preprocessing data and analyses were performed in R 3.4.2 statistical software. HDACs immunostaining were performed using samples from a biobank of PACs and from a local cohort (n=20), with their respective pancreatic and duodenal NT used as controls samples. Differential expression analysis showed that DAC and AVC had equivalent expression profiles for *HDAC1* and *HDAC2*. Additionally, we found similar immunostaining results for all studied HDACs in AVC and NT samples. Nuclear and cytoplasmic staining was detected for the majority of HDACs, with exception of HDAC7 which showed a more pronounced cytoplasmic staining.

Conclusions: The results suggested that *HDAC1* and *HDAC2* similar profiles might be shared between DAC and AVC. Moreover, HDAC1, HDAC2, HDAC3 and HDAC7 equivalent immunostaining in AVC and NT may indicate that these HDACs may not be involved in the malign transformation of this tumor, although these might have a role supporting the cancer phenotype.

Keywords: Periampullary carcinomas, ampulla of Vater carcinoma, epigenetic, histone deacetylases.

Introduction

Periampullary carcinomas (PACs) are rare neoplasms that represent 0.5% of gastrointestinal malignancies [1]. PACs arise within 2 cm of the ampulla of Vater and include four different tumor types: pancreatic ductal adenocarcinoma (PDAC), ampulla of Vater carcinoma (AVC), distal cholangiocarcinoma (dCCA), and duodenal adenocarcinoma (DAC) [2, 3]. Although there is a standardized histopathologic evaluation, the precise diagnosis of the primary site is difficult to classify. In general, advanced PACS involve more than one potential site of origin destructing the normal periampullary anatomy and present epithelial dysplasia in more than a single periampullary portion, which impairs the definition of diagnosis [4–6]. Among the PACs, AVC is the second most frequent malign tumor, with adenocarcinoma being the most prevalent histopathologic pattern, especially of the intestinal (AVCi) and pancreatobiliary (AVCp) subtypes [7, 8].

Modifications in the epigenetic profile, such as histone deacetylase (HDACs) dysregulation, play an important role in cancer development by silencing tumor suppressor genes and creating chromosomal instability [9]. The HDAC family comprises four classes of proteins, namely: class I (HDACs 1-3, and 8), class II (HDACs 4-7, 9 and 10) and class IV (HDAC11), all Zn^{2+} dependent proteins which utilize histones as substrate. Alternatively, class III HDAC is referred to as sirtuin (SIRTs 1–7) and requires NAD^{+} as a cofactor [10]. Until now, PDACs and dCCAs are the periampullary carcinomas that were best characterized regarding tumor HDAC expression [11–13], while in AVCs and DACs data are poor or inexistent. In addition, the majority of the studies focus only on neoplastic tissue and data comparing tumor and surrounding non-tumoral tissue (NT) are scarce. Comparative analysis between these groups may enhance our understanding on the behavior of these tumors and provide insights on the carcinogenesis process, on novel prognostic and/or diagnostic biomarkers and on potential novel therapeutic interventions. In the present study we assessed *HDAC1, HDAC2, HDAC3* and *HDAC7* gene expression profiles in PACs

using publicly-available datasets and results from protein expression data from a cohort of Brazilian patients with AVC.

Materials and Methods

Bioinformatics Analysis

The gene expression profile of the cancer-related HDACs (*HDAC1*, *HDAC2*, *HDAC3*, and *HDAC7)* available in the public datasets *Gene Expression Omnibus* (GEO- GSE39409 and GSE60979) was assessed initially [14, 15]. The GSE60979 dataset includes periampullary carcinomas and surrounding pancreatic non-tumoral (PT) samples, and GSE39409 contains only data regarding periampullary carcinomas. Raw data from GSE39409 (GPL570) were normalized using the RMA method, implemented in *affy BioConductor* R-package [16]. To select a single probe as a representative of a specific gene, we used the JetSet score [17]. As a result, probes *201209_at*, *242141_at*, *216326_s_at*, and *217937_s_at* were assigned to *HDAC1*, *HDAC2*, *HDAC3*, and *HDAC7*, respectively. Raw data from GSE60979 (GPL14550) were background corrected and normalized using *limma BioConductor* R-package [18]. For *HDACs* with more than one probe in the array, we calculated mean expression. To compare *HDAC* gene expression we performed the Kruskal-Wallis test followed by the Benjamini-Hochberg correction for multiple comparisons. All data preprocessing and analyses were performed in R 3.4.2 statistical software. *P*-values of <0.05 were considered statistically significant.

Clinical specimens and tissue microarray

A prospective biological biobank of periampullary carcinoma samples recruited from a public university hospital in Southern Brazil were analyzed. In the present study, HDAC immunostaining was performed in samples from AVC, PDAC, chronic pancreatitis, and samples from patients with other pancreatic diseases who underwent surgical resection. The inclusion criteria were: recent pathology-proven diagnosis and no history of previous or current chemo- or radiotherapy treatment. The study was

approved by the institutional review board, and all patients provided written informed consent (GPPG 14-0526).

Twenty AVC samples were selected and their respective surrounding pancreatic and duodenal non-tumoral tissues retrieved (PT, n=18; DT, n=16). Additionally, 41 PT, 32 DT and 10 surrounding non-tumoral ampullary tissue (AT) samples from PDAC, chronic pancreatitis, and other patients with pancreatic diseases were included in tissue microarrays (TMAs). TMAs were prepared from formalin-fixed paraffin-embedded tissue sections using a 1-mm punch in triplicate.

Immunohistochemistry analysis

Immunostaining of HDAC1 (10E2, 1:600 dilution, Thermo Fisher Scientific), HDAC2 (H-54, 1:300 dilution, Santa Cruz Biotechnology), HDAC3 (Y415, 1:100 dilution, Abcam) and HDAC7 (N18, 1:100 dilution, Santa Cruz Biotechnology) were performed according to the manufacturer's specifications. Nuclear and cytoplasmic staining were evaluated by pairs of independent observers, blinded to the clinical data.

To establish protein expression levels, a semi-quantitative immunoreactivity score (IRS) was applied, as previously described [19, 20], with some modifications. In brief, IRS for each individual case (ranging from 0 to 12) was calculated by multiplication of the intensity and frequency scores. The intensity score was defined according to the following patterns as 0: null, 1: weak, 2: moderate and 3: strong. The sample was also scored according to the percentage of positive cells (frequency score) in four distinct categories: 1 (<10% positive cells), 2 (≥10 - 50%), 3 (≥ 50 - 80%), and 4 (≥ 80 - 100%). Cases exhibiting an IRS between 0 and 6 were combined in the "HDAC negative" group. Cases with an IRS above 6 were combined in the "HDAC positive" group. Discordant cases were reviewed by observers as many times as necessary until consensus was reached.

Results

Bioinformatics Analysis

HDAC1, HDAC2, HDAC3, and *HDAC7* expression was analyzed in two GEO datasets. GSE60979 includes PACs (AVCp, n = 8; AVCi, n = 7; dCCA, n = 8; DAC, n = 9; and PDAC, n = 49) and PT, n = 12. *HDAC1* was overexpressed in AVCi and DAC when compared to PT (p = 0.002, Md= 13.0 and 13.1 *vs* 11.8; respectively) (Figure1a). In addition, *HDAC2* was overexpressed in DAC, in comparison with AVCp, dCCA, and PT (p = 0.015, Md= 10.6 *vs* 10.2, 10.1, 10.3; respectively) (Figure1b). Finally, *HDAC7* expression was higher in PDAC when compared to PT (p = 0.003, Md=10.6 vs 9.5; respectively; Figure1d). No difference in *HDAC3* gene expression analysis was observed (Figure1c). No significant difference in HDAC expression profiles were observed between AVCp and AVCi.

Subsequently, due to lack of data combining all subtypes of PACs, we investigated a second dataset. Although there was a description of pancreatobiliary and intestinal types of AVC in GSE39409 (AVC, n = 14; dCCA, n = 2; DAC, n = 8 and PDAC, n = 8), the dataset did not have information about the specific histology of these samples. This analysis revealed that *HDAC1* and *HDAC2* were overexpressed in AVC and DAC compared to the PDAC samples (p = 0.016, Md=12.4 and 12.2 vs 11.9; p = 0.029, Md=7,1 and 7.2 vs 7.0; respectively). Similar to GSE60979, AVC and DAC samples in this dataset had a comparable expression profile of *HDAC1* and no difference regarding *HDAC3* and *HDAC7* gene expression analyses between groups was observed (Figure1 e-h).

HDAC immunohistochemistry and subcellular localization in AVC tumors

To evaluate HDAC expression and their subcellular localization, we performed immunostaining analyses for HDAC1, HDAC2, HDAC3, and HDAC7 in TMA blocks generated from AVC and NT. Twenty AVC samples were analyzed and all of them were identified as adenocarcinomas, including 10 of the intestinal subtype, 3 of the pancreaticobiliary subtype and one mucinous subtype. In 6 samples specific histologic

subtype was not possible to be determined. Due to mixed epithelium of ampulla of Vater, here we analyzed NT composed of three subgroups: AT (n= 10), PT (n= 59) and DT (n=48). As a result, we observed that most AVC samples had a high IRS scores, being classified as positive for HDAC immunostaining (100% HDAC1; 75% HDAC2; 95% HDAC3 and 65% HDAC7). In addition, both nuclear and cytoplasmic staining for the majority of HDACs was detected in AVC, with the exception of HDAC7 immunostaining which had a more pronounced cytoplasmic staining (Table 1 and Figure 2).

Considering NT samples, HDACs positivity immunostaining was found in all subgroups. Among them, HDAC1 showed more uniform immunostaining in the evaluated subgroups (AT= 70%, PT= 97%, and DT= 92%), while positive HDAC2; HDAC3 and HDAC7 immunostaining was variable among subgroups (Table 1 and Figure 2).

Discussion

HDAC play crucial roles in cancer by regulating the cell cycle, apoptosis, DNA-damage response, metastasis, angiogenesis, and other cellular processes through deacetylation of histone and non-histone proteins [21]. Although HDACs have been investigated in periampullary carcinomas, including PDAC and dCCA, their characterization in AVC and DAC is poor or inexistent. To contribute in reducing this lack of information we performed a combined *in silico* and experimental (immunostaining) study including tumoral and non-tumoral samples from an AVC cohort. The study provides the first data on HDAC1, HDAC2, HDAC3 and HDAC7 immunostaining in AVC and adjacent NT samples.

One of the analyzed datasets (GSE60979) used a microarray platform to identified mRNA prognostic biomarkers in periampullary carcinomas. The authors detected 10 upregulated genes in AVCi, as compared to AVCp, that are regulated by *HDAC1* [15]. We explored this data and showed that *HDAC1* and *HDAC2* expression

is similar between AVC and DAC. Additional analyses of this dataset revealed that dCCA has the same *HDAC2* expression profile of AVCp and PT.

Cholangiocarcinoma (CCA) is a rare malignancy that can occur along the biliary tree and can be classified into three board groups according to the anatomic distribution: 1) intrahepatic, 2) perihilar, and 3) distal [22]. PACs classification includes only dCCA and although HDACs analyses have been reported in CCA, the majority of them did not focus on the distal portion. In our analysis, dCCA was compared with other PACs tumors and PT according to GSE60979, and we did not find HDACs gene expression differences between the non-tumoral tissues and dCCA. HDACs expression in CCA and non-tumoral tissues also was investigated by other groups. Yamaguchi et al., (2010) used chronic cholecystitis samples (gallbladder inflammation) to compare HDAC1 and HDAC2 expression with CCA, as well as our study, they did not find difference on HDACs expression intensity between the samples. In contrast, using matched adjacent non-tumor tissues of CCA, He et al., (2016) found overexpression of *HDAC1*, *HDAC2*, *HDAC3*, *HDAC8*, and *HDAC9* in CCA and the protein levels investigation of these samples confirmed the overexpression for HDAC2, HDAC3, and HDAC8 (53%, 55%, and 53%, respectively [24].

HDACs expression in PDAC is better understood due to the higher frequency of this tumor type [8] and has been determined in tissue samples and cell lines by different methodologies. Herein we investigated *HDACs* gene expression in PDAC and found only *HDAC7* overexpression. The same results were identified in two studies with RT-PCR and immunohistochemistry where just *HDAC7* was overexpressed in PDAC compared to PT samples [11, 25]. To date, the largest cohort of PDAC samples (n= 78) that investigated HDAC expression by immunohistochemistry reported that 32% of samples were positive for HDAC1, 63% for HDAC2, and 79% for HDAC3 [26]. Other studies, with smaller sample sizes, also showed positive staining of these analyzed HDACs, however, none of them compared HDACs staining with normal pancreas tissue [27–30].

Ampulla of Vater is a small and complex anatomic site, composed of the common channel, distal biliary duct, pancreatic duct, and duodenal mucosa and all surrounding tissues share the same embryonic origin [31, 32]. The mixed epithelium of the ampulla of Vater is one of the reasons why AVCs show a large number of histological subtypes and because we used three non-tumoral tissues to compare in our immunohistochemistry analysis. Until now, the only information on HDAC expression reported was obtained from an unusual neoplasm, a high-grade neuroendocrine carcinoma, showing 60% HDAC1, 60.3% HDAC2 and 61% HDAC3 positive immunostaining [33]. The most frequent AVC subtype (adenocarcinoma) was represented in our cohort, and we identified positive levels of these HDACs (100%, 75%, and 95%, respectively) while also reporting HDAC7 positive immunostaining (65%). Moreover, similar to AVC samples, all NT subgroups (AT, PT, and DT) presented HDAC positive immunostaining showing no marked expression difference compared to tumor samples for the studied HDACs.

Conclusions

In summary, we presented the first investigation of HDAC in duodenal adenocarcinoma. The *HDAC1* and *HDAC2* expression similarity between ampulla of Vater carcinoma and duodenal adenocarcinoma might due to ampullary epithelium being formed by intestinal-like cells. It suggests that a similar profile of epigenetic marks may be shared in these tumors. In addition, the results indicate that different HDACs are expressed in AVC and NT samples, with none of them associated exclusively with AVC. Our results may indicate that HDAC1, HDAC2, HDAC3, and HDAC7 may not be involved in malign transformation, although these might have a role supporting the cancer phenotype.

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Author contributions

CG, BA, ABO and PAP conceived the work; CG, ITSS, BA, RCR, SMSM, DMU conducted the analyses, made substantial contributions to acquisition and interpretation of data; CG, ITSS, RCR, SMSM, DMU, ABO and PAP revised the manuscript critically and contributed with interpretation of the findings; PAP and ABO supervised the work; All authors gave final approval of the version to be published.

Compliance with Ethical Standards

Conflicts of interest

All authors declare no potential financial or ethical conflicts of interest regarding the contents of this submission.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (GPPG 14-0526)), and written informed consent was obtained from all patients.

References

1. Albores-Saavedra J, Schwartz AM, Batich K, Henson DE (2009) Cancers of the ampulla of vater: demographics, morphology, and survival based on 5,625 cases from the SEER program. J Surg Oncol 100:598–605

2. Berberat PO, Künzli BM, Gulbinas A, et al (2009) An audit of outcomes of a series of periampullary carcinomas. Eur J Surg Oncol 35:187–191

3. Kamarajah SK (2018) Pancreaticoduodenectomy for periampullary tumours: a review article based on Surveillance, End Results and Epidemiology (SEER) database. Clin Transl Oncol 20:1153–1160

4. Monson JRT, Donohue JH, McEntee GP, et al (1991) Radical resection for carcinoma of the ampulla of Vater. Arch Surg 126:353–357

5. Lüttges J, Zamboni G, Klöppel G (1999) Recommendation for the examination of pancreaticoduodenectomy specimens removed from patients with carcinoma of the exocrine pancreas. Dig Surg 16:291–296

6. Fisher WE, Bakey ME (2007) Differences between ampullary, periampullary and pancreatic cancer. World J Surg 31:144–146

7. Agoff SN, Crispin DA, Bronner MP, et al (2001) Neoplasms of the ampulla of Vater with concurrent pancreatic intraductal neoplasia: a histological and molecular study. Mod Pathol 14:139

8. He J, Ahuja N, Makary MA, et al (2014) 2564 resected periampullary adenocarcinomas at a single institution: trends over three decades. HPB 16:83–90

9. Jayaramayya K, Balachandar V, Santhy KS (2018) Ampullary carcinoma—A genetic perspective. Mutation Research/Reviews in Mutation Research 776:10–22

10. Barneda-Zahonero B, Parra M (2012) Histone deacetylases and cancer. Mol Oncol 6:579–589

11. Ouaïssi M, Silvy F, Loncle C, et al (2014) Further characterization of HDAC and SIRT gene expression patterns in pancreatic cancer and their relation to disease outcome. PLoS One 9:e108520

12. Kwak TW, Kim DH, Jeong Y-I, Kang DH (2015) Antitumor activity of vorinostat-incorporated nanoparticles against human cholangiocarcinoma cells. J Nanobiotechnology 13:60

13. Cai M-H, Xu X-G, Yan S-L, et al (2018) Depletion of HDAC1, 7 and 8 by histone deacetylase inhibition confers elimination of pancreatic cancer stem cells in combination with gemcitabine. Sci Rep 8:1621

14. Overman MJ, Zhang J, Kopetz S, et al (2013) Gene expression profiling of ampullary carcinomas classifies ampullary carcinomas into biliary-like and intestinal-like subtypes that are prognostic of outcome. PLoS One 8:e65144

15. Sandhu V, Lothe IMB, Labori KJ, et al (2015) Molecular signatures of mRNAs and miRNAs as prognostic biomarkers in pancreatobiliary and intestinal types of periampullary adenocarcinomas. Mol Oncol 9:758–771

16. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20:307–315

17. Li Q, Birkbak NJ, Gyorffy B, et al (2011) Jetset: selecting the optimal microarray probe set to represent a gene. BMC Bioinformatics 12:474

18. Ritchie ME, Phipson B, Wu D, et al (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47–e47

19. Weichert W, Denkert C, Schmidt M, et al (2004) Polo-like kinase isoform expression is a prognostic factor in ovarian carcinoma. Br J Cancer 90:815

20. Weichert W, Röske A, Gekeler V, et al (2008) Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis. Lancet Oncol 9:139–148

21. West AC, Johnstone RW (2014) New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124:30–39

22. Blechacz B, Komuta M, Roskams T, Gores GJ (2011) Clinical diagnosis and staging of cholangiocarcinoma. Nat Rev Gastroenterol Hepatol 8:512

23. Yamaguchi J, Sasaki M, Sato Y, et al (2010) Histone deacetylase inhibitor (SAHA) and repression of EZH2 synergistically inhibit proliferation of gallbladder carcinoma. Cancer Sci 101:355–362

24. He J-C, Yao W, Wang J-M, et al (2016) TACC3 overexpression in cholangiocarcinoma correlates with poor prognosis and is a potential anti-cancer molecular drug target for HDAC inhibitors. Oncotarget 7:75441

25. Ouaïssi M, Sielezneff I, Silvestre R, et al (2008) High histone deacetylase 7 (HDAC7) expression is significantly associated with adenocarcinomas of the pancreas. Ann Surg Oncol 15:2318–2328

26. Lehmann A, Denkert C, Budczies J, et al (2009) High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo. BMC Cancer 9:395

27. Fritsche P, Seidler B, Schüler S, et al (2009) HDAC2 mediates therapeutic resistance of pancreatic cancer cells via the BH3-only protein NOXA. Gut 58:1399–1409

28. Wang W, Gao J, Man X-H, et al (2009) Significance of DNA methyltransferase-1 and histone deacetylase-1 in pancreatic cancer. Oncol Rep 21:1439–1447

29. Jiao F, Hu H, Yuan C, et al (2014) Histone deacetylase 3 promotes pancreatic cancer cell proliferation, invasion and increases drug-resistance through histone modification of P27, P53 and Bax. Int J Oncol 45:1523–1530

30. Giaginis C, Damaskos C, Koutsounas I, et al (2015) 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 15:148

31. Kunath U, Hommerding H (1981) Is the duodenal papilla an autonomic sphincter? A contribution to the functional morphology (author's transl). Res Exp Med 178:103–116

32. Kim JH, Kim M-J, Chung J-J, et al (2002) Differential diagnosis of periampullary carcinomas at MR imaging. Radiographics 22:1335–1352

33. Stojsic Z, Brasanac D, Bilanovic D, et al (2010) Large-cell neuroendocrine carcinoma of the ampulla of Vater. Med Oncol 27:1144–1148

Figures

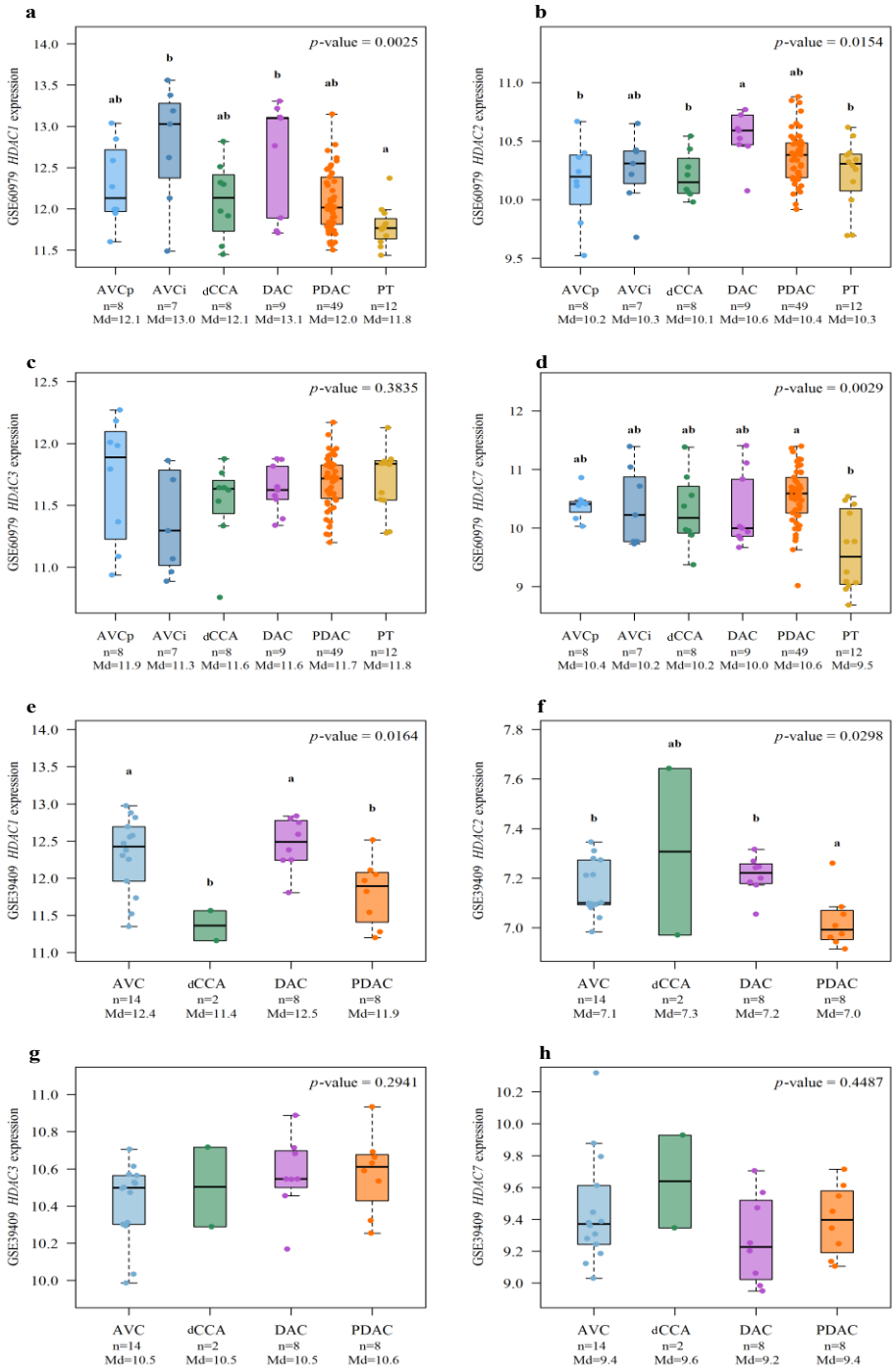

Fig1 Gene expression of HDACs in periampullary adenocarcinomas using expression profiles from *Gene Expression Omnibus.* *HDACs* gene expression comparison: in GSE60979 (a) *HDAC1*, (b) *HDAC2*, (c) *HDAC3* and (d) *HDAC7* and in GSE39409 (e) *HDAC1*, (f) *HDAC2*, (g) *HDAC3* and (h) *HDAC7*. AVC= Ampulla of Vater carcinoma; AVCp= Ampulla of Vater carcinoma pancreatobiliary-type; AVCi= Ampulla of Vater carcinoma intestinal-type AVC; dCCA= distal Cholangiocarcinoma; DAC= Duodenal adenocarcinoma; PDAC= Pancreatic adenocarcinoma; PT= Surrounding Non-tumoral pancreatic tissue. Median values are presented as Md and as solid black lines. Small letters represent post-hoc comparison: equal letters denote that the groups did not differ, while different letters appoint the groups are different. Kruskal-Wallis test followed by Benjamini-Hochberg correction for multiple comparisons. *P*-values of <0.05 were considered statistically significant.

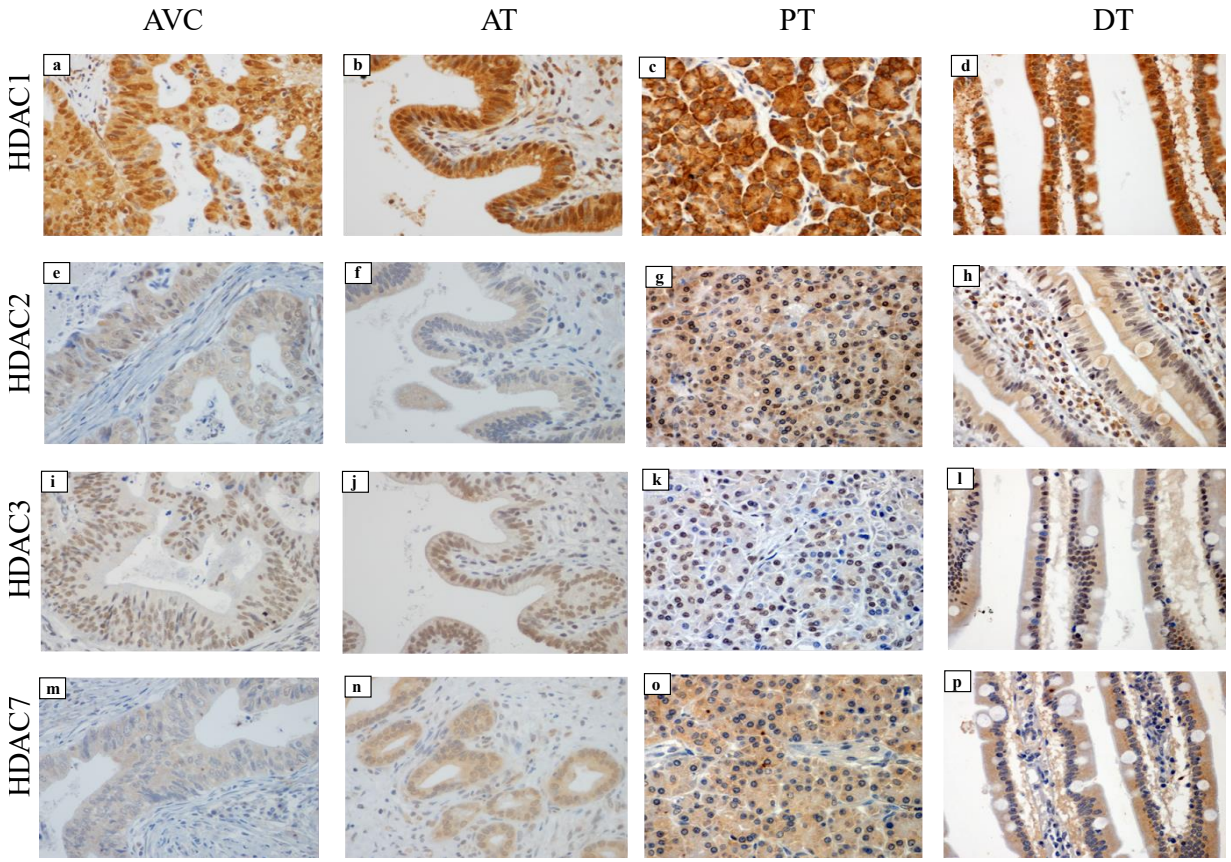

Fig2 HDAC1, HDAC2, HDAC3 and HDAC7 immunostaining in ampulla of Vater carcinoma and non-tumoral tissue samples. (a-d) HDAC1 expression, ×400; (e-h) HDAC2 expression, ×400; (i- l) HDAC3 expression, ×400 and; (m- p) HDAC7 expression, ×400. AVC= Ampulla of Vater Carcinoma, AT= non-tumoral ampullary tissue, PT= Non-tumoral pancreatic tissue, and DT= Non-tumoral duodenum tissue.

Table 1. HDAC1, 2, 3 and 7 immunostaining in carcinomas of the ampulla of Vater and surrounding non-tumoral tissue samples.

		AVC (%)	AT (%)	PT (%)	DT (%)
	All samples	20 (100)	10 (100)	59 (100)	48 (100)
HDAC1	Negative	0 (0)	0 (0)	0 (0)	1 (2)
	Positive	20 (100)	7 (70)	57 (97)	44 (92)
	Nucleous	20	7	57	35
	Citoplasm	19	7	57	44
	NA*	0 (0)	3 (30)	2 (3)	3 (6)
HDAC2	Negative	5 (25)	5 (50)	11 (19)	25 (52)
	Positive	15 (75)	2 (20)	43 (73)	19 (40)
	Nucleous	14	0	43	12
	Citoplasm	15	2	43	19
	NA*	0 (0)	3 (30)	5 (8)	4 (8)
HDAC3	Negative	1 (5)	1 (1)	29 (49)	1 (2)
	Positive	19 (95)	6 (60)	26 (44)	43 (90)
	Nucleous	19	6	26	43
	Citoplasm	19	6	26	43
	NA*	0 (0)	3 (30)	4 (7)	4 (8)
HDAC7	Negative	7 (35)	2 (20)	11 (19)	4 (8)
	Positive	13 (65)	3 (30)	43 (73)	41 (85)
	Nucleous	0	3	27	0
	Citoplasm	13	3	43	41
	NA*	0 (0)	5 (50)	5 (8)	3 (6)

AVC= Ampulla of Vater Carcinoma, AT= surrounding non-tumoral ampullary tissue, PT= surrounding non-tumoral pancreatic tissue and DT= surrounding non-tumoral duodenum tissue. NA* samples not available because of lost tissue cylinders from tissue microarray.

CAPÍTULO VI: Manuscrito 3

Pancreas divisum, multiple gastrointestinal tumors and the co-occurrence of two germline pathogenic variants: unusual presentation in a neurofibromatosis type 1 patient".

Manuscrito submetido à revista *Frontiers in Genetics* (Fator de impacto: 3,5).

Pancreas divisum, multiple gastrointestinal tumors and the co-occurrence of two germline pathogenic variants: unusual presentation in a neurofibromatosis type 1 patient.

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Abstract

In this study, we described for the first time a neurofibromatosis type 1 patient with pancreas divisum, multiple gastrointestinal tumors and the co-occurrence of germline *NF1* and *CFTR* pathogenic variants. **Case report**: A 62-year-old female NF1 patient presented with weakness, choluria, nausea, and diffuse abdominal pain to an emergency room service. Magnetic resonance imaging revealed an abdominal mass involving the periampullary region and pancreas divisum. After surgical resection, three synchronous neoplasms were detected including two ampullary tumors (adenocarcinoma of the major ampulla, and neuroendocrine tumor of the minor ampulla) and a gastrointestinal stromal tumor (GIST). Germline multigene panel testing identified two pathogenic heterozygous germline variants: c.838del in *NF1* and c.1210-34TG[12]T[5] in *CFTR*. **Conclusions**: We report for the first time a phenotype of multiple primary tumors and a developmental anomaly in a patient harboring pathogenic germline variants in *NF1* and *CFTR*, two genes associated with predisposition to pancreatic cancer and pancreatitis, respectively. Identification of two germline variants in this patient may explain the unusual and more severe phenotype and underscores the importance of comprehensive molecular analyses in patients with complex phenotypes.

Keywords: Synchronous neoplasms, GIST, periampullary tumors, neurofibromatosis type 1, pancreas divisum, *CFTR* pathogenic variant, *NF1* pathogenic variant.

1. Introduction

Pancreas divisum is a pancreatic duct developmental anomaly with an incidence of 4.5% (Dimitriou *et al.*, 2018). The anomaly is caused by absent or incomplete fusion of the ventral (main or Wirsung) and dorsal (marginal or Santorini) ducts (Kanth *et al.*, 2014) and results in coexistence of two ampullary systems: the ventral duct drains the pancreatic head through the major ampulla, while the dorsal duct drains the pancreatic body and tail through the minor ampulla (Ferri *et al.*, 2019).

Ampullary neoplasms are rare, comprising 7% of all periampullary malignancies (Adsay *et al.*, 2012), and association of theses tumors with pancreas divisum is considered an episodic event (Singh *et al.*, 2003; Outtas *et al.*, 2004; Kim *et al.*, 2010). Their occurrence has been reported in families with hereditary cancer syndromes, such as Familial Adenomatous Polyposis (Pérez-Cuadrado-Robles *et al.*, 2019) and Neurofibromatosis type 1 (NF1) (Tewari *et al.*, 2014).

NF1 (OMIM 162200) is one of the most common autosomal dominant disorders (incidence estimated at 1 in 2,500–3,000 live births) (Ferner *et al.*, 2007). About one quarter of NF1 patients also have gastrointestinal involvement (Agaimy *et al.*, 2012), with the occurrence of gastrointestinal stromal tumors (GISTs) and an increased incidence of neuroendocrine tumors (NET). The most frequent are somatostatin secreting duodenal NETs usually located in the periampullary region (Klein *et al.*, 1989; Nunobe *et al.*, 2003; Relles *et al.*, 2010), followed by pheochromocytomas (Kalff *et al.*, 1982) and pancreatic endocrine tumors (Thannberger *et al.*, 2001; Fujisawa *et al.*, 2002; Perren *et al.*, 2006). A few NF1 patients with co-occurrence of GIST and NETs have been described in literature (Tewari *et al.*, 2014; Thavaraputta *et al.*, 2019).

In this study, we described for the first time a neurofibromatosis type 1 patient with pancreas divisum, multiple gastrointestinal tumors and the co-occurrence of germline *NF1* and *CFTR* pathogenic variants. Although pancreas divisum is not a feature of NF1, the synchronous occurrence of multiple gastrointestinal tumors in a NF1 background is extremely rare but more frequent than observed in the general population.

Case presentation

A 62-year-old female patient with NF1 presented to the emergency room with symptoms of weakness, nausea, vomiting, choluria, and diffuse abdominal pain. She reported a previous diagnosis of pheochromocytoma and breast cancer. Upon physical examination, the patient was jaundiced and had a palpable and painful gallbladder, and multiple neurofibromas in the abdomen and extremities. Initial laboratory findings

showed unusually high levels of bilirubin (total bilirubin 9.9 mg/dl and direct bilirubin 8.4 mg/dl), elevated serum C-reactive protein levels (29.8 mg/dl) and elevated liver enzymes.

Due to the high levels of bilirubin, the patient was submitted to an endoscopic retrograde cholangiopancreatography for placement of a biliary stent and during the procedure, an ulcerated expansive periampullary lesion was identified and biopsied. Pathology examination of the biopsy specimen revealed a moderately differentiated adenocarcinoma. During further investigation, an abdominal magnetic resonance imaging showed not only a small hypointense nodular mass (T2 sequence) in the periampullary region of the duodenum, but also pancreas divisum **(Figure 1A and 1B).**

The patient underwent a resection of the proximal jejunum, pylorus-preserving pancreaticoduodenectomy (PPPD) with routine reconstruction, and cholecystectomy. Macroscopic examination of the PPPD specimen revealed a 17.0×2.0mm ulcerated and infiltrative tumor located in the major ampulla (**Figure 2A**). In addition, a firm white lesion, 12.0mm in diameter was detected in the duodenum (**Figure 2B**). Microscopic examination of the major ampulla revealed a poorly differentiated adenocarcinoma (pT2 pN1 R0) infiltrating the duodenal wall with focal necrosis (**Figure 3A and B**). Another lesion was identified in the minor ampulla corresponding to a well-differentiated NET (pT1 pN0 R0) 5.0mm in diameter and without lymphovascular invasion (**Figure 3C and D**). The third synchronic tumor identified in the patient, a fusiform low-grade GIST, with a proliferative index of 2% (pT1 pN0 R0) was identified in the duodenal wall (**Figure 3 and F**).

The patient was then referred to the institutional genetic cancer risk assessment program (Hospital de Clínicas de Porto Alegre). She reported clinical diagnosis of NF1 since the age of 14 years. Physical examination identified multiple cutaneous neurofibromas and café-au-lait spots in the gluteal area, lower and upper limbs. Review of the past medical history was significant for diagnosis of viral hepatitis but she denied any previous history of pancreatitis. In addition, review of medical records and pathology reports confirmed previous occurrence of pheochromocytoma (at age 54

years), and breast cancer (first diagnosis at age 58 years and recurrence at age 61 years), all of which are features of the NF1 cancer predisposition syndrome. Review of the patient's family history revealed that her mother also had symptoms consistent with NF1 (café-au-lait spots and neurofibromas) although no formal clinical evaluation or molecular testing had been performed.

1. Materials and methods

Genetic testing

After signing a written informed consent for genetic analysis, a saliva sample was obtained with standard procedures using the Oragene DNA OG-500 self-collection kit (DNA Genotek, Ottawa, CA) and the sample was submitted to germline genetic testing using a multigene panel test (MGPT) in a commercial laboratory (Invitae, San Francisco, CA). The decision to investigate with a multigene panel was prompted by presence of clinical features not consistent with NF1, such as pancreas divisum and by the diagnosis of five distinct primary tumors. Using Illumina technology, the presence of small variants, duplications and deletions in 29 genes (*APC, ATM, BMPR1A, BRCA1, BRCA2, CDKN2A, EPCAM, MEN1, MLH1, MSH2, MSH6, NF1, PALB2, PMS2, SMAD4, STK11, TP53, TSC1, TSC2, VHL, CDK4, FANCC, PALLD, CASR, CFTR, CPA1, CTRC, PRSS1,* and *SPINK1)* associated with hereditary pancreatic cancer and pancreatitis were assessed in the patient. All targeted regions were sequenced at a minimum of 50x depth and reads were aligned to a reference sequence (GRCh37).

2. Results

MGPT revealed a novel heterozygous germline deletion in exon 8 of the *NF1* gene, c.838del, p.(Ile280Ter) (*NF1* chr17:29509630, NM_000267.3) which was reported as pathogenic since it predicts a premature stop codon, resulting in the absence of translation of all NF1 protein functional domains. This variant was not previously reported in the scientific literature, public variant databases (ClinVar and

LOVD) or population databases (ExAC, Abraom, and 1000genomes). Considering the more than 4000 different *NF1* variants reported to date and distributed along its 58 exons described in the ClinVar database, only a small portion are located in exon 8 (77 variants). Of these, only 16 are pathogenic. The significant molecular heterogeneity observed in NF1 renders genotype-phenotype correlations difficult, and no correlation with mutations in *NF1* exon 8 and a specific phenotype have been previously established.

Additionally, one pathogenic germline variant was identified in heterozygosity in intron 9 of the *CFTR* gene, c.1210-34TG[12]T[5], (NM_000492.3). This sequence change, also referred to as TG12-5T or T5TG12 in the literature, consists of 12 TG and 5T sequence repeats on the same chromosome and, although it does not directly change the encoded amino acid sequence of the CFTR protein, it increases alternative splicing of exon 10 (referred to as exon 9 in some publications) from mRNA.

3. Discussion

NF1 is a hereditary cancer predisposition syndrome characterized by increased risk of developing benign and malignant tumors, and a cumulative cancer risk of 20% in affected patients older than 50 years (Friedman and Birch, 1997; Upadhyaya, 2011). The *NF1* gene acts as a tumor suppressor through its Ras-GTPase activity (Cawthon *et al.*, 1990; Boyd *et al.*, 2009) and this activity has a negative regulatory effect on the Ras proto-oncogene signal transduction pathway, which regulates cell proliferation and differentiation (Tewari *et al.*, 2014). Germline heterozygous loss-of-function mutations in the *NF1* gene lead to protein dysfunction and consequently, uncontrolled cell proliferation which has been associated with several of the NF1 clinical features. Loss of heterozygosity has been reported as a necessary step for the development of malignancies in NF1 patients (Ruggieri and Packer, 2001).

It is well known that NF1 patients are predisposed to solid tumors, including those of the gastrointestinal tract (Agaimy *et al.*, 2012), including tumors arising in the ampulla of Vater and the duodenum. GIST, carcinoid tumors and other NETs are frequently reported in NF1 patients, occurring in isolated manifestation or, in rare events, in

synchronism with other tumors (Sørensen *et al.*, 1986; Kim *et al.*, 2014; Park *et al.*, 2019). In this report, the patient was diagnosed with multiple tumors (GIST, NET of the minor ampulla, and adenocarcinoma of the major ampulla) of which GIST and NET could be related to the NF1 , a diagnosis confirmed by germline genetic testing. Among the tumors identified, GIST represents the most common gastrointestinal tumor in NF1 patients (Poredska *et al.*, 2019), accounting for 5 to 25% of all NF1 neoplasms (Zöller *et al.*, 1997; Miettinen *et al.*, 2006). The last update of duodenal and periampullary tumors in NF1 patients reports that most (60%) neoplasms arise in the duodenum, while 31% originate in the ampulla of Vater. Stratification by histology shows that GISTs correspond to 34% of duodenal tumors and the majority of ampullary tumors are neuroendocrine (40%), while only 8% are adenocarcinomas (Relles *et al.*, 2010). In fact, adenocarcinomas associated with NF1 seem to be rare events and have been reported in only a few studies (Deschamps *et al.*, 2010; Tewari *et al.*, 2014). In several documentations, the co-occurrence of periampullary NET tumors and GIST has been proposed as highly suggestive or even pathognomonic of the NF1 diagnosis (Agaimy *et al.*, 2012; Park *et al.*, 2019; Poredska *et al.*, 2019).

Pancreas divisum was reported in some cases of NF1 patients with periampullary tumors and in most of them it is an incidental finding, with no apparent relationship with the development of neoplasia or with the syndrome itself (Waisberg *et al.*, 2006; Bhandari *et al.*, 2015). In general, the majority of patients with pancreas divisum do not develop symptomatic disease and there is no evidence of a direct relationship between this developmental anomaly and cancer (Ferri *et al.*, 2019). Acute and chronic pancreatitis are risk factors for the development of pancreatic cancer (Becker *et al.*, 2014; Kirkegård *et al.*, 2018). Although some studies reported pancreatitis as findings in patients with pancreas divisum, recent works revealed that pancreas divisum is not a cause of pancreatitis, but acts as modulator of risk in carriers of additional genetic variants (Bertin *et al.*, 2012; Gutta *et al.*, 2019), such as the one described here in the *CFTR* gene, suggesting interaction and a cumulative effect of these two risk factors to increase the risk of cancer (as the observed adenocarcinoma).

In our patient, a heterozygous germline pathogenic variant in *CFTR* gene was found. *CFTR* codifies the cystic fibrosis transmembrane conductance regulator, which is a membrane protein and chloride channel. Pathogenic variants in *CFTR* decrease ion channel function and cause extracellular mucus build-up; excessively thick and sticky mucus obstructs airways and pancreatic ducts, resulting in cystic fibrosis (Wang *et al.*, 2014). Although heterozygous carriers of pathogenic *CFTR* variants do not develop cystic fibrosis, they may have an approximately 4-10 fold increased risk for pancreatitis and associated pancreatic injury due to elevated mucus levels, fibrosis, and cyst formation (Noone *et al.*, 2001; Schneider *et al.*, 2011; Steiner *et al.*, 2011; Hegyi *et al.*, 2016). The intronic variant identified in our patient is a reportedly pathogenic variant which ultimately results in a non-functional CFTR protein through abnormal splicing (Delaney *et al.*, 1993; Strong *et al.*, 1993; Niksic *et al.*, 1999; Groman *et al.*, 2004; Bombieri *et al.*, 2011). The specific combination of pancreas divisum and pancreatitis was previously reported in a carrier of the same pathogenic variant (Dray *et al.*, 2007; Montagnani *et al.*, 2013).

4. Conclusions

The current report, to our knowledge, is the first clinical description and documentation of the co-occurrence of germline *NF1* and *CFTR* pathogenic variants in the same patient with pancreas divisum and multiple tumors. The *NF1* variant, predicted to cause severe disruption of NF1 function is likely the cause of the multiple solid tumors (breast cancer, pheochromocytoma, GIST and NET) observed in the patient. Pancreas divisum and the *CFTR* pathogenic variant in heterozygosity, are associated with acute and chronic pancreatitis, which in turn increases the risk for pancreatic cancer. Although the patient did not refer previous pancreatitis, a subclinical event could not be discarded. Finally, whether combination of the two germline variants increases risk for pancreatic cancer in an additive manner remains to be determined. The particular genetic status of this patient will require careful surveillance for lifetime cancer risk as well as appropriate genetic counseling for her relatives and underscores the importance of comprehensive genetic testing in patients with complex phenotypes.

5. Additional Consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal. The study was conducted in accordance with the Declaration of Helsinki and has been approved by the Scientific and Research Committee of Hospital de Clínicas de Porto Alegre (protocol number 13-0260).

6. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

CG conceived the work; conception design of the case report; CG and CR design of the draft the manuscript; GC, CR and CBN were involved in patient recruitment; SM, LA, CN, VB, AO, PAP provided clinical data, were directly involved in the clinical follow-up and helped to draft the manuscript; LA, SS carried out and interpreted the imaging studies; CG, CR, LA, CN, SM, VB, AO and PAP revised the manuscript critically and contributed with interpretation of the findings; PAP supervised the work; All authors gave final approval of the version to be published.

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10. References

Adsay, V., Ohike, N., Tajiri, T., Kim, G. E., Krasinskas, A., Balci, S., et al. (2012). Ampullary region carcinomas: definition and site specific classification with delineation of four clinicopathologically and prognostically distinct subsets in an analysis of 249 cases. Am. J. Surg. Pathol. 36, 1592–1608. doi:10.1097/PAS.0b013e31826399d8

Agaimy, A., Vassos, N., and Croner, R. S. (2012). Gastrointestinal manifestations of neurofibromatosis type 1 (Recklinghausen's disease): clinicopathological spectrum with pathogenetic considerations. Int. J. Clin. Exp. Pathol. 5, 852–862.

Becker, A. E., Hernandez, Y. G., Frucht, H., and Lucas, A. L. (2014). Pancreatic ductal adenocarcinoma: risk factors, screening, and early detection. World J. Gastroenterol. 20, 11182–11198. doi:10.3748/wjg.v20.i32.11182

Bertin, C., Pelletier, A.-L., Vullierme, M. P., Bienvenu, T., Rebours, V., Hentic, O., et al. (2012). Pancreas divisum is not a cause of pancreatitis by itself but acts as a partner of genetic mutations. Am. J. Gastroenterol. 107, 311–317. doi:10.1038/ajg.2011.424

Bhandari, R., Riddiough, G., Lokan, J., Weinberg, L., Efthymiou, M., and Nikfarjam, M. (2015). Somatostatinoma of the minor papilla treated by local excision in a patient with neurofibromatosis type 1. JOP 16, 81–84. doi:10.6092/1590-8577/2906

Bombieri, C., Claustres, M., De Boeck, K., Derichs, N., Dodge, J., Girodon, E., et al. (2011). Recommendations for the classification of diseases as CFTR-related disorders. J. Cyst. Fibros. 10 Suppl 2, S86–102. doi:10.1016/S1569-1993(11)60014-3

Boyd, K. P., Korf, B. R., and Theos, A. (2009). Neurofibromatosis type 1. J. Am. Acad. Dermatol. 61, 1–14; quiz 15–6. doi:10.1016/j.jaad.2008.12.051

Cawthon, R. M., Weiss, R., Xu, G. F., Viskochil, D., Culver, M., Stevens, J., et al. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62, 193–201. doi:10.1016/0092-8674(90)90253-b

Delaney, S. J., Rich, D. P., Thomson, S. A., Hargrave, M. R., Lovelock, P. K., Welsh, M. J., et al. (1993). Cystic fibrosis transmembrane conductance regulator splice variants are not conserved and fail to produce chloride channels. Nat. Genet. 4, 426–431. doi:10.1038/ng0893-426

Deschamps, L., Dokmak, S., Guedj, N., Ruszniewski, P., Sauvanet, A., and Couvelard, A. (2010). Mixed endocrine somatostatinoma of the ampulla of vater associated with a neurofibromatosis type 1: a case report and review of the literature. JOP 11, 64–68.

Dimitriou, I., Katsourakis, A., Nikolaidou, E., and Noussios, G. (2018). The Main Anatomical Variations of the Pancreatic Duct System: Review of the Literature and Its Importance in Surgical Practice. J. Clin. Med. Res. 10, 370–375. doi:10.14740/jocmr3344w

Dray, X., Fajac, I., Bienvenu, T., Chryssostalis, A., Sogni, P., and Hubert, D. (2007). Association of pancreas divisum and recurrent acute pancreatitis with the IVS8-5T-12TG allele of the CFTR gene and CFTR dysfunction. Pancreas 35, 90–93. doi:10.1097/MPA.0b013e318054771f

Ferner, R. E., Huson, S. M., Thomas, N., Moss, C., Willshaw, H., Evans, D. G., et al. (2007). Guidelines for the diagnosis and management of individuals with neurofibromatosis 1. J. Med. Genet. 44, 81–88. doi:10.1136/jmg.2006.045906

Ferri, V., Vicente, E., Quijano, Y., Ielpo, B., Duran, H., Diaz, E., et al. (2019). Diagnosis and treatment of pancreas divisum: A literature review. Hepatobiliary Pancreat. Dis. Int 18, 332–336. doi:10.1016/j.hbpd.2019.05.004

Friedman, J. M., and Birch, P. H. (1997). Type 1 neurofibromatosis: a descriptive analysis of the disorder in 1,728 patients. Am. J. Med. Genet. 70, 138–143. doi:10.1002/(sici)1096-8628(19970516)70:2<138::aid-ajmg7>3.0.co;2-u

Fujisawa, T., Osuga, T., Maeda, M., Sakamoto, N., Maeda, T., Sakaguchi, K., et al. (2002). Malignant endocrine tumor of the pancreas associated with von Recklinghausen's disease. J. Gastroenterol. 37, 59–67. doi:10.1007/s535-002-8135-x

Groman, J. D., Hefferon, T. W., Casals, T., Bassas, L., Estivill, X., Des Georges, M., et al. (2004). Variation in a repeat sequence determines whether a common variant of the cystic fibrosis transmembrane conductance regulator gene is pathogenic or benign. Am. J. Hum. Genet. 74, 176–179. doi:10.1086/381001

Gutta, A., Fogel, E., and Sherman, S. (2019). Identification and management of pancreas divisum. Expert Rev. Gastroenterol. Hepatol. 13, 1089–1105. doi:10.1080/17474124.2019.1685871

Hegyi, P., Wilschanski, M., Muallem, S., Lukacs, G. L., Sahin-Tóth, M., Uc, A., et al. (2016). CFTR: A New Horizon in the Pathomechanism and Treatment of Pancreatitis. Rev. Physiol. Biochem. Pharmacol. 170, 37–66. doi:10.1007/112_2015_5002

Kalff, V., Shapiro, B., Lloyd, R., Sisson, J. C., Holland, K., Nakajo, M., et al. (1982). The spectrum of pheochromocytoma in hypertensive patients with neurofibromatosis. Arch. Intern. Med. 142, 2092–2098.

Kanth, R., Samji, N. S., Inaganti, A., Komanapalli, S. D., Rivera, R., Antillon, M. R., et al. (2014). Endotherapy in symptomatic pancreas divisum: a systematic review. Pancreatology 14, 244–250. doi:10.1016/j.pan.2014.05.796

Kim, I. Y., Cho, M. Y., and Kim, Y. W. (2014). Synchronous multiple colonic adenocarcinomas arising in patient with neurofibromatosis type 1. Ann Surg Treat Res 87, 156–160. doi:10.4174/astr.2014.87.3.156

Kim, Y. G., Kim, T. N., and Kim, K. O. (2010). Carcinoid tumor of the minor papilla in complete pancreas divisum presenting as recurrent abdominal pain. BMC Gastroenterol. 10, 17. doi:10.1186/1471-230X-10-17

Kirkegård, J., Cronin-Fenton, D., Heide-Jørgensen, U., and Mortensen, F. V. (2018). Acute Pancreatitis and Pancreatic Cancer Risk: A Nationwide Matched-Cohort Study in Denmark. Gastroenterology 154, 1729–1736. doi:10.1053/j.gastro.2018.02.011

Klein, A., Clemens, J., and Cameron, J. (1989). Periampullary neoplasms in von Recklinghausen's disease. Surgery 106, 815–819.

Miettinen, M., Fetsch, J. F., Sobin, L. H., and Lasota, J. (2006). Gastrointestinal stromal tumors in patients with neurofibromatosis 1: a clinicopathologic and molecular genetic study of 45 cases. Am. J. Surg. Pathol. 30, 90–96. doi:10.1097/01.pas.0000176433.81079.bd

Montagnani, M., Cazzato, S., Mutignani, M., Cevenini, M., Guidetti, E., Zvi, I. B., et al. (2013). A patient with pancreas divisum, recurrent acute pancreatitis, and homozygosity for the cystic fibrosis transmembrane regulator-associated protein 5T allele. Clin. Gastroenterol. Hepatol. 11, 579–581. doi:10.1016/j.cgh.2013.02.012

Niksic, M., Romano, M., Buratti, E., Pagani, F., and Baralle, F. E. (1999). Functional analysis of cis-acting elements regulating the alternative splicing of human CFTR exon 9. Hum. Mol. Genet. 8, 2339–2349. doi:10.1093/hmg/8.13.2339

Noone, P. G., Zhou, Z., Silverman, L. M., Jowell, P. S., Knowles, M. R., and Cohn, J. A. (2001). Cystic fibrosis gene mutations and pancreatitis risk: relation to epithelial ion transport and trypsin inhibitor gene mutations. Gastroenterology 121, 1310–1319. doi:10.1053/gast.2001.29673

Nunobe, S., Fukushima, N., Yachida, S., Shimada, K., Kosuge, T., and Sakamoto, M. (2003). Clear cell endocrine tumor of the pancreas which is not associated with von Hippel-Lindau disease: report of a case. Surg. Today 33, 470–474. doi:10.1007/s10595-002-2508-x

Outtas, O., Barthet, M., De Troyer, J., Franck, F., and Garcia, S. (2004). [Pancreatic panniculitis with intraductal carcinoid tumor of the pancreas divisum]. Ann. Dermatol. Venereol. 131, 466–469. doi:10.1016/s0151-9638(04)93641-1

Park, E. K., Kim, H. J., Lee, Y. H., Koh, Y. S., Hur, Y. H., and Cho, C. K. (2019). Synchronous Gastrointestinal Stromal Tumor and Ampullary Neuroendocrine Tumor in Association with Neurofibromatosis Type 1: A Report of Three Cases. Korean J. Gastroenterol. 74, 227–231. doi:10.4166/kjg.2019.74.4.227

Pérez-Cuadrado-Robles, E., Piessevaux, H., Moreels, T. G., Yeung, R., Aouattah, T., Komuta, M., et al. (2019). Combined excision and ablation of ampullary tumors with biliary or pancreatic intraductal extension is effective even in malignant neoplasms. United European Gastroenterol J 7, 369–376. doi:10.1177/2050640618817215

Perren, A., Wiesli, P., Schmid, S., Montani, M., Schmitt, A., Schmid, C., et al. (2006). Pancreatic endocrine tumors are a rare manifestation of the neurofibromatosis type 1 phenotype: molecular analysis of a malignant insulinoma in a NF-1 patient. Am. J. Surg. Pathol. 30, 1047–1051. doi:10.1097/00000478-200608000-00018

Poredska, K., Kunovsky, L., Prochazka, V., Dolina, J., Chovancova, M., Vlazny, J., et al. (2019). Triple malignancy (NET, GIST and pheochromocytoma) as a first manifestation of neurofibromatosis type-1 in an adult patient. Diagn. Pathol. 14, 77. doi:10.1186/s13000-019-0848-7

Relles, D., Baek, J., Witkiewicz, A., and Yeo, C. J. (2010). Periampullary and duodenal neoplasms in neurofibromatosis type 1: two cases and an updated 20-year review of the literature yielding 76 cases. J. Gastrointest. Surg. 14, 1052–1061. doi:10.1007/s11605-009-1123-0

Ruggieri, M., and Packer, R. J. (2001). Why do benign astrocytomas become malignant in NF1? Neurology 56, 827–829. doi:10.1212/wnl.56.7.827

Schneider, A., Larusch, J., Sun, X., Aloe, A., Lamb, J., Hawes, R., et al. (2011). Combined bicarbonate conductance-impairing variants in CFTR and SPINK1 variants are associated with chronic pancreatitis in patients without cystic fibrosis. Gastroenterology 140, 162–171. doi:10.1053/j.gastro.2010.10.045

Singh, V. V., Bhutani, M. S., and Draganov, P. (2003). Carcinoid of the minor papilla in incomplete pancreas divisum presenting as acute relapsing pancreatitis. Pancreas 27, 96–97. doi:10.1097/00006676-200307000-00013

Sørensen, S. A., Mulvihill, J. J., and Nielsen, A. (1986). Long-term follow-up of von Recklinghausen neurofibromatosis. Survival and malignant neoplasms. N. Engl. J. Med. 314, 1010–1015. doi:10.1056/NEJM198604173141603

Steiner, B., Rosendahl, J., Witt, H., Teich, N., Keim, V., Schulz, H.-U., et al. (2011). Common CFTR haplotypes and susceptibility to chronic pancreatitis and congenital bilateral absence of the vas deferens. Hum. Mutat. 32, 912–920. doi:10.1002/humu.21511

Strong, T. V., Wilkinson, D. J., Mansoura, M. K., Devor, D. C., Henze, K., Yang, Y., et al. (1993). Expression of an abundant alternatively spliced form of the cystic fibrosis transmembrane conductance regulator (CFTR) gene is not associated with a cAMP-activated chloride conductance. Hum. Mol. Genet. 2, 225–230. doi:10.1093/hmg/2.3.225

Tewari, N., Rollins, K., Gandhi, N., Kaye, P., and Lobo, D. N. (2014). Mixed periampullary adenocarcinoma and somatostatinoma with small bowel gastrointestinal stromal tumour in neurofibromatosis type 1. JOP 15, 600–603. doi:10.6092/1590-8577/2844

Thannberger, P., Wilhelm, J. M., Derragui, A., Saraceni, O., and Kieffer, P. (2001). Von Recklinghausen's disease associated with pancreatic somatostatinoma. Presse Med. 30, 1741–1743.

Thavaraputta, S., Graham, S., Rivas Mejia, A. M., and Lado-Abeal, J. (2019). Duodenal somatostatinoma presenting as obstructive jaundice with the coexistence of a gastrointestinal stromal tumour in neurofibromatosis type 1: a case with review of the literature. BMJ Case Rep. 12. doi:10.1136/bcr-2018-226702

Upadhyaya, M. (2011). Genetic basis of tumorigenesis in NF1 malignant peripheral nerve sheath tumors. Front. Biosci. 16, 937–951. doi:10.2741/3727

Waisberg, J., de Matos, L. L., Waisberg, D. R., dos Santos, H. V. B., Fernezlian, S. M., and Capelozzi, V. L. (2006). Carcinoid of the minor duodenal papilla associated with pancreas divisum: Case report and review of the literature. Clinics 61, 365–368. doi:10.1590/s1807-59322006000400017

Wang, Y., Wrennall, J. A., Cai, Z., Li, H., and Sheppard, D. N. (2014). Understanding how cystic fibrosis mutations disrupt CFTR function: from single molecules to animal models. Int. J. Biochem. Cell Biol. 52, 47–57. doi:10.1016/j.biocel.2014.04.001

Zöller, M. E., Rembeck, B., Odén, A., Samuelsson, M., and Angervall, L. (1997). Malignant and benign tumors in patients with neurofibromatosis type 1 in a defined Swedish population. Cancer 79, 2125–2131.

Figure legends

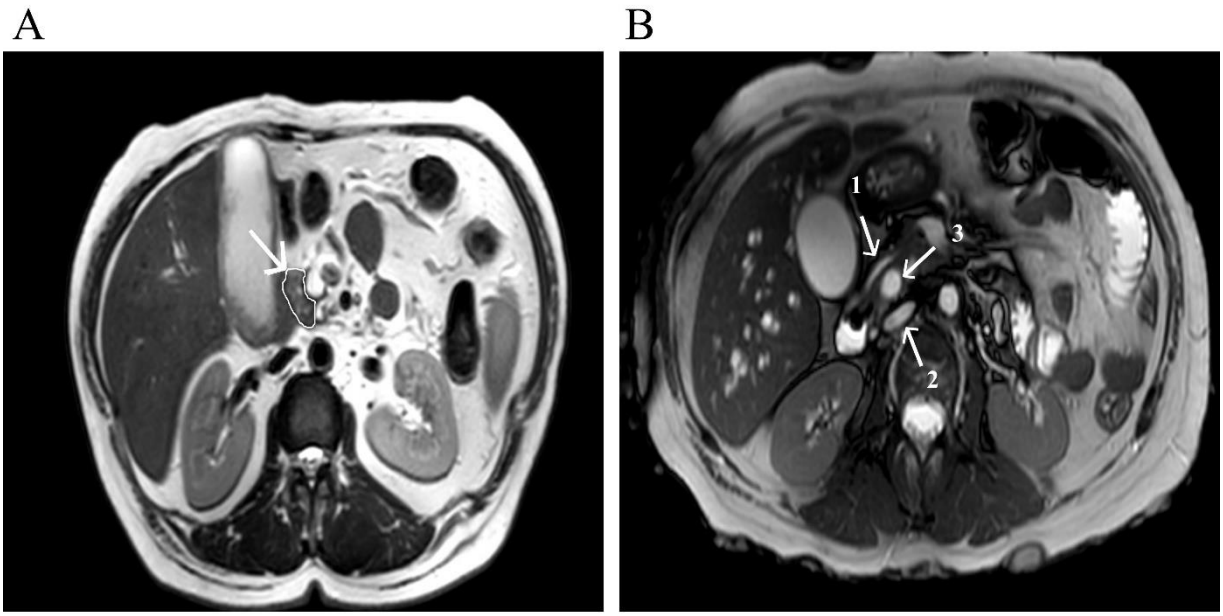

Figure 1. Abdominal magnetic resonance imaging. (A) T2 sequences show a small nodular mass (arrow) with a hypointense signal at the level of the major duodenal ampulla, measuring approximately 10.2 mm in its largest axis; (B) Pancreas divisum diagnosis, arrow 1 shows the ventral pancreatic duct, arrow 2 shows the dorsal pancreatic duct, and between those, the arrow 3 shows the common bile duct.

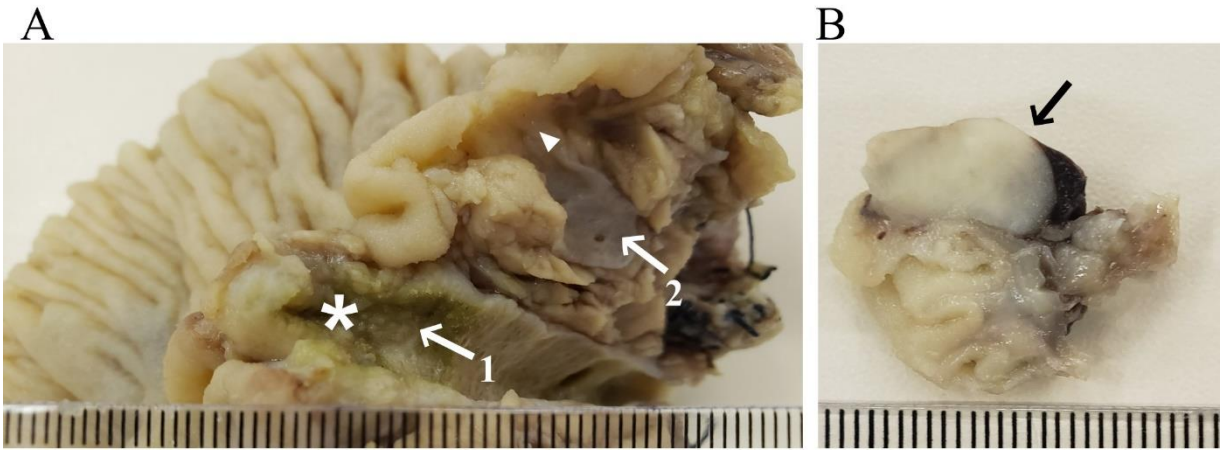

Figure 2. Macroscopy specimens of pylorus-preserving pancreaticoduodenectomy. (A) Pancreas divisum, arrow 1 shows the dorsal duct with a vegetative lesion (17.0×2.0mm) extending to the major ampulla (star); arrow 2 shows the ventral duct and the arrowhead shows a poorly defined densification area near the minor ampulla (5.0mm); (B) Duodenal wall mass (12.0mm).

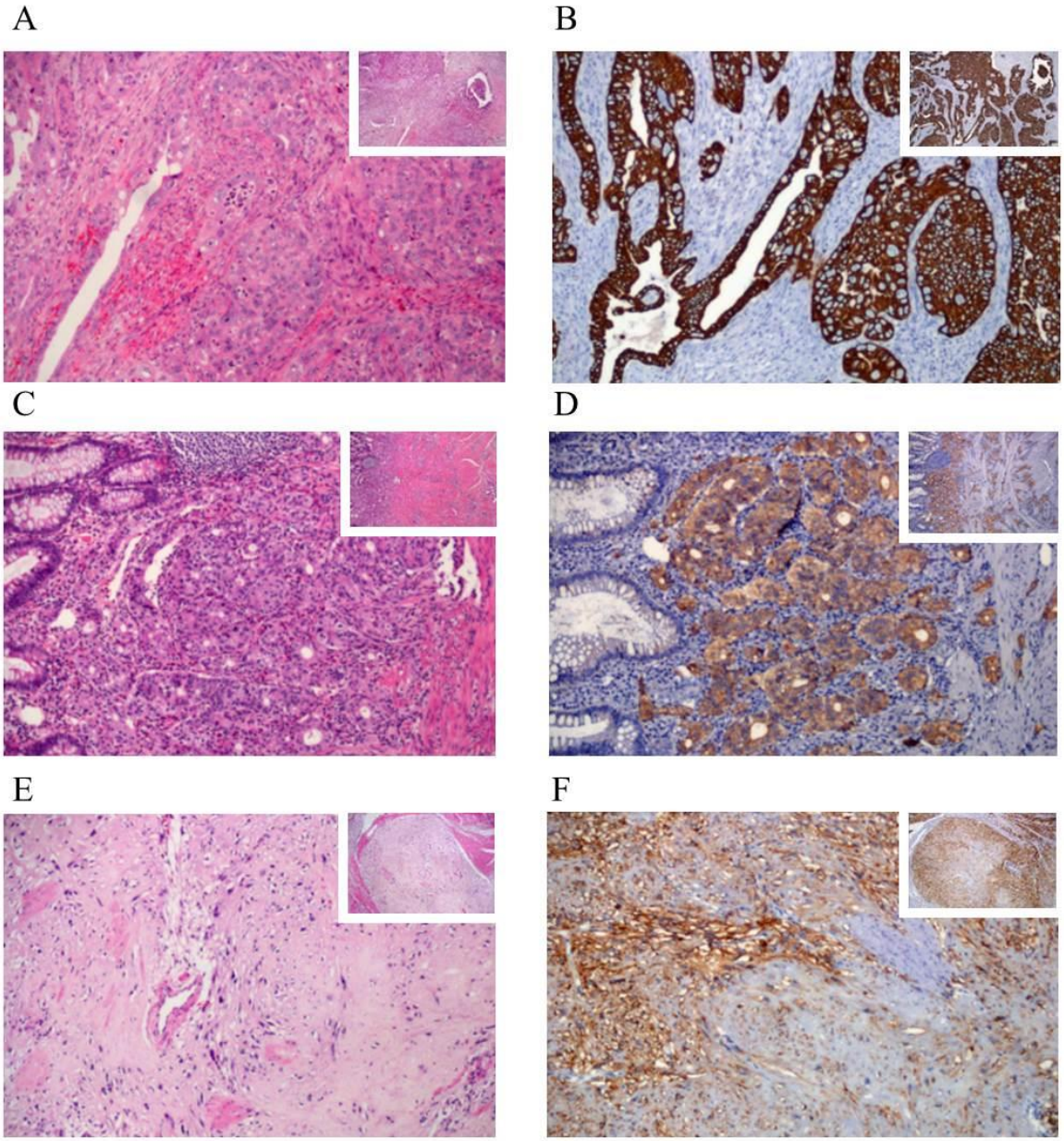

Figure 3. Histological and immunohistochemical features of the synchronic gastrointestinal tumors by hematoxylin-eosin (H&E) and immunohistochemical (IHC) staining. The poorly-differentiated adenocarcinoma of the major ampulla H&E. **A**, and CK7 IHC staining. **B**; The well-differentiated neuroendocrine tumor of the minor ampulla H&E. **C**, and synaptophysin IHC staining. **D**; Spindle cells in the gastrointestinal stromal tumor H&E. **E**, and DOG1 IHC staining. The histological sections stained are presented at x100 magnification and the right small squares represent x40 magnification.

CAPÍTULO VII: DISCUSSÃO

Os tumores pancreáticos são extremamente agressivos e notadamente apresentam mal prognóstico, mesmo o adenocarcinoma ductal pancreático (ADP, tipo tumoral mais estudado) apresenta oportunidades limitadas de tratamento. Tumores mais raros, como os outros carcinomas periampulares, também possuem altas taxas de mortalidade e para estes, há menos estudos que possam embasar terapias inovadoras e modificar significativamente a sobrevida. Desta forma, a busca por novos biomarcadores e alterações moleculares em vias de sinalização pode favorecer o entendimento da biologia tumoral e poderá permitir o desenvolvimento de novas abordagens diagnósticas e terapêuticas.

No manuscrito do capítulo IV, a caracterização do perfil de metilação do DNA em ADP foi realizada em busca de potenciais alvos terapêuticos em vias moleculares associadas à carcinogênese. A plataforma de metilação utilizada neste trabalho (*Infinium HumanMethylation450 BeadChip Kit, Illumina*) é capaz de interrogar 99% dos genes humanos e discriminar padrões de metilação em dinucleotídeos CpG e sua relação com genes e ilhas CpG (Bibikova *et al.*, 2011). Nesta análise, observou-se que os tumores eram predominantemente hipermetilados em comparação às amostras de tecido pancreático normal adjacentes ao tumor (PanN) e que a maioria das sondas hipermetiladas estava localizada nas Ilhas CpG e em promotores gênicos. Esse perfil é comumente observado em várias neoplasias e já foi descrito em trabalhos prévios analisando ADPs com a mesma metodologia (Nones *et al.*, 2014; Mishra & Guda, 2017).

Usando as informações de metilação obtidas na plataforma, investigamos novas vias diferencialmente metiladas e potencialmente associadas à carcinogênese. Observamos que a via de sinalização de Cálcio (Ca^{+2}) apresentava vários genes diferencialmente metilados que tinham interações com vias importantes da carcinogênese pancreática (como por exemplo, as vias de sinalização Ras e Hippo). A via de sinalização de Ca^{+2} atua em diferentes processos biológicos, incluindo regulação do ciclo celular, sobrevivência, apoptose, migração e regulação da expressão gênica (Berridge *et al.*, 2000). Alterações desta via já foram descritas em

outras neoplasias sólidas e regulação positiva ou negativa dos seus genes pode promover a proliferação, migração e metástase (Monteith *et al.*, 2007; Monteith *et al.*, 2012). Um estudo recente e importante foi o de Wang et al., que avaliou o status de metilação do DNA e sua associação com expressão gênica em doze tumores sólidos. A via de sinalização do Ca^{+2} foi descrita como uma das principais vias desreguladas por metilação em nove tumores (mama, bexiga, cólon, cabeça e pescoço, rim, carcinoma hepatocelular, adenocarcinoma de pulmão, carcinoma escamoso de pulmão e útero). Alguns genes silenciados epigeneticamente codificavam proteínas chave da via de sinalização do cálcio como os trocadores Na^{+}/Ca^{+2} e receptores acoplados à proteína G (Wang *et al.*, 2017).

Para aprofundar a análise desta via, a disponibilidade de informações de expressão gênica, metilação e sobrevida do consórcio *The Cancer Genome Atlas* (TCGA) nos permitiu investigar o impacto da metilação sobre a expressão dos genes da via do Ca^{+2}, bem como fazer inferências sobre o seu valor prognóstico. Observamos que 112 sondas apresentavam correlação significativa entre os perfis de metilação e expressão gênica; e entre estas, todas aquelas localizadas nas regiões promotoras apresentavam claramente correlação inversa com a expressão, ao contrário das sondas nos corpos dos genes, em que a correlação entre metilação e expressão gênica foi variável.

Posteriormente, avaliamos o valor prognóstico da expressão de 32 genes (correspondentes às 112 sondas) da via de sinalização do cálcio. Verificamos que os níveis de expressão de dez genes estavam associados com sobrevida dos pacientes com ADP. Pacientes com expressão diminuída dos genes *ADCY8*, *CACNA1A*, *CACNA1B*, *CACNA1D*, *CACNA1H*, *ORAI2*, *PDE1C*, *PLCB1* e *RYR3* apresentavam redução da sobrevida global, enquanto que a expressão diminuída do gene *HRH1* foi associada a maior sobrevida. Dos 10 genes avaliados, apenas os genes *PDE1C*, *PLCB1* e *ADCY8* codificam proteínas citoplasmáticas, os demais genes codificam proteínas que controlam o influxo de Ca^{+2} da membrana plasmática no retículo endoplasmático rugoso. Outros estudos demonstraram que os canais ou bombas de

Ca^{+2} são alvos terapêuticos em potencial em diferentes subtipos de câncer e estão correlacionados com o prognóstico (Monteith *et al.*, 2012; Chen *et al.*, 2013; Raynal *et al.*, 2016; Wang *et al.*, 2017). No ADP, poucos estudos investigaram o papel das proteínas da via de sinalização do Ca^{+2}, principalmente em relação ao controle epigenético mediado pela metilação do DNA de seus genes. O perfil de metilação do gene *S100A4*, que codifica uma proteína de ligação ao Ca^{+2} relacionada ao comportamento metastático (Boye and Maelandsmo, 2010), foi avaliado em amostras de ADP e linhagens celulares (Rosty *et al.*, 2002). Os autores detectaram hipometilação do gene nos tumores, e também associaram os níveis de metilação a um pior grau de diferenciação tumoral. Usando dados do TCGA, Mishra et. al. analisaram o perfil de metilação do DNA em amostras de ADP, embora os genes da via de sinalização de Ca^{+2} ocupassem a quarta posição no enriquecimento funcional das sondas diferencialmente metiladas, esse achado não foi explorado (Mishra & Guda, 2017).

Embora o estudo não tenha sido elaborado com essa finalidade, identificamos padrões desregulados de metilação do DNA em PanN. Ao realizar a clusterização não supervisionada das amostras, verificamos que três PanN apresentavam um perfil de metilação muito semelhante ao ADP em uma pequena parcela das sondas. As amostras foram verificadas em relação ao seu percentual de células normais (> 80%) e apresentaram apenas pequenas regiões de fibrose, sem nenhuma evidência de contaminação celular neoplásica, sugerindo que essas alterações podem ocorrer precocemente na transformação das células pancreáticas, antes mesmo de se observar alteração morfológica identificável por análise histopatológica. Tais alterações podem ser atribuídas ao fenômeno do campo de cancerização, conjunto de alterações genéticas e epigenéticas que indicam que uma área específica do tecido normal está passando por um processo de transformação neoplásica ou tem predisposição para iniciar esse processo, o que pode ocorrer sem alterações morfológicas evidentes (Curtius *et al.*, 2018). A análise específica dessas sondas revelou 23 genes diferencialmente metilados envolvidos na via de sinalização do cálcio

sugerindo que esta via de sinalização pode estar alterada desde etapas muito iniciais da carcinogênese do ADP.

Sendo assim, os resultados deste estudo indicam um papel importante da via de sinalização do cálcio desde cedo no processo de carcinogênese do ADP e abrem uma oportunidade promissora para estudos de pesquisa adicionais. Será importante ampliar e replicar o estudo atual, preferencialmente com um maior número de amostras para confirmar os achados em diferentes estágios do desenvolvimento do ADP.

Quanto aos carcinomas periampulares, as informações genéticas e epigenéticas ainda são limitadas, tanto em relação da sua biologia tumoral como história natural da doença considerando as diferentes origens teciduais. As análises realizadas nessa tese tiveram como objetivo inicial contribuir para o melhor entendimento dos mecanismos de carcinogênese implicados nestes tumores. Neste contexto, a acessibilidade ao DNA mediada pelas desacetilases de histonas poderia ter um papel importante na regulação epigenética dos mesmos. As HDACs atuam modulando a expressão dos genes e removendo grupos acetil de proteínas não-histonas como por exemplo fatores de transcrição, exercendo efeitos diretos em vários processos biológicos (West & Johnstone, 2014). Em neoplasias, as HDACs atuam na indução da transcrição de genes-chave que regulam funções celulares importantes, como proliferação celular, regulação do ciclo celular e apoptose (Ropero & Esteller, 2007). Adicionalmente, inibidores de desacetilases de histonas (HDACi) são uma nova classe de fármacos anti-câncer que tem sido utilizada como terapia complementar à convencional para melhorar o prognóstico de doenças neoplásicas como o ADP (Lakshmaiah *et al.*, 2014). Entre as avaliações iniciais para investigar o potencial terapêutico de um novo agente quimioterápico está a verificação se o seu alvo molecular é expresso. Assim, obter mais informações sobre a expressão das HDACs em CP é um primeiro passo para avaliar se estas proteínas são relevantes no processo de carcinogênese. No manuscrito do capítulo V, caracterizamos o perfil de expressão das desacetilases de histonas HDAC1, HDAC2, HDAC3 e HDAC7 em carcinomas

periampulares, bem como investigamos o possível papel das mesmas no desenvolvimento dos carcinomas de ampola de Vater. Utilizamos bancos de dados de expressão para avaliar o perfil das HDACs e segundo nosso conhecimento, este foi o primeiro estudo a avaliar a sua expressão no adenocarcinoma duodenal. Os carcinomas de ampola de Vater e os adenocarcinomas duodenais apresentaram um perfil de expressão semelhante paras as *HDAC1* e *HDAC2*. Provavelmente porque ambos os tecidos apresentam a mesma origem embrionária e o epitélio da ampola de Vater é formado por células com o perfil intestinal (Kimura *et al.*, 1994; Chang *et al.*, 2013; Yachida *et al.*, 2016). Esse trabalho ainda avaliou, pela primeira vez, a caracterização proteica das HDACs em amostras de adenocarcinoma de ampola de Vater (o subtipo histológico mais frequente), e tecidos normais adjacentes (pancreático, ampular e duodenal). O único relato prévio de expressão de desacetilases de histonas no carcinoma de ampola de Vater era de um subtipo tumoral raro (carcinoma neuroendócrino de alto grau) (Stojsic *et al.*, 2010). Encontramos um perfil de expressão muito semelhante para todos os tecidos analisados, sugerindo que HDAC1, HDAC2, HDAC3 e HDAC7 não estão diretamente envolvidas no desenvolvimento dos carcinomas de ampola de Vater.

Ainda em relação aos carcinomas periampulares, estes tumores não são incomuns em pacientes com neurofibromatose tipo 1 (NF1), um dos distúrbios autossômicos dominantes mais prevalentes (Cimino & Gutmann, 2018). Anomalias do desenvolvimento, como pâncreas *divisum*, foram descritas em pacientes com NF1 e com tumores periampulares e, na maioria deles o diagnóstico foi incidental, sem relação causal aparente da alteração molecular observada na doença (perda de função do gene *NF1*) com o desenvolvimento de neoplasias pancreáticas (Waisberg *et al.*, 2006; Bhandari *et al.*, 2015). No manuscrito do capítulo VI, descrevemos uma apresentação incomum de um paciente com NF1, pâncreas *divisum*, múltiplos tumores gastrointestinais e a co-ocorrência de duas variantes patogênicas na linhagem germinativa (*NF1* e *CFTR*). Este caso é particularmente interessante porque a paciente apresentava dois sistemas ampulares devido ao pâncreas *divisum* e três neoplasias primárias, duas delas tumores ampulares (adenocarcinoma e tumor

neuroendócrino) e um tumor estromal gastrointestinal (GIST). Por conta desta apresentação, foi realizada uma investigação com painel de múltiplos genes relacionados a neoplasias endócrinas e exócrinas do pâncreas. Pacientes com NF1 tem uma predisposição para neoplasias neuroendócrinas pancreáticas e ampulares em especial GIST, entretanto a ocorrência de adenocarcinomas não é comum (Gutmann *et al.*, 1997; Relles *et al.*, 2010; Guilmette & Nosé, 2019). O outro fator de risco para câncer de pâncreas identificado nesta paciente é a presença de uma variante patogênica em heterozigose no gene *CFTR*. O mecanismo de associação de risco de *CFTR* com câncer de pâncreas não é direto, mas sim, indireto, pois em pacientes com variantes patogênicas, pancreatites recorrentes são comuns. A pancreatite, por sua vez, é um dos fatores de risco para neoplasias pancreáticas mais estabelecidos (Raimondi *et al.*, 2010; Kirkegård *et al.*, 2017) e nos casos com etiologia hereditária geralmente cursa com a presença de variantes patogênicas nos genes *PRSS1*, *SPINK1* ou *CFTR* (Zhan *et al.*, 2018; Lee & Papachristou, 2019). Surpreendentemente, a paciente não apresentava história prévia de pancreatite, o que é curioso, pois além de apresentar uma variante patogênica em *CFTR, a* paciente possuía outra característica que favorece o desenvolvimento de pancreatite, o pâncreas *divisum*. Essa malformação pancreática atua como um modulador de risco em portadores das variantes patogênicas (Bertin *et al.*, 2012; Hegyi *et al.*, 2016). Embora não exista relato de sintomas ou claro diagnóstico de pancreatite prévia, a ocorrência de pancreatite subclínica não pode se descartada. Fica também a dúvida em relação a um efeito combinado das duas variantes patogênicas sobre a predisposição aumentada para desenvolvimento de carcinomas periampulares, que poderia ser melhor estudada em modelos experimentais ou nos próprios tecidos tumorais da paciente. Sendo assim, é possível que a identificação de duas variantes germinativas patogênicas possa explicar o fenótipo incomum e mais grave observado na paciente. Este caso ressalta a importância de análises moleculares abrangentes em pacientes com fenótipos complexos, como a presença de múltiplos tumores primários em combinação com anomalias do desenvolvimento.

CAPÍTULO VIII: CONCLUSÕES

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

a. O adenocarcinoma ductal pancreático apresenta genes diferencialmente metilados em relação ao tecido normal adjacente. Entre outros achados, os dados obtidos no presente estudo indicaram que a via de sinalização do cálcio está alterada por esse processo epigenético, e muitos de seus genes são compartilhados com vias chave da carcinogênese pancreática, como as vias Ras e Hippo. Os genes *CACNA1A*, *CACNA1B*, *CACNA1H*, e *RYR3;* que controlam o influxo de Ca^{+2} da membrana plasmática no retículo endoplasmático rugoso estavam mais frequentemente hipermetilados, a redução da expressão desses genes se mostrou associada à redução da sobrevida global nos pacientes. Este achado é relevante, pois pode indicar potenciais alvos terapêuticos para casos selecionados de ADP. Adicionalmente, os dados indicam que a via de sinalização do cálcio parece ter um papel importante desde as etapas muito iniciais da carcinogênese pancreática.

b. A análise de expressão de desacetilases de histonas, embora deva ser considerada uma análise preliminar, indica que que os carcinomas de ampola de Vater e os adenocarcinomas duodenais apresentam perfil de expressão semelhante para *HDAC1* e *HDAC2.* Ainda, os adenocarcinomas de ampola de Vater e tecidos não tumorais adjacentes apresentam também um perfil de expressão semelhante para as HDAC1, HDAC2, HDAC3 e HDAC7. Estes resultados sugerem que as desacetilases de histonas não estão diretamente envolvidas na carcinogênese de tumores da ampola de Vater, embora possam ter um papel auxíliando o fenótipo tumoral, modulando genes envolvidos proliferação celular, regulação do ciclo celular e apoptose.

c. Mediante descrição de um caso clínico apresentamos um exemplo de um caso complexo, com fenótipo atípico e mais grave de neurofibromatose 1 onde a presença de duas variantes germinativas patogênicas

em genes distintos (*NF1* e *CFTR*) relacionados a doenças envolvendo o pâncreas podem ter agido sinergicamente. Em especial, a variante germinativa patogênica do gene *CFTR* associada ao pâncreas *divisum* podem ter contribuído para a ocorrência de múltiplos tumores na paciente portadora de Neurofibromatose tipo 1. Este relato de caso reforça a importância de uma análise molecular mais abrangente, incluindo avaliação de múltiplos genes relacionados ao fenótipo em casos atípicos ou com fenótipo mais grave que o habitual. A informação obtida no estudo de caso será também muito relevante para os familiares do caso índice.

Em suma, os resultados desta tese contribuem para o melhor entendimento do perfil genético e epigenético dos adenocarcinomas ductais pancreáticos e de carcinomas periampulares. Os resultados também indicam importantes linhas de investigação para explorar melhor novas vias de sinalização que possam trazer informações relevantes para o desenvolvimento de biomarcadores prognósticos e novas estratégias terapêuticas.

CAPÍTULO IX: PERSPECTIVAS

Como perspectivas à continuidade desse trabalho, estão as seguintes ações:

1) Investigar o perfil de metilação de genes da via de sinalização do cálcio em um maior número de amostras de adenocarcinoma ductal pancreático para confirmar os achados em diferentes estágios do desenvolvimento do tumor;
2) Analisar o perfil global de metilação do carcinoma de ampola de Vater e do adenocarcinoma duodenal;
3) Analisar a expressão proteica das HDACs em amostras teciduais de adenocarcinoma duodenal e colangiocarcinoma distal por imunohistoquímica;
4) Ampliar a investigação do impacto da combinação das variantes patogênicas de *NF1* e *CFTR* sobre a predisposição aumentada para desenvolvimento de carcinomas periampulares em modelos experimentais e a partir da análise dos tumores identificados em um caso clínico.

CAPÍTULO X: REFERÊNCIAS

ADSAY, V. et al. Ampullary region carcinomas: definition and site specific classification with delineation of four clinicopathologically and prognostically distinct subsets in an analysis of 249 cases. **Am J Surg Pathol,** v. 36, n. 11, p. 1592-608, Nov 2012. ISSN 1532-0979.

ALBORES-SAAVEDRA, J. et al. Cancers of the ampulla of vater: demographics, morphology, and survival based on 5,625 cases from the SEER program. **J Surg Oncol,** v. 100, n. 7, p. 598-605, Dec 1 2009. ISSN 1096-9098

ALEMAR, B.; GREGÓRIO, C.; ASHTON-PROLLA, P. miRNAs As Diagnostic and Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: A Review. **Biomark Insights,** v. 10, p. 113-24, 2015. ISSN 1177-2719.

AMRUTKAR, M.; GLADHAUG, I. P. Pancreatic Cancer Chemoresistance to Gemcitabine. **Cancers (Basel),** v. 9, n. 11, Nov 2017. ISSN 2072-6694.

ASMAR, F.; SØGAARD, A.; GRØNBÆK, K. DNA Methylation and Hydroxymethylation in Cancer. In: (Ed.). **Epigenteic and Cancer Therapy**, 2015.

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.

BATABYAL, P. et al. Association of diabetes mellitus and pancreatic adenocarcinoma: a meta-analysis of 88 studies. **Ann Surg Oncol,** v. 21, n. 7, p. 2453-62, Jul 2014. ISSN 1534-4681.

BAYLIN, S. B.; HERMAN, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. **Trends Genet,** v. 16, n. 4, p. 168-74, Apr 2000. ISSN 0168-9525.

BELTRAN, M. A.; CRUCES, K. S. Primary tumors of jejunum and ileum as a cause of intestinal obstruction: a case control study. **Int J Surg,** v. 5, n. 3, p. 183-91, Jun 2007. ISSN 1743-9159.

BERRIDGE, M. J.; LIPP, P.; BOOTMAN, M. D. The versatility and universality of calcium signalling. **Nat Rev Mol Cell Biol,** v. 1, n. 1, p. 11-21, Oct 2000. ISSN 1471-0072.

BERTIN, C. et al. Pancreas divisum is not a cause of pancreatitis by itself but acts as a partner of genetic mutations. **Am J Gastroenterol,** v. 107, n. 2, p. 311-7, Feb 2012. ISSN 1572-0241.

BHANDARI, R. et al. Somatostatinoma of the minor papilla treated by local excision in a patient with neurofibromatosis type 1. **JOP,** v. 16, n. 1, p. 81-4, Jan 2015. ISSN 1590-8577.

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.

BIBIKOVA, M. et al. High density DNA methylation array with single CpG site resolution. **Genomics,** v. 98, n. 4, p. 288-95, Oct 2011. ISSN 1089-8646.

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

BOYE, K.; MAELANDSMO, G. M. S100A4 and metastasis: a small actor playing many roles. **Am J Pathol,** v. 176, n. 2, p. 528-35, Feb 2010. ISSN 1525-2191.

BRAY, F. et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. **CA Cancer J Clin,** v. 68, n. 6, p. 394-424, 11 2018. ISSN 1542-4863.

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.

BRUNE, K. et al. Genetic and epigenetic alterations of familial pancreatic cancers. **Cancer Epidemiol Biomarkers Prev,** v. 17, n. 12, p. 3536-42, Dec 2008. ISSN 1055-9965.

BRÜCHER, B. L. et al. Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. **Int J Cancer,** v. 119, n. 6, p. 1298-302, Sep 2006. ISSN 0020-7136.

CAI, M. H. et al. Depletion of HDAC1, 7 and 8 by Histone Deacetylase Inhibition Confers Elimination of Pancreatic Cancer Stem Cells in Combination with Gemcitabine. **Sci Rep,** v. 8, n. 1, p. 1621, Jan 2018. ISSN 2045-2322.

CARTER, J. T. et al. Tumors of the ampulla of vater: histopathologic classification and predictors of survival. **J Am Coll Surg,** v. 207, n. 2, p. 210-8, Aug 2008. ISSN 1879-1190.

CHANG, D. K. et al. Histomolecular phenotypes and outcome in adenocarcinoma of the ampulla of vater. **J Clin Oncol,** v. 31, n. 10, p. 1348-56, Apr 2013. ISSN 1527-7755.

CHEN, Y. F. et al. Remodeling of calcium signaling in tumor progression. **J Biomed Sci,** v. 20, p. 23, Apr 2013. ISSN 1423-0127.

CHUNG, S. D. et al. More than 9-times increased risk for pancreatic cancer among patients with acute pancreatitis in Chinese population. **Pancreas,** v. 41, n. 1, p. 142-6, Jan 2012. ISSN 1536-4828.

CICENAS, J. et al. KRAS, TP53, CDKN2A, SMAD4, BRCA1, and BRCA2 Mutations in Pancreatic Cancer. **Cancers (Basel),** v. 9, n. 5, Apr 2017. ISSN 2072-6694.

CIMINO, P. J.; GUTMANN, D. H. Neurofibromatosis type 1. **Handb Clin Neurol,** v. 148, p. 799-811, 2018. ISSN 0072-9752.

COHEN, N. M.; KENIGSBERG E FAU - TANAY, A.; TANAY, A. Primate CpG islands are maintained by heterogeneous evolutionary regimes involving minimal selection. n. 1097-4172 (Electronic)

COLLISSON, E. A.; MAITRA, A. Pancreatic Cancer Genomics 2.0: Profiling Metastases. **Cancer Cell,** v. 31, n. 3, p. 309-310, 03 2017. ISSN 1878-3686.

CURTIUS, K.; WRIGHT, N. A.; GRAHAM, T. A. An evolutionary perspective on field cancerization. **Nat Rev Cancer,** v. 18, n. 1, p. 19-32, Jan 2018. ISSN 1474-1768.

DANIEL, S. K. et al. Hypoxia as a barrier to immunotherapy in pancreatic adenocarcinoma. **Clin Transl Med,** v. 8, n. 1, p. 10, Apr 2019. ISSN 2001-1326.

DELPU, Y. et al. Genetic and epigenetic alterations in pancreatic carcinogenesis. **Curr Genomics,** v. 12, n. 1, p. 15-24, Mar 2011. ISSN 1875-5488.

DEOLIVEIRA, M. L. et al. Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. **Ann Surg,** v. 245, n. 5, p. 755-62, May 2007. ISSN 0003-4932.

DOUGAN, S. K. The Pancreatic Cancer Microenvironment. **Cancer J,** v. 23, n. 6, p. 321-325, 2017 Nov/Dec 2017. ISSN 1540-336X.

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.

EHRLICH, M. DNA hypomethylation in cancer cells. **Epigenomics,** v. 1, n. 2, p. 239-59, Dec 2009. ISSN 1750-192X.

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

ELSHERIF, S. B. et al. Pancreatitis and PDAC: association and differentiation. **Abdom Radiol (NY)**, Nov 2019. ISSN 2366-0058.

ENE-OBONG, A. et al. Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma. **Gastroenterology,** v. 145, n. 5, p. 1121-32, Nov 2013. ISSN 1528-0012.

FANCIULLI, M.; PETRETTO, E.; AITMAN, T. J. Gene copy number variation and common human disease. **Clin Genet,** v. 77, n. 3, p. 201-13, Mar 2010. ISSN 1399-0004.

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.

FERNÁNDEZ PÉREZ, E. R. et al. Epidemiology of Hypersensitivity Pneumonitis among an Insured Population in the United States: A Claims-based Cohort Analysis. **Ann Am Thorac Soc,** v. 15, n. 4, p. 460-469, 04 2018. ISSN 2325-6621.

FISHER, W.; BAKEY, M. Differences between Ampullary, Periampullary and Pancreatic Cancer. **World Journal of Surgery**. 31: 144-146 p. 2007.

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.

FU, T. et al. Methylation of MGMT Is Associated with Poor Prognosis in Patients with Stage III Duodenal Adenocarcinoma. **PLoS One,** v. 11, n. 9, p. e0162929, 2016. ISSN 1932-6203.

FUKUSHIMA, N. et al. Aberrant methylation of preproenkephalin and p16 genes in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma. **Am J Pathol,** v. 160, n. 5, p. 1573-81, May 2002. ISSN 0002-9440.

FUNDATION, C. What is cholangiocarcinoma (bile duct cancer)? , 2020. Accessed on: 02 jan.

GASPAR, B. et al. Current strategies in the therapeutic approach for adenocarcinoma of the ampulla of Vater. **J Med Life,** v. 6, n. 3, p. 260-5, Sep 2013. ISSN 1844-3117.

GENKINGER, J. M. et al. A pooled analysis of 14 cohort studies of anthropometric factors and pancreatic cancer risk. **Int J Cancer,** v. 129, n. 7, p. 1708-17, Oct 2011. ISSN 1097-0215.
GINGRAS, M. C. et al. Ampullary Cancers Harbor ELF3 Tumor Suppressor Gene Mutations and Exhibit Frequent WNT Dysregulation. **Cell Rep,** v. 14, n. 4, p. 907-919, Feb 2016. ISSN 2211-1247.

GRASSO, C.; JANSEN, G.; GIOVANNETTI, E. Drug resistance in pancreatic cancer: Impact of altered energy metabolism. **Crit Rev Oncol Hematol,** v. 114, p. 139-152, Jun 2017. ISSN 1879-0461.

GREER, J. B.; WHITCOMB, D. C.; BRAND, R. E. Genetic predisposition to pancreatic cancer: a brief review. **Am J Gastroenterol,** v. 102, n. 11, p. 2564-9, Nov 2007. ISSN 0002-9270.

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.

GUARNERI, G. et al. Diagnostic strategy with a solid pancreatic mass. **Presse Med,** v. 48, n. 3 Pt 2, p. e125-e145, Mar 2019. ISSN 2213-0276.

GUILMETTE, J. M.; NOSÉ, V. Neoplasms of the Neuroendocrine Pancreas: An Update in the Classification, Definition, and Molecular Genetic Advances. **Adv Anat Pathol,** v. 26, n. 1, p. 13-30, Jan 2019. ISSN 1533-4031.

GUO, R. et al. Aberrant expression of p53, p21, cyclin D1, and Bcl2 and their clinicopathological correlation in ampullary adenocarcinoma. **Hum Pathol,** v. 45, n. 5, p. 1015-23, May 2014. ISSN 1532-8392.

GUTMANN, D. H. et al. The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. **JAMA,** v. 278, n. 1, p. 51-7, Jul 1997. ISSN 0098-7484.

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.

HADDAD, L. B. D. P. Expressão de marcadores imunoistoquímicos de origem tecidual e de carcinogênese nos adenocarcinomas tipo intestinal e pancreatobiliar da ampola de Vate. 2009. 150 (Doutorado). Departamento de Gastroenterologia, Universidade de São Paulo, São Paulo.

HAEBERLE, L.; ESPOSITO, I. Pathology of pancreatic cancer. **Transl Gastroenterol Hepatol,** v. 4, p. 50, 2019. ISSN 2415-1289.

HAREWOOD, G. C.; WIERSEMA, M. J. Endosonography-guided fine needle aspiration biopsy in the evaluation of pancreatic masses. **Am J Gastroenterol,** v. 97, n. 6, p. 1386-91, Jun 2002. ISSN 0002-9270.

HE, J. et al. 2564 resected periampullary adenocarcinomas at a single institution: trends over three decades. **HPB (Oxford),** v. 16, n. 1, p. 83-90, Jan 2014. ISSN 1477-2574.

HE, J. C. et al. TACC3 overexpression in cholangiocarcinoma correlates with poor prognosis and is a potential anti-cancer molecular drug target for HDAC inhibitors. **Oncotarget,** v. 7, n. 46, p. 75441-75456, Nov 2016. ISSN 1949-2553.

HEGYI, P. et al. CFTR: A New Horizon in the Pathomechanism and Treatment of Pancreatitis. **Rev Physiol Biochem Pharmacol,** v. 170, p. 37-66, 2016. ISSN 0303-4240 (Print) 0303-4240.

HERMAN, J. G.; BAYLIN, S. B. Gene silencing in cancer in association with promoter hypermethylation. **N Engl J Med,** v. 349, n. 21, p. 2042-54, Nov 2003. ISSN 1533-4406. HIDALGO, M. Pancreatic cancer. **N Engl J Med,** v. 362, n. 17, p. 1605-17, Apr 2010. ISSN 1533-4406.

HONG, S. M. et al. Genome-wide somatic copy number alterations in low-grade PanINs and IPMNs from individuals with a family history of pancreatic cancer. **Clin Cancer Res,** v. 18, n. 16, p. 4303-12, Aug 2012. ISSN 1078-0432.

HOWE, J. R. et al. Factors predictive of survival in ampullary carcinoma. **Ann Surg,** v. 228, n. 1, p. 87-94, Jul 1998. ISSN 0003-4932.

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.

IAFRATE, A. J. et al. Detection of large-scale variation in the human genome. **Nat Genet,** v. 36, n. 9, p. 949-51, Sep 2004. ISSN 1061-4036.

IARC. (International Agency for Research on Cancer) PRESS RELEASE N° 263: "Latest global cancer data: Cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018". Geneva, Switzerland, 2018. Avaialble at: < https://www.iarc.fr/wp-content/uploads/2018/09/pr263_E.pdf >. Accessed on: 04/jan/2020.

INCA. (Instituto Nacional do Câncer) Atlas On-line de Mortalidade. p. Taxas de mortalidade por câncer, brutas e ajustadas por idade pelas populações mundial e brasileira, por 100.000, segundo sexo, faixa etaria, localidade e por período selecionado., 2014. Avaialble at: < https://www.inca.gov.br/MortalidadeWeb/pages/Modelo03/consultar.xhtml#panelResultado >. Accessed on: 04/01/2020.

INCA. (Instituto Nacional do Câncer) Pâncreas. 2018. Avaialble at: < https://www.inca.gov.br/tipos-de-cancer/cancer-de-pancreas >. Accessed on: 20 dez 2019.

IODICE, S. et al. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. **Langenbecks Arch Surg,** v. 393, n. 4, p. 535-45, Jul 2008. ISSN 1435-2451.

ISHIBASHI, Y. et al. Morphometric study of the sphincter of oddi (hepatopancreatic) and configuration of the submucosal portion of the sphincteric muscle mass. **Clin Anat,** v. 13, n. 3, p. 159-67, 2000. ISSN 0897-3806.

JIANG, Y. et al. Genome-wide distribution of DNA methylation and DNA demethylation and related chromatin regulators in cancer. **Biochim Biophys Acta,** v. 1835, n. 2, p. 155-63, Apr 2013. ISSN 0006-3002.

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.

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.

JUNG, D. E. et al. CG200745, an HDAC inhibitor, induces anti-tumour effects in cholangiocarcinoma cell lines via miRNAs targeting the Hippo pathway. **Sci Rep,** v. 7, n. 1, p. 10921, 09 2017. ISSN 2045-2322.

KAMARAJAH, S. K. Pancreaticoduodenectomy for periampullary tumours: a review article based on Surveillance, End Results and Epidemiology (SEER) database. **Clin Transl Oncol,** v. 20, n. 9, p. 1153-1160, Sep 2018. ISSN 1699-3055.

KIM, J. H. et al. Differential diagnosis of periampullary carcinomas at MR imaging. **Radiographics,** v. 22, n. 6, p. 1335-52, 2002 Nov-Dec 2002. ISSN 0271-5333.

KIM, S. G. et al. Epigenetic and genetic alterations in duodenal carcinomas are distinct from biliary and ampullary carcinomas. **Gastroenterology,** v. 124, n. 5, p. 1300-10, May 2003. ISSN 0016-5085.

KIMURA, W. et al. Different clinicopathologic findings in two histologic types of carcinoma of papilla of Vater. **Jpn J Cancer Res,** v. 85, n. 2, p. 161-6, Feb 1994. ISSN 0910-5050.

KIMURA, W.; FUTAKAWA, N.; ZHAO, B. Neoplastic diseases of the papilla of Vater. **J Hepatobiliary Pancreat Surg,** v. 11, n. 4, p. 223-31, 2004. ISSN 0944-1166.

KIRKEGARD, J.; MORTENSEN, F. V.; CRONIN-FENTON, D. Chronic Pancreatitis and Pancreatic Cancer Risk: A Systematic Review and Meta-analysis. **Am J Gastroenterol,** v. 112, n. 9, p. 1366-1372, Sep 2017. ISSN 0002-9270.

KIRKEGÅRD, J. et al. Acute Pancreatitis and Pancreatic Cancer Risk: A Nationwide Matched-Cohort Study in Denmark. **Gastroenterology,** v. 154, n. 6, p. 1729-1736, 05 2018. ISSN 1528-0012.

KIRKEGÅRD, J.; MORTENSEN, F. V.; CRONIN-FENTON, D. Chronic Pancreatitis and Pancreatic Cancer Risk: A Systematic Review and Meta-analysis. **Am J Gastroenterol,** v. 112, n. 9, p. 1366-1372, Sep 2017. ISSN 1572-0241.

KLAUSS, M. et al. Value of three-dimensional reconstructions in pancreatic carcinoma using multidetector CT: initial results. **World J Gastroenterol,** v. 15, n. 46, p. 5827-32, Dec 2009. ISSN 2219-2840.

KLEEFF, J. et al. Pancreatic cancer. **Nat Rev Dis Primers,** v. 2, p. 16022, 04 2016. ISSN 2056-676X.

KLEIN, A. P. et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. **Cancer Res,** v. 64, n. 7, p. 2634-8, Apr 2004. ISSN 0008-5472.

KLIESER, E. et al. Role of histone deacetylases in pancreas: Implications for pathogenesis and therapy. **World J Gastrointest Oncol,** v. 7, n. 12, p. 473-83, Dec 2015. ISSN 1948-5204.

KOHLER, I. et al. Phenotypic and genotypic characterization of carcinomas of the papilla of Vater has prognostic and putative therapeutic implications. **Am J Clin Pathol,** v. 135, n. 2, p. 202-11, Feb 2011. ISSN 1943-7722.

KUMARI, N. et al. Intestinal and pancreatobiliary differentiation in periampullary carcinoma: the role of immunohistochemistry. **Hum Pathol,** v. 44, n. 10, p. 2213-9, Oct 2013. ISSN 1532-8392.

KUNATH, U.; HOMMERDING, H. [Is the duodenal papilla an autonomic sphincter? A contribution to the functional morphology (author's transl)]. **Res Exp Med (Berl),** v. 178, n. 2, p. 103-16, 1981. ISSN 0300-9130.

KUZMICKIENE, I. et al. Smoking and other risk factors for pancreatic cancer: a cohort study in men in Lithuania. **Cancer Epidemiol,** v. 37, n. 2, p. 133-9, Apr 2013. ISSN 1877-783X.

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, J. H. et al. Significance analysis of histologic type and perineural invasion as prognostic factors after curative resection of ampulla of Vater carcinoma. **Hepatogastroenterology,** v. 57, n. 99-100, p. 646-52, 2010 May-Jun 2010. ISSN 0172-6390.

LEE, P. J.; PAPACHRISTOU, G. I. New insights into acute pancreatitis. **Nat Rev Gastroenterol Hepatol,** v. 16, n. 8, p. 479-496, 08 2019. ISSN 1759-5053.

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.

LEVY, S. et al. The diploid genome sequence of an individual human. **PLoS Biol,** v. 5, n. 10, p. e254, Sep 2007. ISSN 1545-7885.

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, D. et al. Body mass index and risk, age of onset, and survival in patients with pancreatic cancer. **JAMA,** v. 301, n. 24, p. 2553-62, Jun 2009. ISSN 1538-3598.

LI, D. et al. Diabetes and risk of pancreatic cancer: a pooled analysis of three large case-control studies. **Cancer Causes Control,** v. 22, n. 2, p. 189-97, Feb 2011. ISSN 1573-7225.

LIAO, K. F. et al. Diabetes mellitus correlates with increased risk of pancreatic cancer: a population-based cohort study in Taiwan. **J Gastroenterol Hepatol,** v. 27, n. 4, p. 709-13, Apr 2012. ISSN 1440-1746.

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.

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.

LOCKE, W. J. et al. DNA Methylation Cancer Biomarkers: Translation to the Clinic. **Front Genet,** v. 10, p. 1150, 2019. ISSN 1664-8021.

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.

LOPEZ, J. et al. The context and potential of epigenetics in oncology. **Br J Cancer,** v. 100, n. 4, p. 571-7, Feb 2009. ISSN 1532-1827.

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.

LYNCH, S. M. et al. Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium. **Am J Epidemiol,** v. 170, n. 4, p. 403-13, Aug 2009. ISSN 1476-6256.

LÜTTGES, J.; ZAMBONI, G.; KLÖPPEL, G. Recommendation for the examination of pancreaticoduodenectomy specimens removed from patients with carcinoma of the exocrine pancreas. A proposal for a standardized pathological staging of pancreaticoduodenectomy specimens including a checklist. **Dig Surg,** v. 16, n. 4, p. 291-6, 1999. ISSN 0253-4886.

MAFFICINI, A. et al. Ampulla of Vater Carcinoma: Sequencing Analysis Identifies TP53 Status as a Novel Independent Prognostic Factor and Potentially Actionable ERBB, PI3K, and WNT Pathways Gene Mutations. **Ann Surg,** v. 267, n. 1, p. 149-156, Jan 2018. ISSN 1528-1140. MAISONNEUVE, P.; LOWENFELS, A. B. Risk factors for pancreatic cancer: a summary review of meta-analytical studies. **Int J Epidemiol,** v. 44, n. 1, p. 186-98, Feb 2015. ISSN 1464-3685.

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

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.

MCAULIFFE, J. C.; CHRISTEIN, J. D. Type 2 diabetes mellitus and pancreatic cancer. **Surg Clin North Am,** v. 93, n. 3, p. 619-27, Jun 2013. ISSN 1558-3171.

MCGUIGAN, A. et al. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. **World J Gastroenterol,** v. 24, n. 43, p. 4846-4861, Nov 2018. ISSN 2219-2840.

MICHAUD, D. S. et al. Alcohol intake and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium (PanScan). **Cancer Causes Control,** v. 21, n. 8, p. 1213-25, Aug 2010. ISSN 1573-7225.

MIDHA, S.; CHAWLA, S.; GARG, P. K. Modifiable and non-modifiable risk factors for pancreatic cancer: A review. **Cancer Lett,** v. 381, n. 1, p. 269-77, 10 2016. ISSN 1872-7980.

MISHRA, N. K.; GUDA, C. Genome-wide DNA methylation analysis reveals molecular subtypes of pancreatic cancer. **Oncotarget,** v. 8, n. 17, p. 28990-29012, Apr 2017. ISSN 1949-2553.

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 0885-3177.

MIZUNO, S. et al. Smoking, family history of cancer, and diabetes mellitus are associated with the age of onset of pancreatic cancer in Japanese patients. **Pancreas,** v. 43, n. 7, p. 1014-7, Oct 2014. ISSN 1536-4828.

MONSON, J. R. et al. Radical resection for carcinoma of the ampulla of Vater. **Arch Surg,** v. 126, n. 3, p. 353-7, Mar 1991. ISSN 0004-0010.

MONTEITH, G. R.; DAVIS, F. M.; ROBERTS-THOMSON, S. J. Calcium channels and pumps in cancer: changes and consequences. **J Biol Chem,** v. 287, n. 38, p. 31666-73, Sep 2012. ISSN 1083-351X.

MONTEITH, G. R. et al. Calcium and cancer: targeting Ca2+ transport. **Nat Rev Cancer,** v. 7, n. 7, p. 519-30, Jul 2007. ISSN 1474-175X.

MORINE, Y. et al. Role of histone deacetylase expression in intrahepatic cholangiocarcinoma. **Surgery,** v. 151, n. 3, p. 412-9, Mar 2012. ISSN 1532-7361.

MORRISON, A. H.; BYRNE, K. T.; VONDERHEIDE, R. H. Immunotherapy and Prevention of Pancreatic Cancer. **Trends Cancer,** v. 4, n. 6, p. 418-428, 06 2018. ISSN 2405-8025.

MUNIGALA, S. et al. Increased risk of pancreatic adenocarcinoma after acute pancreatitis. **Clin Gastroenterol Hepatol,** v. 12, n. 7, p. 1143-1150.e1, Jul 2014. ISSN 1542-7714.

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.

NONES, K. et al. Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling. **Int J Cancer,** v. 135, n. 5, p. 1110-8, Sep 2014. ISSN 1097-0215.

OHIKE, N. et al. Intra-ampullary papillary-tubular neoplasm (IAPN): characterization of tumoral intraepithelial neoplasia occurring within the ampulla: a clinicopathologic analysis of 82 cases. **Am J Surg Pathol,** v. 34, n. 12, p. 1731-48, Dec 2010. ISSN 1532-0979.

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.

ORTH, M. et al. Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. **Radiat Oncol,** v. 14, n. 1, p. 141, Aug 2019. ISSN 1748-717X.

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.

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.

OVERMAN, M. J. et al. A population-based comparison of adenocarcinoma of the large and small intestine: insights into a rare disease. **Ann Surg Oncol,** v. 19, n. 5, p. 1439-45, May 2012. ISSN 1534-4681.

OVERMAN, M. J. et al.. Prognostic value of lymph node evaluation in small bowel adenocarcinoma: analysis of the surveillance, epidemiology, and end results database. **Cancer,** v. 116, n. 23, p. 5374-82, Dec 2010. ISSN 0008-543X.

OVERMAN, M. J. et al.. Gene expression profiling of ampullary carcinomas classifies ampullary carcinomas into biliary-like and intestinal-like subtypes that are prognostic of outcome. **PLoS One,** v. 8, n. 6, p. e65144, 2013. ISSN 1932-6203.

PARAB, T. M. et al. Gastrointestinal stromal tumors: a comprehensive review. **J Gastrointest Oncol,** v. 10, n. 1, p. 144-154, Feb 2019. ISSN 2078-6891.

PELUCCHI, C. et al. Smoking and body mass index and survival in pancreatic cancer patients. n. 1536-4828 (Electronic),

PENG, D. F. et al. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. **Carcinogenesis,** v. 27, n. 6, p. 1160-8, Jun 2006. ISSN 0143-3334.

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

PEZZILLI, R.; PAGANO, N. Is diabetes mellitus a risk factor for pancreatic cancer? **World J Gastroenterol,** v. 19, n. 30, p. 4861-6, Aug 2013. ISSN 2219-2840.

PFEIFER, G. P. Defining Driver DNA Methylation Changes in Human Cancer. **Int J Mol Sci,** v. 19, n. 4, Apr 2018. ISSN 1422-0067.

PORUK, K. E. et al. Screening for pancreatic cancer: why, how, and who? **Ann Surg,** v. 257, n. 1, p. 17-26, Jan 2013. ISSN 1528-1140.

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.

RAIMONDI, S. et al. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. **Best Pract Res Clin Gastroenterol,** v. 24, n. 3, p. 349-58, Jun 2010. ISSN 1532-1916.

RAIMONDI, S.; MAISONNEUVE, P.; LOWENFELS, A. B. Epidemiology of pancreatic cancer: an overview. **Nat Rev Gastroenterol Hepatol,** v. 6, n. 12, p. 699-708, Dec 2009. ISSN 1759-5053.

RANDI, G. et al. Epidemiology of biliary tract cancers: an update. **Ann Oncol,** v. 20, n. 1, p. 146-59, Jan 2009. ISSN 1569-8041.

RAWLA, P.; SUNKARA, T.; GADUPUTI, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. **World J Oncol,** v. 10, n. 1, p. 10-27, Feb 2019. ISSN 1920-454X.

RAYNAL, N. J. et al. Targeting Calcium Signaling Induces Epigenetic Reactivation of Tumor Suppressor Genes in Cancer. **Cancer Res,** v. 76, n. 6, p. 1494-505, Mar 2016. ISSN 1538-7445.

RAZIN, A. CpG methylation, chromatin structure and gene silencing-a three-way connection. **EMBO J,** v. 17, n. 17, p. 4905-8, Sep 1998. ISSN 0261-4189.

RAZUMILAVA, N.; GORES, G. J. Cholangiocarcinoma. **Lancet,** v. 383, n. 9935, p. 2168-79, Jun 2014. ISSN 1474-547X.

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.

RELLES, D. et al. Periampullary and duodenal neoplasms in neurofibromatosis type 1: two cases and an updated 20-year review of the literature yielding 76 cases. **J Gastrointest Surg,** v. 14, n. 6, p. 1052-61, Jun 2010. ISSN 1873-4626.

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.

RIJKERS, A. P. et al. Risk of Pancreatic Cancer After a Primary Episode of Acute Pancreatitis. **Pancreas,** v. 46, n. 8, p. 1018-1022, 09 2017. ISSN 1536-4828.

ROPERO, S.; ESTELLER, M. The role of histone deacetylases (HDACs) in human cancer. **Mol Oncol,** v. 1, n. 1, p. 19-25, Jun 2007. ISSN 1878-0261.

ROSATO, V. et al. Population attributable risk for pancreatic cancer in Northern Italy. **Pancreas,** v. 44, n. 2, p. 216-20, Mar 2015. ISSN 1536-4828.

ROSTY, C. et al. Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. **Am J Pathol,** v. 160, n. 1, p. 45-50, Jan 2002. ISSN 0002-9440.

SAMOKHVALOV, A. V.; REHM, J.; ROERECKE, M. Alcohol Consumption as a Risk Factor for Acute and Chronic Pancreatitis: A Systematic Review and a Series of Meta-analyses. **EBioMedicine,** v. 2, n. 12, p. 1996-2002, Dec 2015. ISSN 2352-3964.

SANAEI, M.; KAVOOSI, F. Histone Deacetylases and Histone Deacetylase Inhibitors: Molecular Mechanisms of Action in Various Cancers. **Adv Biomed Res,** v. 8, p. 63, 2019. ISSN 2277-9175.

SANDHU, V. et al. Molecular signatures of mRNAs and miRNAs as prognostic biomarkers in pancreatobiliary and intestinal types of periampullary adenocarcinomas. **Mol Oncol,** v. 9, n. 4, p. 758-71, Apr 2015. ISSN 1878-0261.

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.

SCARÀ, S.; BOTTONI, P.; SCATENA, R. CA 19-9: Biochemical and Clinical Aspects. **Adv Exp Med Biol,** v. 867, p. 247-60, 2015. ISSN 0065-2598.

SCHNEIDER, A. et al. Combined bicarbonate conductance-impairing variants in CFTR and SPINK1 variants are associated with chronic pancreatitis in patients without cystic fibrosis. **Gastroenterology,** v. 140, n. 1, p. 162-71, Jan 2011. ISSN 1528-0012.

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.

SCHÖNLEBEN, F. et al. Molecular analysis of PIK3CA, BRAF, and RAS oncogenes in periampullary and ampullary adenomas and carcinomas. **J Gastrointest Surg,** v. 13, n. 8, p. 1510-6, Aug 2009. ISSN 1873-4626.

SEBAT, J. et al. Large-scale copy number polymorphism in the human genome. **Science,** v. 305, n. 5683, p. 525-8, Jul 2004. ISSN 1095-9203.

SHI, C.; HRUBAN, R. H.; KLEIN, A. P. Familial pancreatic cancer. **Arch Pathol Lab Med,** v. 133, n. 3, p. 365-74, Mar 2009. ISSN 1543-2165.

SHIRAKAWA, K. et al. Reactivation of latent HIV by histone deacetylase inhibitors. **Trends Microbiol,** v. 21, n. 6, p. 277-85, Jun 2013. ISSN 1878-4380.

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

SINGH, A. et al. Panobinostat as Pan-deacetylase Inhibitor for the Treatment of Pancreatic Cancer: Recent Progress and Future Prospects. **Oncol Ther,** v. 4, n. 1, p. 73-89, 2016. ISSN 2366-1070 (Print) 2366-1089.

SRIRAKSA, R.; LIMPAIBOON, T. Histone deacetylases and their inhibitors as potential therapeutic drugs for cholangiocarcinoma - cell line findings. **Asian Pac J Cancer Prev,** v. 14, n. 4, p. 2503-8, 2013. ISSN 2476-762X.

STOJSIC, Z. et al. Large-cell neuroendocrine carcinoma of the ampulla of Vater. **Med Oncol,** v. 27, n. 4, p. 1144-8, Dec 2010. ISSN 1559-131X.

TANG, B. et al. Clinicopathological Significance of CDKN2A Promoter Hypermethylation Frequency with Pancreatic Cancer. **Sci Rep,** v. 5, p. 13563, Sep 2015. ISSN 2045-2322.

TEMPERO, M. A. et al. Pancreatic Adenocarcinoma, Version 2.2017, NCCN Clinical Practice Guidelines in Oncology. **J Natl Compr Canc Netw,** v. 15, n. 8, p. 1028-1061, 08 2017. ISSN 1540-1413.

TERADA, T. Malignant tumors of the small intestine: a histopathologic study of 41 cases among 1,312 consecutive specimens of small intestine. **Int J Clin Exp Pathol,** v. 5, n. 3, p. 203-9, 2012. ISSN 1936-2625.

TOZAWA, T. et al. Promoter hypermethylation of DAP-kinase is associated with poor survival in primary biliary tract carcinoma patients. **Cancer Sci,** v. 95, n. 9, p. 736-40, Sep 2004. ISSN 1347-9032.

TSAI, H. J.; CHANG, J. S. Environmental Risk Factors of Pancreatic Cancer. **J Clin Med,** v. 8, n. 9, Sep 2019. ISSN 2077-0383.

VALSANGKAR, N. P. et al. Survival in ampullary cancer: potential role of different KRAS mutations. **Surgery,** v. 157, n. 2, p. 260-8, Feb 2015. ISSN 1532-7361.

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

VINCENT, A. et al. Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma. **Clin Cancer Res,** v. 17, n. 13, p. 4341-54, Jul 2011. ISSN 1078-0432. >.

WAISBERG, J. et al. Carcinoid of the minor duodenal papilla associated with pancreas divisum: Case report and review of the literature. **Clinics (Sao Paulo),** v. 61, n. 4, p. 365-8, Aug 2006. ISSN 1807-5932.

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.

WANG, X. X. et al. Large-scale DNA methylation expression analysis across 12 solid cancers reveals hypermethylation in the calcium-signaling pathway. **Oncotarget,** v. 8, n. 7, p. 11868-11876, Feb 2017. ISSN 1949-2553.

WANG, Y. et al. Role of the microbiome in occurrence, development and treatment of pancreatic cancer. **Mol Cancer,** v. 18, n. 1, p. 173, Dec 2019. ISSN 1476-4598.

WANG, Y. T. et al. Association between alcohol intake and the risk of pancreatic cancer: a dose-response meta-analysis of cohort studies. **BMC Cancer,** v. 16, p. 212, Mar 2016. ISSN 1471-2407.

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.

WESTGAARD, A. et al. Pancreatobiliary versus intestinal histologic type of differentiation is an independent prognostic factor in resected periampullary adenocarcinoma. **BMC Cancer,** v. 8, p. 170, 2008. ISSN 1471-2407.

WHO; SALTO-TELLEZ, M.; RUGGE, M. (World Health Organization) Tumors of the small intestine and ampulla. In: (Ed.). **Classification of tumours of the digestive system**. 5 t^{h}. Lyon, 2019.

WILLIS, J. A. et al. Genome-wide analysis of the role of copy-number variation in pancreatic cancer risk. **Front Genet,** v. 5, p. 29, 2014. ISSN 1664-8021.

WONG, J. C.; LU, D. S. Staging of pancreatic adenocarcinoma by imaging studies. **Clin Gastroenterol Hepatol,** v. 6, n. 12, p. 1301-8, Dec 2008. ISSN 1542-7714.

XUE, Y. et al. Immunohistochemical Classification of Ampullary Carcinomas: Critical Reappraisal Fails to Confirm Prognostic Relevance for Recently Proposed Panels, and Highlights MUC5AC as a Strong Prognosticator. **Am J Surg Pathol,** v. 41, n. 7, p. 865-876, Jul 2017. ISSN 1532-0979.

YACHIDA, S. et al. Genomic Sequencing Identifies ELF3 as a Driver of Ampullary Carcinoma. **Cancer Cell,** v. 29, n. 2, p. 229-40, Feb 2016. ISSN 1878-3686.

YAMAGUCHI, H. et al. The discrete nature and distinguishing molecular features of pancreatic intraductal tubulopapillary neoplasms and intraductal papillary mucinous neoplasms of the gastric type, pyloric gland variant. **J Pathol,** v. 231, n. 3, p. 335-41, Nov 2013. ISSN 1096-9896.

YAMAGUCHI, J. et al. Histone deacetylase inhibitor (SAHA) and repression of EZH2 synergistically inhibit proliferation of gallbladder carcinoma. **Cancer Sci,** v. 101, n. 2, p. 355-62, Feb 2010. ISSN 1349-7006.

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.

YAO, Q.; CHEN, Y.; ZHOU, X. The roles of microRNAs in epigenetic regulation. **Curr Opin Chem Biol,** v. 51, p. 11-17, Aug 2019. ISSN 1879-0402. Available at: <

ZHAN, W. et al. Germline Variants and Risk for Pancreatic Cancer: A Systematic Review and Emerging Concepts. **Pancreas,** v. 47, n. 8, p. 924-936, 09 2018. ISSN 1536-4828.

ZHOU, H. et al. Carcinoma of the ampulla of Vater: comparative histologic/immunohistochemical classification and follow-up. **Am J Surg Pathol,** v. 28, n. 7, p. 875-82, Jul 2004. ISSN 0147-5185 (Print) 0147-5185 (Linking).

ZHU, Y. et al. Tissue-Resident Macrophages in Pancreatic Ductal Adenocarcinoma Originate from Embryonic Hematopoiesis and Promote Tumor Progression. **Immunity,** v. 47, n. 2, p. 323-338.e6, 08 2017. ISSN 1097-4180.

ÖZDEMIR, B. C. et al. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. **Cancer Cell,** v. 25, n. 6, p. 719-34, Jun 2014. ISSN 1878-3686.

CAPÍTULO XI: ANEXOS

Manuscritos em fase preparação desenvolvidos durante o período de Doutorado Sanduíche na International Agency for Research on Cancer:

Identification of tumor-specific differential methylation profile in periampullary carcinomas

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ABSTRACT

Periampullary carcinomas (PACs) are rare neoplasms arising from pancreatic head, ampulla of Vater, distal billiary duct and proximal duodenum. PACs exhibit high mortality due to the lack of efficient therapy and the molecular mechanism underlying the development of these cancers is poorly understood as well as aberrant DNA methylation. We hypothesized that aberrant DNA methylation may be an important event in the tumorigenesis of PACs. To test this hypothesis, we aimed to conduct genome-wide methylation analysis of PACs comparing the methylome profiles of PAC tumors with the adjacent normal tissue (NT). Methylation profiles were investigated using Illumina's Infinium Human Methylation 450 BeadChip array in 17 PAC and 14 NT samples. Differential methylation among the samples was analyzed by robust regression. PAC exhibit distinct global methylation profiles in comparison to their NT. We identified a total of 5622 differentially methylated positions (DMPs) and 1056 differentially methylated regions (DMR) corresponding to 789 genes (FDR ≤ 0.05, Δβ>0.2). Among PAC-specific DMRs, we found 14.2% (112 genes) were hypomethylated and 85.8% (671 genes) were hypermethylated. Some of the identified DMR-associated genes (*ZSCAN18, CDH13, RUNX3, DCLK1, CCND2, SLIT2* and *TWIST1*) were reported in previous studies on PAC, supporting the notion that specific genes may be consistently targeted by differential methylation. To further determine the potential biological relevance of the identified DMRs, pathway analyses were performed using Enrichr that revealed dysregulation in calcium signaling and signaling pathways regulating pluripotency. The present study identified specific differentially methylated genes underscoring the potential role of distinct pathways involved in the development and progression of PAC. These deregulated genes and pathways might be potentially exploited in the development of epigenetics-based strategies for biomarker discovery and therapeutic intervention.

Telomere length and telomerase components evaluation in periampullary carcinomas (ampulla of Vater carcinoma and duodenal adenocarcinoma)

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ABSTRACT

Telomere maintenance is a critical requirement for enabling tumor replicative immortality. Here, telomerase expression, telomere length (TL) and potential regulatory factors that can underlie telomerase machinery alterations in periampullary carcinomas (PA, n= 20) were analyzed. The hTERT immunostaining was detected in 13/14 samples; additionally, we found short relative TL in tumors compared with normal duodenal adjacent tissues (ND, P = 0.01) assessed by qPCR. *hTERT* promoter hotspot mutations were not present, however, the c.-245bp TSS (rs2853669) was detected in 9/20 tumor samples. rs2853669 is located in an ETS (E-twenty six) family transcription factor consensus sequence which can increase *hTERT* expression. The telomere shortening is a common event in 70% of tumors and the rs2853669 may play a role by blocking the progressive telomere shortening by increasing telomerase expression in these tumors.

Colaboração em artigos durante o período de Doutoramento relacionados aos seguintes temas:

Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver.

World Journal of Hepatology

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ORIGINAL ARTICLE

Basic Study

Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver

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Author contributions: Schneider ACR and da Silveira TR conceived the project and designed the experiments; Gregório C, Guizzo R and Longo L carried out genetic assays and zebrafish liver histology; Malysz T and Faccioni-Heuser MC performed ultrastructural analysis; Schneider ACR and Uribe-Cruz C analysed the data; the manuscript was written by the first author Schneider ACR and reviewed by an English grammar professor; all authors contributed clarifications and guidance on the manuscript; all authors were involved in editing the manuscript; all authors read and approved the final manuscript.

Institutional animal care and use committee statement: The protocols were approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre, Brazil (No. 10.0327). The protocols were conducted in accordance with international guidelines for the care and use of laboratory animals. Animal care is described in the manuscript.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at schneiderac@gmail.com. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM

To evaluate the effects of chronic exposure to ethanol in the liver and the expression of inflammatory genes

BRCA1 and *BRCA2* mutational profile and prevalence in hereditary breast and ovarian cancer (HBOC) probands from Southern Brazil: Are international testing criteria appropriate for this specific population?

PLOS ONE

RESEARCH ARTICLE

BRCA1 and *BRCA2* mutational profile and prevalence in hereditary breast and ovarian cancer (HBOC) probands from Southern Brazil: Are international testing criteria appropriate for this specific population?

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Abstract

Background

Germline pathogenic variants in *BRCA1* and *BRCA2* (*BRCA*) are the main cause of Hereditary Breast and Ovarian Cancer syndrome (HBOC).

Methods

In this study we evaluated the mutational profile and prevalence of *BRCA* pathogenic/likely pathogenic variants among probands fulfilling the NCCN HBOC testing criteria. We characterized the clinical profile of these individuals and explored the performance of international testing criteria.

Results

A pathogenic/likely pathogenic variant was detected in 19.1% of 418 probands, including seven novel frameshift variants. Variants of uncertain significance were found in 5.7% of individuals. We evaluated 50 testing criteria and mutation probability algorithms. There was a significant odds-ratio (OR) for mutation prediction ($p \leq 0.05$) for 25 criteria; 14 of these had $p \leq 0.001$. Using a cutoff point of four criteria, the sensitivity is 83.8%, and the specificity is 53.5% for being a carrier. The prevalence of pathogenic/likely pathogenic variants for