Universidade Estadual de Londrina

MARILESIA FERREIRA DE SOUZA

ESTUDO COMPARATIVO DO PERFIL DE MRNAS E MIRNAS RELACIONADOS COM CÂNCER DE PRÓSTATA ENTRE INDIVÍDUOS CONTROLES E PORTADORES DESTA NEOPLASIA

Londrina
2017

Universidade Estadual de Londrina

Instituto Agronômico do Paraná

Empresa Brasileira de Pesquisa Agropecuária

Marilesia Ferreira de Souza

Estudo comparativo do perfil de mRNAs e miRNAs relacionados com câncer de próstata entre indivíduos controles e portadores desta neoplasia

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Orientadora: Profa. *Dra. Ilce Mara de Syllos Cólus*

Londrina
2017

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

729 Souza, Marilesia Ferreira de.
Estudo comparativo fo perfil de mrnas e mirnas relacionados com câncer de próstata entre indivíduos controles e portadores desta neoplasia / Marilesia Ferreira de Souza. - Londrina, 2017.
207 f. : il.

Orientador: Ilce Mara de Syllos Cólus.
Tese (Doutorado em Genética e Biologia Molecular) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Genética e Biologia Molecular, 2017.
Inclui bibliografia.

1. Câncer - Estudo comparativo. - Tese. 2. Próstata - Câncer. - Tese. 3. Ácidos nucleicos circulantes. - Tese. I. Cólus, Ilce Mara de Syllos. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Genética e Biologia Molecular. III. Título.

CDU 574

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Londrina, 03 de março de 2017

APOIO FINANCEIRO

Agradeço à:

Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná, convênio 185/2014, pelo financiamento da pesquisa.

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo financiamento da pesquisa.

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/PQ) pelas bolsas concedidas no Brasil.

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado sanduíche modalidade PDSE.

Agradecimentos

À Universidade Estadual de Londrina por fornecer a estrutura para que a pesquisa pudesse ser desenvolvida e ao Programa de Pós-graduação em Genética e Biologia Molecular pela oportunidade.

À Georgetown University, Lombardi Comprehensive Cancer Center e ao AC Camargo Cancer Center pelas estruturas e oportunidades.

Ao Hospital do Câncer de Londrina e Consórcio Intermunicipal de Saúde do Médio Paranapanema (CISMEPAR) e toda a equipe de médicos urologistas, em especial Dr. Paulo Emílio Fuganti, Nicola Mortati Neto, Miguel Alves Pereira Junior, Rafael Pellegrinelli Moreira de Souza, Alexsandro Koike e Ricardo Brandina; à equipe de enfermeiros, em especial ao Carlão e à Mary Okano; e a todos os funcionários que sempre ajudaram e colaboraram. Também gostaria de agradecer a toda equipe do Laboratório Clínico CETEL por estar prontamente à disposição para a realização de todas as coletas.

A todos os nossos queridos pacientes que aceitaram participar da pesquisa, muito obrigada pela disposição e pela confiança.

À banca examinadora que foi carinhosamente selecionada e que prontamente atendeu ao nosso pedido. Muito obrigada por dispor do seu tempo para contribuir com esse trabalho.

À minha querida orientadora Ilce Mara de Syllos Cólus pela oportunidade de continuar no laboratório. Por ter acreditado em mim e ter me atribuído muitos desafios que fizeram com que crescesse muito profissionalmente. Por todo apoio, amizade, pelos conselhos e dedicação. Obrigada por todas as correções e por ter compartilhado sua sabedoria comigo. Muito obrigada!

À minha co-orientadora estrangeira (brasileira) Profa. Dra. Luciane Regina Cavalli, por ter carinhosamente me recebido em seu laboratório e permitido que eu realizasse um grande sonho. Muito obrigada por todo o incentivo e por ter aceitado trabalhar com um tipo tumoral diferente. Muito obrigada pela gentileza com que me acolheu, por todos os ensinamentos repassados e pela dedicação. Muito Obrigada!

À Profa. Dra. Silvia Regina Rogatto, por ter me recebido em seu laboratório e permitido que eu desenvolvesse uma parte da minha tese com sua maravilhosa equipe. Muito obrigada por todo apoio financeiro e cientifico. A senhora foi fundamental para que esse projeto saísse do papel e se tornasse realidade. Muito Obrigada!

A todos os professores pela dedicação e por todo o conhecimento repassado, em especial às professoras Dra. Roberta Losi-Guembarovski, Dra. Juliana Mara Serpeloni, Dra. Daniela Sartori e Dra. Silvia Helena Sofia.

Ao Prof. Dr. Kumar Depeek pela assessoria cientifica durante a pesquisa realizada no laboratório da Profa. Dra. Luciane Regina Cavalli.

A Deus, por tudo. Pois só Ele sabe todos os desafios que enfrentei para que essa pesquisa tomasse forma.

Aos meus pais por sempre terem me apoiado, por toda a dedicação, pelo amor incondicional, pelos conselhos, pela amizade, por serem as pessoas mais importantes da minha vida. Obrigada por tudo, amo muito vocês.

À minha avó Clari e ao meu avô Bruno, por serem uma segunda mãe e um segundo pai pra mim. Vocês são meus lindinhos que amo muito. Obrigada por tudo.

Ao meu namorado Braian Allievi por ser meu porto seguro, sempre me apoiando, me dando forças. Obrigada pela dedicação, compreensão, carinho e amor. Eu te amo!

Aos meus queridos amigos de laboratório Heloisa Cilião, Milene Roldão e Monyse Nobrega pela ajuda, pelas idas aos hospitais, pelas muitas risadas, pela amizade que quero levar para sempre. Diego Luis Ribeiro pelas muitas risadas, cervejas, caronas, muito obrigada por sua amizade. Maressa Maline por todo o apoio e puxões de orelha, pelo carinho e preocupação, pela amizade que levarei para sempre comigo. À Rossana Godoy Camargo, Katiuska Tuttis, Higor Lopes, Ana Flávia Specian, Lucas Benicio, Beatriz Mattos obrigada por todo tempo de convivência e pela amizade.

A toda a equipe do laboratório de Genética Molecular do AC Camargo Cancer Center, em especial minha querida amiga Dra. Hellen Kuasne que foi determinante para o andamento da pesquisa, sempre disposta a ajudar mesmo quando estava distante, à Dra. Mariana Bisarro dos Reis e à Graziella Spilborghs pela ajuda e companheirismo, ao Dr. Mateus Barros por todos os ensinamentos estatísticos repassados, Dr. Fabio Marchi por toda a análise de bioinformática, Isabela Ortiz, Luísa Matos, Irina Bobrovnitchaia e todos os demais por todo o auxílio e amizade.

Às meninas do E512 Aline Fonseca, Selene Espósito, Akanksha Mahajan e Yara Zabala, por terem me recebido maravilhosamente bem quando cheguei à Georgetown University. Vocês foram verdadeiros anjos na minha vida, muito obrigada por tudo que aprendi com vocês, vocês estarão sempre no meu coração. À Raquel Santana, minha “roommate” e Caroline Dani, a incansável, foi um presente ter conhecido vocês, muito obrigada pela amizade, passeios e conversas, vocês também estarão sempre no meu coração. Á todos os demais integrantes do Lombardi Comprehensive Cancer

Center que auxiliaram de alguma forma nesta pesquisa em especial ao Francisco Sáenz, pelos ensinamentos e altruísmo.

Às minhas mais amadas amigas: Dalva Pereira, Darlene Gris, Marluci Passarin, Vanessa Mattielo e Karen Prokoski que mesmo com a distância estavam sempre presentes de um jeito ou de outro, me ajudando, aconselhando e me dando forças. Obrigada por tudo.

Aos técnicos dos laboratórios Dário Tormena e Melissa por toda ajuda.

Muito Obrigada!

“Tenha coragem e seja gentil”

Cinderela

SOUZA, Marilesia Ferreira de. **Estudo comparativo do perfil de mRNAs e miRNAs relacionados com câncer de próstata entre indivíduos controles e portadores desta neoplasia**. 2017. 206 p. Tese (Doutorado em Genética e Biologia Molecular) Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

O câncer de próstata (CaP) é o segundo tipo de neoplasia mais incidente no mundo. As dificuldades para realização da triagem, assim como a identificação de pacientes com doença agressiva ainda são desafios a serem solucionados. Esta é considerada uma doença heterogênea e, portanto, estudos que visem compreender melhor seu desenvolvimento e progressão são importantes. Os ácidos nucleicos circulantes têm se destacado por sua possível utilização como "biópsia líquida", ou seja, como marcadores tumorais minimamente invasivos. Sendo assim, este estudo teve como objetivo buscar marcadores baseados em mRNAs e miRNAs circulantes com fins diagnósticos, prognósticos e terapêuticos para CaP, assim como, analisar os efeitos de miRNAs no comportamento de células tumorais de próstata *in vitro*. Foi conduzida uma análise a partir dos dados do TCGA (The Cancer Genome Atlas) a fim de definir genes e miRNAs candidatos. Onze genes e dez miRNAs foram avaliados em amostras de plasma de pacientes com CaP virgens de tratamento e de indivíduos controles. Dois genes, *OR51E2* e *SIM2* mostraram-se mais expressos em pacientes quando comparados aos controles e, também, em paciente com antígeno prostático específico (PSA) ≤ 4,0 ng/mL quando comparados com controles com os mesmos níveis de PSA. O miR-200c mostrou-se mais expresso e o miR-200b apresentou menor nível de expressão nos pacientes. A associação destes dois gene e dois miRNAs apresentou 67% de sensibilidade e 75% de especificidade para distinguir pacientes de controles (AUC = 0,71). A análise de predição de agressividade da doença identificou os genes *AMACR*, *BCL2*, *GOLM1 TRPM8* e *NKX3-1*. Destes genes, *NKX3-1*, *TRPM8* e *GOLM1*, quando combinados, foram capazes de identificar corretamente 71,2% das amostras e quando associados com práticas clínicas já utilizadas, como escore de Gleason da biópsia (≥ 8) e níveis de PSA (> 20 ng/mL), mostraram sensibilidade de 88% e especificidade de 58% para identificar pacientes de alto risco para doença agressiva. Dentre os miRNAs avaliados, dois se destacaram e foram selecionados para as análises funcionais. O miR-182 mostrou associação com bilateralidade do tumor. Sua superexpressão *in vitro* foi relacionada com o aumento da proliferação celular, de migração celular, níveis elevados de marcadores de transição epitélio-mesenquimal (EMT) e p-AKT, além de resistência ao docetaxel, indicando que a desregulação deste miRNA é um fator importante para o desenvolvimento do CaP. Já o miR-141 apresentou níveis elevados de expressão em pacientes com metástase óssea, PSA > 10 ng/mL e doença de alto risco. As análises *in vitro* demostraram que, no CaP, desempenha função dupla, como oncogene e supressor de tumor, dependendo do contexto celular. Também foi demostrado que a inibição deste miRNA está relacionada com resistência a quimioterápicos. Assim, o presente estudo demonstrou que mRNAs e miRNAs circulantes podem ser bons marcadores para diagnóstico e prognóstico de CaP e os miR-182 e miR -141 possuem importante papel na modulação das células da próstata e na resistência a quimioterápicos.

Palavras-chave: Câncer de próstata. Ácidos nucleicos circulantes. Diagnóstico. Prognóstico.

SOUZA, Marilesia Ferreira de. **Comparative study of mRNAs and miRNAs profile associated with prostate cancer between controls and patients with this neoplasia**. 2017. 206 p. Thesis (PhD in Genética e Biologia Molecular) Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Prostate cancer (PCa) is the second most common neoplasia worldwide. The difficulties to perform the disease screening, as well as, the early diagnostic of patients with the aggressive disease remain as challenges to be solved. PCa is considered a heterogeneous disease, thus studies to understand its development and progression are needed. Circulating nucleic acids have been highlighted due to its possible use as "liquid biopsy", that means, as minimally invasive tumor markers. Thus, the aim of this study was identify miRNA and mRNA circulating markers for diagnostic, prognostic and therapeutic purposes for PCa, and also, to analyze the effects of miRNAs on the behavior of prostate tumor cells *in vitro*. A TCGA (The Cancer Genome Atlas) data analysis was performed to identify the candidates genes and miRNA. Eleven genes and ten miRNAs were analyzed in plasma patients‟ samples, without previous treatment, and cancer-free controls. Two genes, *OR51E2* and *SIM2* showed overexpression in patients when compared to controls, and were also overexpressed in patients with prostate-specific antigen (PSA) ≤ 4.0 ng/mL when compared to controls that presented the same levels of PSA. MiR-200c showed overexpression, while miR-200b showed downexpression in patients. The association of these two genes and two miRNAs showed 67% of sensitivity and 75% of specificity to discriminated patients from controls (AUC = 0.71). The predicted analysis for aggressiveness PCa identified *AMACR*, *BCL2*, *GOLM1*, *TRPM8* and *NKX3-1* genes. Among those, *NKX3-1*, *TRPM8* and *GOLM1* were able to correctly identify 71.2% of all positive samples when combined, and when associated with previously used clinical practices, such as biopsy‟s Gleason score (≥ 8) and PSA (> 20 ng/mL), showed sensitivity of 88% and specificity of 58% to identify patients with high-risk for aggressive disease. Among miRNA evaluated, two stood out and were chosen for functional analysis. MiR-182 demonstrated association with bilateral tumor. Its ectopic overexpression promoted cell proliferation, migration, increased levels of epithelial-mesenchymal transition (EMT) markers and p-AKT, besides docetaxel resistance, showing that the dysregulation of this miRNA is important to PCa progression. miR-141 were found overexpressed in the plasma of metastatic patients, high-risk PCa and PSA levels > 10 ng/mL. *In vitro* analyses demonstrated that this miRNA can have a dual role in prostate cancer, as oncogene and tumor suppressor, depending on cellular context. Besides that, the miR-141 inhibition promoted resistance to chemotherapeutic drugs. Thus, the present study showed that the circulating mRNAs and miRNAs could be good markers for diagnosis and prognosis of CaP, as well as, miR-182 and -141 play an important role in prostate cell modulation and resistance to chemotherapy.

Key words: Prostate cancer. Cell-free nucleic acid. Diagnosis. prognosis

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AGO	Argonauta
AMACR	Alfa metilacil CoA racemase
AR	Receptor de andrógeno
AUC	Área abaixo da curva
BCL2	Linfoma de células B 2
bHLH/PAS	Do inglês, *Basic helix-loop-helix/per-Arnt-Sim*
BMP6	Proteínas óssea morfogenética 6
CaP	Câncer de próstata
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CDH1	Caderina 1, E-caderina
CDH2	Caderina 2, N-caderina
cfmiRNA	microRNAs circulante
cfmRNA	mRNA circulante
cfNA	Ácidos nucleicos circulante
cfRNA	RNA circulante
CISMEPAR	Consórcio Intermunicipal de Saúde do Médio Paranapanema
CLDN1	Claudina 1
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
COL1A1	Colagenase tipo 1 alpha 1
CRPC	Câncer de próstata resistente à castração
CRT	Controle
CTC	Célula tumoral circulante
CTNNB1	Beta-catenina 1
CYP17A1	Citocromo P450 17A1
DHT	Di-hidrotestosterona
DRE	Toque digital retal
EDTA	Ácido etilenodiamino tetra-acético
EGFR	Receptor do fator de crescimento epidérmico
EMT	Transição epitélio-mesenquimal
ERSPC	Estudo randomizado europeu de triagem para câncer de próstata
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FBS	Soro bovino fetal
FC	Do inglês, *Fold-change*

FDR	Taxa de falsas descobertas
FOXA1	Do inglês, forkhead box A1
GESR	Recursos Genômicos e Epigenômicos Compartilhados
GOLM1	Proteína da membrana Golgi 1
GS	Escore de Gleason
HPB	Hiperplasia prostática benigna
hTERT	Telomerase transcriptase reversa
ICLAC	Comitê internacional de autenticação de linhagens celulares
LCCC	Do inglês, Lombardi comprehensive cancer center
mCaP	Câncer de próstata metastático
mCRPC	Câncer de próstata metastático resistente à castração
miRNA	microRNA
MMP11	Matrix metalloproteinase 11
mRNA	RNA mensageiro
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
OR51E2	Receptor olfativo família 51 subfamília E membro 2
NC	Controle negativo
NKX3-1	NK3 homeobox 1
NPV	Valor negativo preditivo
p-AKT	Proteína quinase B fosforilada
PB	Sangue periférico
PC	Controle positivo
PCA3	Gene 3 do câncer da próstata
PLCO	Ensaio de triagem de próstata, pulmão, câncer colorretal e ovário
PPV	Valor positivo preditivo
PSA	Antígeno prostático específico
PSGR	Receptor acoplado à proteína G específico da próstata
RISC	Complexo de silenciamento induzido por RNA
ROC	Característica de Operação do Receptor
RP	Prostatectomia radical
RT	Transcriptase reversa
qPCR	PCR quantitativo em tempo real
SHGB	Globulina ligadora de hormônio sexual
SIM2	Do inglês, Single-minded family BHLH transcription factor 2
SNAIL1	Do inglês, SNAIL family transcriptional repressor 1

SNAIL2	Do inglês, SNAIL family transcriptional repressor 2
SNT	Tecido normal adjacente
STR	Curta repetição em tandem
suPAR	Do inglês, s*oluble urokinase plasminogen activator receptor*
TCGA	Do inglês, the cancer genome atlas
TCSR	Recursos compartilhados para cultura de tecidos
TNM	Classificação de tumores malignos
TRPM8	Receptor de potencial transitório da melatatina 8
VIM	Vimentina
ZEB1	Do inglês, zinc finger E-box binding homeobox 1

SUMÁRIO

1. INTRODUÇÃO

O câncer de próstata é o segundo tipo de tumor sólido mais incidente no mundo e o quinto em causa de mortes devido a câncer (TORRE et al., 2015). No Brasil, após o câncer de pele não melanoma, o câncer de próstata é o mais incidente em homens, com estimativa de 61 mil novos casos para o ano de 2017 (INCA, 2015). Esta é uma doença considerada de terceira idade, pois a maioria dos casos acomete homens com mais de 65 anos (BAADE; YOULDEN; KRNJACKI, 2009). Possui características heterogêneas, pois os indivíduos podem apresentar a doença com desenvolvimento lento, que na maioria das vezes permanece na sua forma latente, não trazendo prejuízos ao portador, até formas altamente agressivas e metastáticas (CRAWFORD, 2009).

A triagem para esse tipo tumoral vem sendo realizada por dois exames, o Toque Digital Retal e o Antígeno Prostático Específico (do inglês, prostate-specific antigen - PSA). Entretanto, estudos recentes demonstraram que a inclusão do exame do PSA na triagem tem ocasionado, devido à sua baixa especificidade, o diagnóstico e tratamento de milhares de homens com a forma latente da doença, ou seja, milhares de pacientes foram diagnosticados e tratados desnecessariamente (WELCH; ALBERTSEN, 2009; STROPE; ANDRIOLE, 2010; GOMELLA et al., 2011; KLOTZ, 2013). Além disso, foi demonstrado que a inclusão deste exame na triagem praticamente não promoveu impacto na redução da mortalidade da doença. (ANDRIOLE et al., 2012; SCHRÖDER et al., 2012). Todos esses fatores implicaram no não aconselhamento do uso do PSA para o rastreamento do câncer de próstata em vários países, incluindo o Brasil (MOYER, 2012; INCA 2014). Entretanto, até o momento este ainda é o melhor marcador disponível para o rastreamento do câncer de próstata, e até que outro marcador se torne disponível, o teste do PSA deve ser utilizado com cautela (ROOBOL; CARLSSON, 2013). Em vista disso, a descoberta de novos marcadores é imprescindível para tornar o diagnóstico e tratamento desta doença mais preciso, o que tem estimulado pesquisas em todo o mundo (SHEN; ABATE-SHEN, 2010; TORRE et al., 2015).

A busca de marcadores para o câncer de próstata deve sanar os grandes desafios impostos pela complexidade da doença, entre eles, a identificação da doença de alto risco e potencialmente curável, identificação do câncer de próstata

hormônio resistente e a propensão a ocorrer metástase óssea (SHEN; ABATE-SHEN, 2010; ROOBOL; CARLSSON, 2013; ENDZELIŅŠ et al., 2016).

Atualmente a busca por marcadores para neoplasias tem como foco testes minimamente invasivos utilizando amostras biológicas como sangue e urina (PRENSNER et al., 2012). Nos últimos anos, emergiu uma nova classe de marcadores detectáveis nos biofluidos, os “cell-free nucleic acids” (cfNAs), que são moléculas de DNA ou RNA encontradas de forma livre ou associadas a proteínas em diferentes fluidos corporais (SCHWARZENBACH; HOON; PANTEL, 2011; SCHWARZENBACH et al., 2014). Estes marcadores circulantes ou “biópsias líquidas” podem refletir melhor a heterogeneidade do tumor do que as biópsias convencionais (ENDZELIŅŠ et al., 2016). Portanto, a busca de marcadores deste tipo para doenças heterogêneas, como o câncer de próstata, torna-se muito interessante.

Diante dos desafios clínicos tanto para triagem, diagnóstico, prognóstico e tratamento do câncer de próstata, o presente estudo teve como objetivo a busca de novos marcadores de RNA mensageiro (mRNA) e microRNAs (miRNA) circulantes para aprimorar e auxiliar o rastreamento, diagnóstico e prognóstico da doença de alto risco e conduta terapêutica desta neoplasia, assim como compreender fatores biológicos que possam influenciar no desenvolvimento e progressão deste tipo tumoral.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. O CÂNCER DE PRÓSTATA

A próstata é uma estrutura glandular com volume de aproximadamente 20 mL que aumenta de acordo com a idade (RAMSAMY; SUBRAMANIYAN; PATRA, 2016). Sua principal função é a secreção de um fluido para fornecer suporte nutricional aos espermatozoides. O epitélio prostático é composto por três tipos principais de células: secretoras, basais e neuroendócrinas; e possui três zonas anatômicas: periférica, transicional e central (JOSHUA et al., 2008). Dentre as várias doenças que podem afetar esta glândula, como a hiperplasia prostática benigna e a hiperplasia intraepitelial prostática, o câncer de próstata tem se destacado por sua alta incidência e alto impacto na saúde publica, envolvendo métodos de diagnóstico, prognóstico e tratamento (SHEN; ABATE-SHEN, 2010; DE NUNZIO et al., 2011).

O câncer de próstata é considerado uma doença de terceira idade, pois é rara antes dos 50 anos, sendo que a grande maioria dos casos acomete homens com mais de 65 anos (GRÖNBERG, 2003; BAADE; YOULDEN; KRNJACKI, 2009). É o segundo tipo de tumor sólido mais incidente no mundo e o quinto em causa de mortes devido a neoplasias (TORRE et al., 2015). As taxas desta doença variam de acordo com as populações étnicas e os países; a incidência chega a variar em torno de 25 vezes em todo o mundo, sendo que normalmente esta neoplasia apresenta-se mais prevalente em países desenvolvidos, onde a sobrevida é considerada boa, ficando em torno de 76%; entretanto, em países em desenvolvimento é de apenas 45% (PARKIN et al., 2005). No Brasil, para 2017, são esperados cerca de 61 mil novos casos, fazendo do câncer de próstata o mais incidente em homens no país, após o câncer de pele do tipo não melanoma (INCA, 2015). A grande maioria dos casos desta neoplasia, assim como ocorre em outras neoplasias, é esporádico e apenas cerca de 10% dos casos são classificados como familiais (NAROD, 1998).

O surgimento deste câncer é um processo que ocorre em vários estágios. Inicia-se, normalmente, a partir de um epitélio proliferativo ou atrófico, mas aparentemente benigno e a progressão para adenocarcinoma de próstata geralmente ocorre através de estágios histologicamente definidos, tais como hiperplasia adenomatosa atípica e neoplasia intraepitelial prostática de alto grau

(LUO; YU, 2003). No entanto, o conhecimento do desenvolvimento do câncer de próstata está longe de ser completamente elucidado, principalmente devido à sua heterogeneidade, tanto morfológica quanto clínica, ou seja, os tumores variam de forma que abrangem desde o desenvolvimento e progressão lenta, permanecendo muitas vezes na sua forma latente, a formas altamente agressivas e metastáticas (CRAWFORD, 2009).

O câncer de próstata é geralmente considerado multifocal, isto é, a glândula da próstata parece ser um local de múltiplos focos de transformação maligna, que muitas vezes são geneticamente distintos (MEHRA et al., 2007). Muitos destes focos dão origem à doença latente, ou seja, não apresentam manifestações clínicas. Aparentemente a maior parte dos focos tumorais latentes pode não sofrer ativação para se transformarem em doença clínica, ou então, podem permanecer em um estado subclínico. É concebível que a forma clínica inicia-se de um processo com diferentes alterações genéticas (SHEN; ABATE-SHEN, 2010).

Estudos a partir de autópsias de homens que morreram de outras causas, que não câncer de próstata, demonstraram que esta doença é frequentemente encontrada nesses homens, sendo que a incidência aumenta com a idade (STAMATIOU et al., 2006; POWELL et al., 2010). Um dado surpreendente é que cerca de 8-11% dos homens entre 20-29 anos avaliados nestes estudos possuíam a doença. Esses dados refletem o período de latência e o desenvolvimento lento deste tipo tumoral (POWELL et al., 2010). A presença de células prostáticas tumorais em 27-48% dos espécimes de cistoprostatectomia de pacientes diagnosticados com câncer de bexiga também reforçam essas evidências (PETTUS et al., 2007; GAKIS et al., 2010).

Embora apenas uma pequena proporção dos tumores prostáticos mostrem invasão e metástases a distância (LUO; YU, 2003), a forma avançada da doença tem a tendência de desenvolver metástase óssea, e esta é a principal responsável pelas taxas de morbidade e mortalidade. Apesar da relevância clínica da metástase óssea, os mecanismos moleculares que a envolvem ainda não são bem compreendidos, sendo que esta lacuna é devida, em parte, à dificuldade de obtenção de pacientes com câncer metastático, bem como à falta de modelos *in vivo* que mimetizem este processo (SHEN; ABATE-SHEN, 2010).

Aproximadamente 95% dos cânceres de próstata são adenocarcinomas, sendo que carcinomas epidermóides e sarcomas são infrequentes (GRIGNON, 2004; BRACARDA et al., 2005). Os tumores de próstata acometem predominantemente a região posterior da glândula; a grande maioria, cerca de 70-75%, localizam-se na zona periférica da glândula, 15% aparecem na zona central e 10-15% na zona transicional (BRACARDA et al., 2005; JOSHUA et al., 2008).

Os hormônios têm um papel fundamental na biologia da próstata. Os andrógenos são necessários para o desenvolvimento, crescimento e função da glândula e os estrógenos também podem afetar seu crescimento e diferenciação. Os andrógenos sozinhos ou em combinações com estrógenos podem apresentar um papel fundamental na tumorigênese e progressão do câncer de próstata. Durante o curso clínico da doença pode existir uma transição de tumor andrógeno responsivo para um tumor andrógeno não responsivo, sendo que o câncer de próstata refratário a andrógeno geralmente tem um pior prognóstico e é menos sensível à terapia (LUO; YU, 2003; CUNHA et al., 2004). O principal hormônio que tem um importante papel sobre o desenvolvimento desta neoplasia é a testosterona, devido à sua atuação na via do receptor de andrógeno, o qual está diretamente envolvido na regulação de proliferação celular da próstata (CRAWFORD, 2009). A testosterona normalmente encontra-se ligada à albumina ou à globulina ligadora de hormônio sexual (SHBG); o complexo testosterona-SHBG é desfeito e a testosterona livre é internalizada pela célula onde é convertida em di-hidrotestosterona (DHT) pela enzima 5α-reductase, a DHT é a forma mais ativa do hormônio, com afinidade cinco vezes maior ao receptor de andrógeno (AR) que a testosterona. A di-hidrotestosterona se liga ao AR, o qual é dimerizado, fosforilado e pode se ligar a elementos de resposta ao andrógeno de genes alvos, que levam a respostas biológicas como crescimento e sobrevivência celular e produção de PSA (Figura1) (FELDMAN; FELDMAN, 2002).

O câncer de próstata é uma doença complexa, sendo que o acompanhamento e o tratamento da doença enfrentam vários desafios. Os maiores desafios clínicos são a capacidade de discriminar o câncer latente da forma agressiva da doença; a identificação da doença localizada em estágios iniciais, uma vez que os sintomas aparecem apenas em estágios mais avançados; identificação do câncer de próstata hormônio resistente; e a propensão a ocorrer metástase

óssea. Entre os desafios envolvidos no tratamento estão a escolha entre prostatectomia radical, radioterapia e vigilância ativa para câncer localizado e a escolha do tratamento para câncer de próstata metastático (SHEN; ABATE-SHEN, 2010; ENDZELIŅŠ et al., 2016).

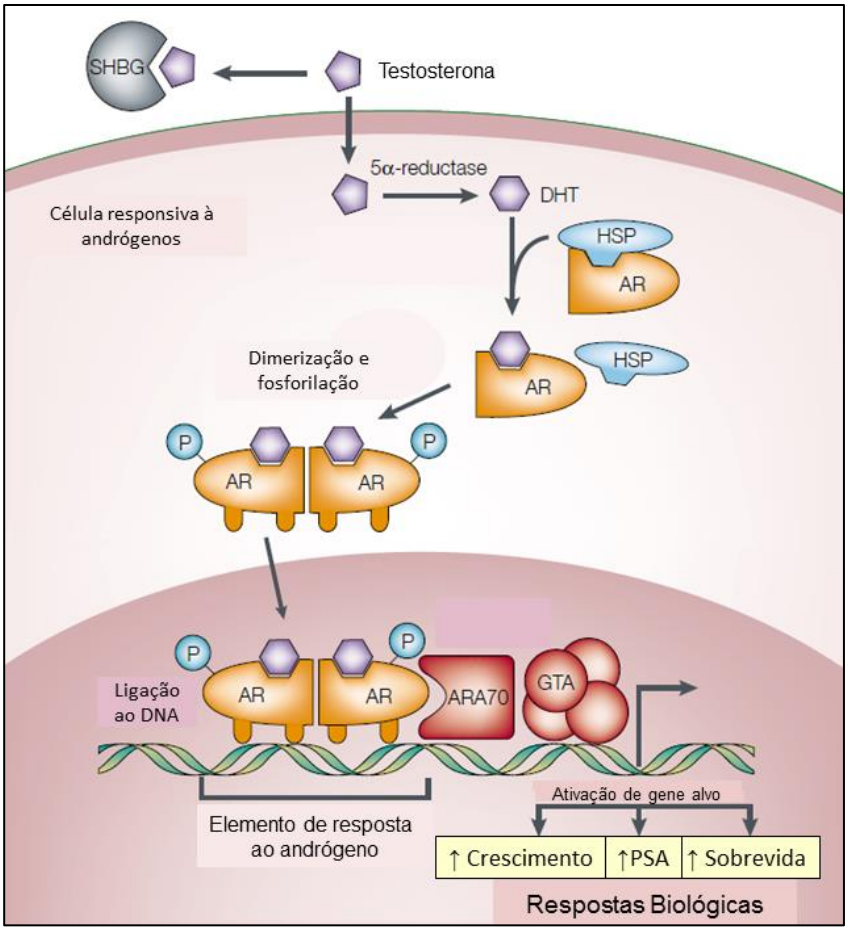

Figura 1. Esquema representando os principais passos da via de andrógenos (Adaptado de FELDMAN; FELDMAN, 2002).

O diagnóstico deste câncer é baseado no exame de espécimes histopatológicos ou citológicos da glândula. A maneira mais comum de se obter a amostra de tecido é por várias biópsias transretais sistemáticas, com orientação por ultrassonografia transretal (DAMBER; AUS, 2008). Ao ser diagnosticada a doença, o tratamento inclui retirada cirúrgica da próstata, radioterapia ou bracterapia. Nos casos de câncer avançado, este tratamento é seguido ou precedido por terapia de privação de andrógenos ou castração (SHEN; ABATE-SHEN, 2010).

O método de classificação histológica mais amplamente utilizado para esses tumores é o escore de Gleason (GLEASON, 1992), calculado a partir da análise histológica do tecido prostático, em que são atribuídos graus numéricos de 1 a 5 baseados no grau de diferenciação histológica do tecido (Figura 2). O escore de Gleason é determinado a partir da soma do primeiro e segundo graus de diferenciação mais frequentes, variando de 2 a 10.

A extensão da doença é classificada de acordo com a classificação de tumores malignos (TNM), que considera três parâmetros: tumores localizados a avançados (T0-T4); sem (N0) ou com invasão de linfonodos (N1) e ausência (M0) ou presença de metástase (M1a-c) (Figura 3) (INCA, 2004; THOMPSON et al., 2007).

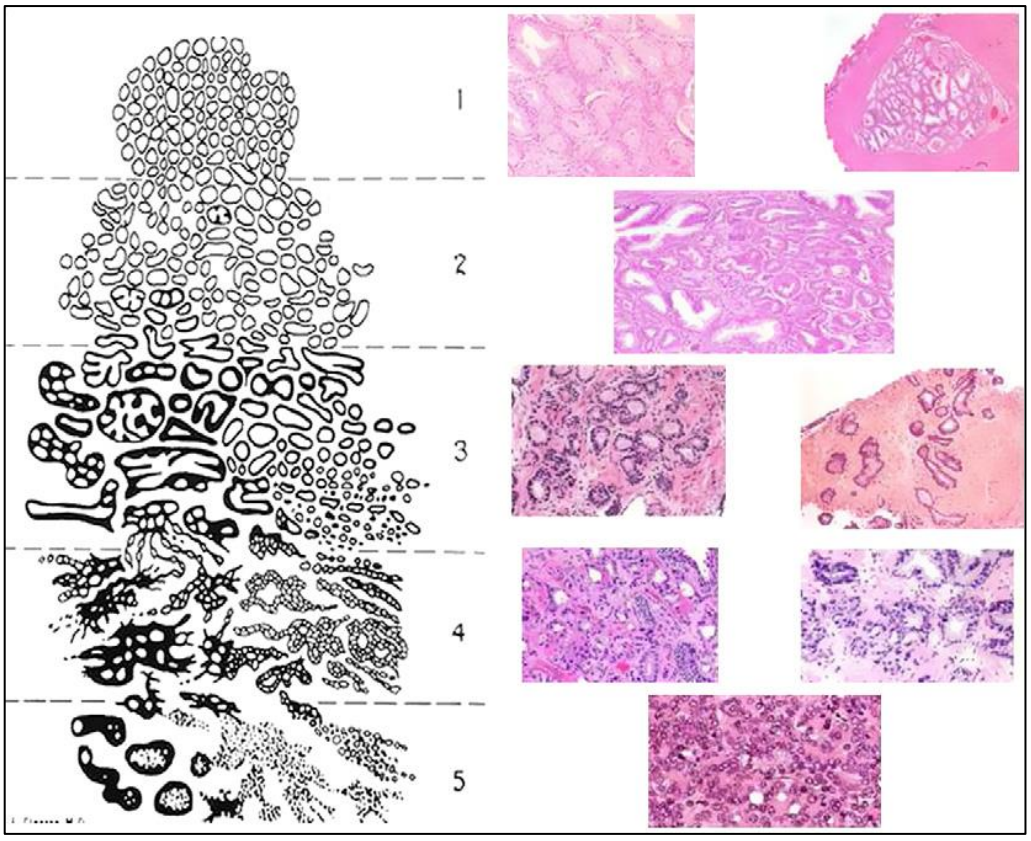

Figura 2. Cinco graus aplicados a padrões de diferenciação histopatológica da próstata para cálculo do escore de Gleason. Grau 1: Muito bem diferenciado; 2: Bem diferenciado; Grau 3: Moderadamente diferenciado; Grau 4: Pouco diferenciado; Grau 5: Indiferenciado (NGUYEN; SARKAR; JAIN, 2012).

TNM - Classificação Clínica	
T - Tumor Primário	
TX	O tumor primário não pode ser avaliado
T0	Não há evidência de tumor primário
T1	Tumor não diagnosticado clinicamente, não palpável ou visível por meio de exame de imagem
T1a	Achado histológico incidental em 5% ou menos de tecido ressecado
T1b	Achado histológico incidental em mais de 5% de tecido ressecado
T1c	Tumor identificado por biópsia por agulha (p. ex., devido a PSA* elevado)
T2	Tumor confinado à próstata
T2a	Tumor que envolve uma metade de um dos lobos ou menos
T2b	Tumor que envolve mais da metade de um dos lobos, mas não ambos os lobos
T2c	Tumor que envolve ambos os lobos
T3	Tumor que se estende através da cápsula prostática
T3a	Extensão extracapsular (uni- ou bilateral)
T3b	Tumor que invade vesícula(s) seminal(ais)
T4	Tumor está fixo ou invade outras estruturas adjacentes, que não as vesículas seminais: colo vesical, esfíncter externo, reto, músculos elevadores do ânus, ou parede pélvica
N - Linfonodos Regionais	
NX	Os linfonodos regionais não podem ser avaliados
N0	Ausência de metástase em linfonodo regional
N1	Metástase em linfonodo regional
M - Metástase à Distância	
MX	A presença de metástase à distância não pode ser avaliada
M0	Ausência de metástase à distância
M1	Metástase à distância
M1a	Linfonodo(s) não regional(ais)
M1b	Osso(s)
M1c	Outra(s) localização(ões)

Figura 3. Classificação clínica de tumores malignos (TNM) (INCA, 2004).

2.1.1. Fatores de risco

O câncer de próstata é uma doença multifatorial e, portanto, torna-se difícil saber exatamente as causas de seu desenvolvimento. Dentre os fatores de risco para esta neoplasia, os três mais determinantes são idade avançada, etnia e hereditariedade (HEIDENREICH et al., 2011).

Um dos mais importantes fatores de risco é a idade, pois a incidência deste tipo tumoral aumenta de acordo com a idade (GRÖNBERG, 2003; BRACARDA et al., 2005; BECHIS et al., 2011). Esta neoplasia é rara em homens com menos de 45 anos, com incidência de 0,4 a cada 100.000 homens/ano; na faixa etária dos 45 aos 54 anos a incidência é de 6 por 100.000 homens/ano; dos 55 a 64 anos o número de casos sobe para 60 e aumenta para 270 por 100.000 homens/ano para homens com mais de 65 anos (BRACARDA et al., 2005).

A etnia também apresenta grande influência neste câncer. Os asiáticos são os que apresentam o menor risco, seguidos pelos caucasianos. Já os homens negros são os que apresentam o risco mais elevado e têm quase duas vezes mais chances de desenvolver a doença e cerca de duas vezes e meia mais chances de morrer devido à essa neoplasia do que os homens caucasianos (CRAWFORD, 2009; JONES et al., 2008).

O histórico familiar de câncer também tem sido relatado como um forte fator de risco, sendo que o grau de risco aumenta conforme o número de parentes em primeiro grau afetados e a idade em que tiveram o diagnóstico, ou seja, quanto mais precoce a idade em que o parente for diagnosticado com câncer de próstata, maior o risco (BRATT, 2002).

Estudos indicam que o risco de desenvolver câncer de próstata pode ser fortemente influenciado pela nutrição e/ou balanço energético durante a infância ou adolescência. Além disso, a suscetibilidade a tais exposições também pode variar entre diferentes grupos étnicos (KOLONEL et al., 2004). Estudos relacionam o aumento de risco de desta doença com ingestão de gordura e uso de tabaco (PLASKON et al., 2003; LOPHATANANON et al., 2010) e diminuição do risco com o consumo de alimentos com licopeno e de vegetais crucíferos (COHEN; KRISTAL; STANFORD, 2000; WCRF; AICR, 2007). Outros fatores como atividade física (FRIEDENREICH; ORENSTEIN, 2002; MILES, 2007), consumo de carne e ovos

(RICHMAN et al., 2010), deficiência de algumas vitaminas e micronutrientes (LIPPMAN et al., 2009); vasectomia (LIU et al., 2015), consumo de álcool (DAMBER; AUS, 2008; ZHAO et. al., 2016; BRUNNER et al., 2017), entre outros, já foram estudados e os resultados não são conclusivos.

2.1.2. Triagem para o câncer de próstata

O principal objetivo da triagem para o câncer de próstata é detectar a doença em seu estágio precoce resultando, assim, em redução da mortalidade e morbidade. Uma triagem eficiente que detecte a doença localizada no seu estágio inicial pode permitir uma diminuição dos índices de mortalidade e também direcionar o tratamento destes pacientes (GOMELLA et al., 2011).

Por muitos anos a triagem para o câncer de próstata era realizada pelo exame do Toque Digital Retal (do inglês, DRE), entretanto, foi demonstrado que este exame apresentava variação entre os avaliadores e detectava apenas o câncer no seu estágio mais avançado (SMITH; CATALONA, 1995). Posteriormente, com a descoberta da glicoproteína denominada Antígeno Prostático Específico (PSA) (WANG et al., 1979), esta foi introduzida como um marcador de soro para o câncer de próstata (STAMEY et al., 1987) e demonstrou ser mais sensível que o toque digital retal (SCHRÖDER et al., 1998). O teste do PSA começou a ser amplamente usado no fim da década de 80 e início dos anos 90 em vários países como Estados Unidos, Canadá, Inglaterra, Austrália e também no Brasil (ANDRIOLE et al., 2009; BAADE; YOULDEN; KRNJACKI, 2009). Atualmente a triagem para este câncer é realizada utilizando-se dois exames, DRE e PSA, os quais são complementares, e em caso de anormalidades é indicada a biópsia (THOMPSON et al., 2007). A confirmação do diagnóstico de câncer é dada pelas avaliações histopatológicas decorrentes da biópsia (DAMBER; AUS, 2008).

Apesar do teste do PSA ser amplamente usado, sua eficácia começou a ser discutida, pois apresenta sensibilidade de 80%, mas os valores de especificidade estão em torno de 37% (SALAMI et al., 2013). Com isso, este marcador não abrange todos os tipos tumorais e é incapaz de distinguir a forma latente da forma agressiva do tumor (STROPE; ANDRIOLE, 2010). Além disso, o teste pode apresentar alterações nos casos de hiperplasia prostática benigna, prostatites ou cistites,

trauma perineural e cirurgias do trato urinário (BAADE; YOULDEN; KRNJACKI, 2009; HOFFMAN et al., 2011). Devido a estes fatos, este teste gera muitos resultados falso-positivos, e também apresenta falsos negativos, pois cerca de 15% dos homens com nível PSA normal apresentam câncer de próstata (THOMPSON et al., 2004).

Um dos principais fatores observado com a inclusão do teste do PSA na triagem foi o aumento significativo de casos de câncer de próstata. Estudos indicam que ocorreu um “super-diagnóstico”, ou seja, que muitos homens que possuíam a forma latente do tumor e não precisavam de exames complementares de diagnóstico, como biópsia, e tratamento, foram diagnosticados e tratados desnecessariamente (WELCH; ALBERTSEN, 2009; GOMELLA et al., 2011; KLOTZ, 2013). Um exemplo disso é a estimativa nos Estados Unidos de que apenas 22% dos casos indicados para biópsia possuíam de fato câncer de próstata (VAN NESTE, 2012). Um modelo matemático indica que cerca de 23 a 42% dos cânceres detectados pelo teste do PSA são “super-diagnosticados” (DRAISMA et al., 2009).

Basicamente três fatores promovem o excesso de diagnóstico deste câncer: a existência relativamente grande de homens com a doença latente; as dificuldades para a identificação destes pacientes e a indolente história natural da mortalidade específica por câncer de próstata (WELCH; BLACK, 2010; SANDHU; ANDRIOLE 2012).

Todos estes fatores fortalecem as discussões sobre a efetividade e o custo-benefício do uso do teste do PSA. Foram publicados dois importantes estudos randomizados para avaliar a eficácia deste teste: o estudo europeu ERSPC (*European Randomized Study of Screening for Prostate Cancer*), com duração de 11 anos, demonstrou que a triagem utilizando o teste do PSA apresentou uma redução moderada na mortalidade (SCHRÖDER et al., 2012) e o estudo norte-americano PLCO (*Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial*), com duração de 13 anos, não encontrou evidências de que este teste tenha implicado em uma redução da mortalidade (ANDRIOLE et al., 2012). Outro estudo em populações menores observou moderadas taxas de redução da mortalidade (HUGOSSON et al., 2010). Entretanto, o preço da redução das mortes relatada nestes estudos é o “super-diagnóstico” desta neoplasia e o tratamento dispensável de milhares de pacientes todos os anos (KLOTZ, 2013). Nos Estados Unidos dados apontam que

mais de um milhão de homens foram diagnosticados e tratados desnecessariamente após a introdução do teste do PSA (WELCH; ALBERTSEN, 2009), fazendo com que o custo-benefício deste teste entrasse em discussão, resultando em recomendações contra seu uso para triagem pela U.S. Preventive Services Task Force (MOYER, 2012). As mesmas diretrizes foram adotadas, em 2013, no Brasil (INCA 2014). Entretanto, Gulati et al. (2014) demonstraram que descontinuar o uso do teste do PSA para todos os homens pode contribuir para a ocorrência de muitas mortes que seriam evitadas, e sugeriram que interromper a triagem a partir dos 70 anos reduziria drasticamente o “super-diagnóstico” em comparação com o contínuo uso do teste do PSA para todas as idades e, ainda, conseguiria impedir aproximadamente 50% da mortes por câncer de próstata que são evitáveis.

Com isso, há necessidade de se desenvolver novos marcadores a fim de tornar o diagnóstico desta doença mais preciso (SHEN; ABATE-SHEN, 2010; TORRE et al., 2015). No entanto, até que uma ferramenta personalizada confiável de avaliação de risco se torne disponível, o teste do PSA deve ser utilizado com cautela e, quando possível, associado a outros marcadores (ROOBOL; CARLSSON, 2013).

2.2. MARCADORES MOLECULARES PARA O CÂNCER DE PRÓSTATA

Biomarcadores são moléculas cuja detecção ou avaliação fornece informações sobre a doença para além dos parâmetros clínicos padrão que são observados pelo médico (PRENSNER et al., 2012). Os biomarcadores são proteínas, metabolitos, RNAs ou DNAs que podem ser detectados em amostras de tecidos de pacientes, obtidos por biópsia ou ressecção cirúrgica, ou de forma minimamente invasiva, por meio do isolamento de células e/ou moléculas, a partir de fluidos corporais, tais como sangue ou urina. Os fatores que podem ser avaliados são diversos, tais como mutações pontuais, amplificações, deleções, modificações epigenéticas, expressão diferencial de RNAs e proteínas, entre outros (SIDRANSKY, 2002; PRENSNER et al., 2012).

O biomarcador ideal para uso clínico deve ter as seguintes características: mensuração segura e fácil; preferencialmente minimamente invasivo; altamente sensível e específico, com valores preditivos positivos e negativos elevados; e que,

em conjunto com os parâmetros clínico-patológicos, melhorem a tomada de decisão. Devido esses múltiplos fatores, há dificuldades na validação de novos biomarcadores para diagnóstico e prognóstico de cânceres (KULASINGAM; DIAMANDIS, 2008).

Os primeiros marcadores para câncer eram baseados nas observações clínicas e patológicas. Os estudos em neoplasias e em lesões pré-neoplásicas indicaram que as alterações genéticas nas fases iniciais da tumorigênese podem ser utilizadas para detectar e também acompanhar o desenvolvimento de tumores, ou seja, serem usadas como marcadores moleculares tumorais (SIDRANSKY, 2002).

A introdução de marcadores moleculares para o diagnóstico e tratamento da doença revolucionou a prática oncológica. Com a recente evolução da biologia molecular o tratamento para o câncer está começando a mudar da abordagem tradicional para uma abordagem individualizada. Isto é possível devido ao desenvolvimento de marcadores (i) de diagnóstico que podem separar de forma eficaz indivíduos com a doença dos que não possuem a doença; (ii) de prognóstico, que podem separar de forma confiável pacientes com doença latente daqueles com formas agressivas, direcionando, assim, seus tratamentos; (iii) que preveem a resposta ou resistência a terapias específicas; (iv) que identificam os pacientes propensos a apresentar efeitos colaterais tóxicos graves; e (v) para monitoramento e progressão da doença (DUFFY; CROWN, 2008). Na prática clínica, poucos tipos de câncer possuem algum dos tipos de marcadores supracitados, portanto, são de extrema importância pesquisas nessa área que ampliem esses marcadores para todos os tipos de câncer (DUFFY; CROWN, 2008; KULASINGAM; DIAMANDIS, 2008).

A busca de um biomarcador para o câncer de próstata deve ser direcionada para a detecção da doença de alto risco e potencialmente curável (ROOBOL; CARLSSON, 2013). Os biomarcadores para este câncer, por conveniência, deveriam ser testes não-invasivos com o intuito de substituir a biópsia como o diagnóstico "padrão ouro". Procedimentos de biópsia apresentam um risco de eventos como hemorragia e sepse; além disso, aproximadamente em 20% dos casos o câncer é detectado apenas na segunda biópsia, provavelmente devido à amostragem ineficiente (ROEHL; ANTENOR; CATALONA, 2002; MOUSSA et al., 2010). Outro problema é que muito frequentemente a somatória de Gleason da

biópsia não é igual à da prostatectomia radical, podendo gerar erros na conduta terapêutica (LATTOUF; SAAD, 2002; SVED et al., 2004). Todos esses fatores contribuem para a busca de biomarcadores do tipo minimamente invasivos, utilizando-se amostras biológicas como sangue e urina, que têm o potencial de aprimorar o procedimento de biópsia. Embora não possam fornecer informações histopatológicas ainda assim poderão melhorar a prática clínica em um futuro próximo (PRENSNER et al., 2012).

A biologia molecular relacionada ao desenvolvimento e progressão do câncer de próstata é complexa e envolve diversas vias, abrindo um leque de possibilidades para o desenvolvimento de um novo marcador; entretanto, a complexidade da doença dificulta o descobrimento de um único marcador especifico. Assim, acredita-se que biomarcadores múltiplos serão necessários para realizar a triagem, diagnóstico e prognóstico da doença, e, também, predição de resposta ao tratamento (KULASINGAM; DIAMANDIS, 2008; PRENSNER et al., 2012). Estudos estão em andamento para avaliar novos testes, com o intuito de identificar homens com maior risco de desenvolver esta neoplasia e permitir a utilização mais eficiente do teste do PSA (TORRE et al., 2015). Entretanto, apesar dos esforços da comunidade científica para encontrar um marcador especifico e eficiente, isso ainda não foi possível, estimulando assim, a continuidade de estudos nesta área (REYNOLDS et al., 2007; SCHROEDER, 2008; SHEN; ABATE-SHEN, 2010; PENTYALA et al., 2016).

2.3. ÁCIDOS NUCLEICOS CIRCULANTES

Muitas moléculas biológicas diferentes originadas de quase todos os tecidos do corpo podem ser encontradas nos fluidos corporais humanos, tais como plasma, soro e urina (WITTMANN; JACK, 2010). Os ácidos nucléicos circulantes, do inglês *cell-free nucleic acid* (cfNAs), são moléculas de DNA e RNA que se encontram de forma livre, associados com proteínas ou dentro de microvesículas, em especial exossomos, no plasma ou soro de indivíduos sadios e doentes. Nestes últimos estão presentes em maiores concentrações (SCHWARZENBACH; HOON; PANTEL, 2011; SCHWARZENBACH et al., 2014) (Figura 4).

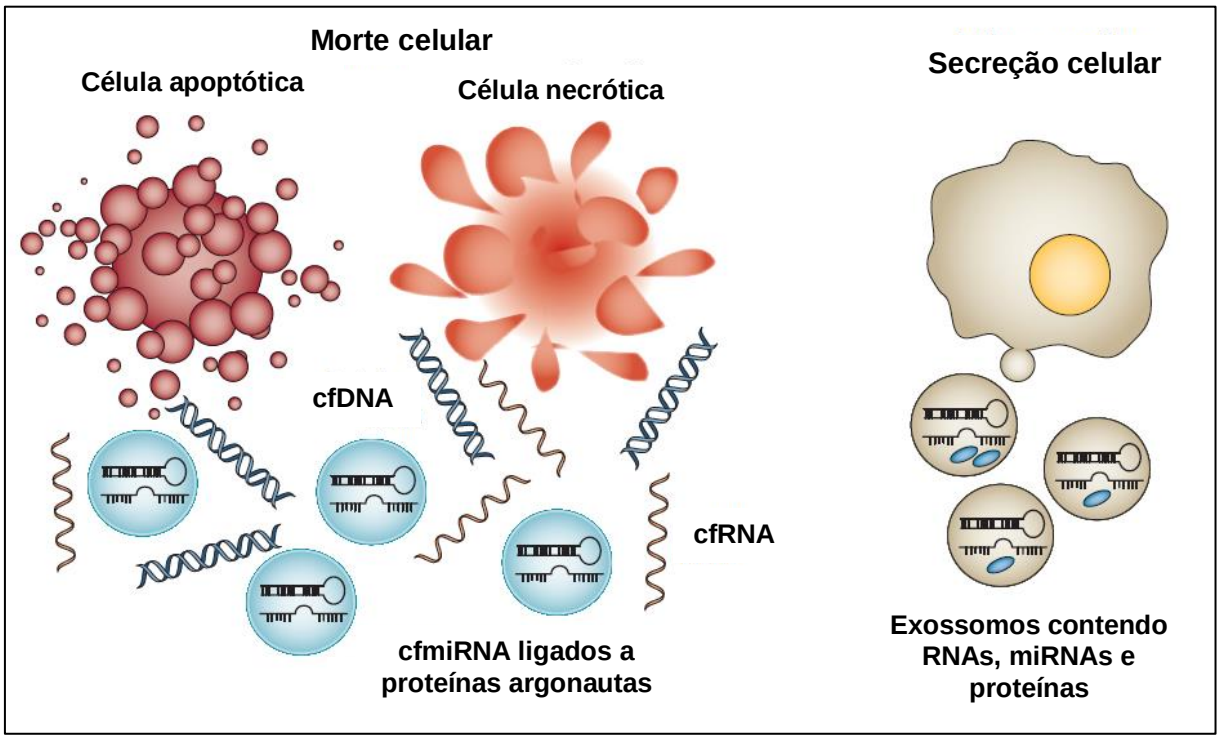

Figura 4. Esquema das vias de liberação dos cfNAs no sangue. Os cfNAs podem ser liberados via apoptose, necrose e secreção celular. Os mesmos podem estar de forma livre no plasma/soro, em exossomos ou associados a proteínas (Schwarzenbach et al., 2014).

Os exossomos representam um modo de comunicação independente de células. São mensageiros extracelulares muito importantes para a transmissão de informações célula a célula, muitas vezes por longas distâncias. São vesículas pequenas, entre 30-100nm de diâmetro, de origem endocítica, que são liberados para o ambiente extracelular por meio de fusão de corpos multivesiculares com a membrana plasmática. Estão envolvidos em diversos sinais celulares, incluindo o de metástase (LAKKARAJU; RODRIGUEZ-BOULAN, 2008).

Os processos de carcinogênese e progressão tumoral são complexos e estão associados a inúmeras alterações genéticas e epigenéticas (JERÓNIMO et al., 2011). Embora o uso das proteínas como marcadores pareça simples, nem sempre o é; existem vários desafios para definir um marcador proteico, principalmente a dificuldade de desenvolver reagentes de elevada afinidade para a captura de proteínas. Em consequência disto, a procura por meios pouco ou não invasivos, levou ao grande interesse no campo de ácidos nucleicos circulantes no plasma e no soro (WITTMANN; JACK, 2010). Desta forma, os cfNAs podem ser excelentes marcadores para o câncer, pois podem ser mais informativos, específicos e

apresentarem maior acurácia que os marcadores proteicos (SCHWARZENBACH; HOON; PANTEL, 2011).

O primeiro relato da presença dos cfNAs no sangue humano aconteceu em 1948 por Mandel e Métais. Em 1977, Leon et al. sugeriram que os níveis de DNA encontrados no soro poderiam ser uma importante ferramenta para avaliar resposta à radioterapia ou comparar diferentes protocolos de tratamento. Todavia, foi na década de 80 que surgiram os primeiros relatos indicando uma relação entre o aparecimento dessas moléculas no plasma/soro com neoplasias (SHAPIRO et al., 1983; STROUN et al., 1989). Na década de 90 os cfNAs ganharam maior atenção; diversos estudos relataram que a presença de alterações genéticas e epigenéticas nestas moléculas estavam associadas a tumores (SORENSON et al., 1994; VASIOUKHIN et al.,1994; NAWROZ et al., 1996; ANKER et al., 1999). Kopreski et al. (1999) detectaram pela primeira vez moléculas de RNA livres no plasma. Entretanto, apenas na ultima década os cfNAs tiveram sua importância reconhecida pela comunidade cientifica. Várias pesquisas têm relacionado os ácidos nucleicos circulantes com várias doenças, incluindo vários tipos tumorais, como mama, pulmão, bexiga e próstata (TONG; LO, 2006; SCHWARZENBACH; HOON; PANTEL, 2011).

Atualmente, acredita-se que estas moléculas encontradas no sangue de pacientes com câncer são derivadas de células tumorais necróticas ou apoptóticas (SCHWARZENBACH; HOON; PANTEL, 2011; SCHWARZENBACH et al., 2014). Também foi sugerido que esses ácidos nucleicos podem ser secretados por células tumorais, sendo que a proporção de cfNAs liberados depende do tamanho e estado do tumor (SCHWARZENBACH; HOON; PANTEL, 2011; SCHWARZENBACH et al., 2014). Diehl et al. (2005) estimaram que em um paciente com um tumor de 100g, aproximadamente 3,3% dos ácidos nucleicos tumorais poderia cair diariamente na circulação sanguínea.

Os estudos envolvendo os cfNAs se concentram em alterações genéticas no DNA (mutações pontuais, amplificações, alterações em microssatélites, integridade de DNA), em alterações epigenéticas (alteração no padrão de miRNA e de metilação de DNA), alterações no DNA mitocondrial e na expressão gênica (BREMNES; SIRERA; CAMPS, 2005; SWARUP; RAJESWARI, 2007; SCHWARZENBACH; HOON; PANTEL, 2011). A identificação da expressão diferenciada dos cfmRNAs (do

inglês *cell-free mRNA*) e dos cfmiRNAs (do inglês *cell-free miRNA*) pode ser realizada por RT-qPCR (do inglês, *real-time reverse transcription polymerase chain reaction*) e microarrays (O'DRISCOLL et al., 2008).

Apesar de sua função biológica não estar completamente estabelecida (TURCHINOVICH; TONEVITSKY; BURWINKEL, 2016), os cfNAs podem ser utilizados como uma “biópsia líquida” para diversos tipos de doenças, incluindo o câncer. A facilidade de obtenção da amostra permite, por exemplo, o acompanhamento da doença ou tratamento. No entanto, ainda não estão bem esclarecidos os eventos fisiológicos que levam ao aumento de cfNAs no sangue durante o processo de tumorigênese (SCHWARZENBACH; HOON; PANTEL, 2011).

A avaliação histológica dos tecidos tumorais obtidos a partir de biópsias é o "padrão ouro" de diagnóstico, mas a maioria dos estudos normalmente realiza estas avaliações apenas uma vez. Os cfNAs permitem avaliar tumores primários e metastáticos do mesmo paciente e verificar como estes podem variar em níveis genômicos, epigenômicos e transcriptômicos. Portanto, os ensaios envolvendo estas moléculas permitem a monitorização repetitiva destes eventos usando amostras de sangue. Também podem ser mais eficientes para avaliar a progressão do câncer em doentes dos quais tecidos tumorais não podem ser avaliados (SCHWARZENBACH; HOON; PANTEL, 2011). Com isto, os cfNAs têm um potencial para revolucionar os diagnósticos atuais de doenças como o câncer (LO, 2001; TONG; LO, 2006).

Dentre os tipos de cfNAs, acreditava-se que os cfRNAs seriam degradados rapidamente ao cair na circulação sanguínea, mas estes são surpreendentemente estáveis no sangue, mesmo em pacientes com câncer, onde a quantidade de RNases é aumentada (SWARUP; RAJESWARI, 2007). Estudos recentes têm demonstrado que os cfRNAs podem ser protegidos por exossomos, tais como micropartículas, microvesículas ou multivesículas (COCUCCI; RACCHETTI; MELDOLESI, 2009; OROZCO; LEWIS, 2010). Assim como os cfmRNAs, os cfmiRNAs presentes no plasma são muito estáveis (BALZANO et al., 2015). Mitchell et al. (2008) demostraram que sessões de congelamento e descongelamento do plasma ou incubação deste a temperatura ambiente por até 24h têm efeitos mínimos nos níveis de cfmiRNAs. Entretanto, quando adicionaram miRNA sintéticos no plasma, os mesmos foram rapidamente degradados, provando que existe atividade RNase no plasma e que os cfmiRNAs endógenos são protegidos deste tipo de

degradação. A principio acreditava-se que a estabilidade dos cfmiRNA era devido á sua associação com os exossomos, mas estudos recentes demostraram que 95-99% dessas moléculas são livres de vesículas e encontram-se associadas apenas a proteínas argonautas (AGO) e ribonucleoproteínas ou ainda a lipídios de alta densidade (TURCHINOVICH; TONEVITSKY; BURWINKEL, 2016).

Em câncer de próstata, os estudos envolvendo cfmRNAs são escassos. Um estudo realizado por March-Villalba et al. (2012) demonstrou que o mRNA transcrito a partir do gene da telomerase (hTERT), encontrado em plasma, pode ser um bom marcador para diagnóstico de câncer de próstata, pois mostra sensibilidade maior que o teste do PSA. Já estudos envolvendo os miRNAs circulantes são mais frequentes na literatura, indicando alguns miRNAs como potenciais marcadores para esta doença (CHENG, 2015; KACHAKOVA et al., 2015).

Deste modo, atualmente o uso de cfNA em diversas análises, incluindo expressão gênica para comparar indivíduos sadios e portadores de neoplasia, vem sendo considerado promissor para o desenvolvimento de novos testes diagnósticos, prognósticos e para terapias mais eficazes. Diversas pesquisas apontam o potencial de uso dessas moléculas como alvo de estudos para esses novos marcadores (JOHNSON; LO, 2002; BREMNES; SIRERA; CAMPS, 2005; TONG; LO, 2006; SWARUP; RAJESWARI, 2007; SCHWARZENBACH; HOON; PANTEL, 2011). Portanto, o investimento nesta metodologia para prevenção e diagnóstico precoce do câncer tem um enorme potencial em reduzir as complicações relacionadas ao desenvolvimento do tumor. Isto possibilitará, assim, uma melhor qualidade de vida desses pacientes e, consequentemente, uma redução dos gastos diretos e indiretos atualmente observados para o combate da doença já em estágios mais avançados (CIMA et al., 2011; ROSS et al., 2011; SCHWARZENBACH; HOON; PANTEL, 2011; CHOUDHURY et al., 2012; CULIG, 2012; SCHWARZENBACH et al., 2014).

2.4. *CELL-FREE* MIRNAS (CFMIRNAS)

Os miRNAs são moléculas de RNA não codificantes de aproximadamente 22 nucleotídeos, envolvidos na regulação pós-transcricional da expressão gênica (JANSSON; LUND, 2012; ACUNZO et al., 2015). O primeiro relato do primeiro pequeno RNA não codificante foi feito por Lee et al. (1993), que descreveram que o

gene *lin-4* codificava um miRNA que controlava o gene *lin-14*, em *Caenorhabditis elegans*. No ano 2000, Reinhart et al. identificaram outro miRNA, o let-7, também em nematoides. A partir de 2001 outros artigos foram publicados e, pela primeira vez, o termo microRNA foi utilizado (LAGOS-QUINTANA et al., 2001; LAU et al., 2001; LEE; AMBROS, 2001). Nos anos subsequentes várias publicações envolvendo diversos tipos de plantas e animais surgiram, incluindo publicações relacionadas com doenças humanas, em especial o câncer.

Os miRNAs têm um papel importante em praticamente todas as vias biológicas de mamíferos e de outros organismos multicelulares (JANSSON; LUND, 2012). Os genes de miRNA representam aproximadamente 1% de todo o genoma humano e são evolutivamente conservados entre diferentes espécies (ACUNZO et al., 2015; CHEN et al., 2014). Podem ser encontrados dentro de introns ou éxons de genes codificadores de proteínas (70%) ou nas áreas intergênicas (30%) (ACUNZO et al., 2015).

Os genes de miRNAs são transcritos pela RNA polimerase II em longos miRNAs primários (pri-miRNA). Ainda no núcleo esses pri-miRNAs são processados em um miRNA precursor (pre-miRNA) de aproximadamente 70-80 nucleotídeos pelo complexo Drosha/DGCR8. A Drosha é uma RNAse do tipo III e o DGCR8 uma proteína de ligação ao RNA. O pre-miRNA é exportado para o citoplasma por uma exportina-5, onde a RNase Dicer, em complexo com o TRBP (do inglês, transactivation-responsive RNA-binding protein), o cliva em um duplex de miRNA de 22 nucleotídeos. Em seguida este duplex é desenrolado, a fita com menor estabilidade termodinâmica é degradada e o miRNA maduro é carregado em conjunto com a proteína Argonauta (AGO2) no complexo RISC (complexo de silenciamento induzido por RNA). O miRNA maduro guia o complexo RISC para silenciar o gene alvo. O miRNA regula o mRNA alvo pareando-se perfeita ou imperfeitamente na região 3'UTR do alvo, e pode silenciar o mRNA alvo por clivagem, inibição da tradução ou deadenilação da cauda poliA (Figura 5) (WIENHOLDS; PLASTERK, 2005; WINTER et al., 2009; CHEN et al., 2014; ACUNZO et al., 2015).

De acordo com o banco de dados MirBase, em novembro de 2016 havia 2588 miRNAs identificados em seres humanos e a lista permanece crescendo. MicroRNAs podem ter como alvos várias centenas de mRNAs, o que os torna reguladores muito

poderosos. Análises de bioinformática e estudos experimentais têm demonstrado que mais de 30% dos genes humanos são alvos diretos de miRNAs, o que implica na função dos miRNAs em quase todos os processos biológicos, incluindo regulação do ciclo celular, crescimento celular, apoptose, diferenciação celular e estresse oxidativo (CHEN et al., 2014).

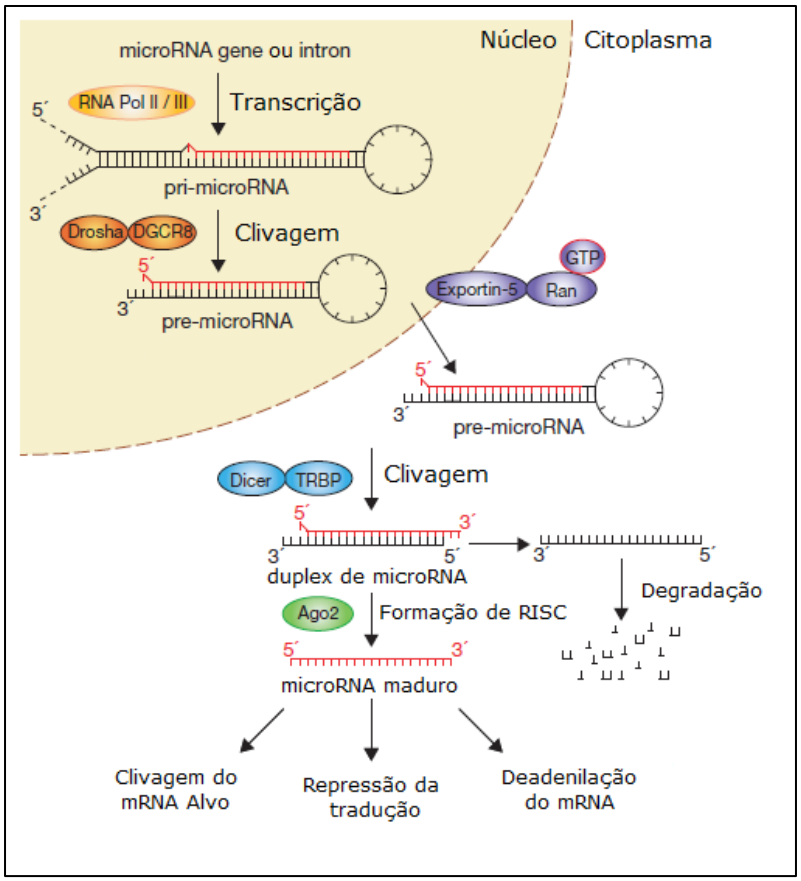

Figura 5: Biogênese dos miRNAs em mamíferos (adaptado de WINTER et al., 2009).

Uma expressão aberrante de miRNA pode perturbar a sinalização celular e influenciar o aparecimento e progressão do câncer (ACUNZO et al., 2015), onde encontram-se maciçamente desregulados, e esta parece ser a regra e não a exceção (CROCE, 2009; CHEN et al., 2014). Em 2002 foi publicado o primeiro estudo de associação entre miRNA e câncer (CALIN et al., 2002) e a partir daí vários estudos foram realizados. Nos últimos anos centenas de estudos foram desenvolvidos para distinguir perfis de miRNA em tecidos tumoral e normal (ACUNZO et al., 2015).

O dados de perfis transcricionais mostraram que os miRNAs são capazes de classificar de forma mais fiel diferentes tipos de tumores que os perfis de mRNA, inclusive para identificar câncer metastático de origem primária desconhecida (WITTMANN; JACK, 2010; JANSSON; LUND, 2012). Entretanto, atualmente não é possível utilizar perfis de miRNAs na ausência de uma massa para biópsia (WITTMANN; JACK, 2010). Portanto, recentemente intensificou-se a busca por marcadores de miRNAs em fluidos corporais como plasma, soro e urina em diversos tipos de câncer (HUANG et al., 2013; SCHWARZENBACH et al., 2014; CHENG, 2015). Apesar dos cfmiRNAs apresentarem um novo e muito atrativo tipo de marcadores para o câncer (ENDZELIŅŠ et al., 2016), os estudos de miRNAs circulantes em câncer de próstata ainda são poucos, havendo aproximadamente 30 artigos na literatura (Tabela 1). Geralmente esses estudos possuem um número amostral pequeno e dentre os candidatos mais promissores estão os miR-141, miR-375, miR-221 e miR-21. Portanto, esta é uma área ainda pouco explorada na ciência e com perspectivas muito boas em relação à descoberta de novos marcadores para fins de aplicação clínica.

Tabela 1: Resumo das publicações de miRNAs circulantes em câncer de próstata

Fluido corporal	Tamanho amostral	Metodologia	miRNAs candidatos	Referência
Plasma	21 CaP	RT-qPCR	miR-141	Gonzales et al., 2011
Plasma	51 CaP, 20 CTR	RT-qPCR	miR-21, miR-221, miR-141	Agaoglu et al., 2011
Plasma	Screening: 25 CaP, 17 HPB Validação: 80 CaP, 44 BPH, 54 CTR	Microarray, RT-qPCR	let-7c, let-7e, miR30c, miR-622, miR-1285	Chen et al., 2012
Plasma	82 CaP	RT-qPCR	miR-21, miR-221	Shen et al., 2012
Plasma	23 CaP, 20 CTR	RT-qPCR	miR-221	Zheng et al., 2012
Plasma	25 CaP, 25 mCRPC	RT-qPCR	miR-16, miR-126, miR-141, miR-151-3p, miR-375	Watahiki et al., 2013
Plasma	Screening: 23 mCRPC Validação: 100 CRPC	Sequenciamento, RT-qPCR	miR-1290, miR-375	Huang et al., 2015

Plasma	59 CaP, 16 HPB, 11 CTR	RT-qPCR	let-7c, miR-30c, miR-141, miR-375	Kachakova et al., 2015
Plasma/ soro	25 CaP, 25 CTR	RT-qPCR	miR-141	Mitchell et al. 2008
Plasma/ soro	78 CaP, 28 CTR; 47 mCaP, 72 CaP	RT-qPCR	miR-107, miR-574-3p; miR-375, miR-141	Bryant et al., 2012
Plasma/ soro	97 CRPC	microarray, RT-qPCR	miR-200b, miR-20a	Lin et al., 2014
Soro	6 CaP, 8 CTR	microarray	vários	Lodes et al., 2009
Soro	Screening: 7mCaP, 14CaP Validação: 45 CaP	RT-qPCR	miR-141 miR-375	Brase et al., 2011
Soro	37 CaP, 8 mPC, 18 HPB, 20 CTR	RT-qPCR	miR-26a, miR-195, miR-let-7i	Mahn et al., 2011
Soro	36 CaP, 12 CTR	RT-qPCR	miR-24, miR-26b, miR-30c, miR-223, miR-874, miR-1274a, miR-1207-5p, miR-93, miR-106a	Moltzahn et al., 2011
Soro	56 CaP, 6 BHP	RT-qPCR	miR-21	Zhang et al., 2011

Soro	24 CaP, 48 CTR	RT-qPCR	miR-21	Sanders et al., 2012
Soro	25 mCRPC, 25 CTR	Microarray, RT-qPCR	hsa-miR-141, hsa-miR-298, hsa-miR-375	Selth et al., 2012
Soro	Screening: 25 mCRPC, 25 CTR Validação:21 mCRPC, 20 CTR	Microarray, RT-qPCR	miR-141, miR-200a, miR-200c, miR-210;miR-375	Cheng et al., 2013
Soro	38 CaP, 40 CTR	RT-qPCR	miR-21, -141	Egidi et al., 2013
Soro	58 CaP, 26 mCRPC	RT-qPCR	miR-375, miR-141, miR-378, miR-409-3p	Nguyen et al., 2013
Soro	Screening: 16 CaP Validação: 70 CTR	Microarray, RT-qPCR	miR-141, miR-146b-3p, miR-194	Selth et al., 2013
Soro	6 HPB, 20 CaP, 30 mCaP	RT-qPCR	miR-141	Zhang et al., 2013
Soro	10 CaP, 10 HPB	RT-qPCR	miR-21, miR-221	Kotb et al., 2014
Soro	Screening: 12 CaP Validação: 72 CaP	Microarray, RT-qPCR	miR-25, miR-101, miR-628-5p	Srivastava et al., 2014

Soro	100 CaP, 50 HPB	RT-qPCR	let-7a, miR-24, -26a, -30c, -93, -103, -106a, -107, -130b, -146a, -223, -451	Mihelich et al., 2015
Soro	146 CaP, 35 HPB, 18 CTR	RT-qPCR	miR-375	Wach et al., 2015

CaP = Câncer de próstata localizado, mCaP = Câncer de próstata metastático, CRPC = Câncer de próstata resistente a castração, mCRPC = Câncer de próstata metastático resistente à castração, HPB = Hiperplasia Prostática Benigna, CTR = Controle.

2.4.1. miRNAs e câncer de próstata

2.4.1.1. miR-133a e miR-133b

A família do miR-133 é composta por miR-133a e miR-133b. Existem poucos estudos na literatura sobre esta família de miRNA em câncer de próstata. Tao et al. (2012) demonstraram que os miR-133a e miR-133b podem regular receptor do fator de crescimento epidérmico (*EGFR*), o qual regula o crescimento, diferenciação, adesão e invasão celulares. Sendo assim, esses microRNAs teriam papel chave na desregulação destes eventos neste tipo tumoral. Entretanto, já foi descrito que o miR-133a apresenta baixa expressão em tecido tumoral de próstata quando comparado com tecido normal (AMBS et al., 2008; KOJIMA et al., 2012). Quando os níveis deste microRNA foram restaurados *in vitro* em linhagens celulares prostáticas tumorais PC-3 e DU145, ocorreu a supressão da proliferação, migração e invasão (KOJIMA et al., 2012; TAO et al., 2012). Um estudo funcional realizado por Kojima et al. (2012) demonstrou que o miR-133a pode ter função de supressor de tumor. Até o momento não foram relatados estudos destes miRNAs em plasma de pacientes com carcinoma prostático.

2.4.1.2. miR-143

O miR-143 é um dos microRNAs mais estudados e foi primeiramente descrito com baixa expressão nas linhagens celulares de câncer de próstata LNCaP e C4-2, quando comparado com linhagens celulares normais. Isso também se mostrou verdadeiro quando analisadas amostras de tecidos tumorais de próstata *versus* normais (CLAPÉ et al., 2014). Este miRNA foi descrito como um supressor tumoral que controla proliferação e a sobrevivência celulares (CLAPÉ et al., 2014). Quando induzida a superexpressão deste miRNA em linhagens celulares de próstata, observou-se a inibição da proliferação e migração e aumento da sensibilidade celular ao docetaxel (XU et al., 2011).

Estudos realizados por Peng et al. (2011) demonstraram uma relação entre baixos níveis de miR-143 e miR-145 com metástase óssea em pacientes com câncer de próstata. Quando esses mesmos autores induziram a superexpressão desses miRNAs em células PC-3, ocorreu um aumento da

expressão de E-caderina e redução da expressão de fibronectina, revelando um fenótipo morfológico menos invasivo. Esta relação entre esses miRNAs e progressão de metástase óssea pode ocorrer potencialmente por repressão de características de células tronco do câncer (HUANG et al., 2012). O miR-143 foi descrito como um candidato a marcador em plasma para osteosarcoma (OUYANG et al., 2013). Em próstata, até o momento, este miRNA não foi indicado como candidato a marcador em plasma.

2.4.1.3. Família do miR-183

A família do miR-183 é composta por miR-96, miR-182 e miR-183 (PIERCE et al., 2008); está superexpressa em tecidos tumorais de próstata e parece contribuir para a carcinogênese da próstata (MIHELICH et al., 2011). miR-182 e miR-183 são os mais estudados desta família.

O miR-182 foi relatado como superexpresso em tecidos e em linhagens celulares tumorais de próstata (LIU et al., 2013; YAO et al., 2016). Também foi descrito que altos níveis deste miRNA estão relacionados com menor sobrevida dos pacientes após prostatectomia radical (HIRATA et al., 2013). O mir-182 comporta-se como um oncogene, sendo que a indução da expressão deste microRNA em células prostáticas normais (RWPE-1) induz a proliferação celular. Em células tumorais, seu silenciamento reduziu a proliferação, migração e invasão celulares (HIRATA et al., 2013), enquanto que sua superexpressão promoveu proliferação, invasão, transição de ciclo celular de G1 para S e, ainda, reduziu a apoptose tardia (LIU et al., 2013).

Este miRNA parece ter um importante papel na proliferação celular (HIRATA et al., 2013; LIU et al., 2013). Em pacientes os níveis de miR-182 estão aumentados tanto em tumores quanto em amostras de urina quando comparados aos controles (CASANOVA-SALAS et al., 2014). Sendo assim, o miR-182 parece ser um bom marcador para câncer de próstata.

O miR-183 foi descrito como superexpresso em câncer de próstata (PIERCE et al., 2008; LARNE et al., 2013; MIHELICH et al., 2011; SCHAEFER et al., 2010) e sua expressão foi correlacionada com níveis elevados de PSA, estadiamento tumoral maior e menor sobrevida global (UENO et al., 2013; LARNE et al., 2015). Em ensaios funcionais a inibição de miR-183 diminuiu o crescimento e a motilidade celular, já em ensaios *in vivo* com ratos "*knockout*"

foi observada diminuição significativa do crescimento do tumor de próstata (UENO et al., 2013).

Um estudo recente demonstrou que o miR-183 tem um papel-chave no câncer de próstata, pois ele tem como alvo o PSA, aumentando diretamente os níveis de mRNA e da proteína. Com isso, é de grande importância melhorar a compreensão dos mecanismos de regulação desta molécula, porque homens com câncer de próstata e baixos níveis de miR-183, possivelmente apresentarão baixos níveis de PSA, e assim, podem passar despercebidos em uma triagem com base no teste do PSA (LARNE et al., 2015). Este miRNA já foi descrito como marcador de tumores uroteliais em urina (YAMADA et al., 2011), abrindo novas possibilidades para testes em outros fluidos corporais.

2.4.1.4. Família miR-200

A família miRNA-200 é composta por cindo membros miR-200a, -200b, -429, -200c, -141. Estes miRNAs encontram-se frequentemente desregulados em câncer e estão relacionados com regulação da transição epitélio-mesenquimal (EMT, do inglês, epithelial-mesenchymal transition), plasticidade celular, apoptose, crescimento celular e medeiam metástase a distância (GREGORY et al., 2008; ANTOLÍN et al., 2015). Em câncer de próstata, os mais estudados são o mir-141, -200b e -200c.

O miR-141 é o mais extensamente estudado e possui alta expressão em câncer de próstata. Como cfmiRNAs, já foi descrito como um marcador em plasma e em soro, o qual tem a capacidade de diferenciar pacientes e indivíduos livres de câncer (HALDRUP et al., 2014). Possui capacidade de distinguir câncer de próstata metastático resistente à castração da doença localizada, por apresentar expressão mais elevada nos primeiros (WATAHIKI et al., 2013). Ainda, está relacionado com metástase óssea (AGAOGLU et al., 2011; ZHANG et al., 2013). O miR-141 também foi descrito como marcador em plasma para ser usado concomitantemente ao teste do PSA, aumentando o poder de diagnóstico (KACHAKOVA et al., 2015). Entretanto, Mihelich et al. (2015) não conseguiram detectar este miRNA em mais de 50% das amostras de soro avaliadas, indicando que mais estudos precisam ser realizados.

O mir-200b tem se mostrado importante para o câncer de próstata, atuando como um supressor tumoral e apresentando-se diferencialmente

expresso em tecidos normal e tumoral (YU et al., 2014). Entretanto, não há consenso na literatura, pois alguns artigos apontam como tendo alta expressão (MITCHELL et al., 2008; HART et al., 2014), enquanto outros indicam baixa expressão (WILLIAMS et al., 2013; YU et al., 2014). Apesar disso, é bem definido que a desregulação do mir-200b tem um papel fundamental na EMT, metástase e no crescimento do tumor primário. Estudos *in vitro* demostraram que a expressão deste miRNA pode diminuir o crescimento tumoral e ter efeito antimetastático, sendo um importante fator para compreensão destes eventos no câncer de próstata e podendo ser usado, no futuro, em terapias por miRNA (WILLIAMS et al., 2013). Como cfmiRNA, o miR-200b foi descrito em soro como um possível marcador para câncer de próstata metastático (BRASE et al., 2010).

Outro miRNA bem estudado desta família é o miR-200c e tem sido indicado como um provável marcador em câncer (WU et al., 2015). Assim como os outros miRNAs desta família possui influência na regulação da EMT (PUHR et al., 2012). Nos tumores de próstata normalmente encontra-se altamente expresso quando comparado ao tecido normal (WACH et al., 2012; GU et al., 2015), mas isso não é um consenso na literatura, pois outros artigos mostram uma baixa expressão em células tumorais (SINGH et al., 2012; SU et al., 2015). Apesar disso, KUMAR et al. (2015) afirmaram que níveis elevados deste miRNA no sangue podem ser utilizados como marcador de diagnóstico de câncer de próstata. Outras publicações mostram que pacientes com carcinoma metastático resistente à castração possuem níveis mais elevados deste miRNA no soro e plasma do que controles ou pacientes com a doença localizada (CHENG et al., 2013; WATAHIKI et al., 2013). Esses resultados apontam o miR-200c como um possível candidato a marcador para câncer de próstata.

2.4.1.5. miR-205

O mir-205 possui característica de supressor tumoral (GANDELLINI et al., 2009). Foi observada baixa expressão em linhagens tumorais *in vitro* quando comparadas às normais (HAGMAN et al., 2013). O mesmo ocorreu com amostras de tecido tumoral de próstata comparado às normais (SRIVASTAVA et al., 2013). Os níveis deste miRNA também estão

relacionados com o desenvolvimento tumoral, quanto mais avançado o tumor, menores são os níveis de miR-205. Além disso, estão relacionados com uma taxa de sobrevida menor (HAGMAN et al., 2013).

Esse miRNA parece ter um importante papel na regulação da EMT e da manutenção na membrana basal. Testes *in vitro* demonstraram que quando a expressão de miR-205 é restaurada há um rearranjo celular e redução da locomoção e invasão celular (GANDELLINI et al., 2009; GANDELLINI et al., 2012). Já a inibição deste miRNA está relacionada com migração e invasão e, consequentemente, metástase (KALOGIROU et al., 2013; NISHIKAWA et al., 2015).

O miR-205 também está relacionado com a regulação de apoptose; quando apresenta baixa expressão, seus genes alvos, como o *BCL2L2* (BCL2-w) e *BCL2*, que são genes antiapoptóticos, mantêm altos níveis de expressão e, assim, resistência à apoptose (BHATNAGAR et al., 2010; VERDOODT et al., 2013). Além disso, este miRNA foi apontado como um candidato a marcador na urina para carcinoma prostático (SRIVASTAVA et al., 2013), o que estimula pesquisas em outros fluidos corpóreos.

2.4.1.6. miR-375

O miR-375 é um promissor marcador para câncer de próstata, pois apresenta alta expressão em tecidos tumorais em relação aos normais de próstata. Também apresenta níveis mais elevados em pacientes com escore de Gleason alto e estágios patológicos mais avançados, como metástase em linfonodos (WACH et al., 2012; COSTA-PINHEIRO et al., 2015). A desregulação deste miRNA afeta várias vias criticas das células, em especial a regulação do ciclo celular. O miR-375, quando superexpresso, atua como um oncomiR nas etapas iniciais deste câncer (COSTA-PINHEIRO et al., 2015).

Dentre os cfmiRNAs, o miR-375 é um dos mais estudados e demonstra associação a risco de câncer de próstata e a estágios patológicos mais avançados (HUANG et al., 2013; SCHWARZENBACH et al., 2014; CHENG, 2015; HALDRUP et al., 2014). Além disso, foi observado um aumento de expressão deste miRNA no plasma de pacientes com câncer de próstata metastático resistente à castração quando comparado com casos da doença localizada (WATAHIKI et al., 2013; HUANG et al., 2015). Um estudo recente

demonstrou que a expressão de miR-375 em soro, quando associado à quantificação da proteína suPAR (do inglês, S*oluble urokinase plasminogen activator receptor*), pode ser um promissor marcador em plasma para diagnóstico e prognóstico neste tipo tumoral (WACH et al., 2015).

Apesar dos vários estudos realizados com esse microRNA, resultados contraditórios foram apresentados por Kachakova et al. (2015), que mostraram relação entre a baixa expressão deste miRNA em plasma e câncer de próstata. Portanto, estudos em diferentes populações são necessários para elucidar essas questões.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Determinar um painel de mRNAs e microRNAs circulantes relacionados ao câncer de próstata para fins diagnósticos, prognósticos e terapêuticos.

3.2. OBJETIVOS ESPECÍFICOS

- Realizar uma análise *in silico* para seleção de miRNAs e genes diferencialmente expressos em câncer de próstata;

- Validar os mRNAs e de miRNAs selecionados em plasma de pacientes com câncer de próstata e em indivíduos livres de neoplasias;

- Associar os parâmetros obtidos em questionários e prontuários com os dados de expressão gênica, a fim de relacioná-los com agressividade e progressão da doença.

- Determinar a função de miRNAs selecionados na modulação da tumorigênese da próstata em linhagens celulares tumorais andrógeno-refratário (PC-3 e DU145) e andrógeno-responsivo (LNCaP).

- Avaliar os efeitos da superexpressão e inibição, *in vitro*, dos miRNAs selecionados na resistência a quimioterápicos.

4. ARTIGO I

CIRCULATING mRNAs AND miRNAs AS NEW MARKERS FOR THE DIAGNOSIS AND PROGNOSIS OF PROSTATE CANCER

Artigo à ser submetido à revista Journal of Molecular Medicine. Fator de impacto 4.885

CIRCULATING mRNAs AND miRNAs AS NEW MARKERS FOR THE DIAGNOSIS AND PROGNOSIS OF PROSTATE CANCER

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ABSTRACT

Circulating nucleic acids are found in free form in body fluids and may serve as minimally invasive tools for cancer diagnosis and prognosis. Only a few studies have investigated the potential application of circulating messenger RNA (mRNAs) and microRNAs (miRNAs) in prostate cancer (PCa). Thus, the aim of this study was identify circulating mRNA and miRNA as diagnostic and prognostic markers for prostate cancer. The Cancer Genome Atlas (TCGA) database was used for an *in silico* analysis to identify circulating mRNA and miRNA as potential markers of PCa. A total of 2,267 genes and 49 miRNAs were differentially expressed between normal and tumor samples. Using integrative analysis (mRNA and miRNA expression data) and target genes prediction among other criteria, we selected eleven genes and eight miRNAs, which were analyzed by RT-qPCR in plasma samples from 102 untreated PCa patients and 50 cancer-free individuals. Two genes, *OR51E2* and *SIM2*, and two miRNAs, miR-200c and miR-200b, showed significant association with PCa. Expression levels of these transcripts distinguished PCa patients from controls (67% sensitivity and 75% specificity). PCa patients and controls with prostate-specific antigen (PSA) ≤ 4.0 ng/mL were differentiated based on *OR51E2* and *SIM2* expression. The miR-200c expression showed association with the Gleason score and miR-200b with bone metastasis, bilateral tumor, and PSA > 10.0 ng/mL. The combination of circulating mRNA and miRNA served useful for the diagnosis and prognosis of PCa.

Key words: Prostate cancer, cell-free nucleic acid, miRNA, mRNA, plasma

INTRODUCTION

Prostate cancer (PCa), the most frequently diagnosed neoplasia of solid organ among men in Brazil and the second most common cancer worldwide, is the fifth leading cause of death by cancer in the world [1, 2]. Digital rectal examination and prostate-specific antigen (PSA) test are widely used for screening of PCa. However, these methods lack the efficiency for the detection of all tumor types and differentiation between advanced and latent disease

forms. In addition, the PSA test exhibits low specificity (37%) [3] and may lead to overdiagnosis in patients with non-neoplastic prostate diseases [4]. As result, several patients are submitted to unnecessary biopsy every year [5].

In USA, more than 1 million men were diagnosed with this disease and received unnecessary treatment since PSA introduction [6]. Long-term studies have shown that the PSA test either caused only a moderate reduction in mortality or was insignificant [7–9]. These evidences resulted in the non-recommendation of the use of PSA test for screening PCa in USA [10]. Several countries, including Brazil, have implemented this recommendation [11], thereby necessitating new biomarkers for better diagnosis and prognosis of PCa [1, 12]. However, PSA test can be used with caution until new markers are introduced in clinical rotine [13, 14].

Circulating nucleic acids (cfNAs) are free DNA and RNA molecules in the plasma, serum, and urine of cancer patients and healthy individuals, which can be used as minimally invasive diagnostic tools [15]. These molecules are found in body fluids in free forms or bound to proteins and exosome-associated. These originate from necrotic and apoptotic cells or are secreted by several cell types [15, 16]. These molecules are stable and exhibit potential to be used as biomarkers; a limited number of studies have focused on circulating RNAs (cfRNAs) in PCa [17, 18]. cfNAs may be served as a "liquid biopsy," which would be useful for diagnostic applications without the need for biopsy. Furthermore, these molecules are powerful tools for monitoring the disease and to evaluate the efficacy of treatment in a rapid non-invasive technique [15].

Among cfRNAs, the circulating microRNAs (cfmiRNAs) have been intensively studied. miRNAs are non-coding RNA molecules approximately 22 nucleotides long; these act as post-transcriptional regulators and exhibit preferential binding to the 3′ untranslated region of mRNAs [19]. A few studies have focused on cfmiRNAs as new attractive cancer biomarkers, with miR-141 and miR-375 being the most promising miRNAs described [20, 21]. However, circulating mRNAs (cfmRNAs) have been poorly explored in cancer research [15]. In prostate cancer, the genes telomerase reverse transcriptase (*hTERT*) [22] and bone morphogenetic protein-6-specific (*BMP6*) [23] are reported as biomarkers for PCa diagnosis.

In this study, the Cancer Genome Atlas (TCGA) database was used to for the identification of genes and miRNAs as potential diagnostic and prognostic markers for PCa. Plasma samples from untreated patients with PCa and cancer-free control subjects were evaluated for the selected genes and miRNAs. Two genes and two miRNAs differentiated cancer patients from controls with sensitivity and specificity higher than that of PSA test. miRNAs were determined as markers for aggressive disease. Our data provide an additional support for the potential use of cfmRNAs and cfmiRNAs as PCa markers.

MATERIAL AND METHODS

Patients

From 2014 to 2015, a total of 102 patients were enrolled at the Londrina Cancer Hospital (Londrina-PR, Brazil). These patients were diagnosed with PCa and failed to receive any treatment before sample collection. Fifty hospital-based cancer-free individuals without urinary disease symptoms and PSA level ≤ 4.0 ng/mL were included as controls. Ethnic groups were categorized based on the ethnic-racial classification by the Brazilian Institute of Geography and Statistics [24]. Individuals who never smoked or quit smoking for 10 years or more were considered non-smokers, while those who never consumed alcohol or quit alcohol for 10 years or more were considered non-alcoholics. Clinical and histopathological data were obtained from the available medical and pathological reports, respectively. The study was approved by the Research Ethics Committee of the State University of Londrina (CAAE19769913.0.0000.5231). All participants provided written informed consent and answered a modified questionnaire based on Carrano and Natarajan [25]. Epidemiological and clinical characteristics of all participants are shown in Table 1.

Selection of miRNAs and candidate genes

Candidate miRNAs and genes were selected *in silico* using gene expression and miRNA data available on TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). The expression data of miRNA (miRNA-seq) and mRNA

(RNAseqV2) were obtained using the Illumina HiSeq platform considering level 3. Data from 425 PCa tissue samples and 52 surrounding normal tissue (SNT) samples were analyzed. The differentially expressed transcripts were selected based on the following parameters: fold-change (FC) > 2, adjusted $P < 0.001$, and false discovery rate (FDR) < 0.001. We used four strategies to define candidate genes and miRNAs as follows: i) integration analyses of mRNA and miRNA data and prediction of target genes using miRWalk [26] and miRTarBase softwares [27]; ii) analysis of clinical (PSA) and histopathological features (Limpy node invasion, Gleason score, tumor stage) of these samples with $P < 0.05$; iii) gross number of reads obtained through the sequencing data, considering values greater than 1,000; and iv) investigation of the candidate genes and miRNAs based on PubMed database. The comparison analyses between groups were performed using the two-sample t-test with the BRB ArrayTools software [28]. The genes and miRNA selected for validation in plasma samples were tested in vitro to assess the secretion by prostate cancer cells.

Sample collection and circulating RNA extraction

Peripheral blood samples were collected through intravenous infusion with needles and disposable BD vacuntainer® tubes containing 6% ethylenediaminetetraacetic acid (EDTA) from all individuals. Blood samples were placed on ice and processed within 2 hours after collection. The whole blood was centrifuged at 700 x*g* for 10 minutes. To avoid cellular contamination, enrichment of cfNAs was performed following protocol described by Duttagupta et al. [29]. Blood plasma was subjected to centrifugation at 2,000 x*g* for 10 minutes at 4°C. Following centrifugation, the cell-free plasma was stored at -80°C until use.

Extraction of total cfRNAs was performed using the miRNeasy Mini kit (Qiagen, Hilden, Germany) with some modifications in the manufacturer's protocol. Briefly, 1 mL of plasma sample was divided into five tubes each containing 200 µL of plasma. Each tube was treated with 1 mL TRIzol™ reagent (Thermo Fisher Scientific), vortexed for 1 minute, and incubated at room temperature for 5 minutes. Following incubation, the mixture was treated with 200 µL chloroform and vortexed for 15 seconds. The solution was incubated at

room temperature for 3 minutes, followed by centrifugation at 12,000 x*g* for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and homogenized using 1.25 volumes of 100% ethanol. A total of 700 µL of the solution was transferred to a binding column with a collection tube and centrifuged at 8,000 x*g* for 15 seconds. The flow-through was discarded and the process repeated for about 12 times. After column saturation, the column was washed and the sample eluted using 25 µL RNase-free water. The sample was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Analysis of mRNA and miRNA expression with quantitative real-time PCR (qPCR)

The expression level of selected genes was determined with qPCR. Briefly, 500 ng of total RNA sample was used for the reverse transcriptase (RT) reaction using oligo-DT, random primers, and 60 Superscript III units (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Each reaction contained 5 µL Sso Advanced Universal SYBR Green Supermix (Bio-Rad, USA), 5 µM of each primer, and 10 ng cDNA. The reaction was performed on the 7900HT Fast Real-Time PCR System Thermocycler (Applied Biosystems, Singapore). Primers were obtained from KiCqStart® SYBR Green Primers (Sigma-Aldrich, Saint Louis, MO, USA). Transcript analyses were performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Singapore) software. The quality of amplification product was verified with the analysis of the dissociation curve.

Expression profiles of miRNAs were evaluated by performing the RT reaction using 5 ng of total cfRNA and TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction. Each reaction was eluted at a 1:4 ratio and contained 5.5 µL of TaqMan 2X Universal PCR Master Mix (Applied Biosystems, Wootston Warrington, WA, UK), 0.45 µL of miRNA-specific TaqMan Probe (Applied Biosystems, Foster City, CA, USA), and 7 µL cDNA of the diluted RT reaction. The reaction was performed on the 7900HT Fast Real-Time PCR System Thermocycler. The reaction was assembled with robot pipetting using QIAgility (QIAGEN, Courtaboeuf, France). Endogenous controls used were

RNU6B and RNU48 for miRNAs and *GAPDH* and *ACTB* for gene transcripts, as described by Bustin et al. [30]. It was also included a pool sample as calibrator.

Statistical analysis

The efficiency of the reaction was considered 100% for all primer pairs and the mathematical model of $2^{-\Delta\Delta ct}$ was applied to obtain the relative expression data [31]. Levene test and Student's *t*-test were used to evaluate the sample distribution and compare means between groups, respectively (GraphPad Prism Software version 5.00, San Diego, California, USA). Descriptive analysis was performed using the IBM SPSS Statistics 22.0 software (IBM Corp., Armonk, New York, USA). The Receiver Operating Characteristic (ROC) curves were constructed using the MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium). A value of $P < 0.05$ was considered statistically significant.

A score was developed for joint analysis of the markers. For each gene, an optimal cut-off point was determined through ROC curve analysis. One point was assigned to the individual who presented levels of expression superior to the cut-off point. The final score was determined by the sum of the points, with a maximum possible score of four points in a blind test. Individuals with three or more points were considered positive for PCa.

RESULTS

Selection of miRNAs and candidate genes

The analysis of TCGA expression data for transcripts and miRNAs from the 425 PCa and 52 SNT samples is shown in Figure 1. A clear difference in the mRNA and miRNA expression profiles was observed between the groups analyzed. Our results revealed a differential expression of 2,267 genes and 49 miRNAs between tumor and normal tissues (FC > 2, $P < 0.001$, FDR < 0.001) (Figure 1, Supplementary Tables 1 and 2). The prediction analyses of target genes, followed by the integrative analysis of the mRNA and miRNA expression data were performed. Only samples with results of both analyses (mRNA and miRNA) were included. An inverse correlation was detected between the expression levels of 81 target genes and 27 differentially expressed miRNAs.

To perform gene and miRNA assortment, clinical and histopathological characteristics; the number of reads obtained from the sequencing data; and PubMed database were used. The eight miRNAs (hsa-miR-143-3p, hsa-miR-183-5p, hsa-miR-200c-3p, hsa-miR-200b-3p, hsa-miR-375, hsa-miR-133b, hsa-miR-205-5p, and hsa-miR-133a-3p) and 11 genes (*AMACR*, *BCL2*, *COL1A1*, *FOXA1*, *GOLM1*, *MMP11*, *OR51E2*, *NKX3-1*, *PCA3*, *SIM2*, and *TRPM8*) obtained, were evaluated in plasma samples by RT-qPCR (Figure 2 and Table 2).

OR51E2, SIM2, miR-200c, and miR-200b are potential circulating diagnostic markers of PCa

Of the 11 genes investigated with qPCR, three (*COL1A1*, *FOXA1*, and *MMP11)* were excluded based on the quality criteria of reactions and six (*AMACR*, *BCL2*, *GOLM1*, *NKX3-1*, *PCA3*, and *TRPM8*) displayed no differences between PCa and controls samples. In comparison to the control subjects, patients with PCa exhibited differential expression of *OR51E2* (FC = 6.98, *P* = 0.002, area under the curve [AUC] = 0.65) and *SIM2* (FC = 1.9, *P* = 0.02, AUC = 0.61) genes. The analysis of cfmiRNA expression revealed differential expression of miR-200b (FC = -3.5, *P* = 0.02, AUC = 0.57) and miR-200c (FC = 1.9, *P* = 0.04, AUC = 0.62) (Figure 3a).

It was performed a joint analysis of the mRNAs and miRNAs differentially expressed in the plasma from patients with PCa and controls. miR-200c, miR-200b, *OR51E2* and *SIM2* were used to construct a score to predict the risk of the disease. Altogether, these circulating markers showed sensitivity of 67% and specificity of 75% for PCa diagnosis (AUC = 0.71, *P* < 0.0001) (Figure 3b).

Correlation of OR51E2 and SIM2 with clinical and histopathological parameters

Patients (n=9) and controls (n=50) with PSA ≤ 4.0 ng/μL, exhibited different expression level of *OR51E2* and *SIM2* genes, and the levels displayed by the patients were higher: *OR51E2* (FC = 47.92, *P* = 0.002, AUC = 0.79) and *SIM2* (FC = 5.21, *P* = 0.002, AUC = 0.85) (Figure 3c). *OR51E2* displayed 100% sensitivity and 50% specificity, while *SIM2* presented the same sensitivity but higher specificity (72%). No correlation was observed between expression

levels of these genes and Gleason scores (GS), bone metastasis, or bilateral tumor (data not shown). Survival analysis could not be performed, owing to the short follow-up time of the patients.

miR-200c and miR-200b as prognostic markers of PCa

The expression analysis and ROC curve values of miRNAs associated with clinical and pathologic parameters are shown in Figure 4. miR-200b was overexpressed in patients with bone metastasis (FC = 12.4, *P* = 0.03, AUC = 0.70), bilateral tumor (FC = 5.22, *P* = 0.03, AUC = 0.64), and PSA level > 10.0 ng/µL (FC = 5.08, *P* = 0.03, AUC = 0.63) (Figure 4a-b).

The overexpression of miR-200c was directly proportional to the increase in the GS observed in PCa biopsies. Patients with GS = 7 exhibited miR-200c level twice that of patients with GS ≤ 6 (P = 0.049). In addition, patients with GS ≥ 8 were 4.8 times more likely to express miR-200c than those with GS ≤ 6 (P = 0.03, data not shown). Patients with GS ≥ 7 showed 2.5 times more circulating miR-200c than those with GS ≤ 6 (P = 0.02, AUC = 0.63) (Figure 4a-b).

DISCUSSION

Circulating nucleic acids are thought to be excellent biomarkers for the diagnosis of cancer [15]. However, their application for the diagnosis of PCa is poorly explored. In this study, we identified cfmRNAs and cfmiRNAs as potential biomarkers for the diagnosis and prognosis of PCa. The two cfmRNAs - *SIM2* and *OR51E2* - found to be overexpressed in plasma from patients with PCa, are known to play an important role in tumor biology as well as development and progression of PCa [32, 33].

The *SIM2* (single-minded 2) gene is a member of the family of transcription factors with *basic helix-loop-helix/per-Arnt-Sim* (bHLH/PAS) domains and has been involved in the pathogenesis of solid tumors [34, 35]. In line with previous studies in prostate cell lines and tissues [32, 36, 37], the present study recorded an increase in the expression of *SIM2* in tumor tissues compared to the SNT (FC = 7.85, *P* < 0.001, FDR < 0.001). Arredouani et al. [36] reported the expression of SIM2 protein in the serum of patients with PCa

and suggested its potential as a target for immunotherapy. Our data confirmed the involvement of *SIM2* gene in PCa.

In agreement with previous studies [38, 39], it was observed that the *OR51E2* gene (olfactory receptor, family 51, subfamily E, member 2) exhibited differential expression in normal and tumor tissue samples (FC = 8.54, $P < 0.001$, FDR < 000.1). *OR51E2* is also known as a prostate-specific G-protein coupled receptor (*PSGR*) [38], and its *in vitro* inhibition retarded cell growth, suggesting its potential as a target for cancer therapy [40]. The use of *OR51E2* as a non-invasive marker is poorly explored. Rigau et al. [41] suggested that *OR51E2* from urine sediment samples collected after prostate massage may be used as a biomarker for PCa screening. Similar results were observed in the study by Sequeiros et al. [42] using similar sample types. In the present study, *OR51E2* overexpression was detected in circulating form in plasma of PCa patients.

To our knowledge, this is the first study showing differential expression of *SIM2* and *OR51E2* transcripts in the circulating form in plasma samples from patients and controls. These genes are potential biomarkers to be evaluated in plasma, allowing minimally invasive sample collection, easy detection, and wide application in clinical practice.

Approximately 15% of patients with PCa present PSA ≤ 4.0 ng/mL, making the diagnosis difficult [43]; in the present study only 9% of patients presented PSA ≤ 4.0 ng/mL. Despite this, our data showed that individuals with PSA level ≤ 4.0 ng/mL can be distinguished into cancer-free or PCa-affected based on the expression levels of *OR51E2* and *SIM2* genes. These two genes had sensitivity of 100% and specificity of 50% and 72%, respectively. We believe that the application of these genes as candidate diagnostic markers for PCa detection may fill the current gap in the diagnosis of PCa.

Of the miRNAs analyzed, miR-200c and miR-200b exhibited differential expression between patients and controls. Members of miR-200 family exert regulatory effect on genes involved in the epithelial-mesenchymal transition [44]. TGCA data analysis revealed that miR-200c was overexpressed in PCa tissues as compared to the SNT, which is in agreement with other studies [45, 46]. Our results are in line with those reported by Cheng et al. [47] wherein high levels of circulating miR-200c were detected in serum samples from patients

with PCa compared to controls. In comparison to controls, patients with PCa exhibited a three-fold downregulation in miR-200b expression. Previous studies reported reduced miR-200b expression in PCa tissue samples and cell lines [48, 49].

Association of *OR51E2* and *SIM2* genes with miR-200b and miR-200c as potential diagnostic markers for the disease was also investigated. Patients with PCa were discriminated from the cancer-free controls with a sensitivity of 67% and specificity of 75%. Salami et al. [3] showed that PSA test exhibits 80% sensitivity and 37% specificity for PCa diagnosis. The combination of four markers used in our study displayed better performance than the PSA test. We were unable to evaluate sensitivity and specificity of the PSA test in our population, owing to the limitation of cancer-free controls included in the experimental design. Nevertheless, the plasma markers *OR51E2* and *SIM2* showed the advantage over the PSA test to identify PCa patients with PSA ≤ 4.0 ng/mL.

A positive correlation was observed between miR-200c and GS; the higher expression levels of miR-200c, the higher of the GS. According to Wu et al. [50] miR-200c is a good candidate marker for PCa detection. We found an association of miR-200c with bone metastasis, PSA level > 10.0 ng/μL, and bilateral tumor. Thus, corroborates the data of Bryant et al. [51] that this miRNA can be used as a potential prognostic marker for PCa.

There is an unmet need for an improvement in the performance of PCa screening [21]. The present study showed that cfmRNAs and cfmiRNAs may be used as efficient diagnostic markers for PCa. In addition, miR-200b and miR-200c are prognostic marker candidates. Our study highlights the potential role of cfRNAs as efficient markers for complexes diseases, such as PCa. The demonstration, for the first time, of a relationship between PCa and some circulating RNAs, might encourage new multicentric studies to be performed.

Table 1 Epidemiological and clinical characteristics of patients with prostate cancer and control subjects

Characteristics		**Patients N (%)**	**Controls N (%)**	***P* value**
Age (years)	< 65	30 (29.4)	26 (52.0)	0.007*
	≥ 65	72 (70.6)	24 (48.0)	
Ancestry	Caucasian	79 (77.5)	39 (78.0)	0.94
	African	23 (22.5)	11 (22.0)	
Smoking habit	Yes	31 (30.4)	15 (30.0)	0.96
	No	71 (69.6)	35 (70.0)	
Alcohol consumption	Yes	58 (56.9)	31 (62.0)	0.55
	No	44 (43.1)	19 (38.0)	
Exposure to xenobiotics	Yes, agrochemicals	54 (52.9)	24 (48.0)	0.68
	Yes, others	22 (21.6)	14 (28.0)	
	No	26 (25.5)	12 (24.0)	
Family history of cancer	Yes	45 (44.1)	23 (46.0)	0.09
	Yes, prostate	16 (15.7)	4 (8.0)	
	No	40 (39.2)	27 (54.0)	
PSA[a] (ng/mL)	≤ 4.0	9 (8.8)	50 (100.0)	
	>4.0 to 10.0	39 (38.2)		
	> 10.0	54 (52.9)		
Gleason score[b]	≤ 6	58 (56.9)		
	7	32 (31.4)		
	≥ 8	10 (9.8)		
Bilateral tumor[b]	P	47 (47.5)		
	A	52 (52.5)		
Bone metastasis	P	11 (11.1)		
	A	88 (88.9)		
Treatment[c,d]				
Prostatectomy		61 (59.8)		
Hormone therapy		16 (15.7)		
Radiotherapy		19 (18.6)		
Orchiectomy		8 (7.8)		
Others[e]		10 (9.8)		

[a]PSA = Prostate-specific antigen; [b]Histopathological parameters from biopsy; [c]Treatment performed after collection; [d]Patients may receive one or more treatment types; [e]Patients who are under active surveillance or refused treatment; P = Present; A = Absent. *Statistically significant difference (Student's *t*-test, $P < 0.05$)

Table 2 Summary of miRNAs and their targets predicted from *in silico* analyses.

MicroRNA	Fold-change[a]	Genes predicted using ***miRWalk***
hsa-miR-133a-3p	0.28	*AMACR*, *BCL2*, *COL1A1*, *NKX3-1*, *SIM2*
hsa-miR-133b	0.27	*AMACR*, *BCL2*, *COL1A1*, *NKX3-1*, *SIM2*
hsa-miR-143-3p	0.40	*AMACR*, *BCL2*, *COL1A1*, *GOLM1*, *MPM11*, *NKX3-1*, *OR51E2*, *SIM2*
hsa-miR-183-5p	4.35	*AMACR*, *COL1A1*, *FOXA1*, *MPM11*, *NKX3-1*, *TRPM8*
hsa-miR-200b-3b[b]	-	*AMACR*, *BCL2*, *GOLM1*, *OR51E2*, *SIM2*, *TRPM8*
hsa-miR-200c-3p	3.33	*AMACR*, *BCL2*, *FOXA1*, *GOLM1*, *OR51E2*, *SIM2*, *TRPM8*
hsa-miR-205-5p	0.36	*AMACR*, *BCL2*, *COL1A1*, *GOLM1*, *MPM11*, *NKX3-1*, *SIM2*, *TRPM8*
hsa-miR-375	6.74	*AMACR*, *BCL2*, *COL1A1*, *FOXA1*, *GOLM1*, *NKX3-1*, *SIM2*, *TRPM8*

[a]Fold-change based on The Cancer Genome Atlas (TCGA) data; tumor tissue versus normal tissue. [b]miRNA selected from the literature data

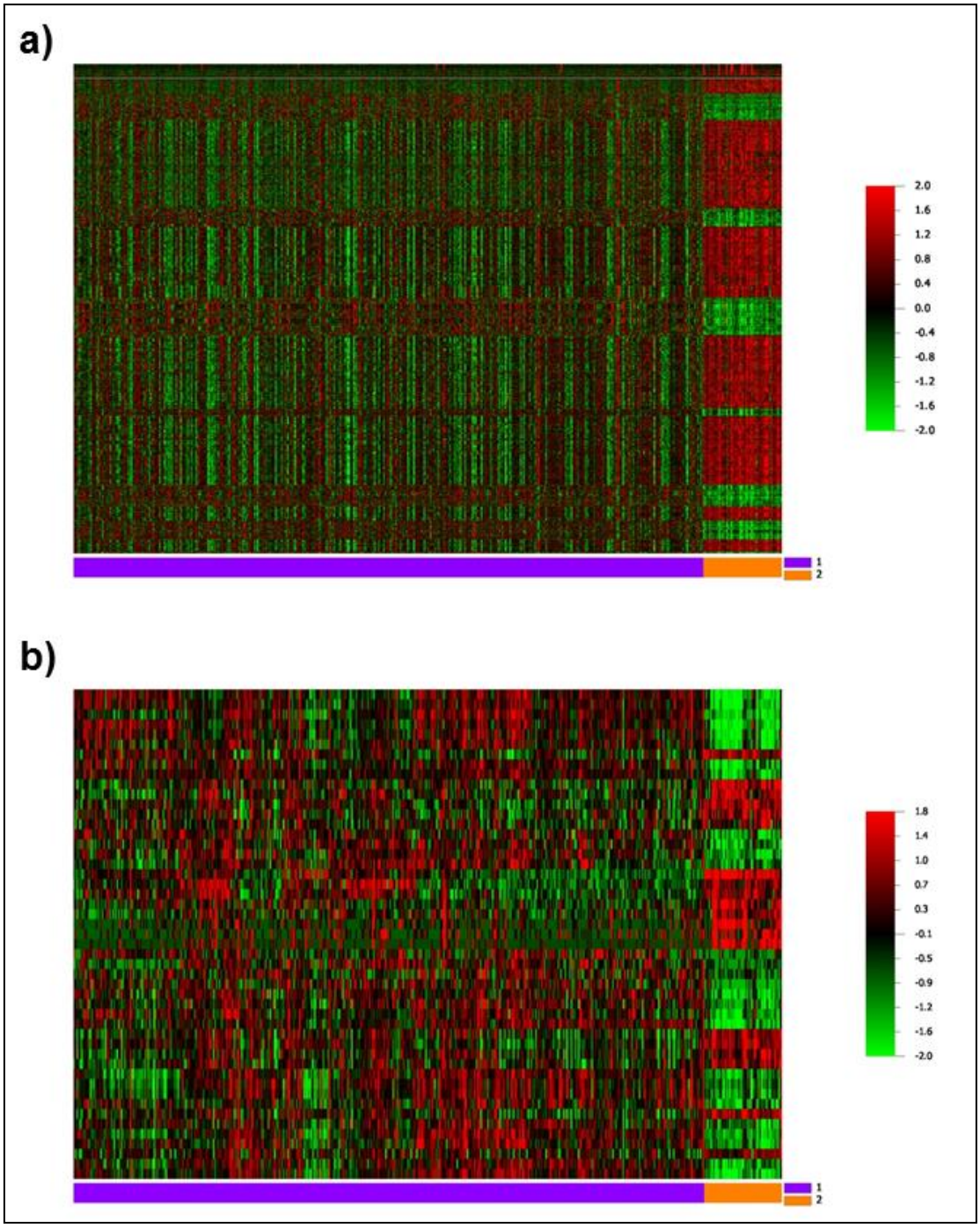

Figure 1 Heatmap showing gene and miRNA differential expression in tumor tissue samples (1: purple) and surrounding normal tissues (2: orange) from TCGA data. **a** Overall gene expression with 2,267 differentially expressed genes ($P \leq 0.001$ and FDR ≤ 0.001); **b** miRNA expression revealing differential expression of 49 miRNAs ($P \leq 0.001$ and FDR ≤ 0.001).

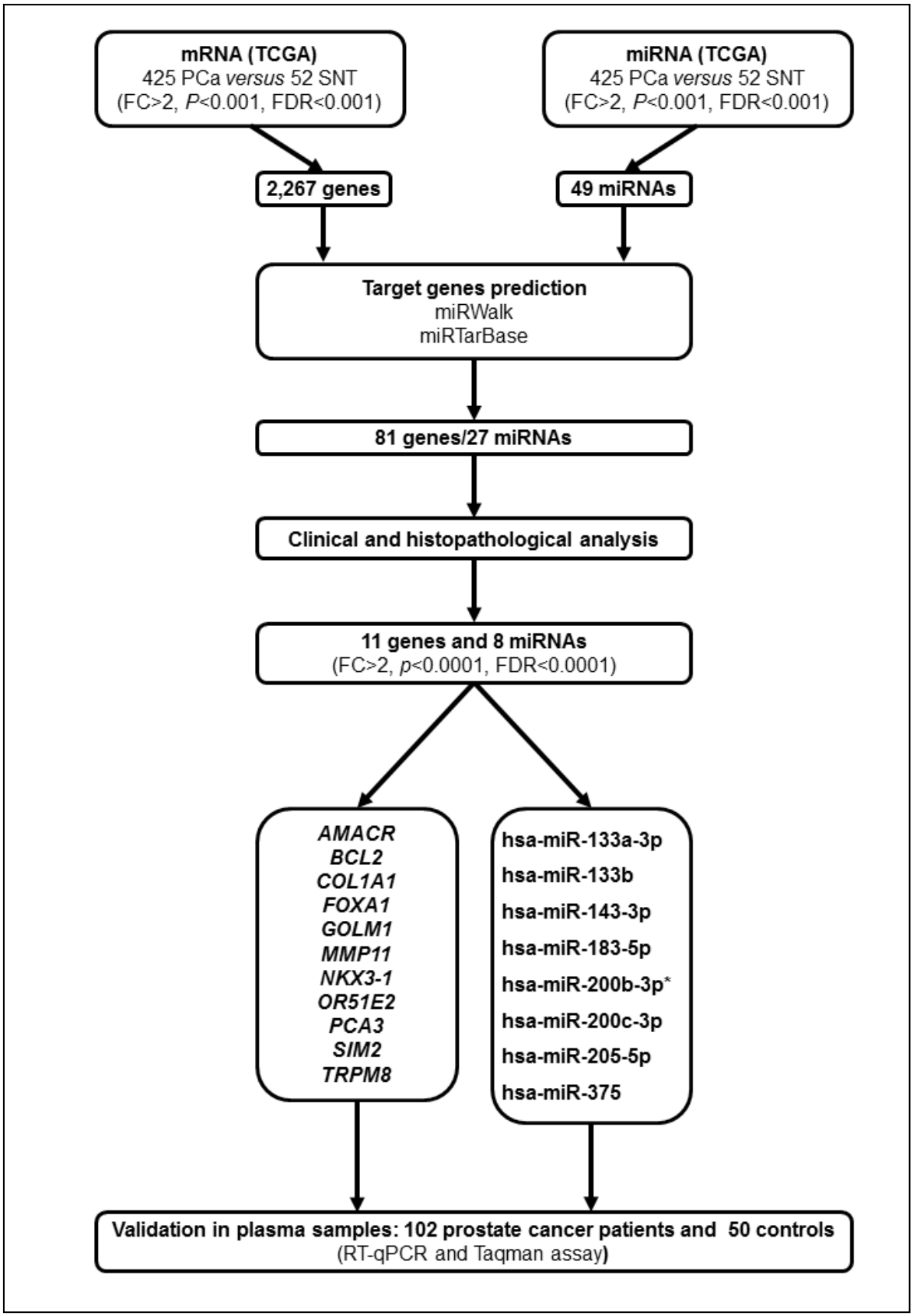

Figure 2 Flowchart of the analyses to define candidate genes.*Gene selected from Pubmed database.

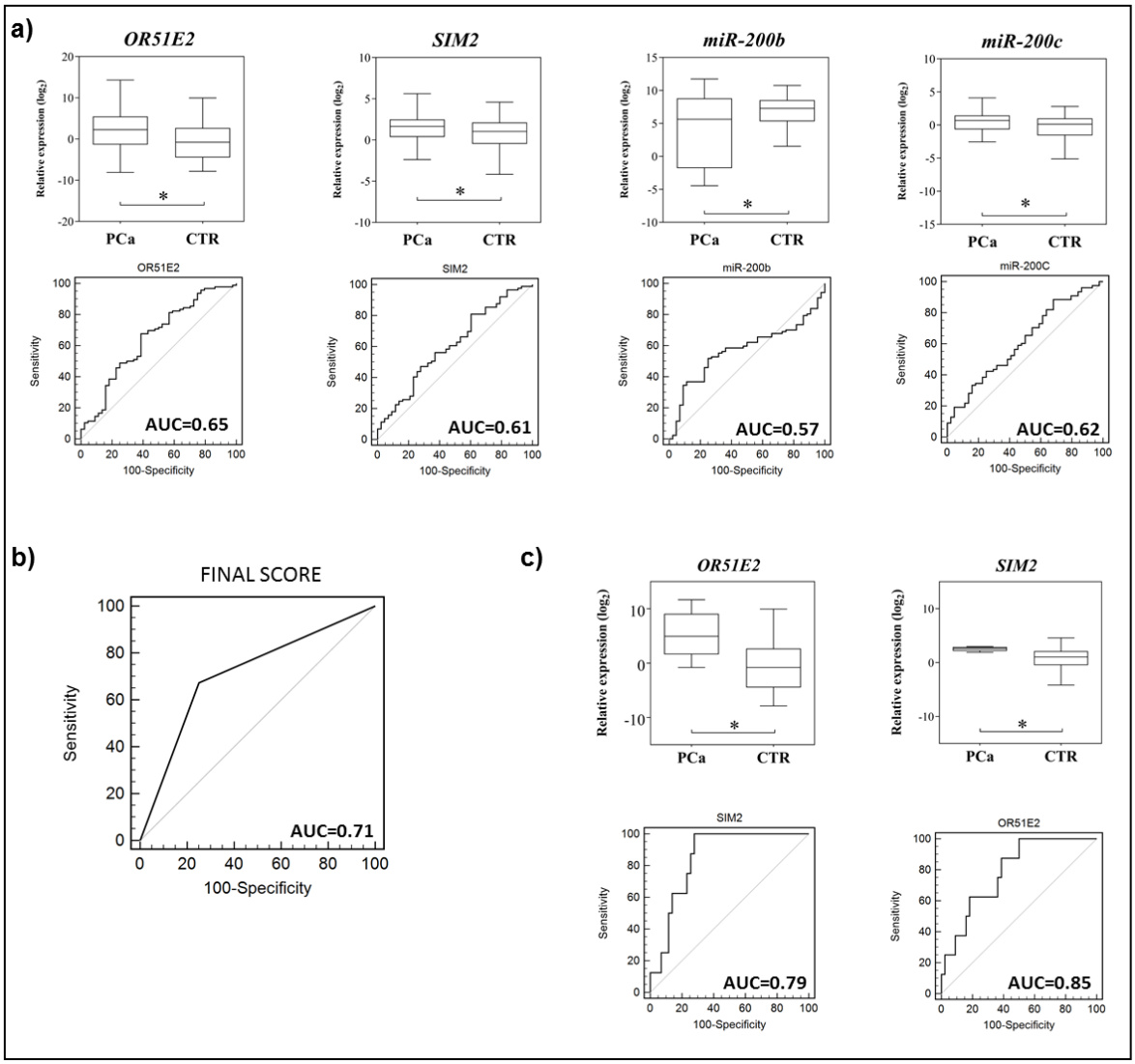

Figure 3 Differential expression of circulating mRNAs and miRNAs in plasma samples from patients with PCa and CTR and their respective ROC curves. **a** Expression analysis of patients versus controls. **b** Differential expression of *SIM2* and *OR51E2* genes in plasma samples from patients versus controls, both with PSA level ≤ 4.0 ng/mL and their respective ROC curves. **c** ROC curve referring to the score developed from the following transcripts: miR-200c, miR-200b, *OR51E2*, and *SIM2* genes; sensitivity of 67%, specificity of 75%. ROC = receiver operating characteristic, PCa = prostate cancer, CTR = controls subjects, PSA = prostate-specific antigen, AUC = area under the curve. *Statistically significant difference (Student's *t*-test $P < 0.05$).

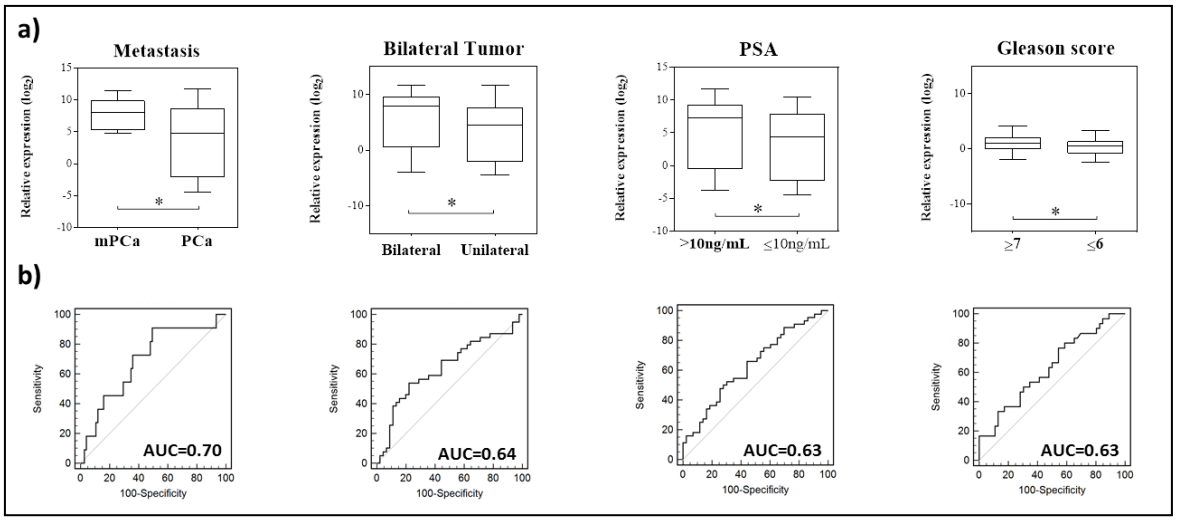

Figure 4 Differential expression of circulating miR-200b for the analysis of clinical and histopathological features. **a** Relative expression. **b** ROC curve analysis. PCa = prostate cancer; mPCa = metastatic prostate cancer; AUC = area under the curve. *Statistically significant difference (Student's *t*-test $P < 0.05$).

ACKNOWLEDGEMENTS

This study was supported by grants from the Araucaria Foundation of Support for the scientific and technological of Paraná (Conv. 185/2014), National Institute of Science and Technology in Oncogenomics (INCITO - Sao Paulo Research Foundation – FAPESP) (2008/57887-9) and the Brazilian National Council for Scientific and Technological Development - CNPq (573589/08-9). The authors thank CAPES (Coordination for the Improvement of Higher Education Personnel), Londrina Cancer Hospital and Intermunicipal Health Consortium of the Middle Paranapanema (CISMEPAR).

CONFLICTS OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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5. ARTIGO II

CIRCULATING mRNA SIGNATURE AS A MARKER FOR HIGH-RISK PROSTATE CANCER

Artigo a ser submetido à revista Carcinogenesis. Fator de impacto 4.874

CIRCULATING mRNA SIGNATURE AS A MARKER FOR HIGH-RISK PROSTATE CANCER

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ABSTRACT

The choice of the best treatment for patients with prostate cancer (PCa) remains a dilemma for physicians. To help resolve this issue, this study aimed to identify minimally invasive molecular markers that are able to diagnose patients with high-risk prostate cancer. Circulating mRNAs were measured in patients' plasma samples before radical prostatectomy. The genes *AMACR*, *GOLM1*, *TRPM8,* and *NKX3-1* were overexpressed in patients with aggressive prostate cancer, such as extracapsular extension and vesicular seminal invasion. Three (*GOLM1*, *TRPM8,* and *NKX3-1*) of them were associated when combined with aggressive prostate cancer and were able to correctly identify 71.2% of all positive samples. Plasma markers associated with such parameters as the biopsy's Gleason score (≥ 8) and prostate-specific antigen (PSA) levels (> 20 ng/mL) showed a sensitivity of 88% and a specificity of 58% with respect to identifying patients with aggressive disease. In conclusion, this study showed that the use of these plasma markers could improve the diagnosis of patients with high-risk PCa and could assist selecting the best choice of treatment for each patient.

Key words: Prostate cancer, cell-free nucleic acid, mRNA, plasma, prostatectomy

INTRODUCTION

Prostate cancer (PCa) is the second most common neoplasia in men in the world (1). Despite its high incidence, its progression is relatively slow (2). PSA-based screening methods can lead to an overdiagnosis, identifying men with low-grade disease and favorable outcomes (3,4). Draisma et al. (5) estimated that the rates of overdiagnosis of PCa range from 23% to 42%. Therefore, every year, many men are diagnosed and treated unnecessarily, increasing the cost of treatment and rates of morbidities (6).

The histopathological examination of the biopsy is considered the gold standard for prostate cancer diagnosis. The clinical decision regarding treatment is guided by PSA levels and biopsy histopathology results, which include the Gleason score (7). However, the biopsy procedure presents risks, such as bleeding and sepsis. Furthermore, approximately 20% of PCa cases are detected only in the

second biopsy (8). In addition, approximately 40% of patients are upgraded before prostatectomy information (9) and radical prostatectomy (RP) treatment has been the treatment option for about 60% of low-risk patients (10). In addition to that, RP causes urinary incontinence in 13.2% to 30.5% of patients and sexual dysfunction in 48.8% to 64.5% (11,12). All of these factors illustrate the urgent need for molecular markers that indicate which patients will present with a more aggressive tumor and would benefit from RP versus those who could simply be monitored (13).

Minimally invasive biomarkers identified in biological samples, such as blood and urine, have the potential to help with treatment decisions. Several biological molecules can be found in plasma (14), including the cell-free mRNA (cfmRNAs), which can be found in a free form or in exosomes (15). These molecules have been shown to be a promising tool for the development of a “liquid biopsy”, which is not an invasive procedure and allows follow-up for the disease and/or treatment (15). Cell-free RNAs have been demonstrated to assist in the diagnosis and prognosis of breast, pancreatic, and gastric tumors (16–18). However, only a few reports have been published showing circulating mRNA as markers for the diagnosis of PCa (19–22), and liquid biopsy studies focused on identifying patients with poor outcomes (23).

Considering all of these challenges in prostate cancer treatment, the present study aimed to identify cell-free RNA markers in the plasma of PCa patients. This research focused on identifying markers associated with histopathological characteristics that have the potential to predict high-grade disease before prostatectomy, thus assisting patients and physicians in choosing the most appropriate treatment regimen.

MATERIAL AND METHODS

Study subjects

The study population included 60 prostate cancer patients, who were subjected to radical prostatectomy (RP) between August 2014 and September 2016 at the Londrina Cancer Hospital, Londrina, Paraná, Brazil. All the patients signed an Informed Consent Form and provided information about their lifestyle and occupational exposures. Their ancestry was determined according to the Brazilian Institute Geography and Statistics (24). All the histopathological data from

prostatectomies were obtained in the Londrina Cancer Hospital. These data included the Gleason score, extracapsular extension, seminal vesicle invasion, perineural invasion, bilateral tumor, lymph node invasion, and tumor stage. The main characteristics of the patients are described in Table 1. The average age was 64.5±6.9 years, and 60% presented with low-grade PCa.

This research was approved by the Ethics Committee on Human Research of the State University of Londrina, Brazil (CAAE19769913.0.0000.5231).

Gene selection

The candidate genes *AMACR*, *BCL2*, *NKX3-1*, *GOLM1*, *OR51E2*, *PCA3*, *SIM2,* and *TRPM8* were selected based on *in silico* analysis of TCGA data according to a previous study from our group (data not published).

Sample collection, RNA total extraction, and RT-qPCR

Samples were collected before the patients underwent RP. From each patient, 10 mL of peripheral blood was collected by an intravenous puncture with needles and BDvacuntainer® tubes containing ethylenediaminetetraacetic acid (6% EDTA). The samples were kept in ice and processed within 2 hours. The cell-free plasma was obtained after two centrifugations of 700 x*g* for 10 minutes and 2000 x*g* for 10 minutes, according to the recommendations of Duttagupta et al. (25). The cell-free plasma samples were stored at -80ºC until use.

Total RNA extraction was performed using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) with modifications. The RNA samples were quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Synthesis of cDNA was carried out using Superscript III (Invitrogen, Carlsbad, CA, USA), and qPCR was performed using Sso Advanced Universal SYBR Green Supermix (Bio-Rad, USA) in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Singapura). The primer sequences were obtained from KiCqStart® SYBR Green Primers (Sigma-Aldrich, Saint Louis, MO, USA). As internal controls, the *GAPDH* and *ACTB* genes were used. It was also included a pool sample as calibrator.

Statistical analysis

The mathematical model $2^{-\Delta\Delta ct}$ was used to perform a relative expression analysis (26). The Student's t-test was used to compare histopathological characteristics with mRNA levels for each gene studied.

In order to identify predictive plasma marker for aggressive tumors, first it were evaluated the association of each gene with the presence of each histopathological characteristic individually. It were also accomplished a division of the patients into two groups after prostatectomy analysis. One group was denominated "High-Risk for aggressive Prostate Cancer," which included patients with Gleason score ≥ 8 and/or tumor stage ≥ pT3a. The other group was denominated "Low-Risk for aggressive Prostate Cancer," which included patients with Gleason score < 8 and tumor stage < pT3a.

A Student's t-test was performed to determine the genes associated with a high-risk for aggressive prostate cancer. The significant genes were included in a discriminative analysis. A ROC curve was used to determine the effectiveness of these markers when compared to the pre-surgery tests (PSA > 20 ng/mL and/or a biopsy's Gleason score between 8–10) (27,28), as well as the effectiveness of all tests combined.

The Student's t-test analyses were performed using GraphPad Prism Software version 5.00 (San Diego, California, USA). The discriminative analysis using leave-one-out cross-validation and ROC curves were performed using IBM SPSS Statistics 22.0 (IBM Corp, Armonk, New York, USA). The sensitivity and specificity were determined using MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgic). *P*-values < 0.05 were considered statistically significant.

RESULTS

In this study, each gene was correlated with each histopathological characteristic. Table 2 shows the relative expression in the plasma for the genes evaluated and their relation to observed histopathological characteristics. The gene expression was compared between patients who presented a certain histopathological characteristic with those who did not presented. The genes *GOLM1*, *NKX3-1*, and *TRPM8* were overexpressed in patients that presented with extracapsular extension (FC = 14.3, *P* = 0.031; FC = 6.0, *P* = 0.008; and FC = 20.1, *P* = 0.004, respectively) and tumor stage ≥ pT3 (FC = 11.0, *P* = 0.041; FC = 4.7, *P* =

0.022; and FC = 16.5, *P* = 0.007, respectively). The *AMACR* gene was overexpressed in patients with extracapsular extension (FC = 4.3; *P* = 0.031). The *GOLM1* gene also was overexpressed in patients that displayed vesicle seminal invasion (FC = 23.2, *P* = 0.004). Furthermore, *BCL2* expression was reduced in patients with bilateral tumors (FC = 0.2; *P* = 0.026). The expression of *OR51E2*, *SIM2*, and *PCA3* were not associated with any histopathological characteristics.

Three genes correlated with a higher risk for aggressive PCa: *GOLM1* (FC = 26.8; *P* = 0.003), *NKX3-1* (FC = 4.5; *P* = 0.021), and *TRPM8* (FC = 16.1; *P* = 0.011) (Figure 1A). The discriminative analysis showed that the co-expression of these genes helped identify 71.2% of all positive samples (Figure 1B). Table 3 shows the performance of the biopsy's Gleason score, PSA levels, and plasma markers (expression levels of *GOLM1*, *NKX3-1*, and *TRPM8* genes), as well the combination of these markers to identify patients at high-risk for aggressive PCa. The association of biopsy's Gleason score, PSA levels, and plasma markers showed the best values for sensitivity (88%) and specificity (58%) when considered together. Figure 2A shows the plot of the ROC curve assay. The plasma markers produced a higher area under the curve (AUC) (AUC = 0.76) compared to the biopsy's Gleason score > 8 (AUC = 0.66) and PSA > 20ng/mL (AUC = 0.66). Figure 2B shows that the markers can be combined to effectively identify patients at high-risk for aggressive PCa.

DISCUSSION

The identification of patients with aggressive prostate tumors is essential for correctly choosing treatment regimens. The proper treatment increases the chances of a cure and improves the quality of life for these patients (29). Currently, the search for minimally invasive methods as prognostic markers has been highlighted in several studies of neoplasias in an effort to minimize patient morbidity (15,30). Among these methods, circulating nucleic acids have been stood out due to their specificity and stability (15,30,31). Thus, the present study focused on the search for additional markers that could predict the aggressiveness of the disease before an RP is ordered by a physician.

Histopathological characteristics obtained after RPs, such as the presence of extracapsular extension, seminal vesicle invasion, and tumor stage ≥ pT3 showed an aggressive phenotype for the tumor. Extracapsular extension is one of the main

characteristics used to determine the aggressiveness of this disease. Studies have shown that patients with extracapsular extension do not benefit from prostatectomy alone and need adjuvant therapy (32,33). Furthermore, patients with seminal vesicle invasion have a high-risk for cancer progression, both regionally and systemically. They also have a high-risk for biochemical recurrence (34,35).

Four genes were found to be overexpressed in prostate cancer patients who presented at least one of the characteristics described above. These genes were *AMACR*, *GOLM1*, *NKX3-1*, and *TRPM8*, indicating that these genes have an important role in the progression of this disease. *NKX3-1* and *TRPM8* have been described as having a critical role in prostate cancer patients (36,37), and they modulate androgen regulation in concert with the androgen receptor during prostate cancer progression (38–40). Consequently, dysregulation of these genes can affect prostate cells and lead to increased tumorigenesis (36,37).

Studies have demonstrated that although *NKX3-1* is a tumor suppressor, surprisingly it also has oncogenic effects. *NKX3-1* overexpression is maintained in metastatic tumors and it is elevated in high-grade prostatic carcinoma (41,42). Our data showed that the upregulation of circulating *NKX3-1* in patients with aggressive prostate cancer corroborates this hypothesis.

Although not fully understood the functions of *AMACR* and *GOLM1* in prostate cells, they have been shown to be overexpressed in tumor tissue when compared to normal tissues, suggesting their potential as markers for prostate cancer (43–45). The overexpression of these genes were also associated with renal and hepatocellular carcinomas (46,47). In addition, the fact that *AMACR* has been associated with extracapsular extension confirms the hypothesis of Box et al. (48) that this gene is associated with a higher risk for disease progression. Thus, the data of the present study strongly supports the use of these genes as markers for this neoplasia.

Despite the importance and advantages of identifying improved circulating markers (15), few studies have been performed. Among the evaluated genes, Varambally et al. (49) showed that *GOLM1* is overexpressed in urine samples of patients. Furthermore, Bai et al. (50) demonstrated that *TRPM8* can be found in the urine and whole blood of patients. However, studies regarding the circulating mRNA have not been done. Therefore, to the best of our knowledge, this is the first time that

these genes have been shown to be possible markers for prostate cancer circulating in the plasma.

In the present study, *BCL2* was the only gene found to be downexpressed in the plasma of patients, and it was associated with bilateral tumors. The role of this gene in prostate cancer is unclear (51). Some studies showed that *BCL2* increases when prostate cancer progresses from an androgen-dependent to an androgen-independent type (51,52). The present study had no patients with androgen-independent prostate cancer. Therefore, this may be why this gene has been shown to be downexpressed.

Molecular markers can be a useful tool for the early identification of patients with aggressive diseases. They may assist medical decisions and have the potential to identify patients who could benefit from RP therapy (29). Low-risk prostate cancer overtreatment involves costs to patients and increased morbidity. Thus, there is a critical need for the development of additional prognostic factors to identify patients with the aggressive type of PCa, even though these cancers may initially present with low-risk biopsy pathology (53,54). In order to solve this problem by identifying genes that correlate with histopathological factors, the present study showed that a combination of three circulating mRNAs (*GOLM1*, *NKX3-1,* and *TRPM8*) can discriminate patients who have the high-risk form of aggressive prostate cancer. The addition of these markers with the PSA and biopsy's Gleason score increased the sensitivity to more than 60% for detecting high-risk patients. As a result, we suggest that after patients undergo the first PSA and biopsy screening, those with PSA < 20 ng/mL and Gleason score < 8 could be screened for the plasma markers identified in this study. Thus, patients could be reclassified as high- or low-risk for aggressive prostate cancer. The high-risk patients would be treated and those with low-risk would undergo active surveillance. This new approach could yield important benefits for patients and the overall health care system.

In conclusion, this study showed that circulating mRNAs used as the basis of a minimally invasive prognosis test can be a good tool for assessing prostate cancer risks. Currently, prostate cancer treatment is a joint decision between a physician and patient, the diagnostic information available today can be helpful, even though it does not make the treatment decision any easier (55). An additional method to screening patients with aggressive prostate cancer can be proposed based on the results obtained in this study of cfmRNA. The combination of these plasma markers

with the data obtained from PSA and biopsy could help the prognosis of aggressive disease and help doctors and patients to making decision regarding prostate cancer treatment. Due to the lack of studies in this area, more research are necessary to validate these results in other populations worldwide.

Table 1: Clinical and histopathological characteristics of prostate cancer patients under radical prostatectomy

Characteristics		**Patients n (%)**
Age (years)	< 65	26 (43.3)
	≥ 65	34 (56.7)
Ancestrally	Caucasian	45 (75.0)
	African	15 (25.0)
Familial history of cancer	Yes	40 (66.7)
	Yes, Prostate	*9 (15.0)*
	No	20 (33.3)
PSA[a] (ng/mL)	≤ 4.0	7 (11.7)
	>4.0 to 10.0	27(45.0)
	> 10.1	26 (43.3)
Histopathological parameters[b]		
Score Gleason	6 - 7(3+4)	50 (83.3)
	7(4+3) - 9	10 (9.8)
Extracapsular extension	Presence	21 (35.0)
	Absence	37 (61.7)
	Missing	2 (3.3)
Seminal vesicle invasion	Presence	5 (8.3)
	Absence	54 (90.0)
	Missing	1 (1.7)
Perineural invasion	Presence	3 (5.0)
	Absence	55 (91.7)
	Missing	2 (3.3)
Bilateral tumor	Presence	38 (63.3)
	Absence	21 (35.0)
	Missing	1 (1.7)
Lymph node invasion	Presence	2 (3.3)
	Absence	40 (66.7)
	Missing	18 (30.0)
Tumor stage	pT1 to pT2c	36 (60.0)
	pT3 to pT4	21 (35.0)
	Missing	3 (5.0)

[a]PSA = Prostate-specific antigen, [b]Histopathological parameters from prostatectomy

Table 2: Differential expression of circulating mRNA in plasma of PCa patients

Histopathological parameters*		Genes							
		AMACR	***BCL2***	***GOLM1***	***NKX3-1***	***OR51E2***	***PCA3***	***SIM2***	***TRPM8***
PSA (> 20 ng/mL)[#]	FC	0.8	0.4	3.5	2.4	0.5	5.0	0.2	0.8
	P	0.810	0.401	0.556	0.453	0.625	0.404	0.378	0.916
Score Gleason (≥ 8)	FC	0.3	2.5	35.1	0.8	2.9	30.6	0.5	0.5
	P	0.815	0.558	0.183	0.888	0.576	0.159	0.715	0.781
Extracapsular extension (positive)	FC	4.3	3.3	14.3	6.0	1.2	4.0	1.4	20.1
	P	***0.031***	0.094	***0.031***	***0.008***	0.868	0.222	0.696	***0.004***
Seminal vesicle invasion (positive)	FC	1.4	2.4	23.2	3.2	1.4	10.4	10.6	16.2
	P	0.755	0.473	***0.004***	0.319	0.817	0.226	0.139	0.178
Perineural invasion (positive)	FC	0.9	6.8	1.7	0.6	1.1	18.0	21.5	40.3
	P	0.916	0.222	0.849	0.744	0.978	0.243	0.131	0.160
Bilateral tumor (positive)	FC	0.6	0.2	0.4	0.3	0.7	3.5	1.0	0.3
	P	0.509	***0.026***	0.474	0.064	0.706	0.327	0.980	0.372
Lymph node invasion (positive)	FC	24.6	21.9	25.0	13.2	4.2	26.6	12.4	20.9
	P	0.110	0.131	0.337	0.203	0.530	0.263	0.181	0.333
Tumor stage (≥ pT3)	FC	3.7	2.0	11.0	4.7	1.6	3.3	1.0	16.5
	P	0.052	0.333	***0.041***	***0.022***	0.576	0.293	0.995	***0.007***

PSA = Prostate-specific antigen; FC = Fold-change; *P* = Student's t-test; Bold and italic = statistically significant $P < 0.05$; *Evaluated in post-surgical material; [#]Measured before surgery

Table 3: Performance of markers differentiating high-risk PCa versus low-risk PCa

Markers	Sensitivity % (IC95%)	Specificity % (IC95%)	PPV % (IC95%)	NPV % (IC95%)
Gleason score[a] (>8)	8 (1-26)	100 (89-100)	100 (20-100)	59 (56-62)
PSA (>20)	15 (4-35)	97 (84-100)	80 (32-97)	59 (55-63)
Plasma mRNA markers	85 (65-96)	58 (39-75)	61 (51-71)	83 (65-92)
Combination	88 (85-98)	58 (22-75)	62 (52-71)	86 (68-95)

[a]Biopsy parameters; PSA = Prostate-specific antigen; PPV=Positive Predictive Value; NPV=Negative Predictive Value.

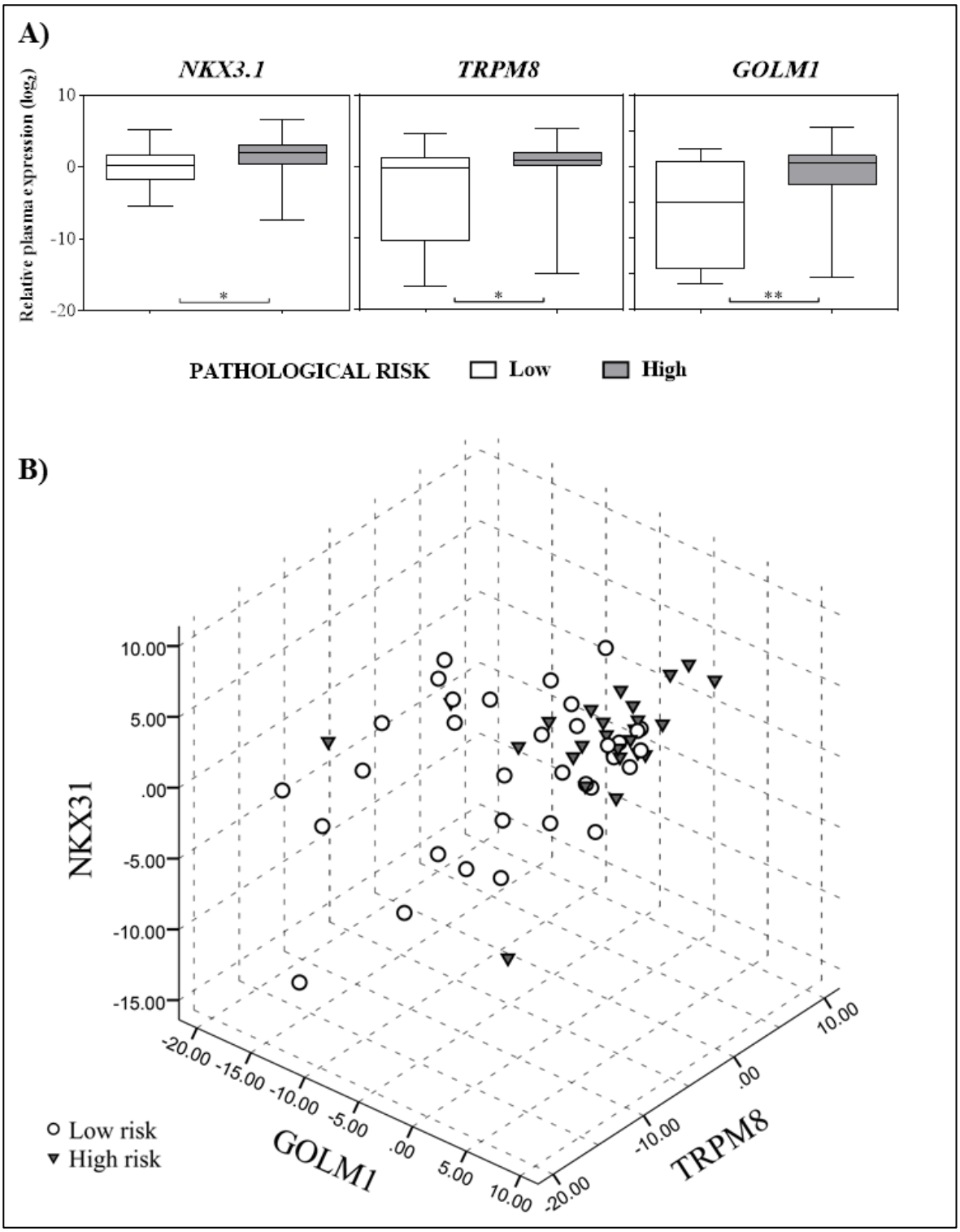

Figure 1: Markers used to differentiate patients who have high-risk PCa versus low-risk PCa. A) Differential expression of circulating mRNAs in plasma samples. B) Three-dimensional graph representing three makers that differentiate high-risk from low-risk PCa patients. Data are shown by relative expression $\log_2$. Statistically significant *P < 0.05; **P < 0.01.

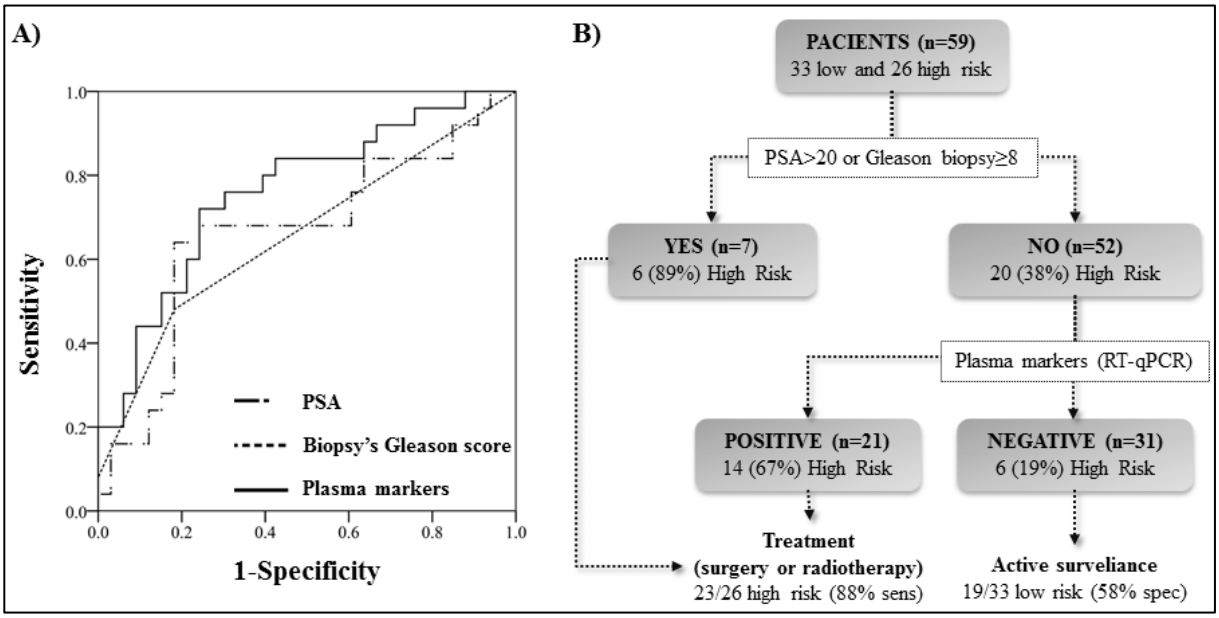

Figure 2: A) ROC curve representations of the markers used to identify high-risk prostate cancer. B) Flowchart illustrating the use of all markers to differentiate high-risk from low-risk PCa.

ACKNOWLEDGEMENTS

This study received financial support from the Araucaria Foundation of Support for the scientific and technological of Paraná (Conv. 185/2014). The authors thank CAPES (Coordination for the Improvement of Higher Education Personnel), CNPq, (National Council for Scientific and Technological Development), the São Paulo Research Foundation, Londrina Cancer Hospital, and Intermunicipal Health Consortium of the Middle Paranapanema (CISMEPAR).

CONFLICTS OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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6. ARTIGO III

THE REGULATORY ROLE OF miR-182-5p IN CONFERRING PROSTATE CANCER AGRESSIVENESS

Artigo a ser submetido à revista Oncotarget. Fator de impacto 5.008

THE REGULATORY ROLE OF miR-182-5p IN CONFERRING PROSTATE CANCER AGRESSIVENESS

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ABSTRACT

Prostate cancer (PCa) is a clinically heterogeneous disease, where deregulations of epigenetic events, such as miRNAs deregulation, are determinant for its development and progression. MiR-182-5p, a member of the miR-183 family, when overexpressed has been associated with PCa tumor progression and with a decreased survival rate. In this study was evaluated the association of circulating levels of miR-182-5p in plasma samples of patients with PCa and determined its regulatory role in conferring the aggressive tumor phenotype in established PCa cell lines. Our data demonstrated that miR-182-5p is overexpressed in patients who present characteristics of aggressive tumor. However, different expression levels of this miRNA were not observed between plasma of PCa patients and controls. Functional assays demonstrated that miR-182-5p when overexpressed induces cell proliferation, increases the number of cells in the G2/M cell cycle phase, and promote cell migration. In addition, an increase in cell resistance to docetaxel was observed in PCa cells. The evaluation of the epithelial-mesenchymal transition (EMT) markers expression, showed an increase in the expression levels of ZEB1 and N-CADHERIN, as well as, increase in the expression of the tumor progression marker p-AKT, in the miR-182 overexpressed cells. Conversely, its inhibition led to a decrease in cell proliferation and cell, adhesion, in the expression of EMT markers B-CATENIN and SLUG, besides a decrease in the expression levels of the tumor progression associated marker, p-AKT. In conclusion, we demonstrated that miR-182-5p acted as oncogenic in PCa cells, conferring an aggressive tumor phenotype. Further analyses in well annotated clinical samples are required to determine its prognostic role in PCa.

Key words: Prostate cancer, cell-free miRNA, plasma, oncogene, miR-182-5p

INTRODUCTION

Prostate cancer (PCa) is one of most incident tumor in men worldwide, with approximately 1.1 million cases diagnosed per year, and the fifth cancer-related death in men [1,2]. Advances in the areas of diagnosis and prognosis have been achieved, with improvement in the imaging technologies [3,4], in the analysis of

circulating tumor cells (CTC) [5,6] and exosomes [7], which have been shown to be superior than the standard prostate-specific antigen (PSA) testing. The PSA test was introduced in 1980's [8], and used as gold standard for PCa screening. However due to its low specificity and false-negative rates [9] it is currently not recommended as a single screening test for PCa [10]. In addition, clinical trials have provided conflicting results in relation to whether PSA screening for prostate cancer reduces mortality [11,12]. Therefore, improvements in the accuracy of PSA testing for screening and/or the development of novel non-invasive biomarkers are critically needed [2,13].

The deregulations of epigenetic events are crucial for the development and progression of several types of cancer, including PCa [14–16]. These events include alterations in miRNAs, short non-coding RNAs of approximately 22 nucleotides, that are involved in the post-transcriptional regulation of cancer driver genes [17,18]. miRNAs play an important role in several biological pathways associated with cancer, such as cell proliferation and differentiation, cell cycle control, apoptosis, migration and invasion [17].

Cancer-specific miRNAs were identified in both hematological and solid tumors, such as prostate cancer, where they can act as oncogenes by repressing targets with tumor suppressor or apoptotic functions and as tumor suppressors, by inhibiting targets with proliferation functions [19]. These cancer specific miRNAs can also be detected in liquid biopsies from cancer patients, including blood fluids, such as plasma and serum, and are called circulating miRNA or cell-free miRNA [20]. The detection of these circulating molecules in blood fluids can provide a "snapshot" of the miRNAs expression patterns of the tumor, and are considered robust minimally invasive tools that can be used as markers for diagnosis, prognosis and therapy [21].

Among several miRNAs with abnormal expression in prostate cancer, the miR-183 family has often been found to be upregulated in this neoplasia [22]. The miR-183 family is highly conserved and consists of miR-96, miR-182 and miR-183 [23]. Mihelich et al. [24] demonstrated *in vitro* that miR-182 is preferentially exported from cells on exosomes and can be found in the cell-free miRNA form, making it a potential candidate for studies on body fluids.

The miR-182-5p is an oncogene and has been reported as overexpressed in tissues and prostate tumor cell lines [25–27]. The high levels of this miRNA have been associated with decreased survival in prostate cancer patients [25,28]. In PCa cell lines, the overexpression of miR-182-5p has been found in association with cell

proliferation and invasion, suggesting its role in modulating these phenotypes [25–27]. However, the role of this miRNA, and the other members of its family, in these cellular process have yielded contradictory results in different tumors, and in some cases, even within the same tumor type. Therefore in this study, our main goal was to evaluate the association of circulating levels of miR-182-5p in the plasma samples of patients with PCa and determine its regulatory role and mechanisms in mediating malignant tumor phenotypes, such as cell proliferation, migration and cytotoxic response to the most commonly used PCa chemotherapeutic agents.

MATERIAL AND METHODS

Prostate clinical cases and controls

Peripheral blood (PB) samples were collected from ninety-three patients with prostate cancer and forty-six cancer-free controls. The patients were recruited from the Londrina Cancer Hospital (Londrina, PR, Brazil) in the period of 2014 to 2015, and PB samples were collected prior to any chemo, radiotherapy or surgery treatment. The control group was obtained from Cismepar Hospital (Londrina, PR, Brazil) and included individuals cancer-free, without urinary symptoms and that presented PSA levels ≤ 4.0 ng/mL. The PB samples were processed for cell-free plasma within 2 hours after collection, according to Duttagupta et al. [29] protocol. This research was approved by Ethics Committee on Human Research of the State University of Londrina, Brazil (CAAE19769913.0.0000.5231). The clinical and histopathological information of the patients are described in **Table 1**. The individuals ancestry was determined according to the Brazilian Institute Geography and Statistics [30].

Prostate cancer cell lines

Prostate cell lines androgen-refractory (PC-3 and DU145) and androgen-responsive (LNCaP) were obtained from the Tissue Culture Shared Resource, Georgetown Lombardi Comprehensive Cancer Center, Georgetown University (Washington, DC, USA). Prior to miRNA analysis all the lines were authenticated by short tandem repeat (STR) profiling assays, following the International Cell Line Authentication Committee (ICLAC) guidelines [31]. Cells were cultured in a 5% CO_2 humidified incubator at 37°C, in RPMI 1640 supplemented with antibiotics

(Invitrogen, USA) and 10% (PC-3 and DU145) and 15% (LNCaP) of fetal bovine serum (FBS).

RNA extraction and qPCR

The RNA extraction of plasma samples was performed by miRNeasy Mini kit (Qiagen, Hilden, Alemanha) with modifications. The RNA was extracted from PC-3, DU145 and LNCaP cell lines using mirVana miRNA isolation Kit (Ambion, Austin, TX, USA), following manufacturer's instructions. RNA quantification was performed in spectrophotometer nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Mature miR-182 expression was analyzed by Taqman miRNA assay (ID: 002334) using miRNA RNU48 (ID:001006) as reference.

Expression of *VIM* (Hs00185584_m1); *CDH2* (Hs00983056_m1); *CLDN1* (Hs00221623_m1); *CTNNB1* (Hs00355049_m1); *SNAIL1* (Hs00195591_m1); *SNAIL2* (Hs00950344_m1); *ZEB1* (Hs01566410_m1); *CDH1* (Hs01023894_m1); O*R51E2* (Hs04231197_m1) and *SIM2* (Hs00894178_m1) was analyzed by Taqman Gene Expression Assay, using *GAPDH* gene as reference. cDNA was performed by High Capacity cDNA (Thermo Fisher Scientific, Baltics, UAB, Lithuania) and qPCR runs were performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Singapure). It was also included a pool sample as calibrator.

In vitro inhibition and overexpression of miR-182-5p

The prostate cancer cell lines were reverse transfected by Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, using has-miR-182-5p mirVana™ miRNA inhibitor (ID:MH12369) and mimic (ID:MC12369) assays (AMBION, USA). MirVana™ miRNA inhibitor let-7c was used as positive control (PC) and mirVana™ miRNA inhibitor as negative control (NC). Inhibition and overexpression of miR-182-5p was achieved using a final concentration of 50 nM and 10 nM, after 24 and 48 hours of transfection, respectively (**Figure 1A-B**). All transfections were performed in triplicate in independent experiments and verified for efficiency prior to each functional assay.

Proliferation Assays

Approximately $3x10^3$ PC-3 and DU145 cells and $5x10^3$ LNCaP cells were reversed transfected with both miR-182-5p inhibitor and mimic assays, in 96-well

plates and exposed to Cell Titer 96®AQ_{ueous} One Solution (Promega, Madison, WI, USA). The proliferation curves were determined at 24, 48, 72 and 96 hours after transfection, in relation to the negative control (NC), by measuring the 490 nm absorbance at an ELISA reader (Bioteck, Winooski, VT, USA). Independent triplicate experiments were performed.

Cell cycle assay

PC-3 cells and DU145 cells were transfected with both inhibitor and mimic miR-182-5p assays, in 6-well plates at $2x10^5$ cells/well and fixed in absolute alcohol for cell cycle analysis. The cells were stained with propidium iodide and analyzed on FACSAria system utilizing FACSDiva and FCS Express 4 softwares (DeNovo Software, Los Angeles, CA) with peripheral blood lymphocyte as an internal control.

Cell adhesion Assay

The PC-3 and DU145 transfected cells were analyzed for cell adhesion using the xCELLigence real-time cell analyzer system (ACEA, Biosciences Inc), Approximately $3x10^3$ of the cells were placed in E-plates, previously equilibrated with 50ul of RPMI at room temperature for 30 minutes, according to the manufacturer's instructions. The analysis was performed from 0 to 3 hours of experiment, according Kho et al. [32]. The Cell Index units were obtained from xCELLigence Software each 15 minutes and represent one point in the curve. The adhesion index was determined in relation to the negative control (NC).

Cytotoxicity assays

Cytotoxicity assays were performed using MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Thermo Fisher Scientific, Eugene, OR, USA) following manufacturer's instructions. PC-3 and DU145 cells were transfected in 96 well plates and treated with Docetaxel (Sigma) and/or Abiraterone (Sigma) in the concentrations of 1 nM, 100 nM and 1000 nM; and/or Metiformine (Sigma) in the concentrations of 1 µM, 10 µM and 100 µM. The most optimal concentrations for each cell line were previously tested in parental cells (not transfected). The absorbance was read at 540 nm in an Elisa reader (Bioteck, Winooski, VT, USA), after 24, 48 and 72 hours.

Western Blot and EMT analysis

PC-3, DU145 and LNCaP transfected cells were lysed using RIPA buffer with Protease and Phosphatase Inhibitor Mini Tablets (Pierce Biotechnology, Rockford, IL, USA). The protein concentrations were measured using Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) following manufactures' recommendations. Total protein (40 µg) was subjected to Bolt Mini Gels^{TM} (Thermo Fisher Scientific), the protein reference was done using the iBlot 2 Gel Transfer Device (Thermo Fisher Scientific) followed by immunoblotting. The proteins were visualized using SuperSignal ECL (Thermo Fisher Scientific). The primary antibodies used were: Epithelial-Mesenchymal Transition (EMT) Antibody Sampler Kit (1:1000, except ZEB1 1:200, Cell Signaling Technology, Inc); HMGA2 (1:750, Cell Signaling Technology, Inc); p-AKT (1:1000, Cell Signaling Technology, Inc); and GAPDH (1:2000, Cell Signaling Technology, Inc). The gels bands were quantified using ImageJ software [33].

Cell migration assays

Cellular migration activity was evaluated by standard wound-healing assays, using Culture-Insert (IBIDI$_{®}$, Germany). Approximately 1.2x10^{4} of PC-3 and DU145 cells were transfected into each side of the inserts. After transfection time, the inserts were removed, and the cells were washed with PBS to allow cell migration. Low concentration (2%) of FBS was used. Photomicrographs were taken in 0 hour and after 6, 24 and 48 hours and the gap distance between the inserts sides was measured by ImageJ [33].

Statistical Analysis

Data were reported as mean values ±SD in at least three replicates. Expression data were obtained from SDS 2.4 software (Applied Biosystems) and the relative expression analysis was performed by mathematical model $2^{-\Delta\Delta ct}$ [34].

All the data were analyzed by Student's t-test or two-way Anova using GraphPad Prism version 7 (La Jolla, California, USA). The descriptive analysis was performed by IBM SPSS Statistics 22.0 (IBM Corp, Armonk, New York, USA). *P*-values <0.05 were considered statistically significant.

RESULTS

miR-182-5p expression patterns in plasma sample

The levels of miR-182 were not different between patients and controls (**Figure 2A**). However, it was higher in patients that presented bilateral tumor when compared with patients that presented tumor in only one side of prostate (FC = 3.9, *P* = 0.04) (**Figure 2B**).

Inhibition of miR-182-5p decreases cell proliferation

In order to determine the role of miR-182-5p in regulating prostate cancer cell growth was performed by MTT. We observed that manipulation of the miR182-5p expression levels, differentially affect proliferation in the cell lines studied (**Figure 3A**). In the PC-3 cells, inhibition of the miR-182-5p expression levels, increased their proliferation rate when compared to the NC (after 96 hours); and its ectopic overexpression increased their proliferation (after 48, 72 and 96 hours); In the LNCaP cells, the inhibition of miR-182-5p also led to the decrease of their proliferation rate, however, the ectopic overexpression showed no effect in the proliferation of the cells after 24, 48, 72 and 96 hours. In the DU145 the inhibition of miR-182-5p expression caused the most significant decreased in the proliferation rate.

Overexpression of miR-182-5p increases the number of cells in G2/M phase in cell cycle analysis.

We observed that the overexpression of miR-182-5p induced a higher number of PC-3 and DU145 cells in G2/M phase (**Figure 3B**). No statistical significant effect in the distribution of the number of the cells in the cell cycle phases was observed upon inhibition of this miRNA in both of the cell lines analyzed in relation to the negative control.

Inhibition of miR-182-5p reduces cell adhesion in RTCA analysis.

The real time PC-3 adhesion assay data performed in xCELLigence instrument are showed in **Figure 4**. This assay was performed in the PC-3 and DU145 cell lines; the LNCaP cells were not evaluated due their poor adhesion capacity. We observed in the PC-3 cells that the inhibition of the expression levels of

miR-182-5p caused a delay in the time of the cells to adhere to the plate. This effect was not reversed, however, with the overexpression of this miRNA. In the DU145 cell line no significant effect in cell adhesion was observed upon manipulation of miR-182-5p levels (data not shown).

Overexpression of miR-182-5p induces cell migration in vitro

As shown for the adhesion assays, wound healing assay was only able to be performed in the PC-3 and DU-145 cells, due to the poor adhesion capacity of the LNCap cells, even after plate treatment with L-lisine. **Figure 5** shows the migration effects of overexpression of miR-182-5p in PC-3 cells; it can be clearly seen that the overexpression of miR-182 reduced the wound gap (as measured by the distance of the gap between the two sides of the insert), reflected by the increase in cell migration; however no effect was evidenced in these cells in cell migration when the miR-182-5p was inhibited. The DU145 cell line showed no significant changes in migration assay, upon manipulation of the miR-182-5p (data not showed).

Overexpression of miR-182-5p in PC-3 cells leads to increase of the expression of EMT promoter markers

Western blot and RT-qPCR analysis were performed to determine whether the alteration of EMT markers is one of the mechanisms by which miR-182-5p regulates tumorigenicity in the prostate cancer cells. We observed that the ectopic overexpression of miR-182-5p increased the levels of the EMT promoter marker ZEB1 and the mesenchymal protein N-CADHERIN. However the same was not observed for the other promoter marker SLUG (*SNAIL2*). At the other hand, inhibition of miR-182-5p expression levels, led to the decrease of E-CADHERIN and B-CATENIN. It was not observed changes in VIMENTIN levels after miR-182 manipulation (**Figure 6A**).

Inhibition of miR-182-5p in PC-3 cells leads to decrease expression levels of p-AKT, a marker for prostate cancer invasion

The expression levels of the protein p-AKT, considered a marker for tumor progression, were determined by Western Blot analysis after manipulation of the miR-182-5p expression levels in the PC-3 cells. Decreased expression levels of this

protein were observed in the cells transfected with miR-182-5p inhibitor, while increased levels were seen with its ectopic expression (**Figure 6B**).

Overexpression of miR-182-5p in PC-3 cells induces docetaxel resistance

The overexpression of miR-182 was able to promote resistance of docetaxel treatment after 72 hours in PC-3 cell when compared to the NC in all tested concentrations (**Figure 7**). No differences were found in the docetaxel cytotoxicity when the miR-182 was inhibited (data not showed). No differences was found in the other drugs evaluated (data not showed).

DISCUSSION

It is well known that miRNA aberrant expression plays a fundamental role in cancer progression [18]. In this study, we evaluated the cellular action of a specific miRNA, miR-182-5p, in modulating the aggressive phenotype of PCa. In addition, we determined the expression levels of this miRNA in plasma samples obtained from PCa patients and control individuals, to determine its diagnostic and prognostic potential.

Circulating miR-182-5p have been shown overexpressed in tumor tissue samples when compared with surrounding normal tissue or benign prostatic hyperplasia [26,35]. In our study, however, it was not possible to differentiate patients from controls individuals based on the plasma levels of miR-182-5p. Nevertheless, patients with aggressive tumor characteristics, such as bilateral tumor, presented higher miR-182-5p expression levels when compared to patients with unilateral tumor. The relationship between miR-182-5p and tumor aggressiveness have been previously reported by Casanova-Sales et al. [35] and Tsuchiyama et al. [36] where overexpression of this miRNA in the tumor tissue was associated with Gleason score. However, our study is the first to detect association of overexpression of miR-182-5p in its circulating form with histopathological parameters.

The functional analysis conducted *in vitro*, using repressors and inducers miRNAs assays, revealed that in general, miR-182-5p acts as oncomiR in PCa cells and contributes to their aggressive phenotype. We observed that inhibition of the expression levels of miR-182-5p significantly decreased the proliferation rate in the LNCap and DU145 transfected cell lines; conversely, its ectopic expression

increased the proliferation rates, however only significantly in the PC-3 cells. These findings are in agreement with previous studies performed by Hirata et al. [25], Liu et al. [26] and Wallis et al. [37] in PCa cells. In addition, we observed that inhibition of miR-182-5p lead to a decrease in cell adhesion, which can be one of the mechanisms that leads to a decrease of cell proliferation [38]. In addition, we observed, by flow cytometry, that in transfected cells with the miR-182-5p mimic, the number of cells in the G2/M phase was increased, supporting our proliferation assay findings and suggesting that miR-182-5p can regulate cell proliferation by controlling cell division events. The involvement of miR-182-5p in cell cycle has been previously observed in PC-3 cells by Liu et al. [26], where they showed that ectopic overexpression of miR-182-5p promoted the G1/S cell cycle transition.

We also shown that manipulation of the miR-182-5p expression levels, more specifically its overexpression, affected the migration capacity of the prostate cancer cells; PC-3 cells with ectopic expression of miR-182-5p presented a higher migratory rate as compared to the negative control, finding also supported by other studies in PCa [25–27]. In addition, inhibition of miR-182-5p, as demonstrated by the real-time adhesion assay conducted, led to the reduction of the capacity of the cells to adhere to the solid substrate. These results support the function of miR-182-5p in regulating cell proliferation, adhesion and migration of the prostate cancer cells, which largely determines their tumorigenic capacity.

In an attempt to elucidate the mechanism(s) by which miR-182-5p exerts this capacity, we assessed the expression levels of the EMT markers, as they play pivotal roles in tumor progression, through intricate and complex regulations of EMT associated genes, such as CADHERINS, SLUG, VIMENTIN, and ZEB1. Alterations of the expression levels of the corresponding genes, lead to changes in the cellular cytoskeleton, connection and polarity of the tumor cells, and directly impacts the tumorigenic behavior of the cells [39]. Interestingly, accumulating studies have shown that the EMT process is also controlled post-transcriptionally by miRNAs [40,41]. A number of miRNAs have been identified to target multiple components of this process [40–42], such as the one investigated in this study. We showed that the PCa cells with miR-182-5p overexpressed showed higher expression levels of ZEB1, an EMT induced marker, and N-CADHERIN, an EMT-associated mesenchymal protein [43,44]. This data was re-enforced by the reduced expression levels of nuclear β-

CATENIN, another EMT-mesenchymal associated protein [43], upon inhibition of miR-182-5p expression.

Another important change during EMT events is the repression of E-CADHERIN, an EMT-epithelial associated protein [45]. We observed that the expression levels of E-CADHERIN were decreased when miR-182-5p was inhibited. This result was not expected, and can reflect either a distinct mode of action of miR-182-5p in regulating E-CADHERIN or an indirect mechanism through the regulation of other downstreams targets. In the present study we also demonstrated that miR-182-5p overexpression led to the reduction of *SNAIL2* (SLUG) expression. This finding corroborates Qu et al. [42] that reported association of miR-182-5p with cell growth by repressing *SNAIL2* expression in PCa cells and Liu et al. [46] showed that the *SNAIL2* affect cell proliferation by regulating cyclin D1. Interestingly, studies in clinical PCa cases, have shown that the downregulation of *SNAIL2* expression is frequent in PCa tissue [47,48].

Finally, we also assessed whether the tumorigenicity of the PCa cells mediated by miR-182-5p could be due to alterations in the p-AKT protein levels, a marker that present an important role in prostate tumorigenesis; activation of p-AKT is reported to be directly associated with PCa cell invasion and tumor progression [49–51]. Indeed, we observed that cells with overexpression of miR-182-5p presented higher p-AKT expression levels, while the inhibition of this miRNA promoted decreased in the p-AKT levels. These data reinforce that miR-182-5p act as oncomiR and is actively involved in prostate tumorigenesis.

As revised by Ma et al. [52] miRNAs can have a role in the development of drug resistance in several types of cancer. Thus, in our study it was tested the most commonly used drugs for chemotherapy in prostate cancer, and we found that miR-182 overexpression can promote docetaxel resistance in PC-3 cell line. This is a metastatic hormone refractory cell line and docetaxel is the most used drug to treatment for advanced prostate cancer, including patients with the metastatic castration-resistant form of disease [53]. Besides that, we found high levels of this miRNA in plasma sample of patients with prostate cancer aggressiveness characteristics. Therefore, the miR-182-5p status seems to have an important role in treatment response. To the best of our knowledge, this is the first time that this miRNA was associated with docetaxel resistance. Thus, studies evaluating the status of this miRNA in patients that present docetaxel resistance are relevant.

In conclusion, the present study showed that miR-182-5p regulates PCa aggressive phenotype by exerting an oncogenic action. Its overexpression was shown to augment tumorigenicity in the PCa cells evaluated, as evidenced by an increased rate of cell proliferation and in the number of cells in G2/M phase and higher migratory capacity. The altered expression of critical EMT markers and the progression tumor marker p-AKT, could be one of the mechanisms by which miR-182-5p exerts its action. However, considering the multiple cancer mRNA targets that are regulated by miR-182-5p, such as *PDGF-D*, *SNAIL2*, *GNA13* [42,54,55], it is likely that additional mechanism(s) are involved in its modulatory role. Nevertheless our data, indicate that the deregulation of miR-182-5p is a determinant factor for PCa progression and warrants further analysis in well annotated clinical samples to determine its prognostic role in PCa.

Table 1: Clinical and histopathological characteristics of prostate cancer patients and controls studied.

Characteristics		**Patients n (%)**	**Controls n (%)**
Age (years)	< 65	28 (30.1)	22 (47.8)
	≥ 65	65 (69.9)	24 (52.2)
Ancestrally	Caucasian	72 (77.4)	35 (76.1)
	African	21 (22.6)	11 (23.9)
Familiar history of cancer	Yes	58 (62.4)	22 (47.8)
	Yes, Prostate	14 (15.1)	4 (8.7)
	No	35 (37.6)	24 (52.2)
PSA[a] (ng/mL)	≤ 4.0	8 (8.6)	46 (100.0)
	>4.0 to 10.0	37 (39.8)	
	> 10.1	48 (51.6)	
Gleason score[b]	3 to 6	55 (59.1)	
	7	30 (32.3)	
	8 to 9	8 (8.6)	
Bilateral tumor[b]	Yes	43 (46.3)	
	No	47 (50.5)	
	Missing	3 (3.2)	
Bone metastasis	Yes	11 (11.8)	
	No	81 (87.1)	
	Missing	1 (1.1)	
Treatment[cd]			
Prostatectomy		57 (58.2)	
Hormone therapy		16 (16.3)	
Radiotherapy		16 (16.3)	
Orchiectomy		8 (8.2)	
Others[e]		1 (1.0)	

[a]PSA = Prostate-specific antigen ; [b]Histopathological parameters from biopsy; [c]Treatment performed after blood collection; [d]Patients could be subjected to one or more treatments; [e]Patient under active surveillance.

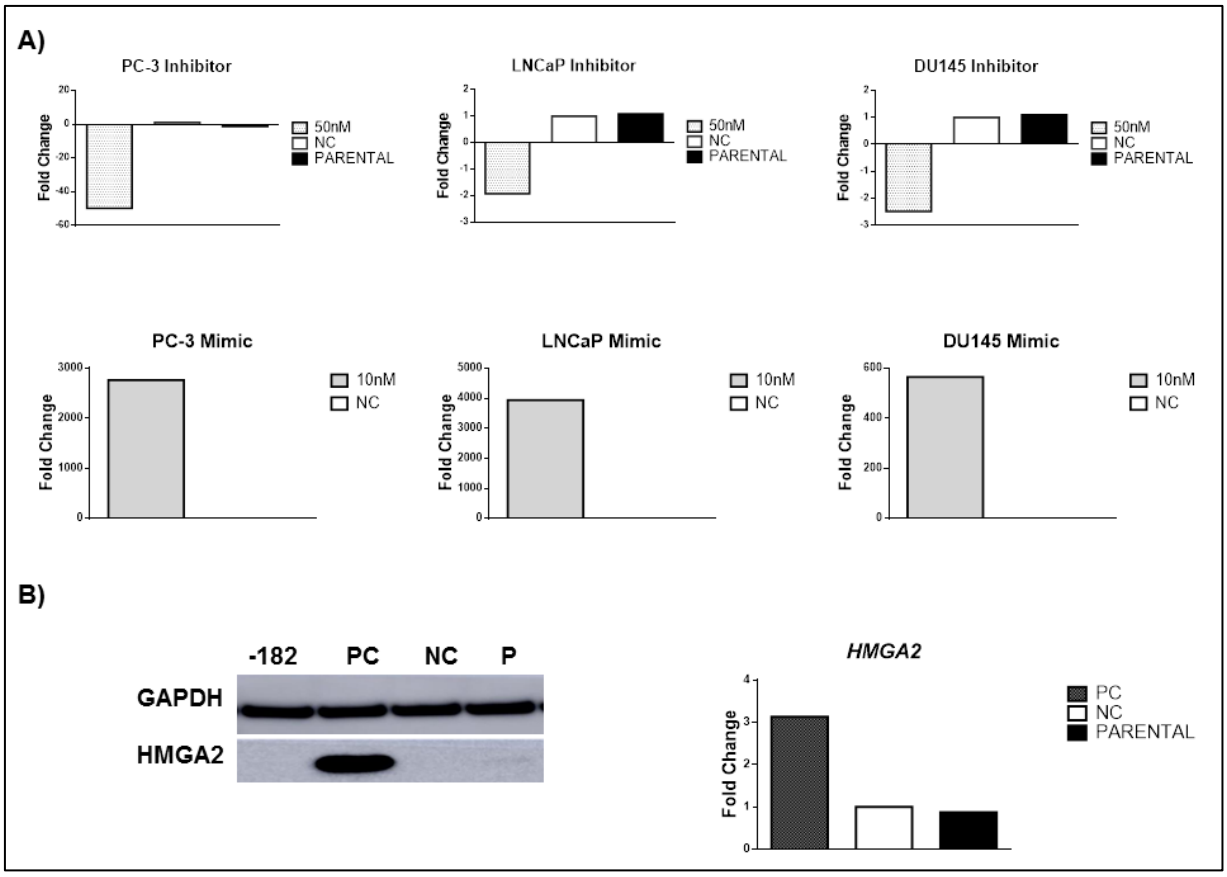

Figure 1: A) Differential expression of the endogenous levels of miR-182-5p in the different cell lines studied and their corresponding expression levels after the miRNA transfections assays; B) HMGA2 (positive control for transfection) assay to determine transfection effectiveness with the miR-182-5p inhibitor. GAPDH used as loading control. PC = Positive control; NC = Negative control; P = Parental; -182 = inhibitor.

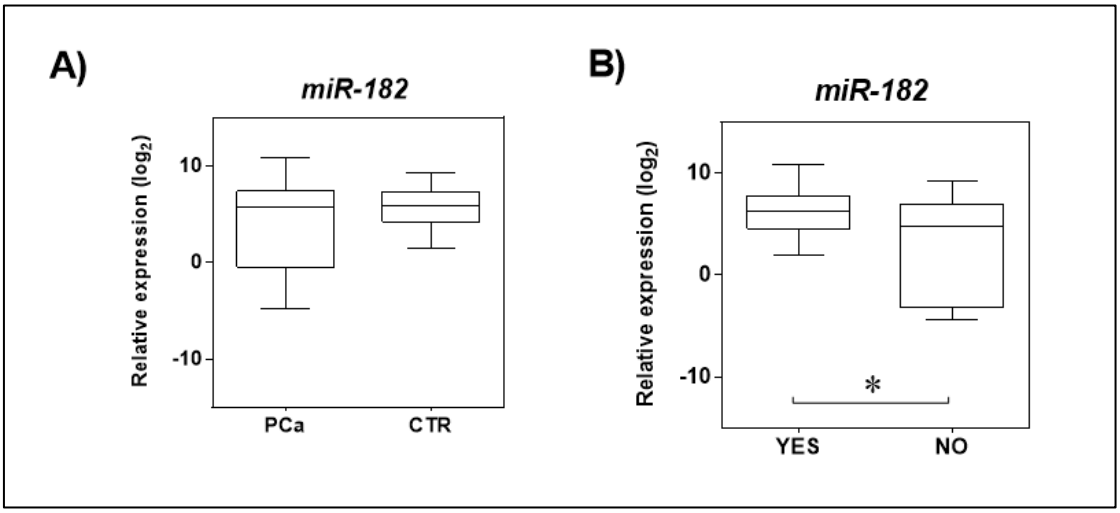

Figure 2: Differential expression of circulating miR-182 in plasma. A) Patients *versus* controls; B) Patients with bilateral tumor *versus* patients with tumor in one side of prostate. PCa = Prostate cancer; CTR = controls. *Statistically significance (Student's test-t $P < 0.05$)

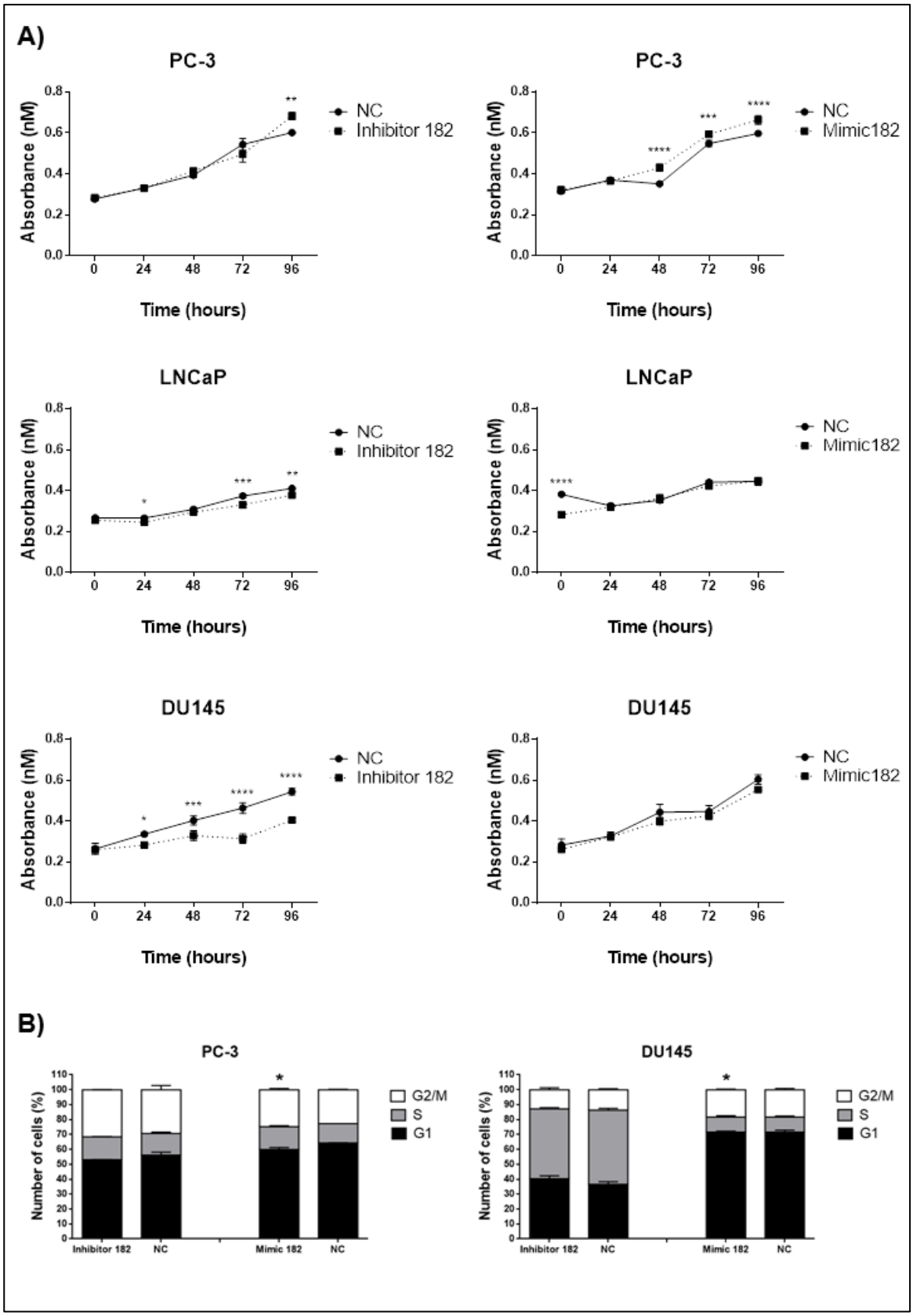

Figure 3: A) Proliferation data after inhibition or overexpression of miR-182-5p in different PCa cell lines; B) Cell cycle analysis after inhibition or overexpression of miR-182 in PC-3 and DU145 cells. NC = Negative control. Statistically significance **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

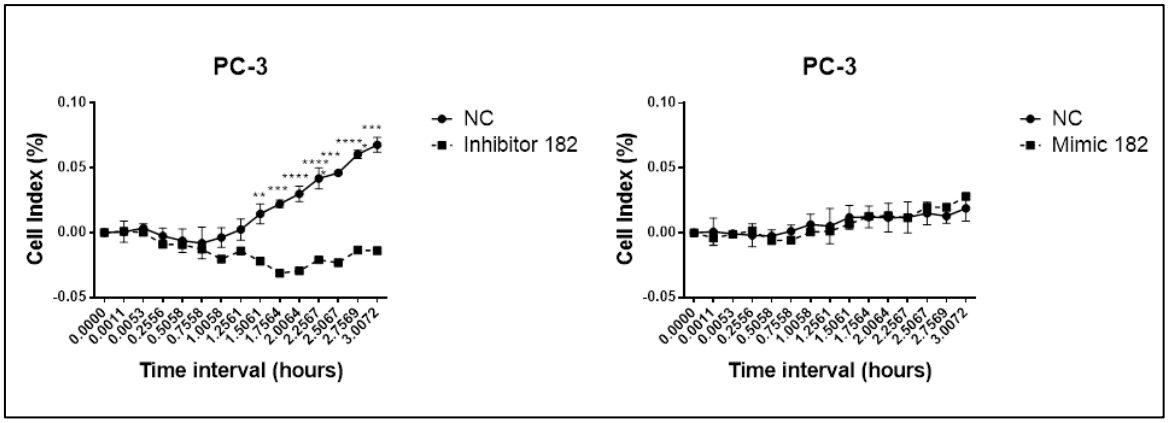

Figure 4: Real time adhesion assay after inhibition or overexpression of miR-182-5p in the PC-3 cells; NC = Negative control. Statistically significance **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

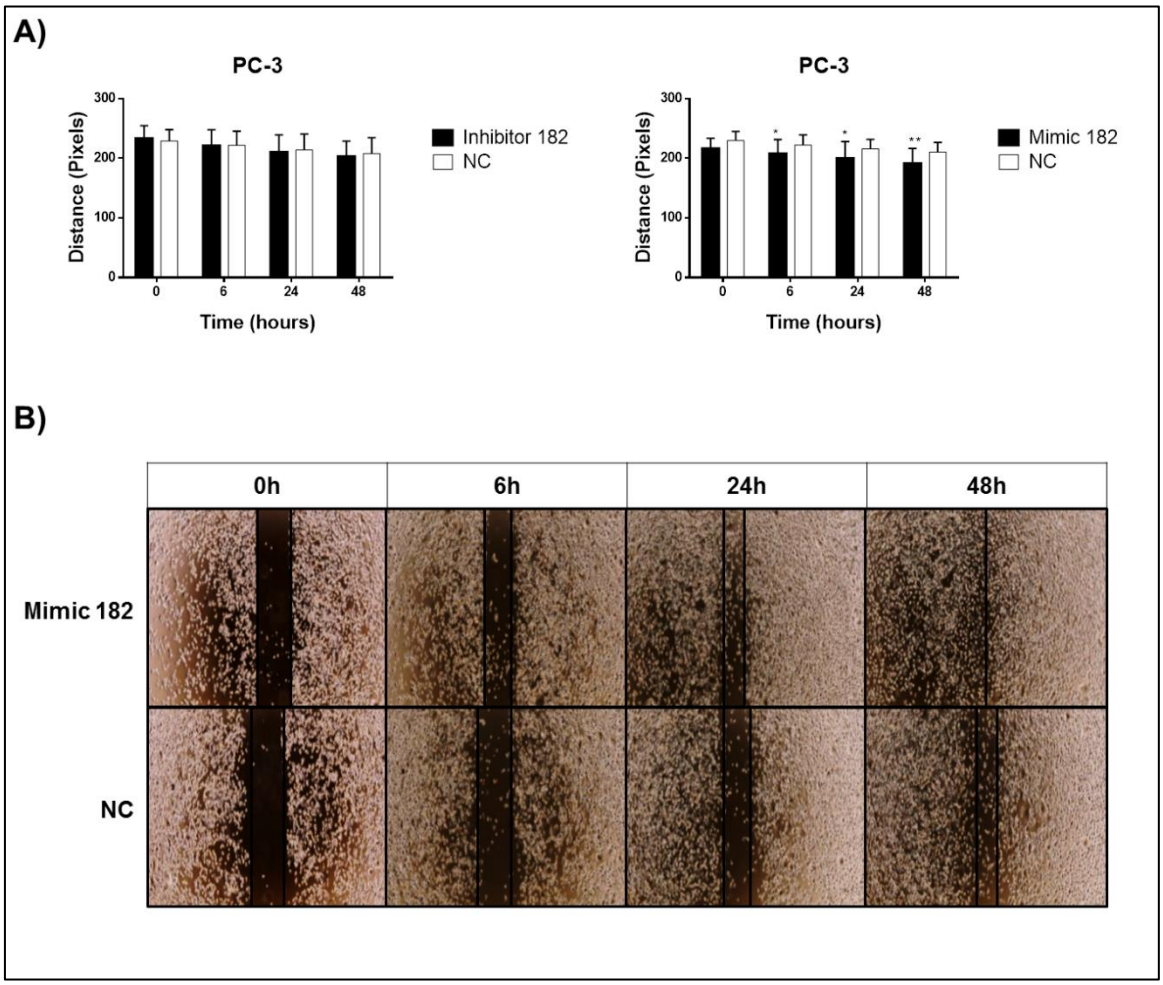

Figure 5: A) Wound healing assays after inhibition or overexpression of miR-182-5p in the PC-3 cells; B) Wound healing assay images after overexpression of miR-182-5p in the PC-3 cells; NC = Negative control. Statistically significance **P* < 0.05; ***P* < 0.01.

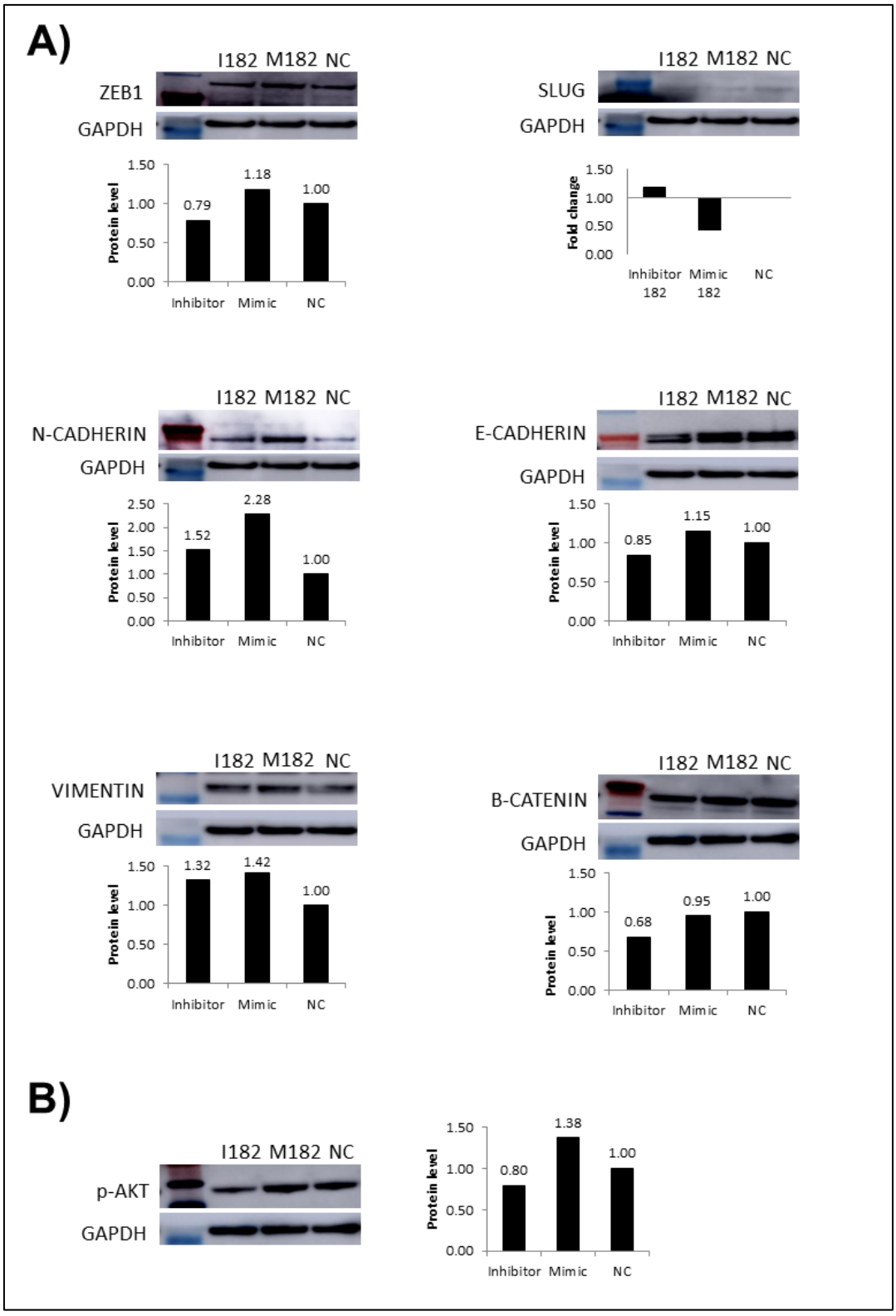

Figure 6: Expression analysis of EMT markers (B-CATENIN, E-CADHERIN, N-CADHERIN, VIMENTIN, ZEB1 and SLUG) and p-AKT proteins by Western blotting and RT-qPCR analysis (*SNAIL2*) after inhibition or overexpression of miR-182-5p in PC-3 cells. GAPDH was used as reference. I182 = Inhibitor; M182 = Mimic.

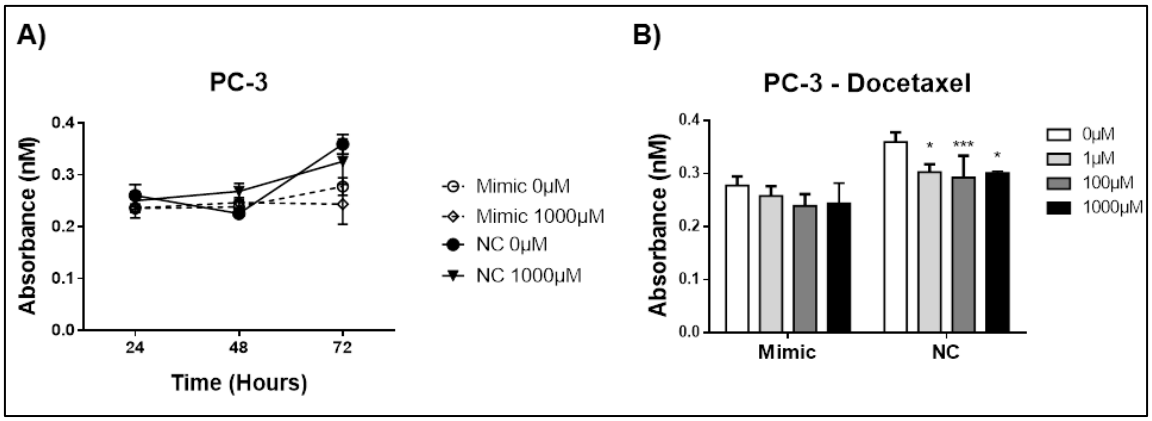

Figure 7: Docetaxel cytotoxicity after overexpression of miR-182 in PC-3 cells. A) All treatment times; B) 72 hours after treatment. Statistically significance **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

ACKNOWLEDGMENTS

This study received financial support from Araucaria Foundation of Support for the Scientific and Technological of Paraná (Conv.185/2014). The authors thank the services provided from the Tissue Culture Shared Resources (TCSR); the Genomics and Epigenomics Shared Resources (GESR) of Lombardi Comprehensive Cancer Center (LCCC); CAPES (Coordination for the Improvement of Higher Education Personnel) for the PDSE scholarship; CNPq, (National Council for Scientific and Technological Development); FAPESP (São Paulo Research Foundation); Londrina Cancer Hospital and Intermunicipal Health Consortium of the Middle Paranapanema (CISMEPAR).

CONFLICTS OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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7. ARTIGO IV

THE ROLE OF miR-141-3p IN MODULATING TUMORIGENICITY IN PROSTATE CANCER

Artigo a ser submetido à revista Oncotarget. Fator de impactor 5.008

THE ROLE OF miR-141-3p IN MODULATING TUMORIGENICITY IN PROSTATE CANCER CELLS

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ABSTRACT

Prostate cancer (PCa) is the second most commonly diagnosed neoplasia in men. Several deregulated miRNAs are found in this disease; among theses, miRNA-141-3p had been found upregulated in patients with aggressive PCa. Therefore, the aim of this study was investigate the potential of miR-141 as circulating marker for diagnostic and prognosis of PCa, as well as, its functional role in modulating events such proliferation, migration and treatment resistance in prostate cancer cell lines. miR-141 were found overexpressed in the plasma of metastatic patients, high-risk PCa and prostate-specific antigen (PSA) levels higher than 10 ng/mL. Our functional studies demonstrated that this miRNA can play different role in prostate cancer, depending on cellular context. In an early stage of PCa, miR-141 act as tumor suppressor, its inhibition promote an increase in the migratory ability, colony formation and higher resistance to docetaxel and abiraterone. Besides this, were observed changes in ZEB1 and E-CADHERIN levels, mechanisms by which miR-141-3p mediates these phenotypes. In the other hand, in a late stage of this disease, miR-141 acts as oncogene. High levels of this miRNA are found in patients with aggressive PCa and the ectopic expression of miR-141 promote increase of proliferation, changes in cell cycle and increase of p-AKT in PC-3 cells. In conclusion, our results showed that miR-141 can be a good marker for PCa aggressiveness; play a dual role in prostate cancer and its status is important to disease progression and drug resistance.

Key words: Prostate cancer, cell-free miRNA, plasma, oncogene, tumor suppressor.

INTRODUCTION

Prostate cancer (PCa) is one of the most frequent diagnosed cancers in men worldwide and the third cause of death, due to cancer, in developed countries [1]. Approximately 10% of the patients are diagnosed with metastatic prostate cancer (mPCa) at first diagnosis, mostly to the bones, which is the ultimately cause of death [2,3]. Although improvements in treatment have been developed for mPCa, including androgen deprivation therapy, they do not adequately predict resistance to therapy [2]. In fact, treatment decisions are still mostly based on histology (Gleason score),

prostate-specific antigen (PSA) levels and local disease state, which have known limitations associated with overtreatment and unnecessary morbidity effects [4,5]. Therefore, molecular biomarkers can augment the diagnosis of patients at an early stage and distinguish the ones with the highest risk of developing metastatic disease is needed, so they can undergo a more aggressive treatment during the course of their disease.

In this sense, microRNAs (miRNAs) have emerged as a new class of disease-specific biomarkers, with important roles in cancer initiation and progression. Their accessibility and high stability in the circulation, make them particularly attractive as potential diagnostic, prognostic, and predictive molecular tools that can be used for surveillance of early stage and presymptomatic diseases and identification of high-risk patients [6–8]. In PCa, this molecules have been involved in multiple processes of the metastasis cascade, including cell migration, invasion, adhesion, and changes in the epithelial–mesenchymal transition (EMT) pathway [9–13].

The miRNA-141-3p is a member of the miR-200 family that presents five members mapped in two independent transcriptional clusters: miR-200a, 200b and 429, on chromosome 1p36; and miR-200c and 141-3p, on 12p13. MiR-141-3p, as other members of the miR200 family, has been shown to be involved in the regulation of the events that promote EMT, apoptosis, cell growth and metastasis in a variety of cancers, including PCa [14–16]. Multiple targets for miR-141-3p are involved in this process, including the EMT promoter gene *ZEB1* [17,18].

In clinical cases, overexpression of miR-141-3p has been reported in patients with aggressive PCa [19,20] and mPCa [21–25]. Interestingly, circulating levels of this miRNA was found to be a potential biomarker in patients with mPCa, including the ones with castration resistance after hormone therapy [22,26,27]. The potential role of miR-141-3p for diagnosis, however, is not clearly defined; while some studies showed that this miRNA is overexpressed in patients with PCa when compared to patients with hyperplasia prostatic benign and healthy controls [25,28], others showed no association [23,29,30].

In this study, we aimed to investigate the expression levels of circulating miR-141-3p in the plasma samples of PCa patients in relation to cancer-free controls, to determine its diagnostic value, as well as its association with metastatic disease. In addition, considering that there are distinct actions of this miRNA in suppressing or promoting metastasis according to cellular contexts in PCa [16,28,31,32], we

evaluated its functional role in modulating cell proliferation, migration, and resistance to treatment in androgen-refractory (PC-3 and DU145) and androgen-responsive (LNCaP) PCa cell lines. Our findings demonstrated a significant higher expression levels of circulating miR-141-3p in the plasma samples of PCa patients that presented bone metastasis, high-risk PCa and PSA levels higher than 10 ng/mL. The functional studies showed that the miR-141-3p can act as an suppressor tumor or as an oncogene in PCa cells, depending on cellular context.

MATERIAL AND METHODS

Sample population

Ninety-two patients with positive biopsy for PCa and forty-three hospital-based cancer-free individuals controls were recruited from the Londrina Cancer Hospital in the period of 2014 to 2015, after informed consent. The control group was cancer-free individuals without urinary symptoms and PSA levels ≤ 4.0 ng/mL.

The patients and controls clinical information was obtained through medical records, and are described in **Table 1**. In the patients' group the average age was 69.4 ± 9.0 and in the control group 64 ± 4.6. The PSA levels of the patients, based in three defined categories (≤ 4.0, from >4.0 to 10.0 and > 10.0 ng/mL), were mostly > 10.0 ng/mL (50.0% of the patients). Gleason score 3 to 6 was observed in 57.6% of the patients, followed by score 7 and 8 to 10, in 32.6% and 9.8%, respectively. Bone metastasis was present in 12% of the PCa patients. The prostate cancer risk was considered high in 43.5% and low in 56.5% of the patients. High-Risk and Low-Risk categorization was performed according to the European Association of Urology and National Comprehensive Cancer Network (PSA > 20 ng/mL, biopsy's Gleason score ≥ 8). Patients with pathologic stage ≥ pT3 and presence of metastasis were also included as high-risk [33,34].

In most of the patients (57.6%) prostatectomy was performed; 9% of the patients performed orchiectomy. 17.4% of patients were treated by hormone deprivation and 19.6% by radiotherapy. 78.3% of the patients were Caucasians and 21.7% were African. Ethnic groups were categorized according to the ethnic-racial categories considered by the Brazilian Institute of Geography and Statistics [35].

Five mL of peripheral blood were collected from each PCa patient and voluntary controls, samples were preserved in ice and processed within 2 hours after

collection. The process for plasma isolation was done according to Duttagupta et al. [36] protocol. The plasma samples were frozen at -80°C until use. This research was approved by Ethics Committee on Human Research of the State University of Londrina, Brazil (CAAE19769913.0.0000.5231).

Prostate cancer cell lines

Prostate cell lines androgen-refractory (PC-3 and DU145) and androgen-responsive (LNCaP) were obtained from the Tissue Culture Shared Resource, Georgetown Lombardi Comprehensive Cancer Center, Georgetown University (Washington, DC, USA). Prior to miRNA analysis all the lines were authenticated by short tandem repeat (STR) profiling assays, following the International Cell Line Authentication Committee (ICLA) guidelines [37]. Cells were cultured in RPMI 1640 supplemented with antibiotics (Invitrogen, USA), in a 5% CO_2 humidified incubator at 37°C. PC-3 and DU145 were supplemented with 10% fetal bovine serum (FBS) and LNCaP with 15% fetal bovine serum (FBS).

MiR-141-3p transfection assays

Indirect reverse transfections were performed in the PCa cell lines studied, using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, for the has-miR-141-3p mirVana™ miRNA inhibitor (ID:MH108660) (Ambion, Austin, TX, USA) and mimic (ID:MC10860) (Ambion). A positive control (mirVana™ miRNA inhibitor let-7c positive control) and a negative control (mirVana™ miRNA inhibitor negative control) for transfection was included in all the experiments.

The inhibition of miR-141-3p was performed at a final concentration of 50nM of inhibitor for 48 hours and the overexpression at a final concentration of 10nM of mimic for 24 hours **(Figure 1).** All assays were performed in triplicate in independent experiments.

RNA extraction and RT-qPCR analysis

The RNA extraction of the plasma samples was performed by miRNeasy Mini kit (Qiagen, Hilden, Alemanha) according to optimization of the manufactors's manual. The RNA from PC-3, DU145 and LNCaP cell lines were extracted using mirVana miRNA isolation Kit (Ambion). RNA quantification was accomplished in

spectrophotometer nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Taqman® miRNA assay (ID: 002334) was used to measured mature miR-141-3p levels. miRNA RNU48 (ID:001006) was used as reference. High Capacity cDNA (Thermo Fisher Scientific, Baltics, UAB, Lithuania) was used for cDNA synthesis for gene expression, which was performed by Taqman® Gene Expression Assay, following manufacturer's instructions in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Singapura). The following genes were analyzed: *VIM* (Hs00185584_m1); *CDH2* (Hs00983056_m1); *CTNNB1* (Hs00355049_m1); *SNAIL1* (Hs00195591_m1); *SNAIL2* (Hs00950344_m1); *ZEB1* (Hs01566410_m1); *CDH1* (Hs01023894_m1); O*R51E2* (Hs04231197_m1); *SIM2* (Hs00894178_m1).

Functional Analysis

Evaluation of cell proliferation, cell cycle, cell adhesion, cell migration, cytotoxicity assays and western blot were done as previously performed by our group (data not published).

Clonogenic assay

PC-3 and Du145 reversed transfected cells were seeded in 6-well plates at a density of 300 cells/well and grown for 4 weeks to allow colony formation. The medium was changed every 4 days. The colonies were fixed in 4% paraformaldehyde and stained with crystal violet 0.5% solution. The number of colonies was counted manually in a light microscope. Cell clusters with more than 50 cells were considered colonies.

Statistical Analysis

Data were reported as mean values ± SD in at least three replicates. The relative expression analysis was performed by the $2^{-\Delta\Delta ct}$ method [38]. Student's t-test was used to compare the relative expression between the groups.

All functional assays were analyzed using two-way Anova test, using GraphPad Prism version 7 (La Jolla, California, USA). The descriptive analysis was performed by IBM SPSS Statistics 22.0 (IBM Corp, Armonk, New York, USA). *P*-values < 0.05 were considered statistically significant.

RESULTS

Circulating miR-141-3p is overexpressed in the plasma samples with high levels of PSA, high-risk and metastatic PCa patients

The analysis of the expression levels of circulating miR-141-3p in the plasma samples of the PCa patients (n = 92) in comparison to controls (n = 43) revealed no significant differences **(Figure 2A).** However, PCa patients that presented bone metastasis (n = 11), high-risk PCa (n = 40) and PSA levels higher than 10 ng/mL (n = 46) showed significant higher expression levels of circulating miR-141-3p when compared with patients without metastasis (n = 78), low-risk of PCa (n = 52) and PSA ≤ 10 ng/mL (n = 46), (FC = 9.1/ *P* = 0.047, FC = 8.4/ *P* = 0.003 and FC = 6.2/ *P* = 0.01, respectively) **(Figure 2B-D).**

Distinct effects in cell proliferation after miR-141 inhibition and overexpression according to the PCa cell line

In order to determine the role of miR-141-3p in regulating prostate cancer cell growth were performed MTT assays followed by flow cytometry after manipulation of miR-141-3p expression levels.

A distinct effect of the manipulation of this miRNA in cell proliferation was observed in the cell lines studied; inhibition of its expression levels showed a significant reduction of cell proliferation in DU145 and LNCaP cells. In PC-3 cells however, a significant increase in cell proliferation was observed after 48 hours, although not maintained at 72 hours (**Figure 3A**). Flow cytometry analysis of these cells showed no changes in the cell cycle phases in PC-3 cells with miR-141-3p inhibitor. However, in DU145 cells, the miR-141-3p inhibition showed an increased in the number of cells in G2/M phase (**Figure 3B**).

Ectopic expression of miR-141-3p, also showed distinct effects in PC-3 and DU145 cells; a significant increase in cell proliferation was observed in PC-3 cells after 48 hours. ($P < 0.0001$) (Figure 3A). Interestingly, the cell cycle analysis also showed a significant change in the content of cells in G2/M, S and G1 phases in these transfected cells (Figure 3B). No changes in cell proliferation were observed in DU145 cells. In LNCaP cells, similarly to the inhibitor effect, overexpression of miR-141-3p caused significant reduction of cell proliferation (**Figure 3A**). Cell cycle analysis was not performed for this cell line.

Inhibition of miR-141-3p increases cell migration and promote colony formation

The effects of miR-141-3p in cell migration were evaluated in standard wound healing assay in PC-3 and DU145 cell lines. Inhibition of its expression in PC-3 cells led to an increase in the migration capability of the cells, most notably after 48 hours **(Figure 4A).** No changes were observed with the ectopic expression of miR-141-3p in these cells **(Figure 4A).** Interestingly, in these cells, inhibition of miR-141-3p promoted colony formation; a higher number of colonies was observed in cells transfected with miR-141-3p inhibitor when compared to NC. No effect was observed in the cells transfected with miR-141-3p mimic **(Figure 4B).** No changes in either migration (data not showed) or colony formation were observed in the transfected DU145 cells **(Figure 4B)**.

The cell-adhesion assays, were also performed in PC-3 and DU145 transfected cells. No effects were observed in any of the cells in the time of cell adherence, after inhibition or overexpression of miR-141-3p (data not shown). The LNCaP cells were not evaluated due their poor adhesion capacity.

Inhibition of miR-141-3p causes increase of the expression of the EMT promoter marker ZEB1

The effects of the manipulation of miR-141-3p expression levels were determined in relation to the expression of EMT markers in PC-3 cells. The inhibition of this miRNA caused significant increased levels of the EMT promoter marker ZEB 1 in all the cell lines analyzed and decreased levels of E-CADHERIN were noted **(Figure 5A).** However its ectopic expression led to the increased expression of SLUG, another EMT promoter **(Figure 5A)** No significant changes were observed in the other markers analyzed: N-CADHERIN, B-CATHENIN and VIMENTIN. Interestingly, the ectopic overexpression of miR-141-3p also led to an increase of the prostate tumor progression associated marker, p-AKT **(Figure 5B)**.

Inhibition of miR-141-3p increases resistance to docetaxel and abiraterone

The cytotoxic effect of docetaxel and abiraterone, first and second line of treatment in PCa, respectively, were evaluated in the PC-3 and DU145 cells after miR-141-3p transfection. In PC-3 cells transfected with the inhibitor, increased number of viable cells was observed after exposure to docetaxel, in comparison to

the NC. However, significance was achieved only after 72 hours and with the highest concentration of the drug (**Figure 6A**). PC-3 cells (NC) are sensitive to docetaxel in a dose dependent manner **(Figure 6A)**. No changes in cytotoxicity to this agent was observed in the inhibitor transfected DU145 cells and/or in the PC-3 and DU145 mimic transfected cells (data not shown).

The effects on abiraterone cytotoxicity were mostly seen in the DU-145 cells transfected with miR-141-3p inhibitor **(Figure 6B).** Increased number of viable cells was observed in 1000μM at 72 hours in relation to the NC. The NC is sensitive to abiraterone in a dose dependent manner (**Figure 6B**) In the PC-3 cell line neither negative control nor inhibited/overexpressed cells were affected by the abiraterone treatment in any of the concentrations tested (1 μM, 100 μM and 1000 μM) (data not shown). Similarly, the treatment with metformin was not cytotoxic for PC-3 cells after the transfection assays, even at the higher concentrations (data not shown).

DISCUSSION

The miR-141 has been highlighted due to its relation with prostate cancer aggressiveness [39]. In this study we showed increased levels of miR-141-3p in the plasma samples of PCa patients in association with high-risk, bone metastasis and high levels of PSA, when compared to patients with low-risk of PCa, absence of metastasis, and with PSA levels ≤ 10 ng/mL. These findings corroborate other studies in prostate tissue and plasma/serum [19–25], indicating the potential role of this miRNA as prognostic marker for PCa. On the other hand, in our study and in agreement with studies conducted by Agaoglu et al. [23], Westermann et al. [29] and Kachakova et al. [30], we did not observe differences of the expression levels of this miRNA between patients and controls. Thus, despite of this miRNA seem to be a good marker for aggressive PCa, it cannot be used as diagnostic marker.

The manipulation of the expression levels of miR-141-3p in our study showed its modulatory role in the metastatic phenotype of PCa, however with distinct types of action in some of the phenotypes analyzed. These conflicting results have been seen in other types of cancers on the role of each miR-200 family member in repressing or enhancing cancer cell migration and invasion as well as the tumor growth and metastasis [40,41].

In the proliferation assays were observed that the ectopic expression of miR-141-3p in PC-3 cells significantly led to an increase in cell proliferation, which was corroborated by an increased frequency of cells in G2/M and S and decreased in G1 phase. This increase was however not observed in the DU145 and LNCaP cells. The same results were observed by Li et al. [17] that showed that the overexpression of miR-141 increased cell proliferation, while the inhibition had the opposite effect. Interestingly however, in the other assays of our study, a consistent tumor suppressor action of this miRNA was observed: inhibition of miR-141-3p increased the migration capability, colony formation and increased resistance to docetaxel and abiraterone of the PCa cells, particularly of the PC-3 cells.

The increase of migration upon suppression of miR-141-3p was also shown in other types of cancer cell lines, such as pancreatic [42] and colorectal [43]. The reverse effect with the ectopic expression, as shown in studies in gastric [44], esophageal [45] and PCa [31], was however not observed in our study.

The miR-141-3p belongs to miR-200 family, knowing as EMT regulatory [15]. *In vitro* studies in breast, cervix and colorectal cancer cell lines demonstrated that miR-141-3p regulate ZEB1 [17,18], which promotes EMT, a pathway critical for cell migration and other tumorigenic properties, such as migration, invasion and stemness-maintenance [46]. In PCa it has been shown that the EMT events that lead to an increase in migration occur in an early stage of tumorigenesis process [40]. In our study, the inhibition of miR-141-3p showed an increase in protein levels of ZEB1, as well as decrease of E-CADHERIN protein levels. These results are in agreement with the findings of Korpal et al. [18] in breast cancer cell line and Burk et al. [17] in colorectal cancer cell line.

In the present study was also showed that patients with low-risk PCa do not presented high levels of miR-141. Thus, our findings support the action of miR-141-3p as a tumor suppressor. On the other hand, we observed high levels of miR-141 in patients with metastatic tumor and high-risk PCa, these events occurs in a late stage of disease progression. Our study also demonstrated that overexpression of this miRNA not affected the migration events, but induced an increase of p-AKT protein levels, a progression tumor marker, and induced proliferation in PC-3 cells. Therefore, it is possible to suggest that miR-141 seems to play as oncogene in late stage of PCa progression.

In relation to drug resistance was showed that miR-141-3p may affect cytotoxicity to docetaxel and abiraterone. Docetaxel is the first line of treatment for patients with mCRPC (metastatic castration-resistant prostate cancer) [47] and combined with androgen deprivation therapy has been shown efficacy for the treatment of metastatic patients that are hormone sensitive [47]. In our analysis using this drug, we observed in agreement with other authors [48], that it caused cytotoxicity to the PC-3 parental cells. However, in the PC-3 cells transfected with miR-141-3p inhibitor, this cytotoxicity was not observed and in fact, an increase in resistance was observed. As mentioned above, high levels of miR-141-3p were found in the plasma samples of metastatic PCa. Considering that half of the patients treated with docetaxel do not respond to treatment [49], it is possible to suggest that high levels of miR-141-3p could impact the efficacy of docetaxel, and be one of the causes of resistance.

Abiraterone, is a potent suppressor of cytochrome P450 17 enzyme (CYP17A1) that mediates androgen production, and that has been used as a second line treatment for mCRPC [47,50]. In our study, in the PC-3 cells, neither the negative control nor the cells transfected were affected by the treatment with this agent, in any of the concentrations tested. Similar results were observed by Bansal et al. [51] in this same cell line, indicating that these androgen-refractory cells are not sensitized by abiraterone treatment. However, in the DU145 cells, also an androgen-refractory line, the inhibited expression of miR-141-3p cells, caused an increase in the number of viable cells after treatment. Interestingly, inhibition of miR-200b-3p in castration–resistant prostate cancer (CRPC) cells was shown to increase resistance to anti-androgen therapy, such as abiraterone [52]. Although there are a limited number of studies assessing the role of miRNAs in modulating abiraterone response in PCa cells [52], these results suggest that miR-141-3p may interfere with drug resistance in hormone resistant PCa cells and could be therefore a potential antisense miRNA for cancer therapy.

In conclusion, in our study was demonstrated that miR-141-3p presents a tumor suppression action, considering that its inhibition promote cell migration, colony formation, and resistance to chemotherapy drugs. Increased levels of the EMT marker ZEB1, one of the miR-141-3p targets, could be one of the mechanisms by which miR-141-3p mediates these phenotypes. However, an oncogenic action was also observed, by a significant increase in cell proliferation, in the number of

cells in the G2/M phase and in the expression of the tumor progression associated marker, p-AKT. In addition, increased expression of miR-141-3p was observed in the plasma of patients with high-risk PCa, metastasis and high PSA levels, showing that this miRNA can be a good marker for PCa prognosis. Although these results are intriguing and warrant further analysis on the functional role of this miRNA in PCa, it supports its involvement as a critical player in this disease.

Table 1: Clinical and histopathological characteristics of prostate cancer patients and controls

Characteristics		**Patients n (%)**	**Controls n (%)**
Age (years)	< 65	27 (29.3)	22 (51.2)
	≥ 65	65 (70.7)	21 (48.8)
Ancestrally	Caucasian	72 (78.3)	33 (76.7)
	African	20 (21.7)	10 (23.3)
PSA[a] (ng/mL)	≤ 4.0	8 (8.7)	43 (100.0)
	> 4.0 to 10.0	38 (41.3)	
	> 10.1	46 (50.0)	
Gleason Score[b]	≤6	53 (57.6)	
	7	30 (32.6)	
	≥8	9 (9.8)	
Bone metastasis[b]	Yes	11 (12.0)	
	No	78 (84.8)	
	Missing	3 (3.2)	
PCa Risk	High	40 (43.5)	
	Low	52 (56.5)	
Treatment[cd]			
Prostatectomy		53 (57.6)	
Hormone therapy		16 (17.4)	
Radiotherapy		18 (19.6)	
Orchiectomy		8 (9.0)	

[a]PSA = Prostate-specific antigen; [b]Histopathological parameters from biopsy; [c]Treatment performed after blood collection; [d]Patients could be subjected to one or more treatments.

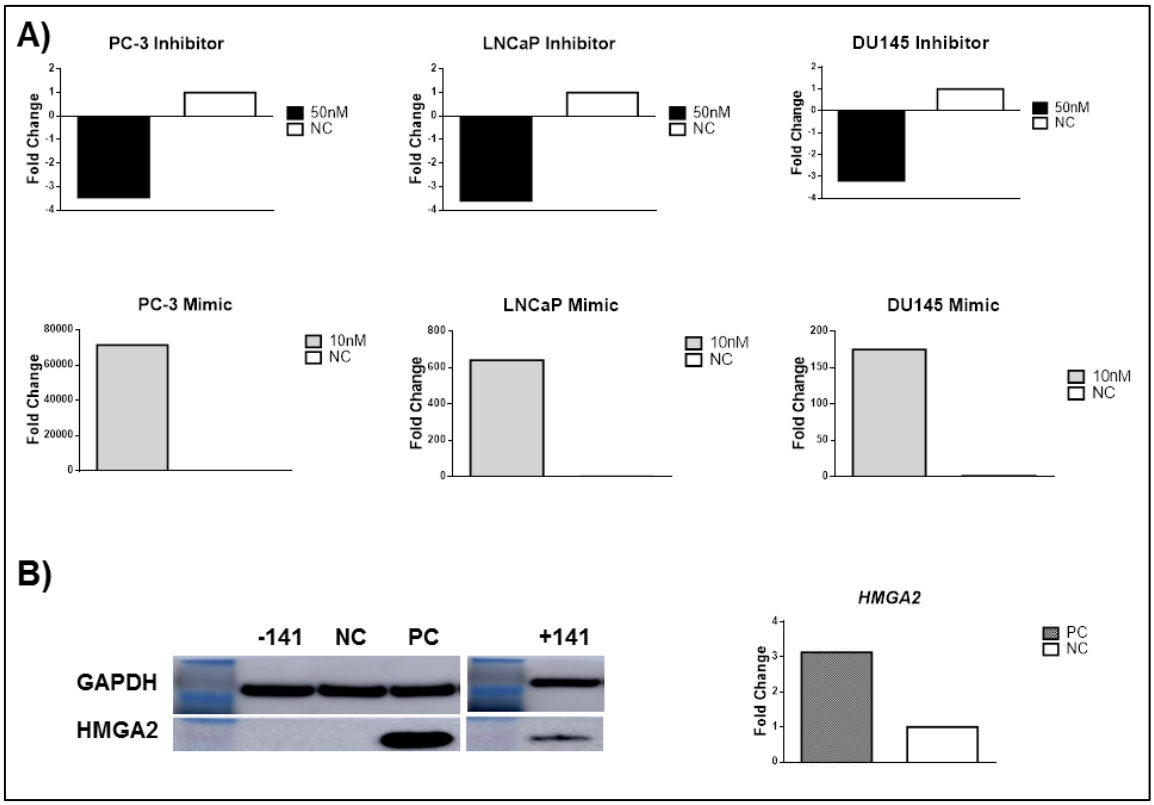

Figure 1: Differential expression of miR-141 in PC-3, LNCaP and DU145 cell lines. A) After inhibition or overexpression of miR-141; B) Transfection effectiveness in positive control. NC=Negative control; PC=Positive control.

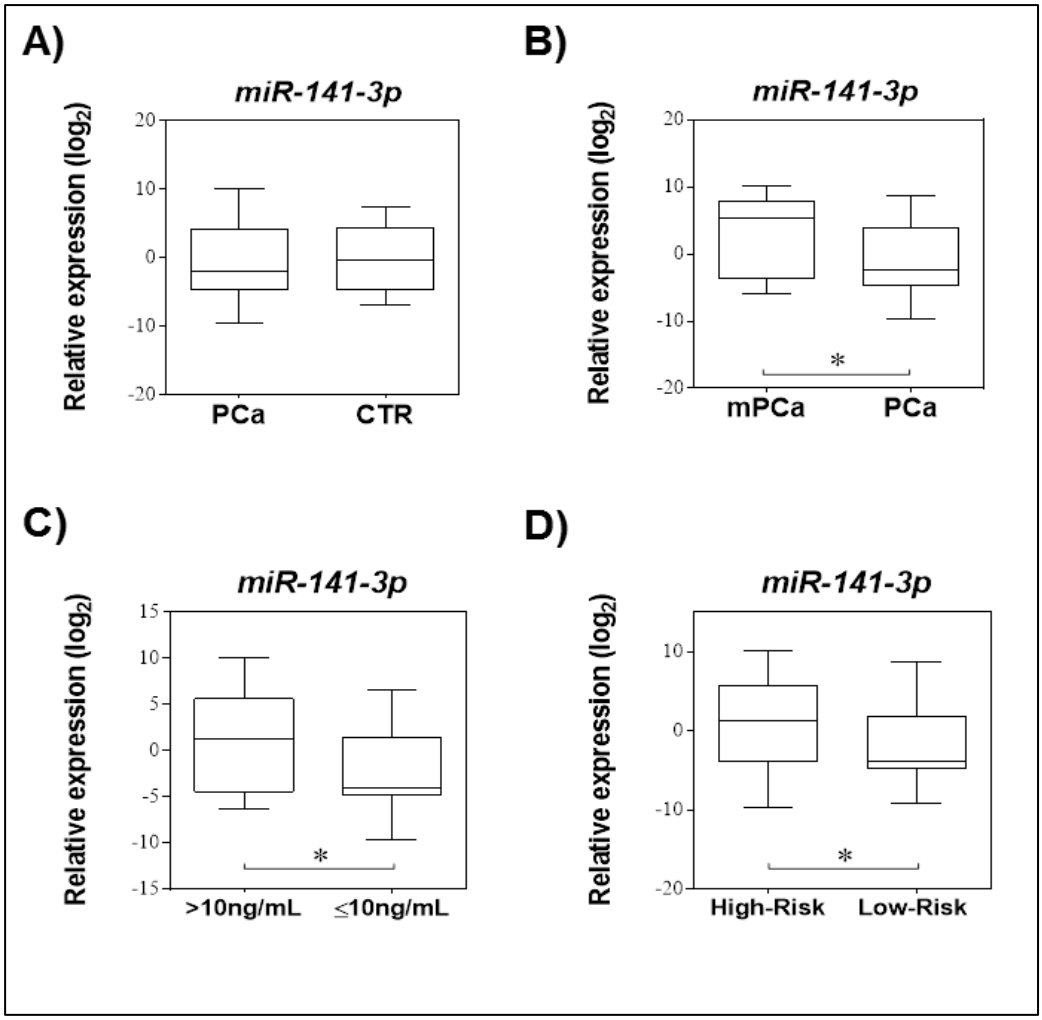

Figure 2: Expression levels of of circulating miR-141-3p, by qRT-PCR analysis, in plasma samples of: A) prostate cancer (PCa) patients *versus* controls, B) patients with metastatic PCa (mPCa) *versus patients with no metastatic* PCa (PCa); C) PCa patients with >10 ng/mL *versus* ≤10 ng/mL of Prostate-Specific Antigen (PSA); D) PCa patients with high-risk *versus* low-risk PCa. *Statistical significant (Student's t-test $P < 0.05$).

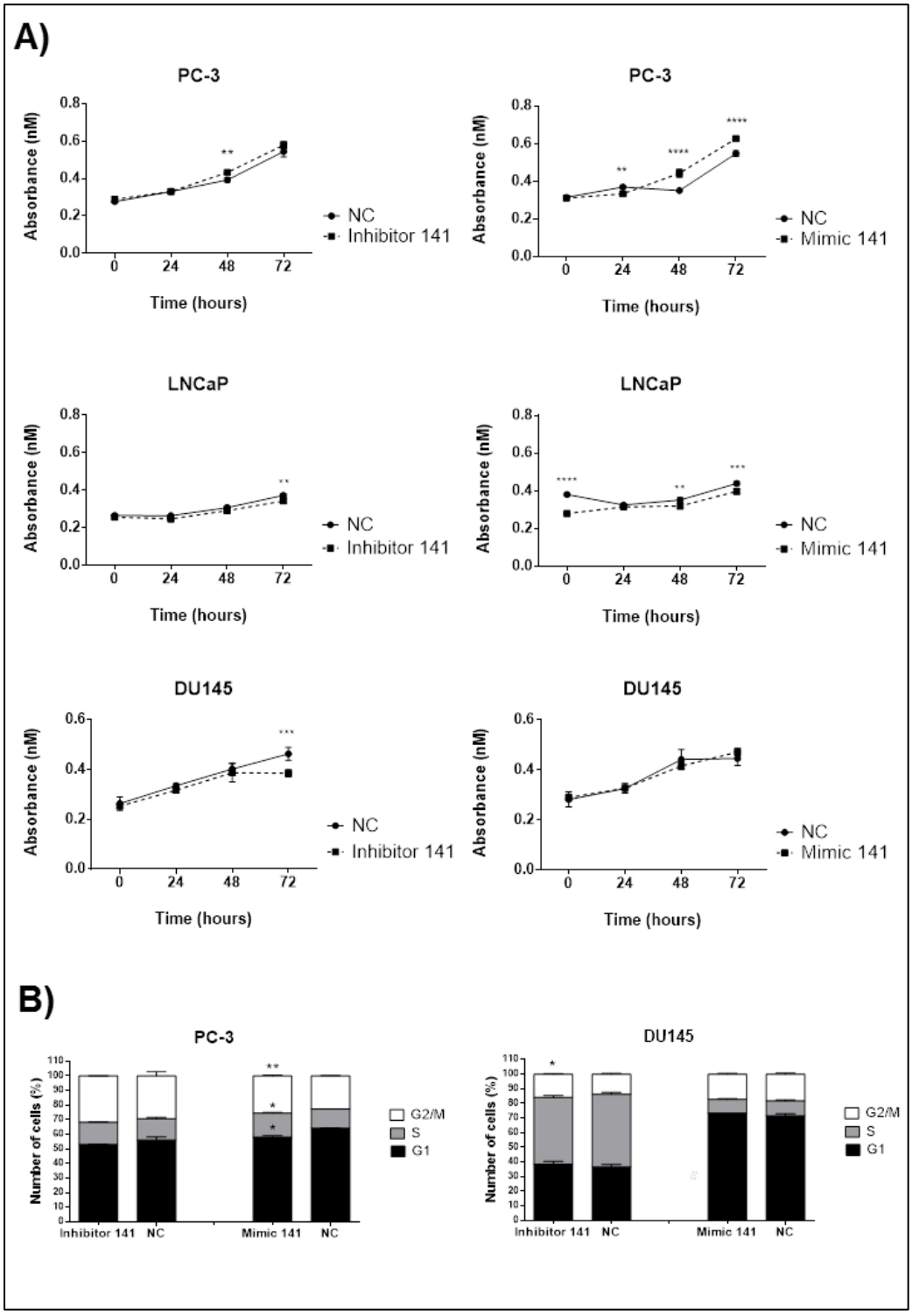

Figure 3: A) Cell proliferation analysis after inhibition (left) and ectopic expression (right) of miR-141-3p in the PC-3, LNCaP and DU145 PCa cell lines; B) Cell cycle analysis by flow cytometry, after inhibition and ectopic expression of miR-141 in the PC-3 and DU145 cells. NC=Negative control. Statistical significant **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

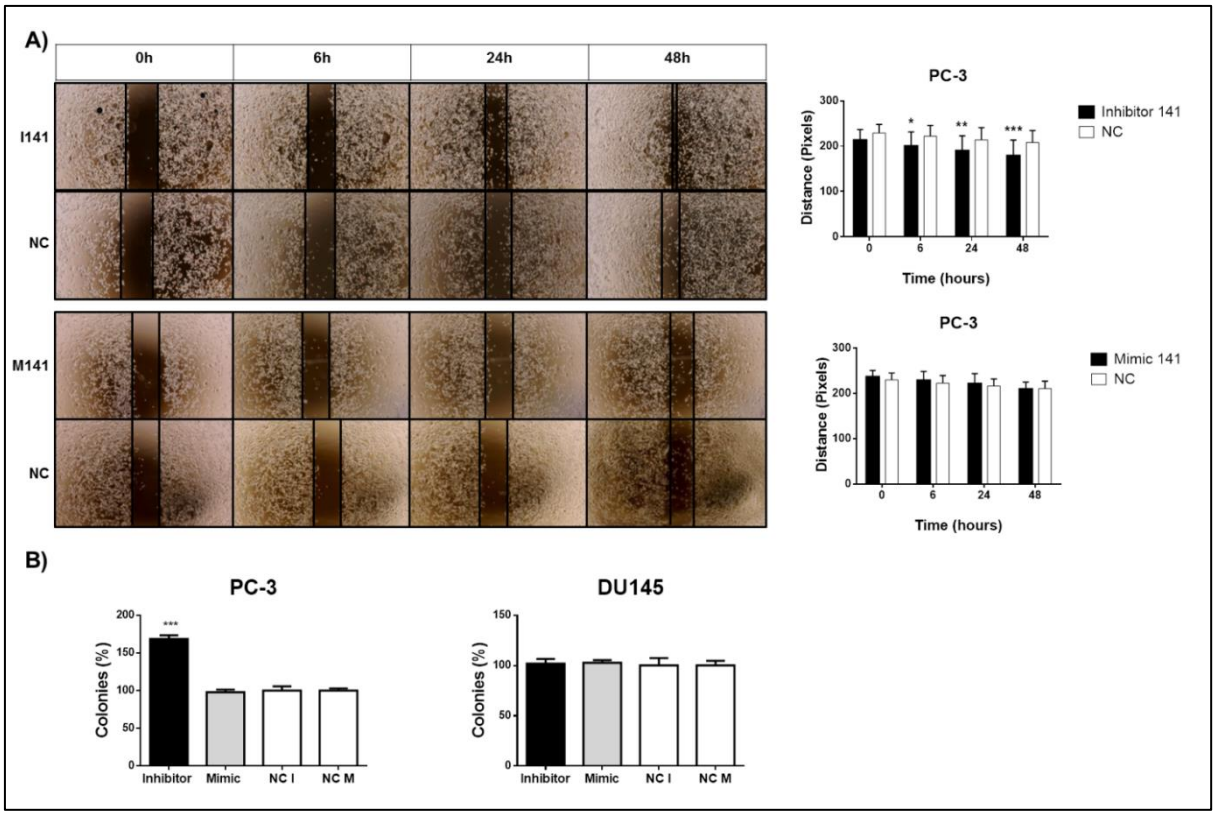

Figure 4: Wound healing assay: A) after inhibition and overexpression of miR-141 in PC-3 cells in different times; B) Clonogenic assay; I141 = inhibitor 141; M141 = Mimic 141, NC = Negative control. Statistical significant **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

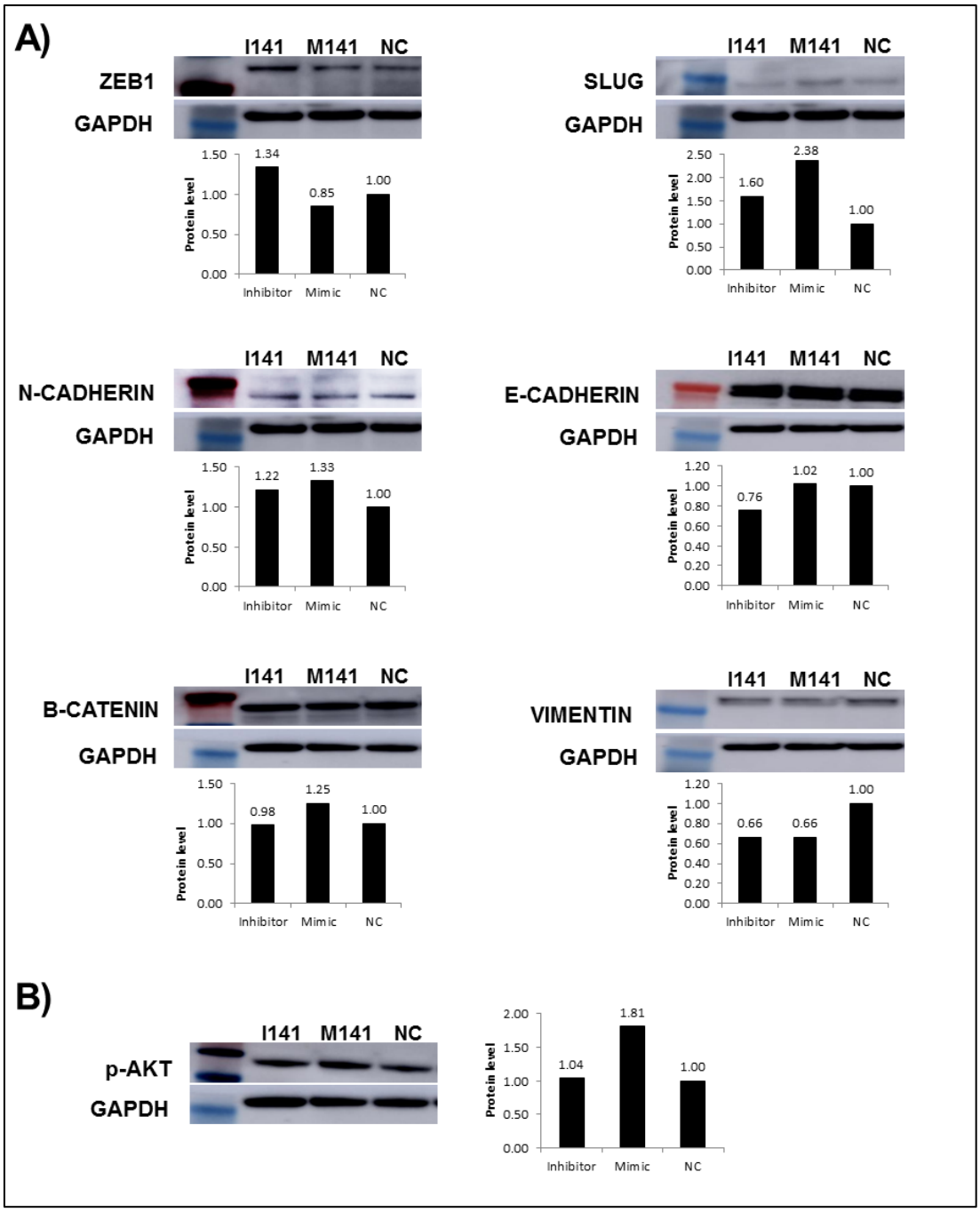

Figure 5: Western blotting analysis of EMT markers and p-AKT in PC-3 cells.

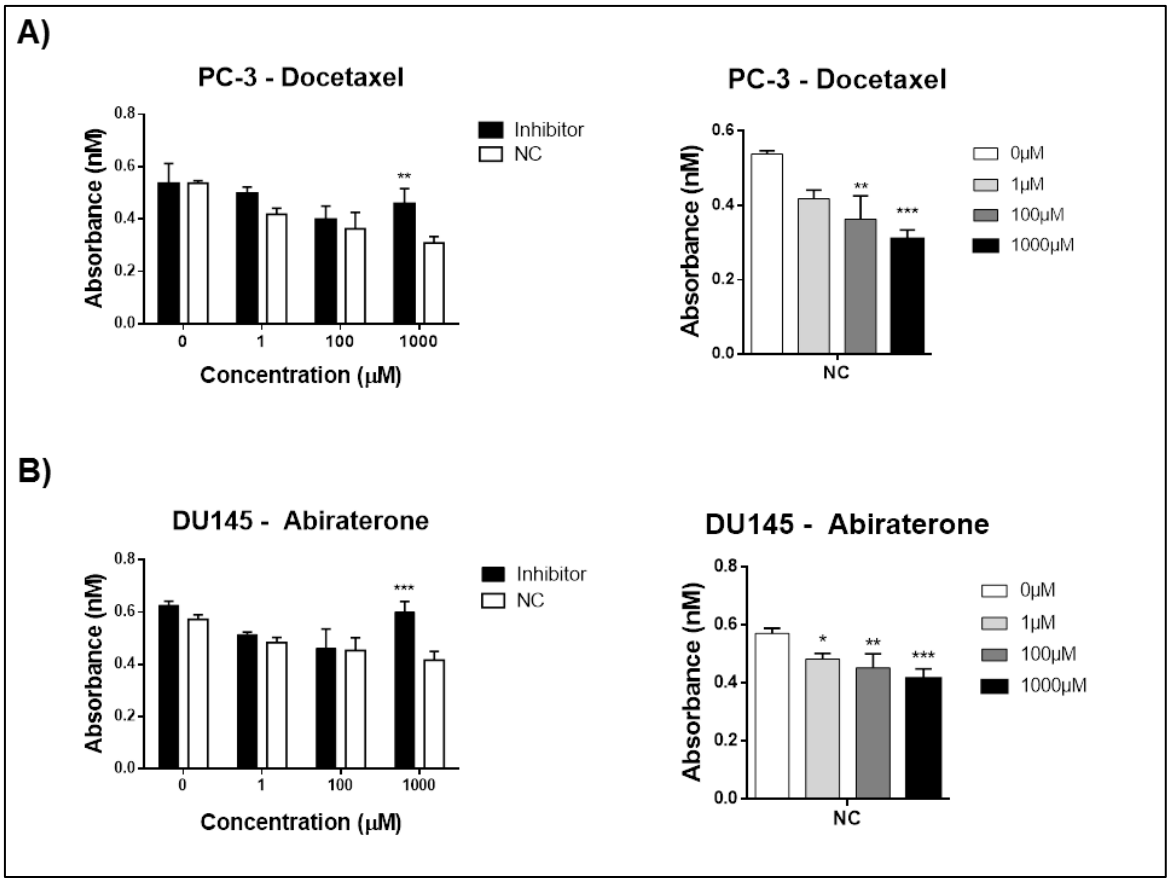

Figure 6: Drug assay after inhibition of 141 after 72 hours of treatment with A) Docetaxel; B) Abiraterone. NC = negative control. Statistical significant **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

ACKNOWLEGMENTS

This study received financial support from Araucaria Foundation of Support for the scientific and technological of Paraná (Conv.185/2014). The authors thank the services provided from the Tissue Culture Shared Resources (TCSR); the Genomics and Epigenomics Shared Resources (GESR) of Lombardi Comprehensive Cancer Center (LCCC); CAPES (Coordination for the Improvement of Higher Education Personnel) for the PDSE scholarship; CNPq, (National Council for Scientific and Technological Development); São Paulo Research Foundation; Londrina Cancer Hospital and Intermunicipal Health Consortium of the Middle Paranapanema (CISMEPAR).

CONFLICTS OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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8. CONSIDERAÇÕES FINAIS

Os resultados do presente estudo permitem concluir que:

- Os mRNAs *OR51E2* e *SIM2* e os miRNAs miR-200b e -200c circulantes quando associados podem ser bons marcadores preditivos para câncer de próstata. Os genes *OR51E2* e *SIM2* apresentam maior especificidade do que o teste do PSA, uma vez que conseguiram distinguir pacientes de indivíduos controles ambos com níveis de PSA ≤ 4.0 ng/mL. Além disso, os miR-200b e -200c apresentaram associação com escore de Gleason, metástase óssea, tumor bilateral e níveis de PSA; e, portanto, podem ser bons marcadores de prognóstico.

- Os mRNA circulantes *AMACR*, *BCL2*, *GOLM1*, *TRPM8* e *NKX3-1* foram associados com características de agressividade tumoral, as quais são obtidas somente após prostatectomia. Destes genes, *NKX3-1*, *TRPM8* e *GOLM1* quando associados apresentam capacidade de identificar pacientes com alto risco para doença agressiva. Quando combinados com métodos já utilizados como PSA e escore de Gleason da biópsia têm o potencial para serem utilizados no aprimoramento da conduta clínica para pacientes com câncer de próstata.

- O miR-182 apresentou maior expressão em plasma de pacientes com tumor bilateral de próstata. Este miRNA parece agir como um oncogene nas células da próstata, promovendo indução de proliferação celular, aumento da migração celular e dos níveis de marcadores de EMT, como ZEB1 e N-CADERINA e resistência ao docetaxel; portanto parece desempenhar um importante papel na progressão da doença.

- O miR-141 apresentou maiores níveis de expressão no plasma de pacientes com metástase óssea, PSA > 10ng/mL e doença de alto risco. As análises de superexpressão e inibição deste miRNA *in vitro* demostraram que ele apresenta função tanto de oncogene quanto de supressor de tumor nas células da próstata, dependendo do contexto celular que estas se encontram. A inibição deste miRNA também mostrou relação com resistência a quimioterápicos. Sendo assim, o miR-141 parece desempenhar importantes funções tanto no inicio quanto na progressão do tumor de próstata.

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10. APÊNDICE

TABELAS SUPLEMENTARES

Table S1: Forty-nine differentially expressed miRNA in the *in silico* analysis.

MicroRNA ID	R-PCa	R-SNT	FC
hsa-mir-375	396601.35	58817.42	6.74
hsa-mir-153-2	109.82	22.2	4.95
hsa-mir-182	72615.27	14835.64	4.89
hsa-mir-96	101.06	23.21	4.35
hsa-mir-183	20969.67	4825.97	4.35
hsa-mir-92a-1	2827.86	698.8	4.05
hsa-mir-3648	16.49	4.67	3.53
hsa-mir-200c	49548.35	14878.65	3.33
hsa-mir-615	4.73	1.45	3.25
hsa-mir-153-1	10.27	3.23	3.18
hsa-mir-93	13173.55	4190.77	3.14
hsa-mir-3074	18.06	6.33	2.85
hsa-mir-20a	925.61	330.75	2.8
hsa-mir-148a	236706.14	85496.45	2.77
hsa-mir-106a	326.64	127.93	2.55
hsa-mir-708	103.83	41.73	2.49
hsa-mir-146b	1028.99	424.64	2.42
hsa-mir-141	5602.93	2331.31	2.4
hsa-mir-19b-2	589.49	254.44	2.32
hsa-mir-3653	23.2	10.13	2.29
hsa-mir-3651	3.59	1.58	2.27
hsa-mir-500a	657.7	290.57	2.26
hsa-mir-25	26109.99	11659.61	2.24
hsa-mir-20b	667.6	300.51	2.22
hsa-mir-425	383.36	174.95	2.19
hsa-mir-7-1	84.86	38.85	2.18
hsa-mir-1304	3.14	1.49	2.11
hsa-mir-92a-2	26858.16	12931.03	2.08
hsa-mir-592	4.95	2.44	2.03
hsa-mir-326	9.57	19.58	0.49
hsa-mir-378c	25.32	52.69	0.48
hsa-mir-889	46.91	97.73	0.48
hsa-mir-488	1.59	3.47	0.46
hsa-mir-222	176.74	415.6	0.43
hsa-mir-450b	17.59	40.8	0.43
hsa-mir-891a	12.78	30.46	0.42
hsa-mir-204	68.42	167.45	0.41
hsa-mir-1274b	5.66	13.69	0.41
hsa-mir-143	1342543.1	3358224.4	0.4
hsa-mir-205	1841.18	5095.87	0.36

hsa-mir-873	1.38	4.04	0.34
hsa-mir-221	693.36	2024.31	0.34
hsa-mir-675	6.67	21.74	0.31
hsa-mir-133a-2	15.82	57.03	0.28
hsa-mir-133b	61.61	226.24	0.27
hsa-mir-184	4.59	21.94	0.21
hsa-mir-490	2.2	11.08	0.2
hsa-mir-187	16.02	104.28	0.15
hsa-mir-23c	3.91	33.94	0.12

R-PCa = Mean of the number reads of the prostate cancer tissue samples; R-SNT = Mean of the number of reads of surrounding normal tissue; FC = Fold-change

Table S2: The 2267 differentially expressed genes in the *in silico* analysis.

Gene ID	R-PCa	R-SNT	FC
DLX1	267.44	6.49	41.22
PCA3	3323.87	109.74	30.29
TDRD1	102.28	6.35	16.1
SLC45A2	38.77	2.57	15.11
ZIC2	34.3	2.37	14.46
DLX2	35.65	2.51	14.21
HOXC6	215.66	15.74	13.7
NKX2-3	20.24	1.81	11.17
FOXD1	44.41	5.19	8.55
OR51E2	6994.5	818.76	8.54
AMACR	7492.86	884.23	8.47
SLIT1	212.4	25.4	8.36
COL10A1	64.88	7.87	8.25
HOXC4	77.51	9.52	8.14
ZIC5	12.98	1.6	8.12
GAL	41.57	5.26	7.91
SIM2	1450.09	184.79	7.85
ONECUT2	102.87	13.33	7.72
TGM3	166.9	22.81	7.32
ACSM1	840.14	115.31	7.29
HPN	4828.04	666.24	7.25
TARP	9205.8	1295.2	7.11
UGT2B4	28.61	4.03	7.1
LOC100130872	650.38	92.3	7.05
FOLH1B	106.83	15.19	7.03
PHGR1	22.76	3.32	6.85
MNX1	25.24	3.9	6.47
COMP	491.15	76.32	6.44
APOF	121.46	18.89	6.43
MMP26	13.92	2.21	6.31
FOLH1	9707.39	1540	6.3
HOXC5	23.13	3.74	6.18
ANKRD34B	25.49	4.17	6.12
CST2	41.19	6.86	6
GLYATL1	976.24	162.89	5.99
LOC145837	428.6	71.82	5.97
ADAM2	9.66	1.65	5.84
DNAH5	658.68	113.09	5.82
LUZP2	474.22	82.04	5.78
ARHGDIG	26.44	4.65	5.68
CHIT1	78.38	14.46	5.42
BEND4	860.24	159.24	5.4
LOC100128675	11.25	2.11	5.34
LMX1B	28.37	5.31	5.34
SERPINA11	43.29	8.2	5.28
B3GNT6	25.49	4.86	5.24
MYBL2	88.42	17.06	5.18
NLRP12	28.79	5.57	5.17
HJURP	59.39	11.59	5.12
LOC728606	323.89	63.22	5.12
KLK15	84.09	16.78	5.01
INSM1	60.79	12.16	5
CGREF1	620.46	124.89	4.97
PRAC	1712.84	345.75	4.95
C2orf72	1000.23	207.15	4.83
FEV	244.25	51.42	4.75
EPHA10	202.77	42.77	4.74
KIF4A	54.69	11.6	4.72
MATK	121.6	25.86	4.7
UNC5A	57.31	12.25	4.68
OTX1	51.16	11.03	4.64
FFAR2	187.66	40.5	4.63
SPON2	13601.35	2950.02	4.61
DNAH8	171.46	37.27	4.6
CCDC78	40.22	8.83	4.55
TRPM8	8371.99	1864.66	4.49
MELK	36.78	8.22	4.48
TMEM178	460.68	102.9	4.48
SLCO1A2	30.22	6.88	4.39
MMP10	81.07	18.92	4.28
ATP8A2	22.26	5.2	4.28
GOLM1	25102.15	5875.83	4.27
ZNHIT2	218.51	51.31	4.26
TMSB15A	599.23	141.61	4.23
B3GAT1	554.25	131.23	4.22
NEK5	19.71	4.69	4.2
CCL18	36.24	8.64	4.2
STX19	182.01	43.45	4.19
BIRC5	49.37	11.78	4.19
CACNA1D	669.03	160.38	4.17
EPR1	55.56	13.38	4.15
SNHG3	82.16	20.03	4.1
UBE2C	60.07	14.7	4.09
APOC1	276.44	67.54	4.09
RRM2	131.3	32.15	4.08
NOX4	24.53	6.01	4.08
THBS4	921.92	226.23	4.08
CCDC108	46.93	11.54	4.07
DLGAP5	29	7.12	4.07
PAX1	7.04	1.74	4.04
NETO2	170.06	42.31	4.02
EN2	10.63	2.66	4

PCSK6	609.53	152.83	3.99
ELAVL2	38.54	9.65	3.99
PDIA2	12.37	3.1	3.99
FGL1	15.01	3.76	3.99
TFF3	1701.5	426.38	3.99
MOV10L1	27.59	6.93	3.98
MMP9	162.26	40.95	3.96
KCNG3	38.92	9.86	3.95
CENPA	17.15	4.37	3.92
SPDEF	14553.22	3776.21	3.85
PLA2G2A	9384.95	2446.88	3.84
ALOX15	35.64	9.27	3.84
ABCC4	11361.91	2964.29	3.83
CPNE7	71.17	18.62	3.82
HIST3H2A	199.21	52.29	3.81
BCL8	48.85	12.83	3.81
ARHGEF38	105.67	27.84	3.8
IRX4	226.89	59.72	3.8
C2CD4C	35.46	9.35	3.79
PRR16	324.04	85.58	3.79
MKI67	305.48	81.44	3.75
GDF15	6158.87	1646.69	3.74
EPHA8	7.62	2.04	3.74
UGT1A3	16.84	4.51	3.74
PAQR6	139.58	37.5	3.72
HAGHL	72.61	19.52	3.72
DNASE2B	148.67	39.96	3.72
CLEC18B	10.28	2.81	3.66
IQGAP3	50.79	13.91	3.65
RAP1GAP	2888.9	793.9	3.64
SMPDL3B	530.53	145.6	3.64
TROAP	35.04	9.63	3.64
GTSE1	35.65	9.9	3.6
FABP5	103.65	28.8	3.6
C19orf45	10.99	3.06	3.59
KLK2	126546.7	35348.81	3.58
CDC25C	14.3	4.02	3.56
TRPM4	9859.89	2779.86	3.55
HOXB13	13287.57	3738.08	3.55
NKAIN1	239.29	67.64	3.54
ROPN1B	27.05	7.67	3.53
TRIM36	1012.5	287.98	3.52
HMMR	52.21	14.86	3.51
LASS1	52.85	15.04	3.51
KIF20A	50.76	14.56	3.49
GHRHR	7.39	2.11	3.49
MS4A8B	232.78	66.6	3.49
SNHG4	17.76	5.1	3.48
CENPM	65.52	18.89	3.47
NCAPG	59.13	17.09	3.46
TMEM132A	985.55	286.58	3.44
LOC100129066	14.64	4.26	3.44
PDLIM5	23478.59	6845.46	3.43
AK5	129.27	37.65	3.43
F2RL2	22.51	6.59	3.42
PODXL2	2517.47	740.32	3.4
LOC100144604	40.59	11.93	3.4
FOXL2	19.67	5.79	3.4
CASKIN1	62.86	18.61	3.38
GNG13	9.34	2.77	3.38
KIAA0087	9.53	2.83	3.37
B4GALNT4	365.11	108.89	3.35
KIF18B	21.1	6.31	3.35
MCOLN2	64.01	19.16	3.34
MGC14436	8.9	2.67	3.33
MUC2	29.84	8.95	3.33
GCNT1	1522.9	458.68	3.32
SKA3	32.15	9.72	3.31
TMC5	1949.66	590.78	3.3
AMH	8.28	2.51	3.29
DAZ1	6.45	1.96	3.29
F5	754.68	229.63	3.29
ADRB1	617.5	188.41	3.28
SLC43A1	1656.01	504.89	3.28
UCN	10.23	3.12	3.28
NEK2	29.22	8.91	3.28
TWIST1	140.36	42.8	3.28
ISX	43.32	13.23	3.27
TBX1	248.01	76.05	3.26
BUB1B	60.42	18.62	3.25
FAM64A	24.78	7.62	3.25
BRSK2	35.19	10.84	3.25
TUBB4	125.07	38.66	3.24
FOXA1	9882.71	3058.19	3.23
TPX2	142.74	44.22	3.23
CDC20	75.84	23.53	3.22
F12	50.66	15.75	3.22
ASPN	188.88	58.92	3.21
HIST1H4E	12.69	3.96	3.21
OR51E1	1024.27	318.9	3.21
RNF157	377.42	117.93	3.2
SDS	51.3	16.08	3.19
C4orf48	19.94	6.27	3.18
TSPAN19	6.76	2.13	3.17
ERG	844.12	267.28	3.16

SDK1	1084.89	343.94	3.15	*EBF2*	10.84	3.67	2.95
SFTPA2	880.46	279.32	3.15	*FOXM1*	129.21	43.95	2.94
CDC45	27.09	8.63	3.14	*CLEC18A*	18.75	6.38	2.94
PRR7	51.4	16.43	3.13	*PYCR1*	4504.9	1537.72	2.93
GDF1	11.86	3.79	3.13	*CPLX3*	258.47	88.24	2.93
PTPRT	189.68	60.52	3.13	*CCNB2*	108.69	37.25	2.92
PLA2G7	936.77	300.55	3.12	*NCRNA00105*	78.47	26.9	2.92
ITGBL1	131.96	42.24	3.12	*RHPN1*	495.52	171.1	2.9
ANGPTL3	5.83	1.87	3.12	*GJB1*	1792.25	619.42	2.89
ASPHD1	105.33	33.85	3.11	*MGAT5B*	19.24	6.66	2.89
SSPO	60.25	19.35	3.11	*MARCKSL1*	9090.39	3155.4	2.88
C19orf48	6512.32	2109.81	3.09	*TOP2A*	234.4	81.38	2.88
PLK1	62.75	20.37	3.08	*SGEF*	1765.13	617.85	2.86
LOC389332	27.21	8.84	3.08	*TBX10*	8.7	3.04	2.86
C17orf93	282.63	92.03	3.07	*GABRB3*	689	241.76	2.85
PPM1E	297.63	96.86	3.07	*TOX3*	185.73	65.3	2.84
FOXB2	3.88	1.26	3.07	*CTHRC1*	74.76	26.35	2.84
CTNND2	294.17	95.98	3.06	*MEX3A*	250.57	88.21	2.84
SPC25	17.47	5.71	3.06	*CXCL11*	109.28	38.5	2.84
KLK3	372282.3	121497.8	3.06	*BICD1*	297.54	104.97	2.83
VSTM2L	1732.5	566.22	3.06	*CAMKK2*	6624.86	2347.84	2.82
PKMYT1	57.81	18.93	3.05	*FASN*	25332.51	8978.49	2.82
AURKB	28.16	9.23	3.05	*CDKN3*	26.88	9.53	2.82
KIAA1875	26.34	8.65	3.05	*NEIL3*	6.28	2.24	2.81
C12orf34	212.4	69.86	3.04	*FAM189A1*	74.74	26.73	2.8
PTCHD2	42.86	14.09	3.04	*C9orf140*	765.41	274.36	2.79
SLC6A11	45.97	15.11	3.04	*KLHL35*	31.52	11.3	2.79
SLC7A11	205.02	67.58	3.03	*PKIB*	292.42	105.04	2.78
APOE	1507.75	497.93	3.03	*HES4*	181.58	65.44	2.77
PRAME	6.12	2.02	3.03	*C19orf51*	21.48	7.78	2.76
EZH2	113.33	37.48	3.02	*SLC22A10*	6.29	2.28	2.76
PTP4A3	1762.89	584.05	3.02	*42805*	4.36	1.58	2.76
PBK	27.78	9.2	3.02	*NKX6-1*	3.59	1.31	2.75
ALB	14.79	4.9	3.02	*KLK4*	17244.13	6293.94	2.74
GSC	28.06	9.33	3.01	*ASPM*	39.29	14.33	2.74
HIST1H2AM	16.62	5.51	3.01	*HIST1H2BG*	119.17	43.5	2.74
RAB3B	421.63	140.24	3.01	*ADAM21*	9.37	3.43	2.73
ZP1	12.17	4.04	3.01	*C4A*	6453.5	2361.02	2.73
C2orf14	36.59	12.15	3.01	*CENPN*	1740.92	639.99	2.72
SLC19A1	874.42	291.9	3	*PCDHGB1*	83.87	30.87	2.72
ELFN2	313.89	104.46	3	*RTN4RL2*	38.04	14.01	2.71
CENPF	155.45	52.09	2.98	*NLRP8*	7.68	2.84	2.71
LOC100287718	4.44	1.49	2.98	*MAK*	22.94	8.48	2.7
RAB17	708.18	238.79	2.97	*C10orf95*	27.85	10.34	2.69
KCNH8	51.73	17.43	2.97	*RGS11*	266.41	98.92	2.69
LRFN1	178.37	60.31	2.96	*NUDT8*	487.69	181.13	2.69
SBK1	498.64	168.41	2.96	*ACCN3*	20.56	7.66	2.68
DEPDC1B	23.44	7.95	2.95	*C9orf163*	15.64	5.86	2.67

TUBB3	296.07	110.87	2.67	*TLCD1*	346.53	137.53	2.52
VGF	15.4	5.76	2.67	*APBA2*	233.77	92.76	2.52
SKA1	22.83	8.58	2.66	*SIX1*	806.72	319.5	2.52
CEP55	35.13	13.19	2.66	*TERT*	3.2	1.27	2.52
FAM111B	79.7	30.01	2.66	*MGC29506*	180.97	71.85	2.52
SRMS	19.29	7.24	2.66	*CHRNA2*	2044.34	812.23	2.52
PABPC1L2B	250.57	94.42	2.65	*CDCA5*	92.83	36.98	2.51
C16orf59	55.22	20.95	2.64	*HIST2H3C*	11	4.39	2.51
LOC153328	9.57	3.62	2.64	*SSTR1*	184.62	73.57	2.51
HIST3H2BB	8.91	3.39	2.63	*CAMK2N2*	36.26	14.49	2.5
NCAPH	35.91	13.63	2.63	*HIST1H3H*	28.98	11.6	2.5
CCDC83	3.52	1.34	2.63	*TMEM184A*	1314.61	527.58	2.49
FOXN4	5.06	1.92	2.63	*GALNT7*	5108.96	2053.21	2.49
VAX2	15.92	6.06	2.63	*TRIP13*	66.63	26.8	2.49
NOL4	29.65	11.28	2.63	*LRRC26*	1116.37	448.32	2.49
BIK	536.25	204.31	2.62	*TSPAN1*	19104.63	7688.8	2.48
DBNDD1	665.09	254.11	2.62	*TTK*	54.46	21.99	2.48
FGFRL1	1526.77	583.2	2.62	*BUB1*	46.2	18.62	2.48
CYorf15B	320.74	122.35	2.62	*TTC36*	5.22	2.11	2.48
KLKP1	117.28	44.83	2.62	*HIST1H1E*	19.76	7.98	2.48
YJEFN3	87.8	33.55	2.62	*TMEM45B*	524.82	211.83	2.48
CHI3L1	83.68	31.88	2.62	*ZDHHC11*	76.9	31.1	2.47
NKX2-2	5.16	1.97	2.62	*RLTPR*	30.01	12.14	2.47
NKX3-1	24547	9414.24	2.61	*LRRC7*	32.53	13.16	2.47
FZD8	303.06	116.02	2.61	*MSL3L2*	308.33	125.44	2.46
OSTalpha	53.26	20.38	2.61	*SLC26A6*	450.2	183.27	2.46
HS3ST4	89.07	34.08	2.61	*EFCAB4A*	1535.77	624.98	2.46
TRIB1	4060.26	1560.43	2.6	*P2RY11*	134.69	54.67	2.46
GPC2	17.45	6.71	2.6	*HIST1H4J*	67.59	27.44	2.46
NEAT1	16249.82	6254.14	2.6	*KIF14*	14.34	5.83	2.46
DLL3	6.78	2.61	2.6	*CLDN3*	5237.65	2137.5	2.45
COL9A2	2300.18	883.38	2.6	*CCNA2*	71.32	29.09	2.45
SLC24A5	17.14	6.61	2.59	*KIAA0101*	67.02	27.31	2.45
KIFC1	95.67	37.08	2.58	*IL11*	11.4	4.65	2.45
ATG9B	39.05	15.12	2.58	*HSF4*	326.67	133.33	2.45
ULBP1	7.18	2.79	2.58	*SFRP4*	936.91	383.01	2.45
GRPR	31.5	12.22	2.58	*LRGUK*	9.62	3.95	2.44
C15orf50	4.48	1.74	2.57	*SLC45A3*	35002.12	14334.86	2.44
LOC644165	19.4	7.55	2.57	*TNN*	7.27	2.98	2.44
STEAP1	2805.13	1094.88	2.56	*SLC13A3*	697.24	287.1	2.43
DIAPH3	17.98	7.03	2.56	*CDKN2A*	46.7	19.19	2.43
ZMYND10	28.63	11.18	2.56	*CYP2J2*	251.78	103.59	2.43
ARMC3	6.24	2.44	2.56	*MESP1*	749.14	308.25	2.43
MYBPC1	2690.54	1050	2.56	*CPLX1*	112.47	46.69	2.41
ADM2	718.94	281.98	2.55	*COLEC12*	1561.52	646.91	2.41
RPRML	4.66	1.83	2.54	*SHCBP1*	24.97	10.34	2.41
LOC149134	46	18.22	2.53	*C14orf39*	11	4.57	2.41
CELSR3	78.52	31.02	2.53	*HIST1H2AE*	258.29	107.68	2.4

GABRD	11.13	4.64	2.4	*RGS10*	1586.16	694.83	2.28
GJA3	16.04	6.69	2.4	*MPP6*	121.53	53.28	2.28
NUP210	1609.96	672.93	2.39	*FHIT*	76.33	33.52	2.28
POU5F1B	53.34	22.33	2.39	*LFNG*	1442.59	633	2.28
RPGRIP1	10.06	4.2	2.39	*HCN2*	22.71	9.97	2.28
TMEM145	10.94	4.58	2.39	*SPTBN2*	3879.21	1710.4	2.27
FLJ12825	5.83	2.45	2.38	*DGCR5*	21.05	9.28	2.27
AGMAT	51.49	21.62	2.38	*DNASE1L2*	19.53	8.61	2.27
SAMD5	376.92	158.26	2.38	*ANO7*	2343.09	1031.59	2.27
PPP1R14B	1134.51	481.33	2.36	*GPRIN1*	42.04	18.62	2.26
GMDS	864.89	366.91	2.36	*RAB26*	179.1	79.35	2.26
ZNF695	9.28	3.93	2.36	*PCDHB2*	191.11	84.54	2.26
TRPM2	152.77	64.6	2.36	*CHTF18*	244.32	108.6	2.25
KRT20	3.22	1.36	2.36	*C1orf182*	6.19	2.76	2.25
LOC440905	173	73.27	2.36	*TMEM52*	50.49	22.43	2.25
SPAG5	120.82	51.39	2.35	*ADPRHL1*	137.71	61.23	2.25
SLC10A4	7.42	3.16	2.35	*SLC25A27*	271.08	120.31	2.25
CECR2	68.41	29.07	2.35	*KRTAP5-1*	4.42	1.97	2.25
SLC4A4	5423.2	2309.32	2.35	*PABPC1L2A*	52.16	23.14	2.25
TRIM58	94.02	40.04	2.35	*MGC87042*	128.6	57.44	2.24
GPR160	1846.59	787.53	2.34	*CCDC154*	22.84	10.22	2.24
RAC3	503.91	215.4	2.34	*P2RX5*	66.37	29.67	2.24
MAPK8IP2	338.04	144.49	2.34	*MIOX*	3.93	1.76	2.24
GPR98	201.29	85.88	2.34	*SLC26A1*	59.3	26.6	2.23
KLB	22.36	9.54	2.34	*PDE3B*	1053.77	473.56	2.23
CNTNAP2	1483.43	634.68	2.34	*CPT1B*	278.61	124.95	2.23
LINGO3	6.07	2.6	2.33	*SCNN1D*	75.46	33.89	2.23
SP5	118.3	50.86	2.33	*FAP*	65.43	29.33	2.23
ELOVL2	478.71	205.08	2.33	*GLOD5*	14.28	6.43	2.22
NCRNA00093	30.2	13.04	2.32	*CABP4*	43.33	19.54	2.22
HPX	33.54	14.43	2.32	*LENG9*	630.08	284.88	2.21
CRYBA2	14	6.04	2.32	*LOC339674*	3.95	1.78	2.21
C19orf23	30.38	13.18	2.31	*FAM72B*	21.62	9.8	2.21
ZP3	258.77	111.83	2.31	*E2F2*	24.05	10.87	2.21
PHF21B	103.25	44.73	2.31	*RPL36A*	57.18	25.84	2.21
DGCR9	8.36	3.63	2.31	*KIF25*	4.73	2.14	2.21
SNHG3-RCC1	302.11	130.74	2.31	*STIL*	145.19	65.87	2.2
ENTPD5	1660.35	721.77	2.3	*MBOAT2*	1916.13	872.4	2.2
C15orf21	1742.31	756.19	2.3	*NSUN5P1*	186.61	85	2.2
LRRC56	196.86	85.86	2.29	*OR13A1*	11.09	5.04	2.2
EPCAM	5719.94	2502.91	2.29	*CBX2*	72.8	33.15	2.2
CDCA8	71.26	31.14	2.29	*HIST1H2BF*	10.03	4.56	2.2
RPL22L1	881.9	384.61	2.29	*SLC6A17*	19.54	8.88	2.2
MYRIP	344.05	150.2	2.29	*LOC100128778*	33.39	15.23	2.19
ROPN1	6.9	3.01	2.29	*MSH5*	253.97	116.12	2.19
SPC24	5.64	2.47	2.29	*CHDH*	789.82	361.41	2.19
NUF2	20.2	8.83	2.29	*SLC10A5*	19.2	8.78	2.19
IGFN1	9.75	4.27	2.29	*TUBB2A*	1543.17	704.64	2.19

ESPL1	29.55	13.49	2.19
HIST1H4H	328.62	150.38	2.19
EXO1	17.54	8.02	2.19
KIAA1324	8310.18	3791.39	2.19
LRIG1	6929.35	3179.53	2.18
MSI1	308.93	141.73	2.18
SH3RF1	2476.56	1135.42	2.18
THPO	9.39	4.3	2.18
CBLC	575.71	264.94	2.17
ALDH3B2	548.44	252.65	2.17
ZNF30	196.97	91.34	2.16
PTTG1	68.89	31.97	2.16
FOXD4	13.74	6.38	2.16
UHRF1	43.37	20.08	2.16
MYO1G	158.13	73.38	2.16
DCST2	25.01	11.6	2.16
TM4SF19	6.13	2.83	2.16
BARX1	3.35	1.55	2.16
CELF5	25.86	11.95	2.16
CXCL10	150.2	69.42	2.16
PLA1A	605.92	281.11	2.16
SLC12A8	442.91	205.6	2.15
LOC100133612	13.86	6.43	2.15
OR52R1	2.72	1.26	2.15
NSUN5P2	472.1	219.34	2.15
HSD17B3	5.15	2.4	2.15
GALNTL6	11.88	5.54	2.15
PMEPA1	17364.15	8109.29	2.14
ALDH1A3	12881.85	6033.08	2.14
CREB3L4	6077.46	2854.96	2.13
C7orf46	199.97	93.89	2.13
CDCA3	45.84	21.48	2.13
THSD1P1	285.14	134.75	2.12
FAM72D	9.51	4.49	2.12
CATSPERB	13.43	6.33	2.12
STEAP4	8422.95	3979.38	2.12
ZYG11A	59.82	28.19	2.12
YPEL1	360.48	171.2	2.11
TP53INP1	3121.08	1480.57	2.11
BAIAP3	714.26	338.16	2.11
TK1	251.14	118.86	2.11
LOC338799	453.71	215.22	2.11
PABPC1L	150.93	71.56	2.11
APOC2	24.33	11.51	2.11
SLC27A2	368.29	175.65	2.1
GDPD1	97.82	46.65	2.1
CLDN8	953.51	454.99	2.1
APLN	159.71	76.07	2.1
SHOX2	5.79	2.76	2.1
TMTC4	1106.09	529.19	2.09
ELL3	364.93	174.86	2.09
DSC2	1804.48	864.76	2.09
GPR37	87.3	41.84	2.09
GAD1	23.39	11.17	2.09
ADRA2A	230.75	110.46	2.09
ESRP2	1832.32	881.37	2.08
DNASE1	189.3	91.18	2.08
NCRNA00115	49.22	23.65	2.08
MLF1IP	137.78	66.35	2.08
?	129.34	62.17	2.08
3-Sep	154.35	74.08	2.08
EME2	27.14	13.08	2.08
C1orf116	7371.16	3559.32	2.07
QRICH2	120.03	58.02	2.07
FBXL8	294.31	142.32	2.07
SOX8	57.52	27.85	2.07
NPW	5.43	2.62	2.07
PCDHB9	67.33	32.55	2.07
RIMKLA	232.39	112.73	2.06
TPRN	841.64	408.13	2.06
BCAM	20777.14	10099.96	2.06
HOMER2	2845.75	1382.08	2.06
PRDM12	3.68	1.79	2.06
CBS	1795.99	871.24	2.06
HES6	110.87	53.84	2.06
THEG	5.75	2.8	2.06
KRT2	4.78	2.32	2.06
MB	681.69	331.51	2.06
KIF2C	50.94	24.79	2.05
ASF1B	78.75	38.4	2.05
ZGLP1	35.59	17.37	2.05
KCNU1	3.6	1.76	2.05
SLC25A10	1562.29	764.72	2.04
CCDC64	296.39	145.09	2.04
SYT7	6046.65	2967.8	2.04
POLN	63.19	30.97	2.04
GSDMA	4.23	2.07	2.04
FAM13AOS	57.03	27.95	2.04
TDRD6	60.47	29.67	2.04
PRG4	8.95	4.38	2.04
HIST1H2BC	294.87	144.29	2.04
SAMD10	329.3	162.42	2.03
C18orf45	196.45	96.75	2.03
PNPLA7	1660.37	818.61	2.03
CCDC110	87.63	43.19	2.03
SPDYA	14.64	7.22	2.03

TTLL6	12.56	6.19	2.03	*WWTR1*	303.81	613.52	0.5
RNFT2	22.86	11.28	2.03	*ANTXR1*	1720.51	3457.97	0.5
SERINC4	12.29	6.06	2.03	*NCRNA00181*	30.57	61.71	0.5
C19orf18	19.21	9.47	2.03	*DPY19L2P2*	15.7	31.65	0.5
C8orf77	23.91	11.8	2.03	*COPZ2*	152.41	307.03	0.5
CYP2D7P1	11.48	5.66	2.03	*SOX30*	4.45	8.9	0.5
DUS1L	3433.72	1697.27	2.02	*CDH16*	1.22	2.44	0.5
SLC25A45	261.44	129.15	2.02	*TMEM200C*	3.02	6.06	0.5
PLXNB3	967.91	478.16	2.02	*KLHL33*	3.58	7.18	0.5
CTU1	83.93	41.63	2.02	*FAM181B*	14.73	29.6	0.5
C17orf96	153.08	75.9	2.02	*REEP2*	96.75	194.89	0.5
BOP1	339.8	168.44	2.02	*GBP1*	313.45	628.66	0.5
REPS2	2701.36	1337.52	2.02	*SDC1*	1743.27	3511.39	0.5
PLEKHN1	69.08	34.14	2.02	*GEM*	268.75	538.19	0.5
SNX22	433.34	214.13	2.02	*CAMK4*	27.44	55.41	0.5
UOX	2.81	1.39	2.02	*NTRK1*	7.73	15.46	0.5
PLAC8L1	3.43	1.7	2.02	*HPDL*	12.99	26.05	0.5
SNHG9	89.13	44.2	2.02	*LCN6*	5.87	11.83	0.5
C8ORFK29	4.14	2.05	2.02	*ABHD6*	148.56	300.26	0.49
WDR62	34.98	17.33	2.02	*TES*	1278.68	2593.06	0.49
LST-3TM12	2.61	1.3	2.02	*RNF180*	204.76	415.02	0.49
KIAA1244	2225.57	1099.55	2.02	*KANK1*	1180.82	2414.12	0.49
PRSS8	6178.07	3072.38	2.01	*TBC1D1*	802.19	1625.61	0.49
PVT1	109.75	54.55	2.01	*PAQR7*	300.1	608.9	0.49
C9orf169	25.09	12.46	2.01	*CLIC4*	2776.89	5648.71	0.49
C7orf4	2.56	1.27	2.01	*TRPV3*	1.58	3.23	0.49
SPOCK1	8148.43	4052.22	2.01	*PGAP1*	253.81	514.17	0.49
CHRNA5	101.34	50.37	2.01	*NR3C1*	848.54	1716.84	0.49
TRAF4	2024.21	1011.46	2	*TEAD1*	945.71	1910.75	0.49
C2orf79	833.98	416.58	2	*GYPE*	2.76	5.63	0.49
TRIB3	819.33	408.82	2	*HCG22*	1.34	2.73	0.49
ZNF697	635.64	317.64	2	*STOM*	1862.96	3830.11	0.49
ADAM21P1	2.93	1.47	2	*MDFIC*	291.6	593.28	0.49
CAMK2B	388.06	193.73	2	*OAT*	1248.9	2570.32	0.49
HIST1H3D	65.29	32.64	2	*FZD7*	588.62	1206.42	0.49
12-Sep	2.56	1.28	2	*SCD5*	398.05	806.44	0.49
IL1F5	3.09	1.54	2	*ACE*	582.03	1181.95	0.49
KRT222	43.16	86.51	0.5	*KCTD17*	91.96	188.69	0.49
ALAD	1001.11	2010.04	0.5	*ZEB1*	439.37	897.55	0.49
C22orf23	46.06	92.14	0.5	*RTP1*	1.84	3.78	0.49
PBX1	1882.95	3793.51	0.5	*PLAU*	185.07	377.77	0.49
PLEKHG3	477.22	959.22	0.5	*GCK*	5.58	11.27	0.49
TTC28	378.48	762.47	0.5	*TSPAN7*	283.61	577.18	0.49
GAS6	1806.17	3615.81	0.5	*SH3BGR*	124.67	253.12	0.49
SLC40A1	1298.76	2618.05	0.5	*LOC283070*	136.38	279.05	0.49
MPO	3.33	6.67	0.5	*EMP3*	200.94	409.39	0.49
KCNJ1	1.71	3.43	0.5	*RNF152*	24.77	50.99	0.49
GJD3	83.81	168.78	0.5	*ACSBG1*	11.24	22.86	0.49

NAT2	2.76	5.58	0.49
MATN2	1796.47	3688.74	0.49
KLRF1	3.91	7.91	0.49
CASP1	99.41	202.6	0.49
CXCR7	145.59	294.92	0.49
ZNF423	148	302.23	0.49
ASS1	1214.38	2469.91	0.49
PDE2A	171	350.55	0.49
AQP10	2.29	4.72	0.49
LGALS3BP	4777.35	9801.84	0.49
ITGA5	1439.12	2955.42	0.49
KCNK10	2	4.06	0.49
AMIGO2	176.05	358.96	0.49
TNFRSF10D	65.31	132.21	0.49
ATP1B2	69.8	142.16	0.49
OLFML1	134.22	272.99	0.49
WIPF3	17.62	36.25	0.49
RIPK3	27.56	55.8	0.49
C1orf51	71.8	146	0.49
C21orf88	2.85	5.83	0.49
AQP4	10.53	21.56	0.49
THSD4	1660.98	3359.87	0.49
FBXO27	102.51	208.27	0.49
TSPYL3	28.6	58.6	0.49
NAV3	74.95	152.78	0.49
ZNF826	11.7	24.1	0.49
SPRR3	1.44	2.96	0.49
HSPB1	6390.69	12938.41	0.49
ROCK2	519.98	1055.55	0.49
BDKRB2	76.35	156.59	0.49
BMP2	55.42	112.93	0.49
CLEC3B	162.15	333.11	0.49
ITM2A	209.17	429.17	0.49
SULT1E1	2.49	5.06	0.49
NAALAD2	19.85	40.9	0.49
NKD1	49.05	100.31	0.49
C13orf39	1.49	3.03	0.49
HAS3	54.58	110.96	0.49
FAM196A	7.84	16.02	0.49
LAMA3	549.22	1113.81	0.49
GABRQ	3.06	6.24	0.49
PCDHGA12	20.3	41.83	0.49
C1orf130	41.4	84.87	0.49
KCTD8	7.27	14.7	0.49
C1orf95	49.88	100.88	0.49
ZNF883	20.4	41.7	0.49
MMRN1	82.33	166.64	0.49
CFD	263.79	541.03	0.49
SLC35F1	32.05	65.07	0.49
PIK3R1	1289.03	2696.89	0.48
RARB	190.61	400.83	0.48
LOC400804	1.56	3.26	0.48
PNMA1	954.11	2004.27	0.48
RNF126P1	2.52	5.22	0.48
ZFP36L1	4321.59	8954.08	0.48
PLCD3	308.13	643.36	0.48
ENO2	283.59	587.61	0.48
HTR1B	1.74	3.6	0.48
GNG11	298.52	622.54	0.48
ZNF483	24.48	50.98	0.48
RERG	195.48	410.92	0.48
CCDC88A	193.93	403.01	0.48
PMP22	514.43	1063.5	0.48
CDK18	167.98	352.98	0.48
KCNA2	2.58	5.34	0.48
BDH2	281.8	588.73	0.48
LOC100128239	8.13	16.94	0.48
SCN11A	3.19	6.67	0.48
ZNF114	3.36	7.02	0.48
DIRC3	4.14	8.56	0.48
TLR2	89.77	188.64	0.48
TULP1	5.23	10.89	0.48
PNMAL2	47.23	98.82	0.48
ADD3	887.48	1836.87	0.48
ETS2	1553.3	3260.74	0.48
FAM46A	407.59	847	0.48
LEPREL2	444.93	929.37	0.48
GPR124	1767.92	3705.17	0.48
EMILIN3	129.83	269.89	0.48
DPYD	223.1	465.44	0.48
HTR2A	2.53	5.31	0.48
PAPL	2.6	5.42	0.48
MAST4	271.18	565.85	0.48
PCDH18	267.54	555.51	0.48
ARSJ	125.24	258.69	0.48
CPNE5	26.14	54.92	0.48
FOXF1	296.33	612.31	0.48
CCDC68	60.36	126.2	0.48
F10	102.93	213.12	0.48
ABCC6P2	8.71	18.01	0.48
C6orf27	19.82	41.7	0.48
FAM38B	68.3	143.21	0.48
NTN1	155.2	324.91	0.48
FBP2	1.66	3.43	0.48
DCAF12L2	17.53	36.72	0.48
BATF	33.88	71.12	0.48

ZNF208	15.7	32.84	0.48	*GPR85*	12.2	25.76	0.47
SIX2	41.03	85.38	0.48	*TMEM158*	197.75	423.97	0.47
DNASE1L3	15.08	31.67	0.48	*LOC400759*	7.05	15.02	0.47
FST	58.14	120.22	0.48	*CLEC9A*	6.29	13.47	0.47
AIM1L	3.89	8.07	0.48	*DCN*	2948.47	6340.62	0.47
PPP1R14C	12.43	25.66	0.48	*MRGPRF*	479.53	1023.7	0.47
CD207	36.42	76.52	0.48	*PLXNA4*	40.95	87.01	0.47
TTC22	45.75	95.7	0.48	*PCDH11Y*	3.08	6.56	0.47
SCN4A	4.38	9.22	0.48	*A1BG*	40.39	86.75	0.47
SALL1	11.49	23.85	0.48	*TNFRSF10C*	19.87	41.86	0.47
ACER1	6.7	13.83	0.48	*ELMOD1*	3.66	7.87	0.47
SEC23A	708.68	1511.66	0.47	*ADORA2B*	22.88	48.63	0.47
KATNAL1	168.3	358.03	0.47	*CNTN2*	6.87	14.48	0.47
MPZL2	1334.3	2825.16	0.47	*HOXD10*	56.94	121.68	0.47
MOBKL2B	94.69	201.99	0.47	*EVX1*	20.72	43.83	0.47
RPS2P32	5.06	10.78	0.47	*SORCS1*	18.56	39.34	0.47
HTR1E	1.36	2.89	0.47	*GRIA2*	5.52	11.66	0.47
RRAS	998.73	2114.95	0.47	*SEMA3E*	130.03	275.28	0.47
RARG	316.49	666.35	0.47	*WNT6*	11.71	25.18	0.47
NAGS	31.5	66.61	0.47	*SLIT2*	176.27	378.02	0.47
SCRN1	1077.71	2293.21	0.47	*CD36*	62.82	133.99	0.47
MTMR11	76.47	163.91	0.47	*PCDHGB7*	101.28	213.79	0.47
POU2F3	28.08	59.96	0.47	*CTSE*	6.32	13.35	0.47
MARVELD1	567	1218.9	0.47	*C10orf116*	1939.98	4133.59	0.47
FBXL22	76.06	160.4	0.47	*SOX2*	47.22	101.08	0.47
C10orf140	11.42	24.41	0.47	*HIPK3*	525.94	1129.28	0.47
C4orf38	8.61	18.46	0.47	*PRDM11*	19.8	42.83	0.46
ANXA2P1	20.83	44.68	0.47	*SGCB*	633.06	1381	0.46
SLC24A4	3.75	7.95	0.47	*MEIS3P1*	231.07	502.36	0.46
PPP4R1L	19.33	40.72	0.47	*NCS1*	922.22	2018.13	0.46
GPR62	4.5	9.54	0.47	*TACC1*	2300.73	4971.2	0.46
CES7	1.15	2.46	0.47	*QSOX1*	1240.82	2685.4	0.46
PABPC4L	60.33	127.06	0.47	*DIXDC1*	678.71	1465.97	0.46
RASGRF2	51.57	108.98	0.47	*TSHZ3*	314.62	679.82	0.46
AHR	591.79	1259.56	0.47	*PDLIM1*	1178.99	2554.15	0.46
GRXCR1	1.06	2.24	0.47	*ISYNA1*	518.81	1132.51	0.46
PDLIM7	1352.99	2862.39	0.47	*C14orf159*	528.9	1155.71	0.46
NAALADL1	48.95	103.82	0.47	*PRICKLE1*	114.41	246.61	0.46
ARHGAP22	40.34	85.58	0.47	*KIAA1377*	73.31	159.21	0.46
UBE2QL1	107.32	227.82	0.47	*TMEM106A*	36.29	79.27	0.46
NID1	708.59	1501.44	0.47	*LAYN*	141.91	309.01	0.46
C10orf72	372.94	785.94	0.47	*FLJ23867*	203.02	439	0.46
ITPKA	6.37	13.59	0.47	*CLDN16*	1.98	4.27	0.46
LOC100192378	2.13	4.53	0.47	*RAB34*	636.73	1371.34	0.46
CFH	409.55	877.15	0.47	*MCAM*	1225.53	2675.13	0.46
MAGEE2	2.93	6.24	0.47	*PHLDA1*	442.83	972.88	0.46
ZSCAN20	23.94	51.05	0.47	*LHFPL1*	4.42	9.72	0.46
PYGL	367.56	786.53	0.47	*FLJ43390*	1.75	3.8	0.46

PIP5KL1	8.75	19.07	0.46	*LHFPL4*	4.27	9.2	0.46
PATE3	1.14	2.49	0.46	*SLMAP*	1488.15	3294.76	0.45
KLF5	715.32	1546.95	0.46	*LARGE*	427.23	939.15	0.45
TRIM22	512.13	1111.75	0.46	*ASPH*	1187.03	2657.9	0.45
ALDH2	2250.38	4873.6	0.46	*C5orf34*	23.16	51.14	0.45
TRERF1	209.76	455.49	0.46	*MBNL2*	737.93	1655.95	0.45
CAP2	287.64	625.72	0.46	*SPINT3*	1.12	2.5	0.45
ZEB2	313.78	682.99	0.46	*ACACB*	365.55	812.66	0.45
C2orf71	1.94	4.18	0.46	*PEG3*	129.61	288.58	0.45
DST	2115.56	4606.45	0.46	*TMEM47*	723.94	1618.05	0.45
AOC3	1347.51	2901.57	0.46	*NPR2*	174.28	387.62	0.45
CLDN11	134.42	289.55	0.46	*PDE4A*	205.31	453.58	0.45
SLC35F4	6.05	13.05	0.46	*GPX8*	191.98	430.62	0.45
BVES	164.17	358.41	0.46	*PLD1*	102.91	228.06	0.45
PDILT	2.15	4.71	0.46	*RBPMS2*	225.68	499.04	0.45
LIMS3	3.85	8.36	0.46	*SV2A*	79.43	174.72	0.45
USP2	33.36	72.91	0.46	*TTBK1*	6.44	14.39	0.45
PPM1L	51.25	110.74	0.46	*ANXA1*	2090.57	4630.48	0.45
OXER1	28.81	63.12	0.46	*ARHGEF19*	185.15	407.08	0.45
KCNH3	12.15	26.46	0.46	*GPR37L1*	3.56	7.95	0.45
NEGR1	110.57	240.51	0.46	*FHL2*	949.39	2094.83	0.45
LEFTY1	11.75	25.68	0.46	*SLC1A3*	108.67	241.72	0.45
SLC28A3	1.4	3.01	0.46	*SPTLC3*	126.89	280.7	0.45
SORCS2	71.48	155.2	0.46	*TMEM229A*	2.1	4.62	0.45
LYPD6B	92.36	201.21	0.46	*DENND2C*	33.14	73.7	0.45
NGFR	285.51	627.02	0.46	*SERPINF1*	1380.98	3051.77	0.45
SERPINF2	271.67	585.16	0.46	*RAG1*	12.28	27.38	0.45
FHL5	19.35	42.2	0.46	*EDA2R*	51.28	113.62	0.45
GPD1	6	12.97	0.46	*CHRNA4*	3.28	7.27	0.45
PADI2	97.1	210.59	0.46	*NOG*	7.12	15.97	0.45
VILL	223.55	480.98	0.46	*RHOF*	37.54	82.79	0.45
COL13A1	40.1	88.1	0.46	*C14orf180*	1.91	4.24	0.45
KNDC1	26.88	58.52	0.46	*IL22RA1*	11.84	26.15	0.45
MAEL	5.7	12.39	0.46	*IFI27L2*	72.46	161.3	0.45
GDF5	13.06	28.52	0.46	*FBXO2*	134.88	298.97	0.45
GAL3ST3	10.86	23.58	0.46	*ATOH8*	311.97	685.83	0.45
PCDH20	26.57	58.05	0.46	*CCDC80*	773.38	1710.59	0.45
ST6GAL2	28.88	63.32	0.46	*FILIP1L*	707.14	1584.7	0.45
INSRR	3.26	7.12	0.46	*KIF5A*	3.96	8.81	0.45
HAS2	14.04	30.8	0.46	*THRSP*	1.99	4.45	0.45
KRT19	3147.45	6784.61	0.46	*DLX3*	12.6	27.86	0.45
EYA1	119.63	257.48	0.46	*FLRT2*	175.25	390.66	0.45
KRT6C	3.84	8.37	0.46	*GDF7*	4.55	9.99	0.45
BCAN	4.55	9.83	0.46	*IGF2*	319.62	714.48	0.45
THSD7B	19.03	41	0.46	*KBTBD12*	5.04	11.21	0.45
ARC	18.96	41.25	0.46	*GFRA1*	128.88	286.38	0.45
HHATL	2.79	6.12	0.46	*PDE6B*	32.83	72.66	0.45
ABCC6	34.96	75.9	0.46	*GRHL3*	35.77	78.73	0.45

ADAMTSL3	187.08	420.18	0.45	*CCDC3*	301.69	683.5	0.44
LOC441666	12.33	27.56	0.45	*CA11*	137.12	309.08	0.44
FERMT1	195.61	432.5	0.45	*LIPC*	2.93	6.66	0.44
CAV3	3.3	7.34	0.45	*EFHA2*	72.89	165.09	0.44
FABP4	5.29	11.85	0.45	*SLFN12*	22.99	52.62	0.44
HOXB5	4.08	9.05	0.45	*GPR75*	14.27	32.8	0.44
C7	965.3	2154.21	0.45	*PRDM16*	17.66	40.32	0.44
DBC1	35.79	79.26	0.45	*LRRC4*	16.18	37.11	0.44
DUSP19	57.77	130.4	0.44	*AMT*	211.41	476.42	0.44
FLJ10357	451.96	1017.98	0.44	*APOBEC3F*	140.46	318.12	0.44
C20orf194	351.51	806.57	0.44	*CYP27C1*	6.44	14.76	0.44
CDC42EP3	1089.75	2504.12	0.44	*PLEKHH2*	61	137.17	0.44
NEURL1B	607.94	1374.8	0.44	*ITGA1*	642.22	1449.9	0.44
SH3PXD2B	458.64	1034.48	0.44	*SLC5A7*	2.82	6.38	0.44
METTL7A	1475.84	3327.7	0.44	*SLFN13*	54.07	123.91	0.44
ABCG4	2.99	6.86	0.44	*GFRA2*	18.84	42.73	0.44
LRRC17	40.65	91.53	0.44	*KCP*	7.02	15.96	0.44
TRIP6	589.05	1326.41	0.44	*GRAMD1B*	8	18.31	0.44
PITPNM3	207.26	470.54	0.44	*JAKMIP2*	6.1	13.86	0.44
RHOB	6589.92	15102.08	0.44	*TWIST2*	31.85	72.01	0.44
CCRL1	6.84	15.66	0.44	*KCNE3*	75.26	171.02	0.44
KLF8	57.83	132.22	0.44	*TGFB3*	764.42	1756.27	0.44
C7orf31	43.55	98.17	0.44	*SRRM4*	2.94	6.66	0.44
ZAK	1357.52	3101.3	0.44	*FABP3*	191.81	440.45	0.44
PTRF	5438.02	12447.89	0.44	*UGT3A2*	3.24	7.38	0.44
GPR161	113.12	257.25	0.44	*IL1RN*	35.1	80.44	0.44
LIPE	56.78	130.32	0.44	*UNC5C*	17.42	39.76	0.44
FCRLB	8.75	19.74	0.44	*CYTL1*	27.8	63.07	0.44
PTPLAD2	95.9	217.83	0.44	*ROBO2*	27.03	60.75	0.44
ADCY8	1.32	3.03	0.44	*LOC643763*	7.25	16.55	0.44
C10orf55	4.08	9.31	0.44	*SCNN1A*	1063.79	2431.6	0.44
GEFT	452.99	1023.61	0.44	*COX7A1*	149.78	337.67	0.44
CHST15	296.82	674.01	0.44	*DUSP4*	158.74	358.76	0.44
C1orf126	34.61	78.22	0.44	*ERBB4*	30.29	69.62	0.44
DDIT4	1529.12	3485.88	0.44	*LPA*	6.38	14.52	0.44
PDGFC	414.69	934.38	0.44	*ISL2*	8.83	19.99	0.44
PLSCR4	190.51	428.64	0.44	*KCNT2*	11.43	25.93	0.44
MYO3A	2.25	5.14	0.44	*COL19A1*	2.55	5.79	0.44
DSCAML1	4.89	11	0.44	*EPHX3*	11.88	26.96	0.44
FADS1	536.42	1213.11	0.44	*PPP1R1C*	2.68	6.15	0.44
ARL10	14.38	32.97	0.44	*DEFB132*	43.33	99.45	0.44
INPP5D	226.32	511.47	0.44	*ABCC3*	165.96	375.99	0.44
SNPH	187.28	423.36	0.44	*GPR133*	82.57	185.71	0.44
EFHD1	173.37	397.2	0.44	*HS3ST6*	3	6.75	0.44
SMTN	2786.63	6366.34	0.44	*HOXB6*	10.22	23.3	0.44
KCNE1L	2.98	6.81	0.44	*NELL2*	133.87	302.39	0.44
SIM1	1.14	2.57	0.44	*ATP6V1B1*	23.87	53.95	0.44
CELF2	406.25	917.62	0.44	*ZFP57*	4.06	9.21	0.44

ANKRD2	3.08	6.97	0.44	*IL33*	314.28	735.18	0.43
FOXA2	5.72	13.05	0.44	*DSG4*	1.49	3.48	0.43
CITED1	81.46	184.3	0.44	*PPAPDC3*	40.19	93.18	0.43
ST5	955.29	2231.17	0.43	*FBXL2*	55.38	128.13	0.43
DOK4	294.24	685.53	0.43	*B4GALNT1*	34.22	80.1	0.43
ZNF157	1.88	4.33	0.43	*ILDR2*	4.39	10.33	0.43
C1orf183	42.67	98.7	0.43	*GPR149*	1.23	2.86	0.43
MST4	367.28	859.49	0.43	*GPR144*	1.79	4.19	0.43
LOC644936	21.51	50.13	0.43	*ABCD2*	7.31	17.18	0.43
KANK2	2125.73	4988.48	0.43	*HEPACAM*	2.44	5.73	0.43
C16orf45	343.83	805.81	0.43	*EFNA5*	140.07	326.58	0.43
DPYSL3	3490.03	8116.15	0.43	*ANKRD35*	90.63	212.61	0.43
LCA5	97.43	225.94	0.43	*SERP2*	37.02	86.47	0.43
TRH	1.41	3.31	0.43	*LOC643008*	13.03	30.08	0.43
CLIP4	306.84	718.34	0.43	*APCDD1L*	4.55	10.51	0.43
ACSL4	379.88	888.34	0.43	*PKHD1L1*	5.13	11.93	0.43
ANXA2	3631.58	8364.25	0.43	*SRPX*	119.24	279.62	0.43
AVPI1	189.26	436.45	0.43	*MUC21*	1.56	3.66	0.43
MESTIT1	2.52	5.86	0.43	*ZNF676*	4.12	9.65	0.43
GLI3	222.11	511.47	0.43	*ADAM28*	58.63	137.65	0.43
ATP1B1	1033.91	2432.47	0.43	*ZNF257*	11.33	26.29	0.43
DZIP1	155.01	364.58	0.43	*ADAMTS15*	9.87	23.09	0.43
SSPN	459.79	1061.03	0.43	*MMP16*	13.67	32.04	0.43
MAMSTR	17.02	39.38	0.43	*TRPM5*	2.35	5.43	0.43
IRAK3	124.78	293.15	0.43	*CACNA2D3*	14.7	33.81	0.43
EHD2	1209.29	2811.87	0.43	*RPRM*	52.04	122	0.43
CHST3	382.73	882.44	0.43	*SIK1*	1340.1	3124.7	0.43
PRELID2	13.26	30.75	0.43	*CHRFAM7A*	18.38	43.15	0.43
PRDM5	34.98	82.01	0.43	*HSD11B1*	53.86	126.44	0.43
ELF4	139.46	321.63	0.43	*HOXB4*	14.79	34.02	0.43
RAPGEFL1	151.49	349.59	0.43	*NPAS4*	4.89	11.41	0.43
EDDM3B	1.04	2.41	0.43	*TSHZ2*	58.25	135.37	0.43
ZNF454	15.59	36.39	0.43	*FCER1A*	36.49	84.23	0.43
GJC1	183.26	426.68	0.43	*RIMS4*	34.74	81.44	0.43
KCNJ13	2.43	5.7	0.43	*LRRTM1*	4.62	10.85	0.43
LOC441869	189.95	440.08	0.43	*IL17RE*	128.43	295.95	0.43
SPARCL1	6726.06	15666.46	0.43	*HAPLN2*	15.2	35.1	0.43
FAM5B	1.93	4.44	0.43	*CGB7*	5.18	11.91	0.43
OLFML2A	290.49	669.67	0.43	*DAPP1*	32.3	74.33	0.43
PTPLA	75.14	174.73	0.43	*FOXE1*	8.25	19.14	0.43
TUBA4A	553.23	1280.51	0.43	*CCKBR*	5.62	13.16	0.43
STARD8	145.41	342.08	0.43	*ZNF727*	6.16	14.41	0.43
C14orf132	285.58	658.63	0.43	*C11orf93*	38.12	91.03	0.42
C14orf37	31.95	73.47	0.43	*ANP32E*	363.76	860.53	0.42
SCHIP1	122.91	287.92	0.43	*LRRC3B*	1.62	3.88	0.42
ROBO1	603.01	1418.29	0.43	*KRT9*	1.53	3.68	0.42
REEP1	137.98	324.09	0.43	*ANXA6*	2006.79	4758.27	0.42
C15orf51	3.69	8.62	0.43	*CERK*	270.73	646.17	0.42

ZNF98	2.06	4.94	0.42	*ZNF486*	19.28	45.94	0.42
CALD1	10161.41	24258.2	0.42	*GPR173*	8.53	20.44	0.42
DMC1	4.27	10.26	0.42	*DEPDC7*	13.2	31.72	0.42
RIMS3	94.35	225.81	0.42	*NTRK2*	132.57	316.5	0.42
KLHL13	124.32	294.97	0.42	*POMC*	9.65	22.75	0.42
SNX7	173.04	409.1	0.42	*C6orf123*	3.61	8.55	0.42
SLC16A4	54.88	130.38	0.42	*CLIC3*	19.28	46.16	0.42
EID3	27.13	64.04	0.42	*ASAM*	41.35	98.08	0.42
GPM6B	223.85	536.58	0.42	*AQP9*	10.58	25.16	0.42
HOXD12	1.84	4.34	0.42	*PCSK9*	3.34	7.9	0.42
PARM1	2838.97	6749.2	0.42	*FAM19A1*	6.9	16.49	0.42
C14orf176	6.68	15.77	0.42	*DUSP15*	18.16	43.45	0.42
RASSF5	258.57	608.42	0.42	*YBX2*	8.81	21.03	0.42
MR1	75.22	180.61	0.42	*CYP26B1*	37.98	90.88	0.42
SPG20	699.79	1662.31	0.42	*FAM65B*	111.13	263.72	0.42
KSR1	139.29	332.2	0.42	*FMO2*	81.71	196.55	0.42
CHST11	49.57	119.22	0.42	*PLD5*	5.74	13.54	0.42
TRPC1	84.33	199.25	0.42	*RARRES1*	299.05	705.51	0.42
CAND2	90.88	217.72	0.42	*PTPRQ*	6.53	15.39	0.42
EFEMP2	624.91	1480.13	0.42	*NFATC2*	22.26	53.57	0.42
BCL2	384.32	913.53	0.42	*GFRA3*	19.3	46.39	0.42
RBPMS	1045.85	2461.65	0.42	*DPP6*	5.86	14.11	0.42
CCDC158	4.12	9.8	0.42	*PCDHAC2*	10.12	24.08	0.42
SGCE	195.37	461.27	0.42	*HPSE2*	32.3	76.02	0.42
SLC6A2	2.6	6.12	0.42	*SCNN1B*	58.57	138.67	0.42
GALNT6	42.66	100.51	0.42	*AKAP2*	295.45	708.37	0.42
NRP2	305.04	727.03	0.42	*NDRG2*	1806.4	4389.1	0.41
RCAN2	337.31	808.17	0.42	*TIMP3*	4995.58	12113.62	0.41
AMOT	496.87	1189.56	0.42	*FRMD7*	1.97	4.8	0.41
GPX7	95.18	228.73	0.42	*PAQR5*	17.42	42.74	0.41
DYNC1I1	81.46	195.95	0.42	*TMEM169*	7.07	17.11	0.41
PAPPA2	2.04	4.81	0.42	*HCG11*	184.55	449.15	0.41
FAM90A1	5.22	12.49	0.42	*PRKCA*	553.95	1335.48	0.41
ETV5	162.25	384.31	0.42	*SP6*	75.67	186.3	0.41
NHLH2	2.31	5.53	0.42	*LRP4*	128.11	309.43	0.41
ANKRD53	10.34	24.62	0.42	*PRTFDC1*	41.97	101.36	0.41
LOC149837	16.57	39.08	0.42	*AMPH*	15.71	38.2	0.41
LIMS2	887.76	2138.76	0.42	*LOC100126784*	22.7	55.18	0.41
SLC4A11	65.35	155.84	0.42	*FAM124A*	44.57	108.04	0.41
LPP	490.2	1165.77	0.42	*MRAP2*	88.58	218.02	0.41
CDKL2	11.08	26.54	0.42	*SERPINB1*	210.92	519.64	0.41
SLIT3	197.94	470.29	0.42	*HLF*	303.18	746.72	0.41
HAPLN4	4.73	11.24	0.42	*LGALS3*	1714.54	4157.92	0.41
STARD4	85.01	200.61	0.42	*SLC24A3*	249.08	601.98	0.41
EGOT	3.27	7.72	0.42	*NACAD*	77.44	187.28	0.41
ABCC9	176.75	421.11	0.42	*RNF182*	6.09	14.67	0.41
SPINK13	1.45	3.47	0.42	*XKR4*	2.6	6.29	0.41
SCUBE1	18.44	43.88	0.42	*INMT*	441.5	1075.3	0.41

ATP10B	2.14	5.26	0.41	*PRRG4*	118.64	295.17	0.4
NTN4	1024.94	2488.95	0.41	*BEND5*	43.14	108.75	0.4
F7	5.1	12.5	0.41	*GPRASP1*	332.81	823.48	0.4
HS3ST3A1	32.7	80.66	0.41	*KCNIP3*	81.14	205.14	0.4
MET	303.63	745.62	0.41	*C11orf87*	1.59	3.97	0.4
HSPB2	56.11	137.88	0.41	*SGK269*	365.39	904.64	0.4
ZNF711	46.57	114.53	0.41	*CCDC89*	13.34	32.98	0.4
ITGB4	1059.84	2593.77	0.41	*DKK3*	1235.15	3068.76	0.4
FAM178B	5.6	13.75	0.41	*CORO2B*	31.19	77.93	0.4
CNR1	15.86	38.3	0.41	*NCRNA00087*	56.27	141.05	0.4
TRPC4	62.85	154.74	0.41	*EPB41L3*	219.87	553.15	0.4
DTNA	284.97	689.65	0.41	*FHOD3*	397.24	1004.93	0.4
HR	120.32	292.79	0.41	*ASB12*	3.19	7.97	0.4
ZCCHC12	6.24	15.08	0.41	*ANXA2P3*	3.02	7.64	0.4
LOC134466	27.17	66.41	0.41	*TSPAN2*	214.94	543.58	0.4
SLC38A5	38.48	93.91	0.41	*BNC2*	118.6	296.79	0.4
IGF2BP2	23.91	57.8	0.41	*EVC2*	62.06	156.01	0.4
TNIK	67.08	163.07	0.41	*MAP1B*	1474.21	3666.47	0.4
DNAJC15	172.53	415.92	0.41	*TMEM154*	35.37	89.31	0.4
KLHL30	10.14	24.94	0.41	*REC8*	82.97	206.75	0.4
CXCR1	3.25	7.96	0.41	*WFIKKN2*	9.3	23.09	0.4
C2orf43	139.67	337.81	0.41	*HEPH*	302.44	752.54	0.4
TMPRSS11A	1.71	4.18	0.41	*CHST6*	11.88	29.44	0.4
SERTAD4	32.53	79.25	0.41	*FAM19A2*	26.07	65.19	0.4
C9orf24	9.05	22	0.41	*PLIN4*	376.53	946.65	0.4
ESPNL	10.4	25.61	0.41	*ZNF655*	430.13	1086.63	0.4
C9orf4	5.66	13.9	0.41	*TACR2*	38.63	97.33	0.4
RUNX1T1	16.27	39.79	0.41	*PTGER3*	55.15	139.52	0.4
OGN	423.47	1027.81	0.41	*PDE5A*	1049.68	2615.02	0.4
CPXM1	111.86	275.96	0.41	*TMEM114*	1.19	3.02	0.4
ROR1	47.4	115.96	0.41	*CHAD*	7.22	18.21	0.4
HAND2	5.65	13.92	0.41	*PTGIS*	516.2	1290.81	0.4
PRPH	6.3	15.49	0.41	*ITPR1*	1086.95	2711.22	0.4
MYOZ1	18.15	44.54	0.41	*SGCA*	140.74	351.9	0.4
GALNT5	18.14	43.76	0.41	*NTRK3*	33.21	84.02	0.4
MFAP5	26.49	63.99	0.41	*LSAMP*	189.49	476.85	0.4
TBX5	26.54	65.26	0.41	*RSPO3*	67.24	167.5	0.4
GALNT13	17.48	42.21	0.41	*HHIPL2*	7.32	18.14	0.4
SCNN1G	125.27	308.7	0.41	*LGALS4*	9.31	23.16	0.4
C19orf33	56.09	136.72	0.41	*NOX1*	10.17	25.54	0.4
GLRA4	1.32	3.32	0.4	*CALN1*	8.26	20.81	0.4
FERMT2	929.5	2345.89	0.4	*GSTA2*	3.47	8.72	0.4
FEZ1	140.7	354.58	0.4	*PITX2*	5.81	14.59	0.4
B3GNT9	239.89	595.13	0.4	*CFL2*	893.76	2298.94	0.39
LOC401093	153.62	383.26	0.4	*PLEKHA2*	393.7	1012.01	0.39
PLS3	851.46	2118.62	0.4	*TPM1*	8608.86	21899.03	0.39
CCDC129	1.8	4.45	0.4	*PLP2*	617.24	1590.99	0.39
PDGFD	226.43	563.39	0.4	*ANXA2P2*	1220.41	3126.6	0.39

SVIL	2362.98	6133.24	0.39	*SLC7A14*	3.11	8.05	0.39
C4orf35	1.28	3.28	0.39	*PNCK*	127.83	331.31	0.39
TCF7L1	228	587.84	0.39	*PNMAL1*	141.78	363.45	0.39
SLC9A4	1.45	3.77	0.39	*WNT9B*	2.91	7.37	0.39
RNASE12	1.49	3.82	0.39	*NHSL2*	24.28	62.68	0.39
MXRA7	1991.5	5078.55	0.39	*FAT4*	161.21	413.89	0.39
FAM92A1	44.63	113.52	0.39	*LGALS7*	3.09	7.95	0.39
DBNDD2	333.61	848.04	0.39	*PCDH10*	250.51	638.56	0.39
PPP1R12B	3696.87	9521.78	0.39	*LOC284837*	17.9	45.81	0.39
KCNH4	3.35	8.51	0.39	*C1orf175*	26.54	68.79	0.39
TPRG1	12.18	31.49	0.39	*GPR39*	19.52	49.6	0.39
SOX7	119.96	309.19	0.39	*LCN15*	2.09	5.3	0.39
CD40	190.08	487.24	0.39	*FCGR3B*	6.6	16.77	0.39
LOC644538	84.51	216.7	0.39	*FCGBP*	379.09	964.07	0.39
KCNJ8	157.01	400.83	0.39	*PREX2*	22.28	57.02	0.39
S100A16	381.04	984.83	0.39	*RSPO2*	43.5	112.02	0.39
ZNF660	10.01	25.48	0.39	*NR4A3*	163.56	420.49	0.39
HOXA7	66.92	170.05	0.39	*EFNB1*	369.02	961.46	0.38
TCEAL7	43.04	109.08	0.39	*C15orf41*	89.43	234.25	0.38
RAB40AL	6.86	17.39	0.39	*ETNK2*	188.06	501.13	0.38
LOC399959	218.07	555.6	0.39	*GSTM4*	294.53	782.98	0.38
ITIH5	607.39	1544.15	0.39	*GPR156*	3.03	7.96	0.38
EDNRA	533.43	1378.12	0.39	*KITLG*	397.85	1058.02	0.38
GIPC2	26.43	68.15	0.39	*TINAGL1*	531.88	1394.52	0.38
NTF3	17.78	45.91	0.39	*JAZF1*	328.04	855.72	0.38
PDPN	197.55	501.8	0.39	*JUB*	233.82	611.49	0.38
FAM162B	18.97	48.37	0.39	*CCDC69*	505.29	1343.53	0.38
ID3	913.13	2324.11	0.39	*VCL*	2865.2	7487.61	0.38
EFHC2	25.14	64.2	0.39	*C8orf31*	4.69	12.36	0.38
NCRNA00092	30.58	78.5	0.39	*LPAR1*	187.5	494.2	0.38
RASL10B	35.28	90.21	0.39	*C7orf51*	18.09	47.22	0.38
FGF2	171.51	435.99	0.39	*NUDT10*	210.82	559.02	0.38
KCNS2	3.89	9.87	0.39	*KIAA1614*	31.91	84.92	0.38
CDKL1	21.27	54.01	0.39	*TNNI3K*	7.41	19.3	0.38
TGFB1I1	678.39	1720.03	0.39	*MEIS1*	253.89	665.61	0.38
PAPPA	30.15	77.85	0.39	*TAF7L*	3.68	9.66	0.38
ZDHHC15	37.38	95.59	0.39	*ADRA2B*	12.4	32.42	0.38
CDH18	2.05	5.19	0.39	*C1QTNF1*	496.66	1304.4	0.38
RASL11B	47.17	121.52	0.39	*NLGN3*	118.75	314.82	0.38
CAMK1D	33.03	85.01	0.39	*ACOT11*	46.95	124.89	0.38
TP53AIP1	10.51	27.22	0.39	*ATAD3C*	52.31	136.71	0.38
WNT7A	2.68	6.8	0.39	*SSTR2*	17.46	46.02	0.38
ST8SIA1	98.59	249.61	0.39	*XKR5*	8.7	23.04	0.38
RNASE7	4.17	10.78	0.39	*NMUR1*	21.73	57.26	0.38
ATP13A4	2.1	5.42	0.39	*RGNEF*	127.66	335.13	0.38
SPEG	380.93	987.34	0.39	*ANKRD43*	49.03	127.66	0.38
UGT8	4.81	12.21	0.39	*STK32A*	2.71	7.05	0.38
UGT2B7	1.63	4.13	0.39	*CAMK1G*	19.54	51.52	0.38

ACE2	7.79	20.42	0.38	*DDO*	22.56	61.38	0.37
ODZ3	120.82	317.52	0.38	*TRO*	71.57	195.12	0.37
MATN4	2.51	6.63	0.38	*ITGA2*	340.88	915.96	0.37
HUNK	46.04	121.52	0.38	*S100A6*	2318.9	6189.94	0.37
WFDC1	470.48	1230.34	0.38	*CCNJL*	24.86	66.48	0.37
HOXB3	72.15	188.62	0.38	*PRPH2*	11.99	32.48	0.37
SLC16A7	9.17	23.87	0.38	*OR7C1*	2.6	7.07	0.37
SCRG1	56.57	149.25	0.38	*SULT2A1*	1.13	3.05	0.37
LY75	74.28	197.65	0.38	*ADRB3*	1.83	4.89	0.37
PNMA2	16.32	42.4	0.38	*ANKRD45*	6.82	18.22	0.37
CNTNAP4	1.82	4.73	0.38	*MRVI1*	850.35	2267.7	0.37
PCDH11X	4.54	12.09	0.38	*C3orf54*	28.2	76.66	0.37
WNT16	6.68	17.45	0.38	*SEMA5A*	196.63	533.74	0.37
WNT2B	53.73	142.62	0.38	*ACTA2*	16864.81	45162.18	0.37
LGALS7B	1.95	5.19	0.38	*KIF26B*	31.62	85	0.37
FMN2	21.66	56.51	0.38	*STK33*	40.19	108.11	0.37
C6orf174	34.85	91.15	0.38	*HRH2*	13.66	37.05	0.37
TGM5	2.84	7.47	0.38	*TAGLN*	22535.4	60700.26	0.37
HCG4	20.25	53.92	0.38	*ZFPM2*	14.35	39.11	0.37
S1PR5	23.41	61.89	0.38	*C6orf186*	7.44	20.24	0.37
MGAM	3.29	8.54	0.38	*PCDP1*	3.29	8.93	0.37
IVL	2.07	5.43	0.38	*PRSS1*	1.26	3.45	0.37
NEURL3	27.1	70.85	0.38	*KCNQ4*	15.35	41.37	0.37
C4orf31	126.33	334.32	0.38	*IER3*	406.35	1096.82	0.37
PCDHGA9	28.64	75.78	0.38	*C1orf186*	8.38	22.69	0.37
FXYD1	100.37	262.98	0.38	*KCNH1*	8.77	23.57	0.37
POU2AF1	26.7	69.62	0.38	*PLN*	248.71	669.95	0.37
SGK1	1034.09	2710.54	0.38	*GPR109A*	45.35	121.46	0.37
ANXA13	3.12	8.29	0.38	*HSPB7*	258.61	695.11	0.37
CDH19	8.76	23.18	0.38	*MACC1*	67.02	182.24	0.37
SOX10	15.98	41.75	0.38	*TMEM100*	56.93	154.04	0.37
COL27A1	94.33	246.19	0.38	*SLC8A2*	17.48	46.91	0.37
BMPER	32.69	85.41	0.38	*NLGN1*	26.97	72.28	0.37
CYP2W1	9.8	25.77	0.38	*VAT1L*	3.77	10.24	0.37
CYP4F12	29.75	78.06	0.38	*DUSP2*	181.15	484.6	0.37
PRKG2	59.72	157.36	0.38	*AKR1C2*	24.27	66.19	0.37
CDO1	119.91	313.59	0.38	*CES1*	472.11	1274.1	0.37
KCNH5	1.29	3.47	0.37	*BNC1*	5.97	16.17	0.37
LOC283856	2.99	8.19	0.37	*SRL*	36.75	99.26	0.37
ANO6	852.34	2300.48	0.37	*RNF112*	36.72	99.24	0.37
SLCO3A1	141.04	379.61	0.37	*HBB*	97.45	261.14	0.37
RTDR1	3.1	8.29	0.37	*MAOB*	931.5	2526.64	0.37
DNAJB5	371.73	1002.08	0.37	*PRSS12*	21.34	57.39	0.37
C4orf49	12.1	32.78	0.37	*LRRC2*	15.92	42.54	0.37
DNAJB4	176.87	480.59	0.37	*BCL11A*	73.45	200.33	0.37
KIAA1161	74.94	200.11	0.37	*CXCL6*	31.16	84.7	0.37
CAPG	640	1747.88	0.37	*P2RX2*	22.57	61.38	0.37
CCND2	715.72	1913.92	0.37	*TIMP4*	29.29	79.09	0.37

WNT3A	19.18	52.22	0.37	*MAML2*	177.54	492.21	0.36
CA4	16.7	44.55	0.37	*TPM2*	8313.18	23108.98	0.36
CCDC60	22.6	61.45	0.37	*CAMK2A*	6.14	17.25	0.36
CDH2	44.53	120.12	0.37	*MBNL3*	27.87	78.4	0.36
FUT6	3.3	8.88	0.37	*FUT2*	31.6	88.81	0.36
S100A2	64.56	174.21	0.37	*SCGB2A1*	24.65	68.87	0.36
GABRP	133.78	366.42	0.37	*ENPP6*	31.62	87.79	0.36
PALLD	3478.9	9763.85	0.36	*BMP7*	175.12	490.06	0.36
CLDN19	2.04	5.71	0.36	*GRIA3*	12.06	33.41	0.36
PDE7B	87.04	244.97	0.36	*ITLN1*	3.3	9.22	0.36
NHS	86.67	242.33	0.36	*RBP4*	15.78	43.99	0.36
CD200	115.43	317.6	0.36	*CASQ2*	50.83	140.65	0.36
CLIP3	422.95	1180.33	0.36	*CDH22*	22.63	63.21	0.36
TRIM6	41.44	114.1	0.36	*PROK1*	40.5	111.55	0.36
DOCK3	41.36	113.54	0.36	*KIAA0408*	40.25	112.51	0.36
ADCYAP1R1	1.82	5.03	0.36	*KRT17*	1440.52	3959.27	0.36
VWA5A	262.21	727.25	0.36	*WFDC12*	5.92	16.29	0.36
NT5E	185.1	518.05	0.36	*MGAT4C*	1.77	5.08	0.35
SDPR	529.66	1468.41	0.36	*TRIM61*	2.41	6.79	0.35
VAV3	92.6	258.02	0.36	*ST6GALNAC4*	113.88	323.52	0.35
HOXB2	62.96	174.99	0.36	*PLBD1*	107.45	307.04	0.35
PPARGC1B	16.03	44.75	0.36	*LPCAT2*	245.44	699.76	0.35
APOBEC3D	32.64	91.23	0.36	*TGFBR3*	567.58	1603.87	0.35
KCNMA1	994.51	2744.36	0.36	*MGC16703*	3.52	10.15	0.35
C3orf70	184.07	508.13	0.36	*USH1G*	2.21	6.27	0.35
GPLD1	19.87	55.6	0.36	*TMIE*	5.04	14.39	0.35
SH3GL3	3.77	10.32	0.36	*CSRP1*	12935.04	37332.22	0.35
IL1R2	8.19	22.55	0.36	*TRPC3*	2.94	8.39	0.35
P2RY12	8.57	24.13	0.36	*LOC100130238*	2.63	7.56	0.35
WISP3	2.71	7.53	0.36	*ATP1A4*	2.12	6.06	0.35
KIRREL3	3.36	9.38	0.36	*SLC1A6*	2	5.77	0.35
CPAMD8	779.42	2136.24	0.36	*POPDC2*	162.49	462.53	0.35
NEXN	541.93	1523.82	0.36	*CACHD1*	178.88	516.2	0.35
OR7A5	2.7	7.39	0.36	*MAP1A*	276.26	796.74	0.35
COL21A1	42.7	117.27	0.36	*INSC*	3.33	9.42	0.35
LRRK2	78.85	216.81	0.36	*RND3*	431.06	1223.63	0.35
LRMP	30.1	84.54	0.36	*TMEM139*	15.52	44.46	0.35
GAS1	193.9	545.97	0.36	*ITGB8*	376.72	1091.24	0.35
GPX3	1168.3	3215.77	0.36	*FGF7*	131.33	373.96	0.35
SOX5	13.09	36.74	0.36	*ADRA1B*	4.54	13.03	0.35
CLIC6	151.99	426.78	0.36	*ZBTB7C*	103.12	294.15	0.35
HAAO	90.2	250.54	0.36	*RGN*	81.28	232.01	0.35
RNF175	9.95	27.92	0.36	*FLNA*	25172.05	72124.97	0.35
TLR3	92.53	257.72	0.36	*SPATA6*	34.7	98.91	0.35
POU3F3	1.17	3.28	0.36	*TMEM200B*	158.56	459.02	0.35
LRFN5	30.92	84.76	0.36	*HPCAL4*	3.67	10.4	0.35
EGFL6	4.92	13.8	0.36	*CHD5*	24.31	69.12	0.35
CRHBP	10.84	30.2	0.36	*WNT10A*	23.86	68.08	0.35

EDNRB	249.72	707.09	0.35	*SPATA18*	105.05	312.72	0.34
CKMT2	44.98	127.24	0.35	*EFS*	398.81	1157.52	0.34
SLC16A12	7.12	20.41	0.35	*LRCH2*	134.18	391.04	0.34
ODZ2	61.31	175.25	0.35	*MYL9*	13698.76	40814.65	0.34
RXRG	4.09	11.75	0.35	*SCUBE3*	32.28	94.24	0.34
GLT1D1	7.6	21.92	0.35	*PHYHIP*	80.74	236.32	0.34
ELAVL3	5.06	14.55	0.35	*IGDCC4*	9.49	27.63	0.34
GRIN2A	8.25	23.75	0.35	*XG*	5.29	15.64	0.34
C2orf40	158.03	452.73	0.35	*ITGA9*	119.52	356.51	0.34
APOB	7.55	21.78	0.35	*RASAL1*	5.71	16.99	0.34
CSTA	67.48	190.7	0.35	*KANK4*	31.02	90.33	0.34
SLC2A4	133.96	384.48	0.35	*NPPC*	2.54	7.37	0.34
ANXA8L2	35.08	100.22	0.35	*PCP4*	563.6	1646.53	0.34
IL1RL1	7.11	20.39	0.35	*CES4*	8.13	23.95	0.34
C16orf89	36.99	107.17	0.35	*ATCAY*	52.25	153.84	0.34
KCNK3	330.19	931.98	0.35	*MYOM1*	76.79	224.08	0.34
RNF128	50.05	143.78	0.35	*PTGDS*	2032.68	6004.09	0.34
KIRREL	66.06	190.77	0.35	*CDH17*	10.42	30.25	0.34
CACNA2D1	65.8	189.49	0.35	*DDIT4L*	26.63	77.2	0.34
CAV2	356.74	1048.21	0.34	*ASCL2*	24.28	70.84	0.34
C12orf75	362.41	1070.56	0.34	*MME*	1879.18	5586.02	0.34
OVCH2	1.83	5.35	0.34	*L1CAM*	38.77	115.55	0.34
ARHGAP23	387.31	1142.59	0.34	*CHGB*	73.12	214.49	0.34
SVOPL	2.73	8.02	0.34	*STON1-GTF2A1L*	4.85	14.12	0.34
MECOM	187.01	552.43	0.34	*SLC38A4*	16.46	47.99	0.34
ENTPD3	61.75	179.64	0.34	*FGFBP1*	8.51	25.4	0.34
EFEMP1	739.93	2192.1	0.34	*ARSF*	1.66	5.01	0.33
GLIS3	103.2	299.38	0.34	*GABRA3*	1.59	4.84	0.33
ZSCAN4	1.91	5.55	0.34	*GBP6*	2.75	8.37	0.33
ATP2B4	1487.99	4436.27	0.34	*TMEM37*	36.56	110.7	0.33
RAB38	21.74	64.15	0.34	*MTMR8*	7.55	22.72	0.33
CTTNBP2	60.4	177.36	0.34	*MCC*	386.79	1160.48	0.33
PLA2G4A	139.8	405.96	0.34	*CYP19A1*	1.91	5.85	0.33
GJA1	1266.32	3718.06	0.34	*C15orf59*	76.31	231.5	0.33
GPRC6A	1.62	4.77	0.34	*MYOF*	1270.01	3822.84	0.33
KCNF1	4.31	12.87	0.34	*NRXN3*	112.91	346	0.33
CHST2	201.26	583.65	0.34	*MSRB3*	943.83	2832.77	0.33
FXYD6	528.46	1570.46	0.34	*USP44*	7.06	21.35	0.33
CA12	154.59	450.46	0.34	*TNS1*	5955.67	17843.23	0.33
RGS22	14.27	42.32	0.34	*BHMT2*	66.72	203.32	0.33
PPP2R2B	13.89	40.65	0.34	*ZNF488*	6.13	18.73	0.33
MEIS2	429.26	1263.24	0.34	*ARMCX1*	294.72	902.84	0.33
DCAF12L1	2.56	7.52	0.34	*RGS13*	7	21.41	0.33
ANK2	133.17	390.11	0.34	*RAB40A*	12.11	36.71	0.33
LOC283392	7.13	20.98	0.34	*RASGRF1*	8.42	25.18	0.33
C11orf70	19.92	57.88	0.34	*JPH2*	424.68	1272.26	0.33
ITGB1BP2	23.08	67.41	0.34	*ACSL6*	7.48	22.57	0.33
DRD2	6.34	18.53	0.34				

C20orf200	25.31	77.59	0.33	*RGS9*	29.13	91.75	0.32
COL23A1	41.1	122.71	0.33	*PRSS35*	6.05	19.08	0.32
FRMD3	14.21	43.36	0.33	*HSPB6*	1225.84	3803.71	0.32
KIAA1644	96.39	292.27	0.33	*EFCAB1*	31.35	97.16	0.32
HOXB8	3.59	10.99	0.33	*ZNF536*	12.71	40.2	0.32
FLJ45983	4.15	12.61	0.33	*CYP27A1*	662.31	2068.8	0.32
ID1	627.09	1924.99	0.33	*WSCD2*	73.74	227.13	0.32
IL1RL2	7.88	23.6	0.33	*PYGM*	169.83	537.82	0.32
CHRNA7	4.85	14.78	0.33	*MASP1*	489.82	1534.99	0.32
SMTNL2	9.9	29.63	0.33	*ANO4*	67.73	208.68	0.32
PRSS16	107.66	322.26	0.33	*S100B*	26.61	83.06	0.32
SEMA3D	123.88	376.37	0.33	*HOXD11*	33.35	105.09	0.32
SCN7A	72.72	222.57	0.33	*LOC440173*	3.8	11.9	0.32
KCNJ16	3	9.05	0.33	*APOBEC2*	3.6	11.4	0.32
HECW1	21.51	65.36	0.33	*CALML3*	29.93	92.71	0.32
IL6ST	482.2	1444.96	0.33	*FAM55D*	6.79	21.29	0.32
DMBT1	5.84	17.59	0.33	*AFAP1L2*	282.17	902.12	0.31
PRICKLE2	270.04	843.31	0.32	*ATP6V1G3*	1.39	4.42	0.31
C14orf64	4.27	13.16	0.32	*FRMD6*	573.72	1822.26	0.31
UNC5B	414.16	1278.1	0.32	*GATM*	134.14	428.15	0.31
C1orf114	11.93	37.54	0.32	*POU3F1*	4.44	14.18	0.31
G0S2	71.91	225.67	0.32	*PLLP*	94.95	301.78	0.31
CCDC8	147.93	464	0.32	*FOXI2*	2.34	7.45	0.31
PIPOX	26.78	83.79	0.32	*SOX15*	47.61	154.48	0.31
CTF1	100.18	312.41	0.32	*RBP1*	153.05	487.61	0.31
VWCE	11.5	35.91	0.32	*BAI3*	6.68	21.69	0.31
ME1	131.35	408.11	0.32	*RASL12*	503.67	1640.66	0.31
SLC47A1	42.81	132.12	0.32	*TRPC6*	31.73	101.96	0.31
B3GNT8	34.06	107.48	0.32	*FBXO17*	135.48	430.48	0.31
PGF	70.03	219.98	0.32	*LONRF3*	14.2	46.55	0.31
ACSF2	202.45	627.71	0.32	*DMD*	412.8	1335.39	0.31
CDH23	26.73	82.59	0.32	*HCN4*	4.75	15.51	0.31
NAV2	209.89	650.76	0.32	*PCDH9*	29.26	93.57	0.31
ZNF516	171.24	534.62	0.32	*AHNAK2*	497.95	1597.06	0.31
OPCML	2.81	8.72	0.32	*TEPP*	2.7	8.81	0.31
FAM163A	2.51	7.75	0.32	*EPHA2*	191.98	619.37	0.31
ASXL3	15.57	47.96	0.32	*KCNMB1*	515.45	1682.47	0.31
ROR2	313.38	969.45	0.32	*SLC8A1*	387.64	1250.65	0.31
KIT	116.36	360.84	0.32	*PKD1L2*	1.8	5.71	0.31
PGR	138.89	437.31	0.32	*MAT1A*	3.11	9.88	0.31
BDNF	25.28	78.9	0.32	*ZNF804A*	14.74	47.63	0.31
SPON1	305.97	960.14	0.32	*GSTM5*	142.14	455.28	0.31
ASB2	209.66	655.97	0.32	*GALNT9*	5.56	17.67	0.31
RNF165	29.4	91.21	0.32	*FGF10*	3.99	12.91	0.31
CEND1	21.09	64.95	0.32	*VEPH1*	7.95	26.02	0.31
C7orf58	363.4	1132.64	0.32	*PRIMA1*	75.71	241.69	0.31
ADCY5	193.19	594.61	0.32	*PRODH*	21.83	70.4	0.31
FAM110C	7.02	22.25	0.32	*ITGB3*	76.89	247.62	0.31

SPOCK3	435.95	1412.02	0.31	*SLC46A2*	4.01	13.47	0.3
ATRNL1	50.71	161.03	0.31	*P2RY2*	14.9	50.09	0.3
LASS3	5.3	16.95	0.31	*FHL1*	2216.95	7499.49	0.3
TGM1	26.67	86.08	0.31	*C18orf34*	15.82	52.56	0.3
IQSEC3	15.06	48.38	0.31	*DGKG*	20.27	67.86	0.3
LIPG	36.02	115.96	0.31	*PCYT1B*	17.73	58.58	0.3
CNN1	5877.94	18979.55	0.31	*SEMA3A*	26.35	88.6	0.3
LGI3	19.02	60.87	0.31	*CYSLTR2*	6.34	20.85	0.3
SYT9	23.8	75.84	0.31	*GLIS1*	49.08	164.05	0.3
CYP4F3	5.06	16.48	0.31	*PTCHD1*	29.15	97.58	0.3
SLITRK2	9.48	30.85	0.31	*FOXP2*	15.91	53.47	0.3
PALM3	23.98	76.44	0.31	*ADH1B*	42.79	143.64	0.3
P2RX1	76.26	244.24	0.31	*CRISP1*	1.38	4.67	0.3
GJB3	33.72	109.33	0.31	*BCHE*	46.54	154.02	0.3
SGCG	8.34	26.92	0.31	*DES*	18000.5	60861.15	0.3
FADS2	1854.01	5895.36	0.31	*NCCRP1*	12.6	41.69	0.3
C4orf19	38.78	126.5	0.31	*NRXN1*	28.39	94.43	0.3
NTF4	22.16	71.54	0.31	*PMP2*	9.95	33.4	0.3
SV2B	52.43	167.24	0.31	*SLC13A2*	2.1	7.06	0.3
POF1B	36.38	118.49	0.31	*DSC3*	232.83	788.39	0.3
TG	42.17	135.43	0.31	*SLITRK3*	10.48	34.97	0.3
KRT14	327.82	1073.51	0.31	*EDIL3*	46.83	154.81	0.3
WFDC5	5.46	17.67	0.31	*CRABP1*	6.27	20.99	0.3
ITGA8	128.28	419.6	0.31	*B3GNT3*	31.97	106.42	0.3
CARTPT	9	29.35	0.31	*MYOT*	11.14	36.91	0.3
SHISA9	42.42	137.38	0.31	*SLPI*	237.7	803.14	0.3
GNAZ	163.01	535.1	0.3	*CXCL17*	42.02	139.29	0.3
CCDC85A	31.56	105.31	0.3	*SLC7A4*	52.45	173.34	0.3
MAMLD1	67.32	223.04	0.3	*GPRC5B*	258.2	899.9	0.29
KRT24	1.3	4.28	0.3	*PAQR8*	155.33	528.63	0.29
ZNF185	601.06	2021.16	0.3	*CAV1*	1095.54	3769.82	0.29
C11orf45	23.46	78.83	0.3	*ZNF154*	25.31	86.97	0.29
GCOM1	235.2	790	0.3	*ANO1*	490.74	1708.07	0.29
TSPAN18	284.43	939.75	0.3	*ARL4D*	99.33	346.74	0.29
PLAG1	30.29	100.56	0.3	*AKR1B1*	391.09	1334.95	0.29
CBLN4	2.17	7.27	0.3	*PIP5K1B*	38.51	134.94	0.29
C5orf4	370.96	1224.69	0.3	*C16orf74*	24.12	82.52	0.29
NPFFR2	1.52	5.13	0.3	*TPRXL*	5.72	19.72	0.29
ABCG2	162.64	541.76	0.3	*SNAI2*	176.92	610.52	0.29
CXorf57	10.7	35.58	0.3	*KCNQ5*	25.62	88.46	0.29
TRPM3	3.01	9.91	0.3	*AFF2*	17.25	58.98	0.29
MPP2	110.71	364.36	0.3	*P2RX6*	6.46	22.41	0.29
MAMDC2	73.09	240	0.3	*LMOD1*	2650.61	9033.56	0.29
ITPRIPL1	16.98	56.26	0.3	*OCA2*	3.96	13.51	0.29
ZNF204P	98.3	325.09	0.3	*TCEAL2*	142.96	499.8	0.29
GALR1	4.22	14.09	0.3	*NIPAL4*	11.79	41.28	0.29
KCNAB1	163.67	538.7	0.3	*PLCL1*	137.99	481.63	0.29
SORBS1	1944.46	6398.85	0.3	*B3GALT2*	40.76	140.1	0.29

LPHN3	19.31	67.59	0.29	*DMKN*	99.65	351.34	0.28
EDDM3A	1.08	3.68	0.29	*PPP1R1A*	32.22	116.76	0.28
KCNB1	21.93	74.54	0.29	*D4S234E*	122.38	438.41	0.28
FAM46B	116.01	406.2	0.29	*ASTN1*	7.96	28.68	0.28
FAM83C	2.6	8.88	0.29	*ZNF750*	100.35	356.63	0.28
FOXQ1	68.21	231.59	0.29	*GGT6*	99.69	352.82	0.28
CCNI2	5.27	18.17	0.29	*PLAC9*	29.95	107.04	0.28
STXBP5L	47.86	165.81	0.29	*PTPRZ1*	21.16	76.27	0.28
GPR109B	12.92	44.31	0.29	*KRT7*	480.04	1742.24	0.28
FLNC	3292.3	11270.19	0.29	*DPYS*	19.28	69.83	0.28
RGS7BP	37.25	127.75	0.29	*C1orf190*	29.49	107.37	0.27
C1QL1	7.41	25.21	0.29	*KLHL4*	5.68	20.66	0.27
CEL	20.74	71.24	0.29	*ALDH1L2*	53.55	197.97	0.27
FLRT3	223.56	776.93	0.29	*LOC284276*	15.8	57.52	0.27
BEX1	23.09	78.27	0.29	*TMEM108*	10.76	40.21	0.27
GATA5	24.89	84.57	0.29	*PHYHIPL*	55.7	205.56	0.27
DDR2	92.95	315.75	0.29	*RND2*	50.79	185.56	0.27
CNTN1	148.9	510.31	0.29	*HSPA4L*	48.22	181.09	0.27
NEFM	9.11	31.79	0.29	*IQCA1*	17.74	66.1	0.27
DSG3	9.17	31.7	0.29	*FABP7*	1.98	7.25	0.27
WDR72	4.58	15.64	0.29	*UGT3A1*	1.78	6.54	0.27
SLC5A8	9.21	31.52	0.29	*CPEB1*	9.07	34	0.27
KRT15	960.97	3295.65	0.29	*DAAM2*	287.92	1060.09	0.27
NKAPL	8.24	29.63	0.28	*TSLP*	35.84	132.26	0.27
COLEC10	1.58	5.68	0.28	*CSRNP3*	6.04	22.65	0.27
SCARA3	513.2	1805.36	0.28	*ST6GALNAC2*	82.14	305.45	0.27
TRIM9	8.56	30.34	0.28	*CDHR1*	19.89	74.7	0.27
MRO	7.62	26.99	0.28	*LOC84856*	3.8	13.9	0.27
EVX2	2	7.08	0.28	*LOC554202*	5.57	20.62	0.27
FAM176A	9.52	33.51	0.28	*ANO5*	86.31	318.34	0.27
APOBEC3G	106.83	384.05	0.28	*LYVE1*	27.42	102.21	0.27
SAMD12	120.24	422.31	0.28	*LOC255167*	23.9	87.12	0.27
PPP1R3C	164.72	581.91	0.28	*NRG1*	12.25	45.74	0.27
FAM107A	624.57	2207.26	0.28	*PEG10*	69.61	259.83	0.27
RBMS3	21.84	77.47	0.28	*C1orf106*	107.98	396.26	0.27
HFE	54.84	196.52	0.28	*CRIP3*	10.46	39.19	0.27
EYA4	68.05	240.1	0.28	*ATP6V0D2*	8.18	30.22	0.27
PDZRN4	126.11	456.99	0.28	*EDAR*	11.1	40.87	0.27
SYT10	3.31	11.76	0.28	*VIT*	17.74	65.67	0.27
ALDH1A2	260.93	921.06	0.28	*SYT8*	4.9	18.24	0.27
ACSS3	72.78	259.89	0.28	*C21orf62*	7.31	27.31	0.27
C9orf125	96.27	343.03	0.28	*MLC1*	13.89	51.56	0.27
FILIP1	34.24	122.64	0.28	*DEFB1*	30.89	116.57	0.27
FBXL21	2.17	7.79	0.28	*MUC4*	87.57	321.45	0.27
GPR126	123.9	441.39	0.28	*GSTM3*	628.53	2387.74	0.26
ACTG2	13024.46	46902.35	0.28	*NECAB1*	70.76	275.88	0.26
NRK	44.16	155.81	0.28	*RHBDL3*	21.91	83.81	0.26
ITGB6	127.03	450.69	0.28	*CLCNKB*	5.04	19.07	0.26

HS3ST5	1.63	6.3	0.26	*H19*	111.06	447.72	0.25
KCNJ5	29.68	115.34	0.26	*UPK1A*	35.66	141.05	0.25
CRYAB	609.85	2361.45	0.26	*LOC642587*	125.86	499.64	0.25
ARHGAP20	59.2	224.72	0.26	*C20orf56*	14.75	58.42	0.25
GNAL	84.05	328.37	0.26	*SLC2A9*	36.2	149.24	0.24
ADAMTS5	81.64	311.85	0.26	*TMLHE*	33.71	141.21	0.24
GLDN	11.12	43.14	0.26	*NYNRIN*	236.55	996.09	0.24
DCHS2	14.66	57.16	0.26	*CLU*	5721.79	23950.17	0.24
SCN2B	23.56	89.07	0.26	*LINGO2*	2.34	9.89	0.24
SYNM	4075.02	15637.49	0.26	*CDH8*	2.71	11.27	0.24
TRHDE	19.04	73.19	0.26	*SEMA6D*	55.66	228.57	0.24
MYH11	41607.11	159735.6	0.26	*DHDPSL*	10.96	46.53	0.24
FAM167A	37.16	140.82	0.26	*RAB9B*	12.61	52.28	0.24
NDP	36.67	138.38	0.26	*CIDEC*	2.85	12.11	0.24
ANGPTL1	46.64	178.23	0.26	*SNCG*	144.45	605.57	0.24
MPZ	22.5	85.76	0.26	*PCDH7*	304.61	1262.47	0.24
VGLL1	11.67	44.5	0.26	*SNAP25*	29.87	125.48	0.24
CLDN2	3.26	12.43	0.26	*ACOX2*	85.36	361.38	0.24
SLC14A1	549.53	2132.46	0.26	*SLC16A5*	101.09	429.86	0.24
DLK2	78.08	298.19	0.26	*LMO3*	97.23	401.5	0.24
TNNT3	3.44	13.31	0.26	*CYP11A1*	7.2	30.15	0.24
ACTN2	12.06	46.28	0.26	*LY6G6D*	4.43	18.79	0.24
DCC	1.74	6.97	0.25	*LRRTM3*	2.37	10.04	0.24
SCN5A	10.34	40.96	0.25	*PDK4*	965.07	4078.11	0.24
DMRT2	1.99	7.99	0.25	*PGM5*	1571.43	6438.87	0.24
L3MBTL4	22.21	89.1	0.25	*HMGCLL1*	8.91	36.7	0.24
PLA2G3	2.18	8.76	0.25	*SMOC1*	302.15	1246.46	0.24
ID4	281.31	1119.28	0.25	*ADRA1D*	23.75	99.23	0.24
CHRM2	1.6	6.42	0.25	*PTGS2*	447.15	1869.65	0.24
STOX2	13.8	54.78	0.25	*EDN3*	19.57	82.3	0.24
CMTM5	5.15	20.43	0.25	*SFRP5*	6.42	26.65	0.24
PRKCB	135.53	533.84	0.25	*HSPB3*	3.78	15.82	0.24
KCTD14	81.91	330.22	0.25	*RHCG*	5.65	23.28	0.24
SLITRK6	86.2	343.29	0.25	*ERN2*	12.57	53.13	0.24
SYNPO2	5222.65	20514.12	0.25	*TBX4*	21.19	90.14	0.24
FGFR2	421.54	1693.22	0.25	*CD38*	184.33	760.75	0.24
EVPLL	4.41	17.46	0.25	*C10orf99*	1.7	7.43	0.23
LRP1B	9.96	39.09	0.25	*GSTP1*	1211.02	5186.69	0.23
HIF3A	52.47	209.89	0.25	*ADAMTS18*	2.21	9.62	0.23
GPR172B	10.99	44.22	0.25	*MYLK*	8050.93	35450.37	0.23
GPM6A	49.93	200.38	0.25	*UBXN10*	41.01	176.74	0.23
TNMD	2.68	10.85	0.25	*LRRN3*	17.99	77.79	0.23
A2BP1	7.12	28.02	0.25	*GSTM2*	312.92	1350.4	0.23
PRDM8	229.17	923.19	0.25	*CD300LG*	6.12	26.26	0.23
GDF10	7.31	29.03	0.25	*CYP3A7*	2.83	12.28	0.23
PCSK2	8.39	34.21	0.25	*CRABP2*	215.45	930.58	0.23
CHST9	63.43	257.79	0.25	*CHRDL1*	891.01	3881.95	0.23
ANXA8	54.22	215.2	0.25	*LDB3*	246.01	1073.89	0.23

BNIPL	42.85	189.06	0.23	*PROM1*	44.65	216.94	0.21
ATP1A2	355.74	1549.12	0.23	*SLC18A2*	14.72	69.97	0.21
GJB4	10.12	43.69	0.23	*CCK*	38.93	187.97	0.21
PRKG1	24.8	109.72	0.23	*KLHL14*	16.09	80.19	0.2
IP6K3	22.96	98.51	0.23	*SLCO4C1*	2.5	12.3	0.2
ODAM	4.57	19.51	0.23	*SOSTDC1*	15.49	79.43	0.2
NOS1	9.25	40.02	0.23	*EPHB1*	19.24	95.53	0.2
ABO	12.55	53.51	0.23	*HPD*	6.14	30.46	0.2
CAPNS2	7.12	30.48	0.23	*COL4A6*	183.66	933.28	0.2
GNAO1	123.18	566.17	0.22	*JPH4*	144.31	733.52	0.2
PTGS1	189.98	863.8	0.22	*CCBE1*	11.68	59.42	0.2
PPARGC1A	63.07	292.29	0.22	*TMEM132C*	22.27	110.32	0.2
HSPB8	1099.08	4990.97	0.22	*MAL*	17.45	85.23	0.2
PAK7	8.26	36.89	0.22	*TMEM35*	123.2	609.32	0.2
FAM196B	2.92	13.1	0.22	*DUOXA2*	6.59	32.61	0.2
CNTFR	28.34	131.05	0.22	*FAT2*	98.54	481.84	0.2
NRG2	15.34	68.97	0.22	*SPINK2*	2.65	13.2	0.2
HOXD13	79.49	368.64	0.22	*SCARA5*	10.88	55.58	0.2
FAT3	59.75	274.43	0.22	*LOC339535*	3.43	16.87	0.2
NCAM1	124.46	558.61	0.22	*LY6D*	6.24	30.77	0.2
SRD5A2	134.09	612.09	0.22	*TNS4*	248.13	1239.71	0.2
CLVS2	7.07	32.16	0.22	*CXCL13*	20.85	105.92	0.2
PAEP	1.26	5.8	0.22	*ACTA1*	25.25	128.84	0.2
MUC15	16.11	74.84	0.22	*C8orf84*	98.72	526.67	0.19
SLC2A5	110.4	512.22	0.22	*APOBEC3C*	121.46	653.6	0.19
DPT	117.07	542.46	0.22	*PAK3*	6.08	32.16	0.19
KCNS1	11.79	54.67	0.22	*ANGPT1*	167.11	868.6	0.19
LAMB3	561.93	2540.51	0.22	*AQP5*	6.83	35.5	0.19
CASQ1	14.63	66.13	0.22	*GATA3*	122.15	626.63	0.19
WISP2	37.63	171	0.22	*FUT3*	6.93	36.74	0.19
PLP1	49.54	222.75	0.22	*FOLR1*	9.6	50.55	0.19
VTCN1	33.1	149.16	0.22	*TMEM40*	10.04	53.98	0.19
CPA6	23.14	107.39	0.22	*GJB5*	19.8	101.59	0.19
TRIM29	686.13	3179.27	0.22	*GPR87*	36.9	196.13	0.19
NEFL	17.91	80.23	0.22	*SERPINB5*	64.22	330.59	0.19
CA3	14.96	69.54	0.22	*WFDC2*	385.25	2052.11	0.19
NDRG4	46.62	226.99	0.21	*LOC572558*	48.8	256.07	0.19
QPRT	52.16	251.9	0.21	*KRT16*	20.84	108.84	0.19
HRASLS5	11.59	54.17	0.21	*KIAA1210*	100.53	522.63	0.19
TMEM213	2.63	12.48	0.21	*HSD17B13*	26.85	138.58	0.19
FAM83A	2.5	11.92	0.21	*PI16*	68.54	351.73	0.19
ADRA1A	24.17	116.67	0.21	*CLCA4*	9.52	49.04	0.19
MYOCD	131.34	628.36	0.21	*KRT4*	27.16	140.51	0.19
ALDH3A1	13.03	61.14	0.21	*GCNT4*	11.59	62.89	0.18
VSNL1	20.52	96.19	0.21	*ASPA*	9.59	54.53	0.18
PATE4	1.43	6.82	0.21	*DAB1*	8.39	45.57	0.18
HRNBP3	101.42	488.51	0.21	*CAPN6*	62.92	341.95	0.18
STAC2	34.37	165.48	0.21	*LIX1*	12.57	68.08	0.18

KRT23	109.63	617.35	0.18
KRT5	1448.77	8018.04	0.18
SLC26A3	12.62	70.72	0.18
PIP	16.2	88.78	0.18
PON3	2.96	17.21	0.17
PGM5P2	15.31	89.76	0.17
C10orf82	3.71	21.81	0.17
MFSD2A	16.43	96.1	0.17
DUOX1	128.96	780.88	0.17
FAM83B	12.88	75.29	0.17
STAC	56.92	332.7	0.17
TP63	252.16	1522.22	0.17
ABP1	74.93	431.63	0.17
C2orf88	51.81	329.91	0.16
PDE1C	4.25	27.17	0.16
AOX1	266.43	1674.83	0.16
CXCR2	7.59	47.23	0.16
PCP4L1	32.83	202.45	0.16
NPY6R	5.11	32.1	0.16
DUOXA1	31.6	202.93	0.16
ATP6V0A4	3.74	23.65	0.16
PENK	30.84	197.83	0.16
S100A14	46.94	300.39	0.16
COL17A1	226.33	1413.02	0.16
WIF1	27.29	173.13	0.16
MSLN	15.44	95.83	0.16
CKM	29.69	185.69	0.16
KY	15.75	103.8	0.15
CYP4F22	10.73	69.35	0.15
CYP4B1	105.09	699.35	0.15
CYP3A5	40.48	270.32	0.15
PATE1	1.39	9.45	0.15
DAPL1	7.12	46.05	0.15
SCGB1A1	42.46	282.95	0.15
IGSF1	17.14	122.38	0.14
LGR6	35.2	260.55	0.14
RPE65	5.79	41.21	0.14
KCNJ3	13.57	94.75	0.14
DUOX2	28.6	203.65	0.14
CHP2	13.58	94.69	0.14
SCGB3A1	19.54	136.75	0.14
BMP5	5.79	42.23	0.14
SMR3B	1.31	9.87	0.13
CRTAC1	22.94	170.96	0.13
KCNJ15	19.27	146.7	0.13
PNMT	5.64	42.6	0.13
EMX2	4.69	35.38	0.13
GPX2	34.63	271.77	0.13
GSTM1	28.03	220.81	0.13
MCF2	4.43	36.6	0.12
CA14	15.86	133.33	0.12
EMX2OS	8.89	73.8	0.12
CPNE6	17.38	149.28	0.12
FOXI1	7.32	63.54	0.12
MYH6	3.77	32.67	0.12
PADI3	2.52	23.71	0.11
KRT13	62.62	621.05	0.1
ACTC1	133.69	1357.44	0.098
CLCA2	11.2	120.66	0.093
SERPINA5	20.65	225.23	0.092
SLC39A2	7.02	88.86	0.079
PIK3C2G	3.38	45.51	0.074
AQP2	1.92	26.03	0.074
SEMG2	7.61	105.3	0.072
SEMG1	5.99	193.96	0.031

R-PCa = Mean of the number reads of the prostate cancer tissue samples; R-SNT = Mean of the number of reads of surrounding normal tissue; FC = Fold-change

11. ANEXOS

ANEXO I: Questionário pessoal

QUESTIONÁRIO PESSOAL

Por favor, leia as questões seguintes cuidadosamente e responda-as da forma mais completa e precisa possível. A informação que você der não será associada a seu nome em nenhum documento público, e será conhecida somente dos principais pesquisadores deste estudo. As informações que você der podem ter influência direta na interpretação de nossos resultados, portanto, pedimos que coopere gentilmente, fornecendo informações corretas. Obrigado pelo interesse.

1- Nome: _____

último primeiro do meio

2- A ser preenchido pelo pesquisador:

Código:_____

Data: ___/___/___

Esta folha deve ser destacada do restante do questionário e preenchida pelo pesquisador. Somente o código será usado como identificação para as páginas subsequentes. Se for necessário espaço adicional para complementar a sua resposta, por favor escreva no verso da página e identifique a parte restante da questão com seu respectivo número.

Código n°_____

HISTÓRICO PESSOAL

1- Registro hospitalar: _____

2- Sexo: ( ) masculino ( ) feminino

3- Qual a cor da sua pele?

Negro ( ) Branco( ) Amarelo ( ) Outros ( )

4- Idade:_____ Data de Nascimento:_____/_____/_____

5- Local de nascimento: Paraná ? ( ) SIM ( ) NÃO

Se NÃO: Que região brasileira ? Norte ( ) Sul ( ) Nordeste ( ) Centro-Oeste ( ) Sudeste ( )

6- Sua moradia é na zona rural ou urbana? ( ) Rural ( ) Urbana

7- Quanto tempo vive neste local? _____anos _____meses

8- Qual o seu grau de instrução?

( ) analfabeto ( ) 1° grau incompleto ( ) 1° grau completo ( ) 2° grau incompleto

( ) 2°grau completo ( ) técnico ( ) profissional ( ) superior

Histórico de exposição relacionado ou não ao trabalho

9- Você já se expôs a alguma destas substâncias abaixo em seu trabalho?

Se SIM, por quanto tempo e a quanto tempo foi isso:

Derivados de petróleo (querosene, gasolina, solventes,...)	( )sim	( )não
Tintas/ corantes	( )sim	( )não
Indústrias têxteis ou tecelagem	( ) sim	( ) não
Praguicidas / Herbicidas	( )sim	( )não
Radiação	( )sim	( )não
Metais pesados (Pb, Ni, Cr,...)	( )sim	( )não
Processamento de madeira	( )sim	( )não
Papel ou celulose	( )sim	( )não
Mineração	( )sim	( ) não
Fábrica de sapatos ou curtume	( )sim	( )não
Metalúrgica	( )sim	( )não
Usina de açúcar ou álcool	( )sim	( )não
Plástico ou borracha	( )sim	( )não
Outras substâncias químicas	( )sim	( )não

10- Se SIM para a pergunta acima: Você utilizava equipamentos de proteção individual para trabalhar com essas substâncias químicas? (máscaras, luvas, óculos, etc.)

a) ( )sim b) ( )não

Histórico Tabagista

11- Você fuma atualmente? ( ) SIM ( ) NÃO

12- Se SIM, quanto você fuma por dia? ( ) menos de ½ maço
( ) de meio a 1 maço
( ) mais de um maço

13- Se SIM, há quanto tempo você fuma? _____

14- Se NÃO, mas já fumou algum dia: há quanto tempo parou de fumar?
a) ( ) 0-5 anos b) ( ) 5-10 anos c) ( ) >10

15- Você convive/conviveu em seu trabalho ou em casa com pessoas que fumam?
a) ( ) SIM b) ( ) NÃO

Histórico de Etilismo

16- Você consome bebidas alcoólicas? ( ) SIM ( ) NÃO

17- Se SIM, que tipo de bebida alcoólica você costuma consumir?
a) ( )Destiladas b) ( )Não-Destiladas c) ( )Outra d) ( )Ambas

18-Quanto você costuma beber por semana?
( ) no máximo um copo ( ) de 2 a 5 copos ( ) de 6 a 10 ( ) mais de 10

19- Se já parou, há quanto tempo parou de consumir esta bebida?
a) ( ) 0-5 anos. b) ( ) 5 –10 anos. c) ( ) mais 10 anos.

20- Quanto você costumava beber por semana?
( ) no máximo um copo ( ) de 2 a 5 copos ( ) de 6 a 10 ( ) mais de 10

21- Durante a sua vida, já consumiu ou consome alguma bebida diariamente por mais de 6 meses continuamente?
( ) SIM ( ) NÃO

Histórico de Saúde

22- Nos últimos 12 anos você automedicou-se ou recebeu medicamentos?

( ) SIM ( ) NÃO ( )não sabe

Se SIM

( ) Hormônio

( ) Antinflamatório

( ) Analgésicos

( ) Antipertensivos

( ) Anabolizantes

( ) Outros

23- Você toma vitaminas ou tem tomado nos últimos seis meses?

( )SIM ( ) NÃO ( )não sabe

24 – Você já foi submetido a cirurgia nos testículos?

( ) SIM ( ) NÃO

Qual?_____

25- Você foi tratado anteriormente de algum tipo de câncer? ( ) SIM ( ) NÃO

Qual? _____

26- Você tem antecedentes de câncer na sua família? ( ) SIM ( ) NÃO

27- Em casos de câncer na família, qual era o vínculo de parentesco?

( ) Pai ( ) Mãe ( ) Irmão ( ) Filho ( ) Tio ( ) Primo ( ) Outro

28- Qual foi a localização do tumor?

( ) Próstata ( ) Mama ( ) Bexiga ( ) Outro (qual?) _____

29 – Você já teve alguma doença sexualmente transmissível?

( ) SIM ( ) NÃO

Se sim, qual?_____

Histórico alimentar: (refira-se somente a hábitos frequentes)

30- Você segue uma dieta vegetariana? ( ) SIM ( )NÃO

31- Você come carne? ( ) SIM ( )NÃO

32- Se SIM, com que frequência você come estes alimentos:

	Dias/Semana			
	1-2	3-4	5-6	Diariamente
Carne de gado	( )	( )	( )	( )
Peixe	( )	( )	( )	( )
Frango	( )	( )	( )	( )
Porco	( )	( )	( )	( )
Outros	( )	( )	( )	( )

Histórico genético

33- Você possui algum irmão idêntico? ( ) SIM ( ) NÃO

Incluir informações das amostras do tumor (localização, laudo histo-patológico)

ANEXO II: Parecer do Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina.

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	176/2013
CAAE:	19769913.0.0000.5231
Data da Relatoria:	19/09/2013
Pesquisador(a):	Ilce Mara de Syllos Cólus
Unidade/Órgão:	CCB - Departamento de Biologia Geral

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo comparativo do perfil transcricional e genotípico de genes relacionados ao câncer de próstata entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 27 de setembro de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL